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REVIEW

Esophageal lichen planus: Current knowledge, challenges and future perspectives

Annegrit Decker, Franziska Schauer, Adhara Lazaro, Carmen Monasterio, Arthur Robert Schmidt, Annette Schmitt-Graeff, Wolfgang Kreisel

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Abstract

Lichen planus (LP) is a frequent, chronic inflammatory disease involving the skin, mucous membranes and/or skin appendages. Esophageal involvement in lichen planus (ELP) is a clinically important albeit underdiagnosed inflammatory condition. This narrative review aims to give an overview of the current knowledge on ELP, its prevalence, pathogenesis, clinical manifestation, diagnostic criteria, and therapeutic options in order to provide support in clinical management. Studies on ELP were collected using PubMed/Medline. Relevant clinical and therapeutical characteristics from published patient cohorts including our own cohort were extracted and summarized. ELP mainly affects middle-aged women. The principal symptom is dysphagia. However, asymptomatic cases despite progressed macroscopic esophageal lesions may occur. The pathogenesis is unknown, however an immune-mediated mechanism is probable. Endoscopically, ELP is characterized by mucosal denudation and tearing, trachealization, and hyperkeratosis. Scarring esophageal stenosis may occur in chronic courses. Histologic findings include mucosal detachment, T-lymphocytic infiltrations, epithelial apoptosis (Civatte bodies), dyskeratosis, and hyperkeratosis. Direct immuno-fluorescence shows fibrinogen deposits along the basement membrane

zone. To date, there is no established therapy. However, treatment with topical steroids induces symptomatic and histologic improvement in two thirds of ELP patients in general. More severe cases may require therapy with immunosuppressors. In symptomatic esophageal stenosis, endoscopic dilation may be necessary. ELP may be regarded as a precancerous condition as transition to squamous cell carcinoma has been documented in literature. ELP is an underdiagnosed yet clinically important differential diagnosis for patients with unclear dysphagia or esophagitis. Timely diagnosis and therapy might prevent potential sequelae such as esophageal stenosis or development of invasive squamous cell carcinoma. Further studies are needed to gain more knowledge about the pathogenesis and treatment options.

Key Words: Lichen planus; Esophagitis; T-lymphocytes; Budesonide; Dysphagia; Precancerosis

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Core Tip: Lichen planus (LP) is a frequent, chronic inflammatory disease involving the skin, mucous membranes and/or skin appendages. Esophageal involvement in lichen planus (ELP) is an underdiagnosed inflammatory condition. ELP mainly affects middle-aged women. The principal symptom is dysphagia. Aymptomatic cases may occur. An immune-mediated pathogenesis is probable. Endoscopy shows mucosal denudation and tearing, trachealization, and hyperkeratosis. Scarring esophageal stenosis occurs. Histology includes mucosal detachment, T-lymphocytic infiltrations, epithelial apoptosis, dyskeratosis, and hyperkeratosis. Direct immuno-fluorescence shows fibrinogen deposits along the basement membrane zone. Treatment with topical steroids or immunosuppression may induce symptomatic and histologic improvement. ELP can be regarded as a precancerous condition.

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INTRODUCTION

Inflammatory esophageal diseases comprise a broad spectrum of differential diagnoses [1-3]out of which reflux esophagitis is the most frequent condition[4]. Infectious etiologies include Candida or viral esophagitis which are mainly linked to compromised immune function[5]. Esophageal disorders based on immunological background include Crohn's disease[6], Behçet's disease[7], graft-versus-host disease after allogeneic stem cell transplantation[8], and eosinophilic esophagitis (EoE)[9-12]. The spectrum of differential diagnoses ranges to less defined subtypes such as lymphocytic[13] or sloughing esophagitis [14]. These differential diagnoses as summed up in Table 1 encompass additional manifestation of autoimmune bullous diseases such as mucous membrane pemphigoid or pemphigus vulgaris[2,3,15] as well as lichen planus (LP). Esophageal lichen planus (ELP), a mucocutanous manifestation of LP, should be considered in patients with signs and symptoms corresponding to esophageal inflammation. Since many aspects of this disease are still poorly understood, ELP tends to be underreported and often misdiagnosed. However, in the last decade, gastroenterologists and researchers provided more emphasis to this condition. Likewise, proposals for macroscopic and histopathologic diagnostic criteria were made and data on therapy has been increasingly available[16-20].

This narrative review aims to summarize current knowledge on ELP in order to increase awareness about this clinically important esophageal inflammatory disease and make it more accessible in clinical practice.

MAIN BODY

LP

LP is a frequent mucocutaneous disease whose pathogenesis is only partly understood[21-24]. It affects 0.5%–2% of the general population and has female predominance (65%)[21,23,24]. Lesions of skin, oral, and genital mucosa are the most frequent manifestations, however involvement of nails, scalp, genitoanal mucosa, eyes, ears, urinary bladder, or nasal mucosa are also seen. Classic exanthematic,

Table 1 Inflammatory diseases of the esophagus

Chemical or physical damages
Reflux esophagitis
Chemical esophagitis (acids, leach)
Radiation induced esophagitis
Drug-induced esophagitis *e.g.* NSAID, bisphosphonates, tetracyclines, KCl, ferric sulfate, ascorbinic acid
Infectious esophagitis
Candida *spp.*
Viruses, *e.g.* Cytomegalovirus, Herpes simplex, HIV
Immune-mediated esophagitis
EoE
Crohn's disease
GVHD
Behçet's disease
Systemic sclerosis
Lymphocytic esophagitis
Lichen planus
Mucus membrane pemphigoid
Pemphigus
Congenital skin disease
Esophageal involvement in epidermolysis bullosa
Others
EIPD
Sloughing esophagitis

NSAIR: Non-steroidal anti-inflammatory drug; KCl: Kalium chloride; HIV: Human immunodeficiency virus; GVHD: Graft-versus-host disease; EIPD: Esophageal intramural pseudodiverticulosis; EoE: Eosinophilic esophagitis.

cutaneous LP manifests as flat, reddish, itching papules in the face, arms, wrists, with a tendency to develop postinflammatory hyperpigmentation. In two-thirds of patients, an oral manifestation is observed with reticular, erythematous, and erosive subtypes. Patients with oral LP complain of oral discomfort or pain, exhibit characteristic fine white buccal lines (Wickham striae) and often have visible ulcerations on gingiva and palate, tongue and/or labial mucosa. Genital LP may cause itching lesions on glans penis, prepuce or scrotum in men, and on vulva or vagina in women. Involvement of genital mucosa may show all stages of inflammation, starting with erythema, progressing to erosions, plaque formation, and scarring. LP pemphigoides is a rare, mostly IgG-mediated autoimmune variant of LP, exhibiting characteristics of bullous pemphigoid (reactivity against collagen XVII)[25]. As LP may involve multiple organ systems, this disease requires multidisciplinary approach involving dermatologists, dentists, gynaecologists, and gastroenterologists[26-29]. The European guidelines for therapy of LP have recently been published[30,31].

Pathogenesis

A T-cell mediated inflammatory reaction involving antigen-specific and antigen-unspecific mechanisms is regarded as the basic mechanism of pathogenesis[21,28,32]. A recent review about the immunogenetics of LP reported that multiple imbalances of cytokines or interleukins are involved[33]. In addition, genetic influences and MHC associations were found. Micro-RNAs might also be implicated in LP. Antigen-specific mechanisms include antigen presentation of an unknown trigger by basal keratinocytes, activation of CD4+ Th1-helper cells, cytokine production, and CD8-positive cytotoxic reaction against basal epidermal cells. On the other hand, antigen-unspecific mechanisms could involve upregulation of proinflammatory mediators such as interferon-γ, tumour necrosis factor-alpha, interleukins, and matrix metalloproteases, leading to T-cell infiltration in the epidermal cell layer.

The cytokine profile suggests a Th1/Th2-imbalance, whereas B-cells, plasma cells, or antibodies may play a minor role[33]. Similar to psoriasis or pemphigus[34-36], a disturbance in the IL17/IL23 axis was observed[37,38]. Bacterial or viral antigens may trigger LP. An association with chronic hepatitis C was described, however data remains controversial[39,40]. An association with IgG4-related disease is possible[41]. LP may be triggered by several drugs, *e.g.* NSAIDs, beta-blockers, ACE-inhibitors, and check-point inhibitors[42]. Amalgam or mercury are regarded as trigger for oral LP[43], while concomitant diabetes or smoking influence the clinical severity[44]. There are associations with systemic diseases and autoimmune disorders such as primary biliary cholangitis, autoimmune thyroiditis, myasthenia, alopecia areata, vitiligo, thymoma, and autoimmune polyendocrinopathy[28,45-47]. As in other immune-mediated diseases, psychological component may influence the disease progression[48-50].

ELP

Involvement of the esophagus in LP as another possible site of mucosal affection was first described in 1982[51,52], followed by case reports and small case series presenting this new type of esophageal inflammatory disease with lichenoid features[53-64]. ELP was regarded then as a rarity, likely because its clinical, endoscopic, and histologic features were not yet clearly understood. In recent years, interest about this "new" disease was growing and consequently, larger case series and studies[16-20,58,60,65-69],as well as two comprehensive reviews were published[70,71]. For this narrative review, studies were collected using PubMed/Medline and single case reports were excluded. Table 2 presents an overview of these studies and their key findings.

Epidemiology

The population-based prevalence of LP was estimated to reach an average of 1.3%[21,73]. Oral LP is considered the most predominant mucosal manifestation affecting two-thirds of patients with cutaneous LP[26-28]. A recent metaanalysis showed a varying global prevalence of oral LP (0.57% in Asia, 1.68% in Europe, and 1,39% in South America)[72,73]. Esophageal involvement was initially regarded as a rarity, however further studies showed an esophageal manifestation in up to 50% of patients presenting with cutaneous or oral LP[16,74]. Since the number of cases in these studies were limited and the patient groups non-randomized, the true prevalence of ELP might be overestimated. Surprisingly, ELP does not necessarily correlate with oral disease[20]. However, oral LP is found in most of the cases of severe ELP. Esophageal manifestation also correlates with the occurrence of other mucosal involvement such as genital LP. The median age at presentation is 60 years and 80% of patients are female[71,75]. Determining the true prevalence of ELP remains a challenge, as it would require endoscopic screening in a large group of patients with LP regardless of localization and symptoms. Focusing on patients with esophageal symptoms only, *e.g.* dysphagia, would underestimate the true prevalence of ELP. A previous study showed that more than 50% of patients with mild ELP did not report dysphagia[20]. Moreover, cases where the esophagus is the only affected site of LP could still be missed. Hence, the prevalence of ELP on a population-based level can only be roughly estimated thus far. Furthermore, assuming that about 10% of all LP patients would have an esophageal involvement, the prevalence could be as high as 0.1% in the general population, thus outnumbering the prevalence of eosinophilic esophagitis which has been reported to reach 0.04%–0.05% in Western countries[76].

Diagnostic features of ELP

Clinical symptoms: Dysphagia is the leading symptom found in 80%–100% of patients with ELP. Other symptoms include odynophagia, heart burn, regurgitation, weight loss, hoarseness, and chronic unproductive cough. In some studies, approximately 20% of patients with ELP did not manifest any esophageal symptoms[77]. Development of esophageal symptoms might be influenced by severity of disease. In a previously published study, 94% of patients with endoscopically severe ELP presented with dysphagia. However, only 44.4% of patients with mild ELP complained about dysphagia[20]. On the other hand, up to 6% of LP patients had symptoms of dysphagia without esophageal involvement. In clinical practice, ELP should be investigated in patients presenting with the above-mentioned symptoms, especially in patients with known LP. Moreover, ELP should be considered in all patients where other common causes of esophagitis (see Table 1) have been ruled out.

Diagnosis: Similar set of macroscopic and histologic features of ELP has been repeatedly described in literature (see Table 2). Alongside some findings which can be considered typical of ELP, some similarities with other esophageal disorders such as eosinophilic esophagitis, lymphocytic esophagitis, and sloughing esophagitis can be found[3,9-11,13,78-82], hence, making the diagnosis challenging. Based on published data and experience from our cohort of patients, a diagnostic score combining endoscopic and histopathologic findings, as well as direct immunofluorescence (DIF), and a severity grading (no ELP, mild ELP, and severe ELP) has been previously proposed by our group[20]. These criteria are not completely new, however existing criteria and our own findings were integrated into a comprehensive and reproducible scoring system. Examples for endoscopic, histopathologic, and DIF findings are shown in Figures 1-4.

Table 2 Studies on esophageal lichen planus (numbers in braces indicate number/percentage of patients from the cohort to which the criterion applies)

Ref.	Study design	Number of ELP cases	Further manifestation sites of LP	Macroscopic findings as described in the manuscript	Histologic findings as described in the manuscript	Signs and symptoms	Therapy
Keate *et al* [53], 2003	Case series	3 (all)	Cutaneous oral genital	Mucosal sloughing stenosis	Band-like infiltrate hyperkeratosis acanthosis	Dysphagia	Tacrolimus intralesional Pred. Response 3/3; Etretinate (no effect)
Donnellan *et al*[116], 2011	Case series	5 (all)	Oral (all), genital (2), cutaneous (1)	Ulcerations, strictures	Band-like lymphocytic infiltrate, civatte bodies	Dysphagia (all)	Dilation (4) Flut; Response 3/5
Franco *et al* [69], 2015	Case series	6 (83%)	Cutaneous and oral (4)	Ulcerations, strictures (5)	Band-like lymph infiltrate, civatte bodies, fibrinogen + in DIF	Dysphagia (all), food impaction (2)	Dilation (3) Flut/Pred/Triam. Response 5/5
Dickens *et al* [74], 1990	19 LP patients	5	Cutaneous (19), oral (4)	Papular lesions, mucosal detachment on biopsy, erosions	Submucosal lymphocytic infiltrate	Dysphagia (1)	
Harewood *et al*[65], 1999	Retrospective search in patient register	6 (100%)	Oral (5), genital (3), cutaneous (2), ELP as initial manifestation (5)	Proximal strictures (4)	Lymphocytic infiltration (4)	Dysphagia (6); odynophagia (2)	Dilation of strictures (6); Prednisone (40-60mg). Response 3/4
Quispel *et al* [16], 2009	24 LP patients	12	Oral and/or cutaneous (all)	Whitish papules (10), hyperemic lesions (3), mucosal detachment (2), submucosal plaques (3)	Lymphohistiocytic infiltrations para-/hyperkeratosis, hyperplasia, civatte bodies, glycogen akanthosis	Dysphagia (4), odynophagia (3), heart burn (3), regurgitation (2)	
Katzka *et al* [17], 2010	Retrospecitve review (10 years) of data base/ esophageal biopsies from patients with dysphagia	27 (92%)	Oral (19), genital (13), cutaneous (3), ELP as initial manifestation (13)	Strictures (18): Proximal (11), distal (3), both (4), mucosal detachment (11), erythema, plaques, whitish mucosa, superficial ulcerations, Koebner effect after dilat	Lichenoid lymphocytic infiltration, damage of ephithelial basal layer civatte bodies squamous cell carcinoma (1)	Dysphagia (27); odynophagia (2)	Dilation of strictures (17). Dilation + Fluticasone Response 10/11. No dilation plus intralesional corticosteroids (2) or swallowed Futicason/ Budesonide (2). Response 6/6
Fox *et al*[77], 2011	Review of published ELP cases until 2009 (including 4 own cases)	72 (87%)	Oral (89%), genital (42%), cutaneous (38%), scalp (7%); nails (3%), eyes (1%), ELP as initial manifestation (14)	Pseudomembranes, bleeding, fragility, inflammation; proximal (64%); distal (11%); Both (26%); Stenosis (47%)	Lichenoid lymphocytic infiltrates; dysplasia/squamous cell carcinoma (6%)	Dysphagia (81%); odynophagia (24%); weight loss (14%); heart burn (8%); regurgitation (3%); hoarseness (1%); asymptomatic (17%)	
Linton *et al* [66], 2013	Retrospective analysis of esophageal biopsies from 273 patients out of a large cohort	1 typical ELP; 6 possible ELP	No data	Inflammation (7); stricture (5); trachealization (4) mucosal fragility (1); ulcerations (3); nodules (3)	Lymphocytic infiltration (7); Civatte bodies (1); parakeratosis (6); mixed infiltration (6) elongation of lamina propria papillae (7) hyperplasia of basal cells (4); widened intercellular space (3); neutrophilic inflamm (1)	Dysphagia (7); odynophagia (4)	Dilation of stenosis (3). Topical Fluticasone (2). Response 2/2. Proton pump inhibitors (7). Sucralfate (2). 5-HT4-RA (1)
Podboy *et al* [19], 2017	Retrospective analysis of a cohort of ELP-patients	40 (80%)	Cutaneous (4), oral (19), genital (15), ELP as only; manifestation (13)	Strictures (29), ring formation (29), ulcerations (8), mucosal detachment (6), other mucosal, lesions (14), squameous cell carcinoma (2)	Common findings (> 5): Esophagitis (20), focal ulcerations (13), mucosal hyperplasia (10), intraepithelial lymphocytic infiltrate (13), eosinophilia < 5 (13) dyskeratosis (11). DIF positive: Lichenoid (2) equivocal (5) not evaluable because of mucosal detachment (13)	Dysphagia for solid food (32) even for fluids (8); odynophagia (6); reflux (1)	Topical corticosteroids: Budesonide in honey 2 x 3mg (32). Fluticasone spray 880 μg 2x/d (8). Response rate: Endoscopic (72,5%), clinical (62%)

Ravi *et al* [101], 2019	Retrospective analysis of ELP patients	132 (80%)		Clinical diagnosis (77)	"Specific histology" (55); Esophageal carcinoma (8)		Response to topical steroids 84/132 63.6%. Immunosuppressive; therapy necessary 38/132. Response: No data
Kern *et al* [18], 2016; Schauer *et al* [20], 2019	52 patients.with proven LP on other site (75%)	34mild (18); severe (16)	Oral 78-100% (*vs* 78% in non-ELP), genital 44-61% (*vs* 6% non-ELP). Cutaneous 25-44% (*vs* 28% non-ELP)	Mucosal detachment iatrogenic (12); spontaneous (16); hyperkeratosis (7); trachealization (10); stenosis/strictures (7)	Epithelial detachment, lymphocytic infiltration, Civatte bodies, dyskeratosis; DIF: Fibrinogen deposits (17); (85% in severe ELP)	Dysphagia. severe ELP: 15; mild ELP: 8	Topical corticosteroids (12). Budesonide gel 3x0.5mg. Fluticasone. Response 11/12. Stenosis: Topical corticosteroids dilation

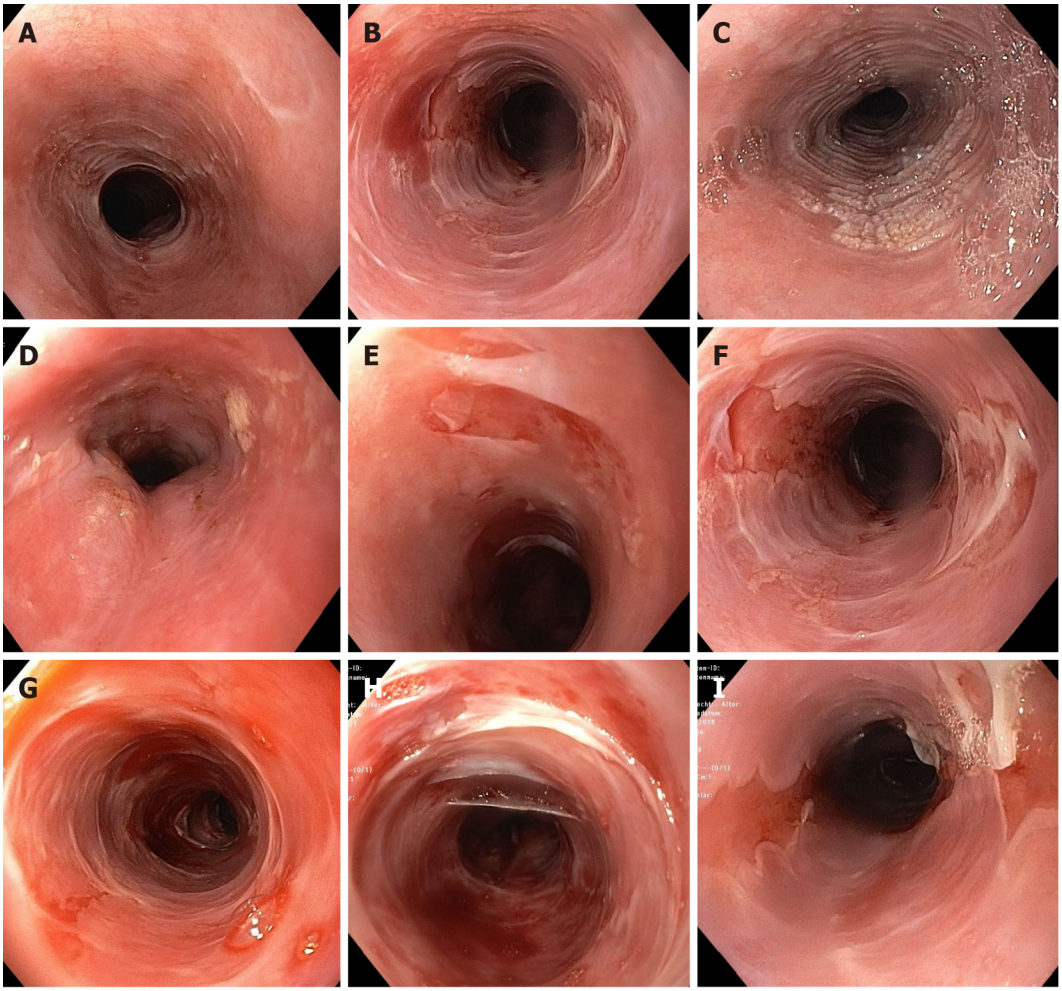
LP: Lichen planus; ELP: Esophageal lichen planus;DIF: Direct immunofluorescence; Flut: Fluticasone; Bud: Budesonide; Pred: Prednisolone; Triam: Triamcinolone.

Macroscopy

The endoscopic hallmark in nearly all studies analysed (see Table 2) is denudation or sloughing of the esophageal mucosa. It may occur spontaneously or during the endoscopic procedure. Less specific indicators of ELP are "trachealization" (an endoscopic sign well known in EoE) and presence of a rough and whitish surface of the mucosa which is the macroscopic correlate of hyperkeratosis as seen in histology. Stenoses or strictures may occur as sequelae of chronic inflammation in ELP as in other chronic inflammatory esophageal disorders. Endoscopic images of mucosal alterations are shown in Figure 1. Endoscopic changes may be observed in all parts of the esophagus, but mainly in the middle third. As reflux esophagitis often occurs simultaneously, macroscopic and histologic alterations directly above the gastroesophageal junction may be ambiguous. Thus, biopsies should be taken at least 5 cm above the gastroesophageal junction. To evaluate microscopic changes in patients with known LP, we recommend to perform at least two biopsies (in the lower and upper third of the esophagus) regardless if the above-mentioned endoscopic signs are not present.

Histopathologic Features

Esophageal biopsies provide a reliable assessment of mucosal lesions characteristic of ELP (Figure 2). Band-like inflammatory infiltrates are observed at the interface between the squamous epithelium and the lamina propria corresponding to a lichenoid esophagitis pattern. The predominant cell type in the inflammatory infiltrate of ELP are CD3+ T cells which spill over into the adjacent epithelium involving the lower third or lower half of the epithelial thickness. CD4+ cells are the main T-cell subset reported in cutaneous LP while ELP also frequently harbors abundant intraepithelial CD8+ lymphocytes. Intraepithelial lymphocytosis is associated with scattered squamous cell apoptosis designated as Civatte bodies. The epithelium may become partially or completely detached from the tunica propria or show intraepithelial splitting reminiscent of sloughing esophagitis. However, superficial necrosis and neutrophilic aggregates seen in sloughing esophagitis are not a feature of ELP. The squamous epithelium may be hyperplastic and exhibit acanthosis similar to the saw-toothed rete ridges of cutaneous LP especially in long-standing esophageal involvement. In contrast to the normal esophageal epithelium, hypergranulosis is frequently observed in the superficial epithelium of ELP. Surface orthokeratosis, also termed esophageal epidermoid metaplasia (EEM), is the histologic correlate of the rough and whitish mucosal surface with leukoplakia (Figure 3). This lesion is referred to as uncomplicated EEM as long as epithelial maturation is preserved and dysplasia/intraepithelial neoplasia (IEN) is absent. Chronic



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Figure 1 Endoscopic findings in esophageal lichen planus. A: Trachealization; B: Trachealization and fragile mucosa; C: Hyperkeratosis; D: Hyperkeratosis and stenosis; E and F: Tearing and localized denudation of the mucosa; G-I: Tearing and spacious denudation of the mucosa. Endoscopic images were taken from our cohort of patients.

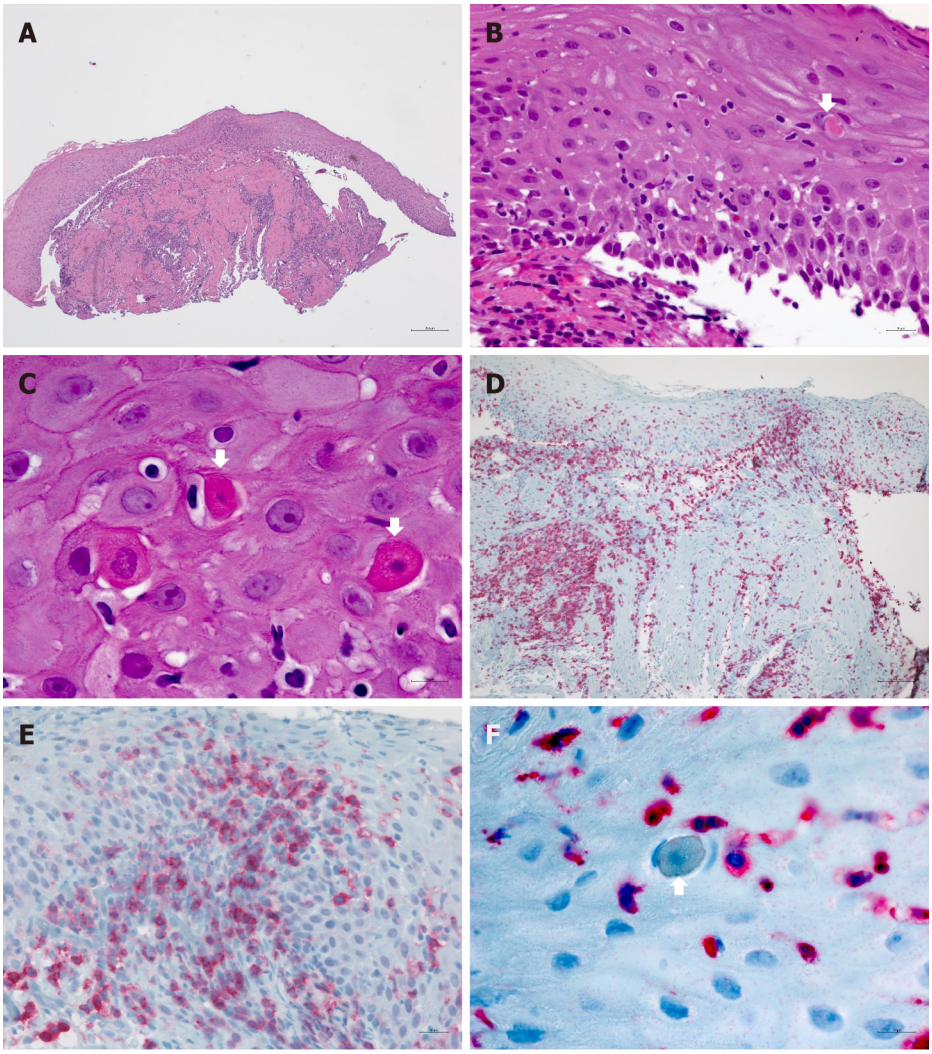
inflammation may lead to fibrosis and scarring of the tunica propria resulting in strictures and dysphagia.

DIF

In ELP, DIF often highlights fibrinogen deposits along the basal membrane as another important criterion (Figure 4). This is based on the data on oral LP, where linear fibrinogen deposition (or granular IgG and IgM deposits) in DIF could discriminate the diagnosis from other lichenoid lesions[83] and mucus membrane pemphigoid[15,27]. Therefore, positive results in DIF support the diagnosis of ELP yielded by conventional histopathology and, in turn, differentiate the findings from diseases like mucous membrane pemphigoid or pemphigus vulgaris in erosive stages.

Therapy

In contrast to cutaneous and oral LP[30,31], there are no generally accepted guidelines for therapy of ELP. Conventional management of cutaneous LP with retinoids does not seem to prevent the emergence of ELP, nor is it suitable for therapy of ELP[20,53,84,85]. However, a few case reports described successful therapy using alitretinoin[62] . Good therapeutic response was reported with topical corticosteroids such as fluticasone or budesonide leading to clinical and/or endoscopic response rate of 62% up to 74% in ELP[17-20]. The type of budesonide preparation might play an important role for its efficacy. Viscous syrups or gels offer better adherence to the esophageal mucosa than swallowed sprays, and led to good response rates[20]. However, for a comparison of response rates based on specific preparation, case numbers in literature are too limited (see Table 2). Orodispersible tablets designed for eosinophilic esophagitis might play an interesting role but have not yet been studied in ELP. Intralesional injection of triamcinolone has also been described in literature[53,69,86]. Systemic corticosteroids have been proposed to induce rapid response in severe cases[66]. However, they are not suitable for maintenance therapy and tapering may lead to reoccurrence of symptoms. Therefore, more severe cases not



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Figure 2 Histologic findings in esophageal lichen planus. A and B: Lichenoid lymphocytic infiltrate of the lamina propria spilling over to the partially detached squamous epithelium; B and C: Intraepithelial lymphocytosis associated with apoptotic squamous cells (Civatte bodies, arrows); D: Dense CD3+ T-cell rich inflammation of the lamina propria involving 2/3 of surface epithelium and muscularis; E: Presence of a CD4+T-cell subset in the infiltrate; F: Civatte body rimmed by CD3+ T-cells.

responding to topical corticosteroids require therapy with systemic immunosuppressants. Different types of immunosupressors such as adalimumab, hydroxychloroquine, mycophenolate, azathioprin, cyclosporine, tacrolimus or rituximab have been used[24,53,54,63,67,68,87,88]. In one of our patients, cyclophosphamide was the only drug which effectively induced at least a partial remission. Refractory cases also exist[64].

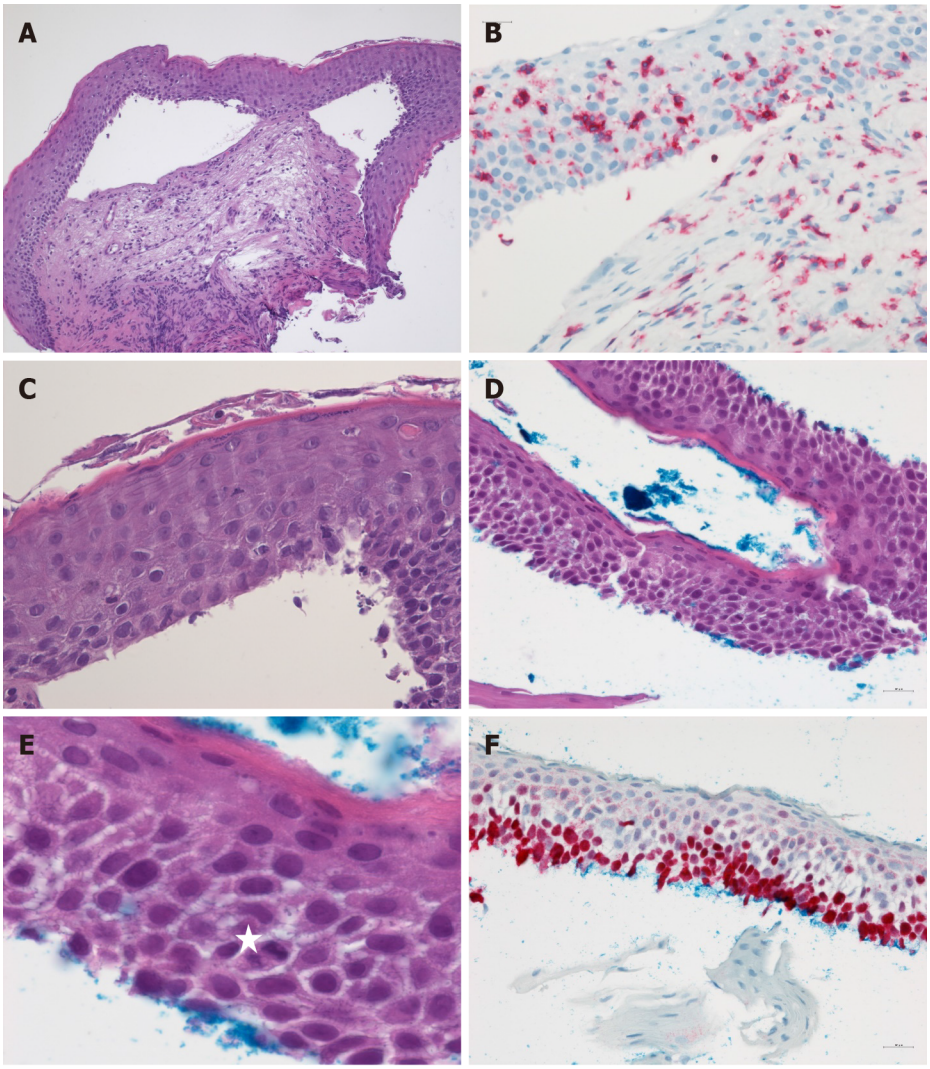
Since ELP mainly occurs as part of a systemic or multilocular LP, treatment should always be initiated in a multidisciplinary approach involving at least gastroenterologists and dermatologists, especially when topical therapy is not effective and systemic immunosuppressive therapy is necessary.

Complications

Esophageal stenosis/Food impaction: As with other inflammatory esophageal diseases, inflammatory or scarring stenosis can be a sequela of chronic untreated or refractory course leading to typical complications such as dysphagia, odynophagia, food impaction, and weight loss[17]. Therefore, ELP should be considered as one of the potential causes of food impaction[89], together with achalasia or eosinophilic esophagitis, or of unexplained esophageal stenosis[90-92]. This applies, not only, but especially to patients with known LP on other site or to patients presenting with signs of undiagnosed mucocutaneous disease.

Treatment of esophageal stenosis

In symptomatic esophageal stenosis, endoscopic dilation may be necessary and has been successfully performed in multiple cases[17,93]. The possibility of considerable mucosal denudation, the main feature of florid ELP, prompted some authors to advice against endoscopic dilation in the past.



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Figure 3 Esophageal epidermoid metaplasia in esophageal lichen planus. A-C: Atrophic squamous epithelium showing extensive detachment from the lamina propria, subtle hyperkeratosis (A, C) and mild intraepithelial CD3+ T-lymphocytosis (B) associated with scattered Civatte bodies (C, arrow); D, and E: Low-grade squamous orthokeratotic dysplasia in detached epithelium of ELP. Presence of basal-type cells in the lower half of the flat epithelium, note presence of scattered mitosis (E, star); F: An increased Ki67+ proliferation index.

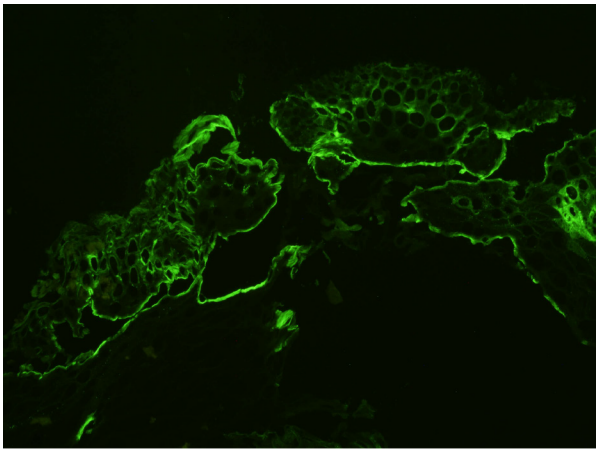
However, this can be overcome by simultaneously treating the underlying inflammation as recommended in other esophageal inflammatory conditions. Anti-inflammatory treatment can reduce mucosal fragility, making it more resistant to physical stress, consequently preventing the reoccurrence of stenosis and inducing remission. The need for endoscopic dilation has been reported to decrease under anti-inflammatory therapy[71] and in a few cases, budesonide alone led to relief of symptomatic stenosis [20]. However, vis-a-vis therapy of stenosis in Crohn's disease, this may only apply for inflammatory and not for scarring stenosis.

Precancerous lesions and esophageal squamous cell carcinoma

Several factors may limit the life expectancy of patients with LP[94,95]. Oral squamous cell carcinoma is one of them, as oral LP is widely regarded as a precancerous condition, even though the exact rate of malignant transformation is a matter of debate[55,96-99].

Accordingly, correlation between ELP and development of esophageal squamous cell carcinoma (ESCC) has been well documented. The number of case reports has been increasing in which esophageal inflammatory and hyperkeratotic lesions have progressed to squamous cell dysplasia/IEN and even to invasive ESCC. In some studies, development of ESCC has been reported in up to 4.5% of ELP patients [100,101].

ELP-associated esophageal precancerous squamous lesions are generally detected in areas of EEM [102-104]. In low-grade dysplasia, cytologic and structural epithelial abnormalities are confined to the lower half of the esophageal epithelium, while high-grade dysplasia involves more than half of the epithelial cell layers with lack of surface maturation. Therefore, endoscopically detected areas of EEM/leukoplakia should be systematically sampled for histologic evaluation since these constitute a



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Figure 4 Direct immunofluorescence. Fibrinogen deposits in the basal membrane as a characteristic feature of Lichen planus. Direct immunofluorescence image was taken from one of our patients.

hallmark of orthokeratotic dysplasia (Figure 3). It should be noted that invasive ESCC may be detected underneath or adjacent to EEM. Our experience showed uncomplicated hyperkeratosis/EEM in a considerable number of patients with severe ELP (37.5%), while predominantly low-grade orthokeratotic dysplasia was rare (6%) and the transition to an early invasive ESCC was diagnosed in only one patient[20]. Anti-inflammatory therapy did not lead to regression of hyperkeratotic areas in this cohort. New therapeutic strategies should aim to either slow down or arrest the development of EEM.

According to Singhi *et al*[103], mutation in TP53 correlates with occurrence of or progression to ESCC in ELP. *p53* overexpression in immunohistochemistry has been frequently observed in our cohort. Additional molecular analyses have yet to be performed to gain more knowledge on risk stratification. Future advances in identifying the molecular landscape which drives the development of precancerous lesions and overt invasive carcinoma may help establish prognostic biomarkers for early detection of ELP cases at high risk of progression to overt ESCC.

Translating this knowledge to clinical practice, we recommend regular endoscopic surveillance of ELP patients for development of dysplasia. Detection of suspicious areas may be assisted by chromoendoscopy. Patients with known hyperkeratotic regions or florid inflammation should be assessed more often. In cases of low grade dysplasia, we recommend further endoscopy every six months; in cases of transition to high grade dysplasia, endoscopic mucosal ablation should be performed similar to patients developing dysplasia in Barrett's esophagus. Furthermore, other known risk factors for development of ESCC such as nicotine or alcohol intake should be discouraged.

Proposal for management of ELP

Figure 5 presents a proposal for clinical management of ELP. We recommend esophago-gastro-duodenoscopy (EGD) in every patient with known LP (skin or mucosal manifestation) and with any associated esophageal symptoms as described above. Diagnosis can be established using the above-mentioned criteria (Table 3). We recommend to treat every newly diagnosed ELP initially with topical steroids and then to reevaluate therapeutic response after a certain time interval (*e.g.* three months). In our clinical experience, 0.5 mg budesonide in 5 mL viscous solution three time a day for the initial treatment period is used. Further therapy would depend on whether a clinical and/or histological remission has been established. Otherwise, systemic immunosuppressive therapy may be necessary as described above. At present, there is not enough data on recommended immunosuppressant. Every patient diagnosed with ELP with no known LP on other sites should also be assessed by a dermatologist.

To date, there is still no consensus on how to identify and treat asymptomatic ELP patients, specifically patients with asymptomatic hyperkeratosis, a potential precursor of ESCC. A wait-and-see strategy seems to be warranted[20,70]. However, in patients with EEM, we recommend EGD every six months to screen the emergence of dysplasia.

Future perspectives

Investigation of pathogenesis and search for targeted therapy: Current data on the pathogenesis of LP suggest an (auto)-immunological background with T-cells as key players. As in other diseases triggered by overactive immune system, environmental or lifestyle factors may play an important role, as well as psychological circumstances. Further investigation of mucosal lymphocyte populations in ELP might yield more insights on pathogenesis and establish new options for targeted therapies. Evaluation of environmental factors might lead to identification of triggers (*e.g.* dental fillings with gold or amalgam).

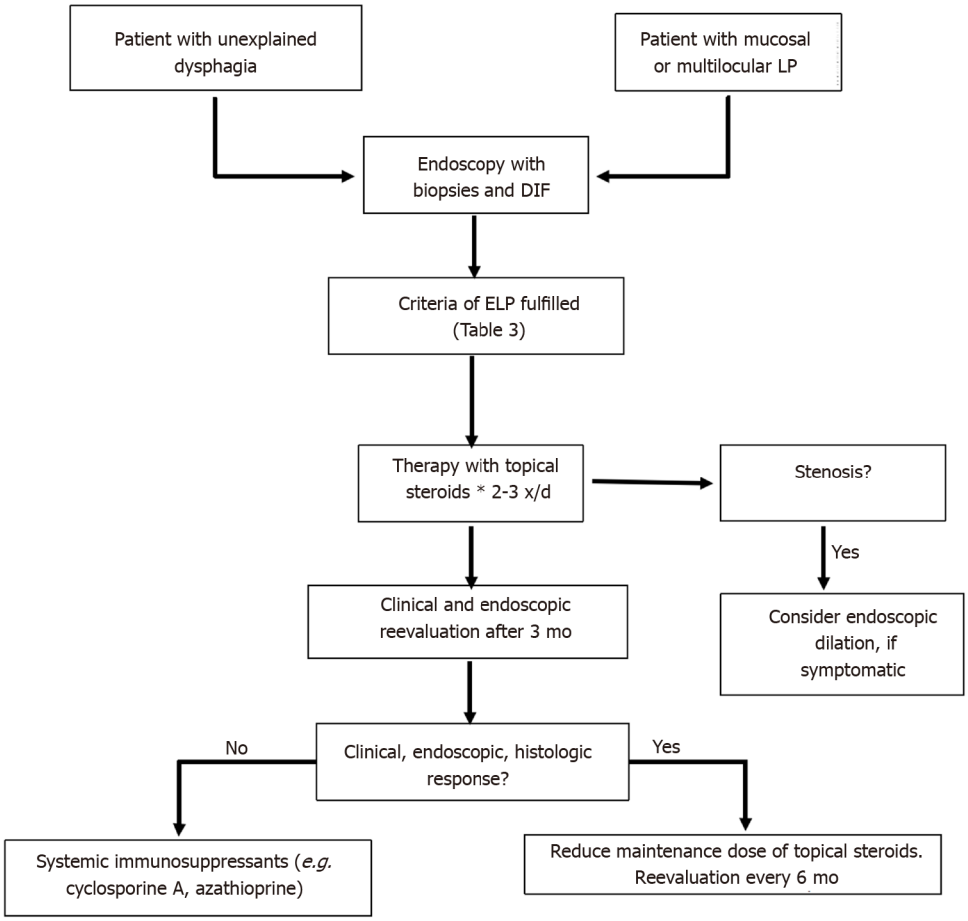
Table 3 Diagnostic criteria to establish diagnosis and assess disease severity (modified from Schauer *et al*[20])

Macroscopic-endoscopic criteria	
Specific signs	
D	Denudation/sloughing of the mucosa
D1	Iatrogenic denudation (caused by biopsies)
D2	Spontaneous localized denudation < 1 cm^2
D3	Spontaneous spacious denudation > 1 cm^2
Possible signs	
S	Stenosis/stricture
S1	Passable with standard endoscope
S2	Not passable with standard endoscope
H	Hyperkeratosis (whitish, rough mucosa)
T	Trachealization
N	None of the criteria fulfilled
Microscopic criteria- histopathology and direct immunofluorescence	
HP	Sloughing of the epithelia (subepithelial, intraepithelial)
	Lymphocytic infiltrate, mainly T-lymphocytes subepithelial, intraepithelial, junctional (region of the basal membrane)
	Intraepithelial apoptosis of keratinocytes (Civatte bodies)
	Dyskeratosis
HP0	Negative
HP1	Weakly positive
HP2	Positive
HP3	Strong positive
F	Fibrinogen deposition along the basal membrane
F0	No visible reaction
F1	Weak positive, discrete depositions visible
F2	Marked fibrinogen depositions along the basal membrane
Severity grading	
Severe ELP	≥ D2 and HP ≥ 1 and/or F ≥ 1
Mild ELP	D1 and HP ≥ 1 and/or F ≥ 1; S, H, T, N and HP ≥ 1 and F ≥ 1
No ELP	Criteria not fulfilled in a patient with LP on other localization

HP: Histopathology; F: Immunofluorescence; ELP: Esophageal lichen planus.

As no therapeutic option has been universally approved for ELP so far, there is a need for further investigation in larger cohort of patients. Although several studies had demonstrated beneficial effects of topical glucocorticoids, duration and maintenance of treatment still need to be defined. In terms of galenics, an orodispersible preparation of budesonide has recently been licensed for eosinophilic esophagitis[105-107] and should be evaluated in ELP.

New therapeutic approaches may be chosen vis-a-vis contemporary therapy of inflammatory bowel disease[108]. A favorable candidate could be ozanimod, an SP-1-modulator recently licensed for therapy of ulcerative colitis[109,110]. Available data suggest a disturbance in the IL12/23 cytokines and/or IL-17 axis in ELP quite similar to psoriasis[34-38], promising possible targeting of these regulatory factors[24]. A candidate influencing the interleukin 12 and 23 pathways would be tyrosine-kinase 2-inhibitor deucravacitinib[111] which has been already used in other diseases with an autoimmune background (*e.g.* Crohn's disease, ulcerative colitis) and localized or systemic lupus erythematosus[112-116]. In patients with precancerous lesions, new endoscopic mucosal resection techniques can prevent progression to invasive carcinoma.



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Figure 5 Proposal for management of esophageal lichen planus. * As topical steroids (*e.g.* budesonide or fluticasone), swallowed spray, viscous solution, or orodispersable tablets might be adminstered. LP: Lichen planus; DIF: Direct immunofluorescence; ELP: Esophageal lichen planus.

CONCLUSION

ELP is an underdiagnosed yet clinically important inflammatory disease of the esophagus which should be considered in patients with unclear dysphagia or esophagitis, especially but not limited to those with history of mucocutaneous LP. Its diagnosis may be based on endoscopic features and typical findings in histopathology and immunofluorescence. Management and treatment of ELP patients is a multidisciplinary challenge. Further understanding of the pathogenesis and new options for targeted therapies need to be established.

FOOTNOTES

Author contributions: Decker A, and Kreisel W designed the manuscript; Schmitt-Graeff A performed the histological evaluation; Schauer F evaluated dermatological findings and performed direct immunofluorescence; All authors collected and interpreted data from patients of their own cohort and from literature; All authors contributed to writing the text. The final stylistic corrections were made by Lazaro A.

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REVIEW

Epidemiology of liver cirrhosis and associated complications: Current knowledge and future directions

Yuan-Bin Liu, Ming-Kai Chen

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Abstract

Cirrhosis causes a heavy global burden. In this review, we summarized up-to-date epidemiological features of cirrhosis and its complications. Recent epidemiological studies reported an increase in the prevalence of cirrhosis in 2017 compared to in 1990 in both men and women, with 5.2 million cases of cirrhosis and chronic liver disease occurring in 2017. Cirrhosis caused 1.48 million deaths in 2019, an increase of 8.1% compared to 2017. Disability-adjusted life-years due to cirrhosis ranked 16th among all diseases and 7th in people aged 50-74 years in 2019. The global burden of hepatitis B virus and hepatitis C virus-associated cirrhosis is decreasing, while the burden of cirrhosis due to alcohol and non-alcoholic fatty liver disease (NAFLD) is increasing rapidly. We described the current epidemiology of the major complications of cirrhosis, including ascites, variceal bleeding, hepatic encephalopathy, renal disorders, and infections. We also summarized the epidemiology of hepatocellular carcinoma in patients with cirrhosis. In the future, NAFLD-related cirrhosis will likely become more common due to the prevalence of metabolic diseases such as obesity and diabetes, and the prevalence of alcohol-induced cirrhosis is increasing. This altered epidemiology should be clinically noted, and relevant interventions should be undertaken.

Key Words: Causes; Cirrhosis; Complications; Cost; Epidemiology; Burden; Feature

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Core Tip: The global burden of liver cirrhosis continues to rise. In 2017, there were 520000 new cases of cirrhosis and chronic liver disease. In 2019, cirrhosis caused 1.48 million deaths, an increase of 8.1% compared to 2017, and its disability-adjusted life-years ranked 16th among all diseases. The global burden of cirrhosis due to hepatitis B virus and hepatitis C virus infection is decreasing, while the burden of cirrhosis due to alcohol and nonalcoholic fatty liver disease is increasing rapidly. We also outlined the recent epidemiology of the major complications and hepatocellular carcinoma in cirrhosis.

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INTRODUCTION

Cirrhosis is a consequence of chronic liver damage and inflammation, and it is characterized by diffuse hepatic fibrosis and normal liver structures being replaced by regenerative liver nodules[1,2]. As the end stage of chronic liver disease (CLD), it can be caused by a variety of conditions, such as alcohol consumption, nonalcoholic fatty liver disease (NAFLD), hepatitis viruses, and autoimmune diseases. The progressive course of cirrhosis can generally include asymptomatic stages, such as compensated cirrhosis, and decompensated stages, which are frequently associated with the development of a range of complications, such as ascites, gastro-esophageal variceal (GEV) bleeding, and hepatic encephalopathy (HE); furthermore, cirrhosis may advance to liver failure and lead to death[3]. These complications impose a heavy burden on global public health in terms of significant quality of life impairment and associated high mortality in patients[4].

Despite the global prevalence and disease burden of cirrhosis, there is less public awareness and concern regarding cirrhosis than for other common chronic diseases, such as congestive heart failure, chronic obstructive pulmonary disease, and chronic kidney disease[5]. Currently, there remains an insufficient understanding of the clinical relevance of cirrhosis, which can therefore lead to unnecessary disease progression and outcomes[5]. In this review, we comprehensively overview and synthesize the recent epidemiological features of cirrhosis and its complications and discuss the changing trends in epidemiology. This could provide definite value for the clinical diagnosis and management of cirrhosis.

METHODS

The PubMed electronic database was manually searched to obtain relevant literature. The reference lists of the primary included literature were also searched to identify potentially eligible articles. Only articles published in English were included. There was no restriction regarding the publication year. The index terms included "cirrhosis", "ascites", "spontaneous bacterial peritonitis", "variceal bleeding", "hepatic encephalopathy", "acute kidney injury", "hepatorenal syndrome", "infection", "hepatocellular carcinoma", "epidemiology", "prevalence", "incidence", "mortality", "disease burden", "hospitalization", and "cost". A critical evaluation was carried out for all studies included in this paper.

CURRENT EPIDEMIOLOGY OF LIVER CIRRHOSIS

Prevalence

The most recent data available on the global prevalence of cirrhosis are from the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2017[6]. The GBD 2017 reported the burden of cirrhosis based on pooled epidemiological data from 195 countries and territories stratified by cause, age, and sex from 1990 to 2017. The results for prevalence are presented as numbers and age-standardized or age-specific rates per 100000 populations with 95% uncertainty intervals (UIs). In 2017, there were an estimated 112 (107-119) million cases of compensated cirrhosis and 10.6 (10.3-10.9) million cases of decompensated cirrhosis prevalent worldwide. This represented a huge increase compared to the prevalence figures for 1990, when 65.9 (63.4-68.7) million cases of compensated cirrhosis and 5.20 (5.08-5.32) million cases of decompensated cirrhosis were observed. The age-standardized prevalence of compensated cirrhosis increased from 1354.5 (1300.6-1411.7) per 100000 in 1990 to 1395.0 (1323.5-1470.5) in 2017, while decompensated cirrhosis increased from 110.6 (108.0-113.0) per 100000 in 1990 to 132.5 (128.6-136.2) in 2017. In 2017, 58.8% of compensated cirrhosis cases and 60.3% of decompensated cirrhosis cases were observed in males, suggesting that men suffer from cirrhosis at higher rates. In

males, the age-standardized prevalence of compensated cirrhosis increased by 2.9% from 1990 to 2017; the prevalence of decompensated cirrhosis increased by 21.1%. In females, these figures were 3.5% and 18.1%, respectively. Overall, the prevalence of liver cirrhosis increased by 74.53% from 1990 to 2017[7].

At the regional level, the GBD 2017 also provided relevant epidemiological characteristics[6]. In 2017, the high-income Asia-Pacific region had the highest age-standardized prevalence of both compensated [2455.0 (2344.9-2575.8) per 100000] and decompensated [267.4 (259.8-275.1) per 100000] cirrhosis. Most cases in this region were from Japan and were largely due to hepatitis C. In contrast, Australia reported the lowest age-standardized prevalence of both compensated and decompensated cirrhosis, with hepatitis C also being the main etiology. High-income regions in North America showed the lowest age-standardized prevalence of compensated cirrhosis (mainly caused by hepatitis C), while the lowest prevalence of decompensated cirrhosis was found in South Asia. At the country level, Moldova, Taiwan (Province of China), and Slovakia had the highest prevalence of compensated cirrhosis, while for decompensated cirrhosis, the Philippines had the lowest prevalence, and Slovakia had the highest.

Etiology-specific statistics on the prevalence of cirrhosis are also currently available. In a recent systematic review (retrieved until August 1, 2021) that included 520 studies from 86 countries or territories (reporting a total of 1376503 patients with cirrhosis), 42% of patients with cirrhosis worldwide had hepatitis B virus infection (HBV), and 21% had hepatitis C virus infection (HCV)[8]. The prevalence of HBV infection in cirrhosis was higher in Africa and Asia (8%-61%) than in Europe, the Americas, and Oceania (3%-14%). In contrast, the prevalence of HCV infection in cirrhosis was considerably heterogeneous by country and region (12%-83%). However, in general, the overall prevalence of HBV and HCV exceeded 50% in most parts of Asia and Africa. In China, 68% [95% confidence interval (CI) 60%-74%] of patients with cirrhosis had HBV infection, while only 7% (95%CI 5%-9%) reported HCV infection. In 2017, the age-standardized prevalence of HBV-related compensated cirrhosis did not change significantly compared to 1990, but the prevalence of decompensated cirrhosis increased from 30.9 (95%UI 29.3-32.2) to 36.6 (95%UI 34.7-38.4) per 100000 population[6]. The age-standardized prevalence of HCV-associated compensated cirrhosis increased to 341.1 (314.1-368.7), and the prevalence of decompensated cirrhosis increased to 32.5 (30.6-34.5) per 100000 population in 2017[6]. Regarding cirrhosis due to alcohol consumption, the highest prevalence was recorded in Europe (16%-78%), the Americas (17%-52%), and Oceania (15%-37%), while the lowest was reported in Asia (0%-41%)[8]. In 2017, the global age-standardized prevalence for alcohol-related compensated cirrhosis remained stable compared to 1990 (288.1 in 2017 compared to 290 per 100000 in 1990). However, the global prevalence of alcohol-related decompensated cirrhosis increased from 1990 (30 in 2017 compared to 25.3 per 100000 in 1990)[6]. Another important cause that should not be overlooked is NAFLD-related cirrhosis. According to the aggregate data of the GBD 2017, there were 9.42 million (8.57-10.34) cases of compensated cirrhosis and 917000 (850000-986000) cases of decompensated cirrhosis attributed to non-alcoholic steatohepatitis (NASH) in 2017, showing an impressive increase compared to 1990[6]. The age-standardized prevalence of NASH-related compensated cirrhosis was 115.5 (105.0-126.5) per 100000 in 2017, indicating a 33.2% increase compared to 1990; the prevalence of decompensated cirrhosis showed a 54.8% increase to 11.3 (10.4-12.1) per 100000. However, there is a lack of recent global or regional reported epidemiological data on other causes of cirrhosis, such as drugs, autoimmune liver disease, and metabolic disorders.

Additional profiles were also provided by some newer regional or organizational epidemiological studies (Table 1). A recent study from the United States reported summary statistics on the prevalence and disease burden of digestive diseases in a commercially insured adult population for the period 2016 to 2018. Of the total population, 7297435 (24%) individuals had a diagnosis of digestive disease, and the prevalence of nonalcoholic cirrhosis in the digestive disease population was 0.389% compared to 0.090% in the overall population[9]. Gu *et al*[10] evaluated all hospital admissions within the diagnosis-related group (diagnosis based on ICD-10-GM codes) in Germany from 2005 to 2018. A total of 248085936 admissions were recorded during this period, of which 2302171 admissions were diagnosed with cirrhosis, reflecting a prevalence of 0.94%[10]. A cross-sectional study conducted in Japan in 2020 randomly selected 6000 general citizens from 2 cities, and 488 individuals underwent fatty liver and advanced fibrosis screening. The prevalence of cirrhosis based on liver stiffness measurement (LSM) was 1%, with a markedly higher prevalence in men than in women (1.6% compared to 0.4%)[11]. Finally, a study using a commercial medical claims database yielded an adult prevalence of 0.21% for cirrhosis in 2018 (estimated at 536856 cases)[12].

In specific at-risk populations, there were significant increases in the prevalence of cirrhosis (Table 2). In a 2017-2018 cross-sectional study using data from the National Health and Nutrition Examination Survey (NHANES), which included 825 United States adults with type 2 diabetes who had reliable transient elastography results, the prevalence of cirrhosis was 7.7% (95%CI 4.8%-11.9%)[13]. Another study also analyzed NHANES data from 2017-2018 and found a 4.4% prevalence of suspected cirrhosis among patients with fatty liver disease[14]. A recent study utilizing NHANES data from 2001 to 2018 included 3115 HBsAg-negative/HBcAb-positive subjects in which the prevalence of cirrhosis/advanced liver fibrosis based on FIB-4 diagnosis was 3.76% (95%CI 2.80%-4.72%), notably higher than in the general US population[15]. A systematic review and meta-analysis summarized the prevalence of cirrhosis in HBV-infected populations in sub-Saharan Africa. A total of 17 studies were included, including 22 cohorts from 13 countries (13 with HBV infection alone and 9 with HIV/HBV coinfection).

Table 1 Recent local epidemiological data on liver cirrhosis in the general population

Ref.	Country	Study population	Study period	Diagnostic methods	Presented data
[9]	US	7297435 patients with a GI diagnosis in a commercial insurance database	2016-2018	ICD-10 code	Nonalcoholic cirrhosis prevalence: 0.389%; average inpatient cost: 43733 dollars; annualized total costs: 53214 dollars
[10]	Germany	All hospital admissions (248085936 patients)	2005-2018	ICD-10 code	Prevalence: 0.94%
[11]	Japan	488 randomly selected individuals underwent fatty liver and advanced fibrosis screening	From October to November 2020	Liver stiffness measurement	Prevalence: 1%
[12]	US	Adult patients in a commercial medical claims database	2018	ICD-9 or ICD-10 code	Prevalence: 0.21%
[28]	China	503993 participants prospectively included in China Kadoorie Biobank	2004-2008 (10 years of follow-up)	ICD-10 code	Incidence: 756.4 and 397.4 per 100000 among diabetic patients and nondiabetic patients, respectively
[30]	Korea	Adult patients in the HIRA-NPS database	2012-2016	ICD-9 code	Alcoholic cirrhosis incident cases: 7295 cases
[31]	Sweden	All patients at Halland Hospital	2011-2018	ICD-10 code and SNOMED code	Age-standardized incidence: 23.2 per 100000 person-years
[32]	Korea	Adult patients in the NHIS database	2011-2015	KCD-7 code	Primary biliary cirrhosis average annual cumulative incidence: 68.32 cases per 10000000
[33]	Canada	Adult patients in the ICES databases	1997-2016	ICD-10 code	Age-standardized incidence: 70.6 in 1997 and 89.6 per 100000 person-years in 2016
[34]	Canada	Children in the ICES databases	1997-2017	ICES-validated algorithm	Age- and sex-adjusted incidence: 2.7 in 1997 and 10.6 per 100000 person-years in 2017
[44]	US	NIS	2003-2017	ICD-10 code	Alcoholic cirrhosis deaths in women: 14330 cases
[45]	Mexico	National Institute of Statistics and Geography	2000-2017	ICD-10 code	Alcoholic cirrhosis mortality rate: From 20.55 to 10.62 per 100000
[54]	US	Adult patients in the NIS	2008-2014	ICD-9 code	Hospitalization costs: 7.37 billion dollars in 2014

US: United States; ICD: International classification of diseases; HIRA-NPS: Health insurance review and assessment-national patient samples; SNOMED: Systematized nomenclature of medicine; NHIS: National Health Insurance Service; KCD: Korean standard classification of diseases; ICES: Institute for clinical evaluative sciences; NIS: National inpatient sample.

The prevalence was 4.1% (95%CI 2.6%-6.4%) in primary care or the general population and significantly higher at 12.7% (95%CI 8.6%-18.3%) in referral or tertiary care settings, with no effect of HIV coinfection on cirrhosis[16]. In a recent Spanish population survey on the prevalence of NASH-related liver fibrosis, the prevalence of cirrhosis in the current biopsy-proven NASH cohort (a total of 501 patients from 2015-2020) was 0.70% (95%CI 0.10%-4.95%)[17]. A survey analyzing the Korea National Health and Nutrition Examination Survey (KNHANES) between 2015 and 2019 reported prevalence rates of cirrhosis in metabolically healthy obesity and metabolically unhealthy obesity populations of 0.5% and 0.4%, respectively[18]. In the TARGET-NASH study conducted from August 2016 to March 2019, researchers revealed that lean participants had a lower prevalence of cirrhosis (22.6% *vs* 40.2% of nonlean participants), with almost half of the lean subjects being Asian. Lean Asians were half as likely to develop NAFLD-related cirrhosis as nonlean individuals [odds ratio (OR) 0.47; 95%CI 0.29-0.77][19]. Furthermore, different diagnostic tools can have varied diagnostic accuracy and cost-effectiveness for cirrhosis. A cost-effectiveness study found high diagnostic accuracy and cost-effectiveness of fibrosis-4 (FIB-4), followed by either vibration-controlled transient elastography (VCTE), magnetic resonance elastography (MRE), or liver biopsy[20]. In terms of the combination of diagnostic tools, FIB-4 + VCTE was the least costly combination, whereas the incremental cost-effectiveness ratios (ICERs) for the combination of FIB-4 and MRE were lower than those for FIB-4 and liver biopsy[20]. The prevalence of cirrhosis in older patients with multiple comorbidities has recently been addressed. In a study that included a cohort of 6,193 elderly patients admitted to acute medical wards between 2010 and 2018, 315 patients (5%) were diagnosed with cirrhosis[21]. Finally, a pooled meta-analysis including 15 studies with a total of 320777 patients suggested that among those receiving dialysis, the prevalence of cirrhosis was 5% and associated with higher mortality, with further analysis showing that hepatitis B and C,

Table 2 Epidemiology of cirrhosis in specific at-risk populations

Ref.	Country	Study population	Study period	Diagnostic methods	Presented data
[13]	US	825 adults with type 2 diabetes who had reliable TE results from the NHANES	2017-2018	TE	Prevalence: 7.7%
[14]	US	Patients with NAFLD from the NHANES	2017-2018	TE	Prevalence: 4.4%
[15]	US	3115 HBsAg-negative/HBcAb-positive subjects from the NHANES	2001-2018	FIB-4	Cirrhosis/advanced liver fibrosis prevalence: 3.76%
[16]	13 countries in sub-Saharan Africa	HBV-infected population	2012-2019	TE, APRI and Fibrotest	Prevalence: 4.1% in primary care or the general population and 12.7% in referral or tertiary care settings
[17]	Spain	501 biopsy-proven NASH patients with paired TE data from tertiary centers	2015-2020	TE	Prevalence: 0.70%
[18]	Korea	27629 adults with MHO or MUHO from the KNHANES	2015-2019	Self-report survey or by an AST level ≥ 23.5 IU/L	Prevalence: 0.5% and 0.4% in MHO and MUHO, respectively
[19]	US	3386 patients with NAFLD in the TARGET-NASH study	2016-2019	Pragmatic case definitions	Prevalence: 22.6% in lean patients
[21]	Italy	6193 older subjects admitted to acute medical wards and included in the REPOSI registry	2010-2018	ICD-9 code	Prevalence: 5%
[22]	10 countries in the world	320777 dialysis patients	1980-2019	TE, histopathology, radiology, and ICD codes	Prevalence: 5%
[36]	NR	902 patients with a Fontan circulation	NR	NR	Cumulative incidence: 27.5%
[39]	Japan	1260 patients who underwent the Fontan procedure and survived to discharge from 9 institutions	From before 2011 to 2021 (median10.2 of years follow-up)	Biopsy or imaging or extrahepatic features	Cumulative incidence at 10, 20, and 30 years after the Fontan procedure: 0.9%, 11.6%, and 25.7%, respectively
[41]	Italy	All adults aged 30 years or older without cirrhosis in Rome	From 2001 follow up to 2015	A validated algorithm	Crude incidence rate: 67 per 100000 person-years

US: United States; TE: Transient elastography; NHANES: National Health and Nutrition Examination Survey; NAFLD: Nonalcoholic fatty liver disease; HBsAg: Hepatitis B surface antigen; HBcAb: Hepatitis B core antibody; FIB-4: Fibrosis-4; HBV: Hepatitis B virus; APRI: Aspartate transferase-to-platelet ratio index; NASH: Nonalcoholic steatohepatitis; MHO: Metabolically healthy obesity; MUHO: Metabolically unhealthy obesity; KNHANES: Korea National Health and Nutrition Examination Survey; AST: Aspartate transferase; IU/L: International unit per liter; REPOSI: Registro Politerapia Società Italiana di Medicina Interna; NR: Not recorded.

rather than diabetes, contributed to the increased risk of cirrhosis[22].

Incidence

The global number of incident cases of cirrhosis and CLD in 2017 was 5.2 million according to the GBD 2017, although the incidence was not available[23]. However, the latest global incidence of NASH-associated cirrhosis has been mentioned in recent publications using data from the GBD 2017[24,25]. In 2017, the global incidence of cirrhosis due to NASH was 367780 cases, an increase of approximately 105.56% compared to 1990 (178430 cases in 1990). The age-standardized incidence rate (ASR) was 4.81 (95%UI 4.38-5.28) per 100000 population in 2017 compared to 3.31 (95%UI 3.02-3.63) per 100000 population in 1990, with an estimated annual percentage change (EAPC) of 1.35% (95%CI 1.28-1.42%). Gender and regional differences in incidence were also observed. The incidence was higher in males than in females [5.54 (5.06-6.07) compared to 4.08 (3.69-4.49) per 100000 population], although the EAPC was higher in females. The middle-high sociodemographic index (SDI) region featured the highest incidence, while that of the low SDI was the lowest [6.14 (5.60-6.70) compared to 2.72 (2.43-3.05) per 100000 population]. A more pronounced increase in the ASR was recorded in Eastern Europe, Andean Latin America, and Central Asia, while the Asia Pacific region showed a decline. In terms of HBV and HCV-associated cirrhosis, the overall incidence has shown a relatively encouraging trend both in men and women. Veracruz *et al*[26] exploited GBD statistics from 2010-2019 to analyze the global incidence and mortality trends in HBV and HCV infection, cirrhosis, and hepatocellular carcinoma (HCC) over this period. The worldwide incidence of HBV-related cirrhosis decreased by 15% from 5.78 (95%CI 4.3-7.3) in 2010 to 4.91 (95%CI 3.5-6.5) in 2019 per 100000 individuals, with the greatest reduction occurring

in Eastern Europe at 36%. This trend may be related to widespread HBC vaccination[27]. However, the 2019 incidence of HCV-associated cirrhosis amounted to 6.7 (95%CI 5.0-8.6) per 100000 population, an increase of 5.6%, with the greatest increase of 27.8% in central sub-Saharan Africa and the greatest decrease of 13.5% in the high-income Asia-Pacific region.

The China Kadoorie Biobank prospectively included 512891 adults (210222 men and 302669 women) aged 30-79 years in 10 geographically disaggregated sites. During the 10-year follow-up period, 2082 cases of cirrhosis occurred in 503993 participants with an excluded history of CLD. The incidence of cirrhosis among diabetic patients was 756.4 per 100000 compared to 397.4 per 100000 among nondiabetic patients, and the Cox regression yielded a hazard ratio (HR) of 1.81 (95%CI 1.57-2.09) for cirrhosis among diabetic patients[28]. Another modeling study employed a multicohort state-transition (Markov) model to predict the epidemiological trends in alcohol-related liver disease in the US from 2019 to 2040[29]. In this model, researchers modeled prevalence and mortality trends of decompensated cirrhosis in three projection scenarios, including a status quo scenario (current trends continued), a moderate intervention scenario (high-risk alcohol intake receded to 2001 levels), and a strong intervention scenario (high-risk alcohol intake trends decreased by 3.5% per year). In the status quo scenario, the age-standardized incidence of alcohol consumption-related decompensated cirrhosis was projected to increase from 9.9 (95%CI 9.3-10.9) cases per 100000 person-years in 2019 to 17.5 (15.8-18.4) cases per 100000 person-years in 2040, which would be a 77% increase. In the moderate intervention scenario, the age-standardized incidence of alcohol drinking-related decompensated cirrhosis would be expected to increase by 69% to 16.7 (95%CI 14.2-16.4) cases per 100000 person-years in 2040. Conversely, the incidence of alcohol-related decompensated cirrhosis associated under the strong intervention scenario would decrease by 11% compared to 2019, which is encouraging. From 2019 to 2040, the cumulative incidence was projected to reach 1118200 cases (95%CI 1005400-1123500), 1067000 (943400-1084600) and 786400 cases (711200-819300) for the status quo, moderate and strong intervention scenarios, respectively, with the strong intervention scenario achieving a 30% reduction compared to the status quo scenario. In a large national cohort study conducted in Korea, the incidence of alcoholic cirrhosis showed an overall increase between 2012 and 2016, from 1463 cases in 2012 to 1530 cases in 2016[30]. A Swedish population-based cohort study including 310000 inhabitants analyzed epidemiological trends in the incidence, causes, and complications of cirrhosis in the last decade (2011-2018). The incidence of cirrhosis was assessed at 23.2 per 100000 person-years, with a higher rate of 30.5 in men and 16.4 in women[31]. Stratifying by age showed the highest incidence in the 60-69 age group, and alcohol was the leading cause of all cases (50.5%). In a study conducted in South Korea using the National Health Insurance Service database, trends in the incidence of rare diseases were explored for the period 2011-2015. The average annual cumulative incidence of primary biliary cirrhosis was 68.32 cases per 10000000 and was increasing at an annual trend of 6.32[32]. A retrospective study conducted in Ontario, Canada, used health data from the period 1997-2016 to determine the incidence of cirrhosis in young birth cohorts. During this period, 165979 cases of cirrhosis were diagnosed, with an increasing trend in age-standardized incidence from 70.6 in 1997 to 89.6 per 100000 person-years in 2016. The incidence was higher in the younger birth year cohort than in the middle-aged birth cohort and was more evident in women[33].

In pediatric populations, the incidence and current trends of cirrhosis have also been reported in the recent literature. A population-based study from Ontario, Canada, analyzed changes in the incidence of cirrhosis in children from 1997-2017[34]. Over the past two decades, 2966 new cases of cirrhosis were diagnosed in children (median age 9 years), and the age- and sex-adjusted incidence of cirrhosis increased significantly by nearly fourfold (from 2.7. in 1997 to 10.6 per 100000 person-years in 2017). Notably, the most marked increases were identified in infants < 1 year and adolescents > 11 years old. After the age-period-cohort study, the authors found that children born in 2010 had twice the risk of developing cirrhosis than those born in 2001. Dong *et al*[35] prospectively included 139 children with biopsy-proven cirrhosis (median age at initial diagnosis 2 years) from January 2010 to January 2020, 93 of whom had a definite cause. HBV infection was the most common cause (33.3%), followed by glycogen storage disease (17.2%) and Wilson disease (15.1%).

The incidence of cirrhosis has also been investigated in specific populations in recent studies. A recent meta-analysis pooled 14 studies including 902 patients with Fontan circulation and estimated the incidence of cirrhosis in this population[36]. Fontan circulation is characterized by an increase in central venous pressure, which in turn affects back-stream veins and can lead to congestive hepatopathy known as Fontan-related liver disease[37,38]. There were 241 patients with reported cirrhosis following a mean follow-up period of 17.9 ± 4.5 years, with a cumulative incidence of 27.5% (95%CI 16.9%-34.4%). Another updated study included 1250 patients (median age 3.6 years, 47.5% female) who underwent their first Fontan procedure, with cirrhosis diagnosed in 5.8% of patients over a median follow-up period of 10.2 years. The cumulative incidence of cirrhosis at 10, 20, and 30 years after Fontan surgery was 0.9%, 11.6%, and 25.7%, respectively[39]. The high incidence of cirrhosis in this population can be a substantial disease burden and therefore should be taken seriously. A nested case control study conducted in Taiwan found that the presence of diabetes and associated extrahepatic complications, such as hypertensive cardiovascular disease and chronic kidney disease, were associated with an increased incidence of treatment-naïve HCV-related cirrhosis[40], suggesting an important role for metabolic risk factors in the increased risk of developing cirrhosis. A nationwide cohort study including

approximately 1.2 million people aged 30 years or older in Rome analyzed the association between long-term exposure to air pollution and the incidence of cirrhosis, of which 10111 cases occurred during a 14-year follow-up period, yielding a crude incidence of 67 per 100000 person-years[41]. Long-term exposure to PM10, PM coarse, PM2.5, and NO2 was associated with the incidence of cirrhosis.

Mortality

The most recent GBD 2019 describing global mortality from cirrhosis is available. A recent systematic analysis of the GBD 2019 highlighted that the total number of deaths from cirrhosis worldwide was 1.43 million in 2019, an increase of 8.1% compared to the number of deaths in 2017 according to the GBD 2017 (1323000 cases)[42]. In a recent report, the GBD 2019 assessed the health progress of subnational regions in Ethiopia in 2019. In 2019, the all-cause age-standardized mortality rate was 993.52 (95%UI 914.97-1070.55), and for cirrhosis and other CLDs, it was 52.18 (95%UI 44.17-62.07) per 100000 population[43].

In 2017, 31.5% of cirrhosis deaths among men were caused by HBV, 25.5% by HCV, 27.3% by alcoholic liver disease (ALD), 7.7% by NASH, and 8.0% by other factors[6]. Among women, the proportion of deaths from cirrhosis due to HBV, HCV, ALD, NASH, and other causes was 24.0%, 26.7%, 20.6%, 11.3%, and 17.3%, respectively[6]. Deaths from hepatitis B-related cirrhosis were 321000 in 2019, representing 22% of all cirrhosis deaths. In 2017, the number of associated deaths was 384000 (29%), which indicated a 16.4% decrease in the mortality rate[42]. A previous study investigated trends in the incidence and mortality of acute infections, cirrhosis, and HCC by exploring the GBD for HBV and HCV from 2010-2019[26]. The 2019 global mortality rate for HBV-associated cirrhosis was 4.03 (95%CI 3.39-4.76) per 100000 population, showing a 23.2% reduction over this decade. The highest death rate for cirrhosis due to HBV infection was recorded in western sub-Saharan Africa at 16.49 (95%CI 12.69-21.35), while the lowest was seen in high-income North America at 0.35 (95%CI 0.29-0.42). The largest reduction in mortality compared to 2010 was in East Asia at 46.5%. The global mortality rate for HCV-related cirrhosis was 4.82 (95%CI 4.09-5.57) per 100000 in 2019, a 7.4% decrease compared to 2010. The highest mortality rate was 15.4 (95%CI 12.52-19.04) in Eastern sub-Saharan Africa, and the lowest was 1.79 (95%CI 1.41-2.25) per 100000 population in Western Europe. The greatest decrease of 23.9% was seen in the high-income Asia Pacific region, although several regions, such as the Caribbean and high-income North America, experienced an upward trend. In 2017, the global age-standardized mortality rate for ALD was 6.2 (5.7-6.9) and 2.1 (1.9-2.6) per 100000 for men and women, respectively[6]. The estimated number of deaths due to NASH cirrhosis worldwide in 2017 was 118,030, an increase of 90.7% compared to 1990, with an age-standardized death rate of 1.5 (1.3-1.6) per 100000 population, which was not significantly different compared to that of 1990[25].

The trend in the in-hospital burden of ALD among women was analyzed using data from the 2003 to 2017 National Inpatient Sample (NIS). In 2017, there were 14330 deaths from alcoholic cirrhosis (2.05% of all deaths), a relative decrease of 21.27% compared to 2003, although deaths from alcoholic hepatitis have increased rapidly[44]. Another study analyzed trends in mortality from alcohol-related cirrhosis in Mexico from 2000-2017 and found a decrease in mortality in all age groups, with the associated mortality rate falling from 20.55 to 10.62 per 100000 for all populations during this period[45]. However, there has been a rapid increase in alcohol consumption in the United States and other regions in recent years, and consequently, the mortality rate from ALD has shown a marked increase[46-49]. A study included underlying cause of death public-use data from January 1, 2017, to December 31, 2020, a dataset that contains death data for all United States citizens. Age-adjusted mortality from ALD increased from 13.1 (95%CI 12.9-13.3) to 16.9 (16.7-17.1) in men and 5.6 (5.4-5.7) to 7.7 (7.6-7.9) per 100000 in women[50].

Public health burden

In the latest analysis of the burden of 369 diseases and injuries in 204 countries and territories, the percentage of disability-adjusted life-years (DALYs) for cirrhosis and CLD at all ages in 2019 was 1.8 (95%UI 1.6-2.0), ranking 16th. The percentage increase in the number of DALYs compared to 1990 was 33.0 (22.4-48.2), while the age-standardized DALYs decreased by 26.8%[51]. In the age-stratified analysis, DALYs for cirrhosis in 2019 were ranked 12th at 2.8% of all diseases and injuries among individuals aged 25-49 years, 7th at 2.7% among individuals aged 50-74 years, and 19th at 1.1% among individuals aged 75 or older. Another recent study analyzed the impact of HBV and HCV infections on DALYs using data from the GBD 2010-2019. The 2019 DALYs for HBV cirrhosis decreased by 23% from 168.6 (95%CI 146.9-191.3) in 2010 to 129.8 (108.3-153.0), while the DALYs for HCV cirrhosis decreased by 8.2% to 146.2 (124.4-169.8) compared to that in 2010[52]. In 2019, HCV infection, alcohol, and HBV infection-related etiology were the most predominant sources of DALYs for cirrhosis, with prevalences of 26%, 24%, and 23%, respectively, and NAFLD contributed a relatively small proportion (8%) but showed a rapidly increasing trend[53]. In poorer countries, DALYs were higher, and cirrhosis due to HBV was the main source, whereas in wealthier countries, HCV and alcohol were the primary contributors. DALYs due to NAFLD cirrhosis are expected to become mainstream in the future in parallel with the epidemic of diabetes and obesity. Furthermore, the authors critically highlighted the current underestimation of the disease burden of cirrhosis (as compensated cirrhosis is currently considered no disability and decompensated cirrhosis only mild disability)[53].

The financial burden associated with hospitalized patients with cirrhosis was addressed in a recent study using data from the 2008-2014 NIS. Hospitalization costs for cirrhosis increased by 30.2% to $7.37 billion over the study period. Admissions for compensated and decompensated cirrhosis increased by 24% and 36%, respectively, while noncirrhotic populations dropped by 7.7%. The median length of stay (LOS) in the hospital was longer for cirrhosis than for other diseases. Implementing mechanical ventilation and complications associated with cirrhosis were the main drivers of the increased costs. More specifically, mechanical ventilation increased costs by 15%-152% in hospitalized patients with cirrhosis, and infection and nonhypertensive gastrointestinal bleeding led to increased costs in patients with compensated cirrhosis, while renal and infectious events were contributors to decompensated cirrhosis[54]. Jepsen *et al*[53] suggested that although the prevalence of cirrhosis was increasing, it would be simplistic to assume that costs for patients with cirrhosis were increasing, as the treatment currently provided for cirrhotic patients may be less or cheaper than before. However, the cost of NAFLD-related cirrhosis will likely continue to rise until an effective treatment becomes available[55].

EPIDEMIOLOGY OF MAJOR COMPLICATIONS

Ascites and ascites infection

Ascites is the most common complication in patients with cirrhosis and is defined as pathological fluid accumulation in the peritoneal cavity[56]. Ascites occurs only in the presence of portal hypertension, and approximately 75% of the occurrence of ascites is due to cirrhosis and portal hypertension[56,57]. The pathophysiological mechanisms may involve a complex interaction of the endogenous vasoactive system, portal hypertension, and renal dysfunction[58]. As a hallmark of decompensation, ascites has a prevalence of approximately 10% in patients with cirrhosis[58]. Approximately 60% of patients with compensated cirrhosis can develop this complication over a 10-year period, and it is associated with a high mortality rate of up to 50% within 3 years of onset[59,60]. A recent population-based study analyzed the epidemiology of ascites infection among patients with cirrhosis in Queensland, Australia, from 2008-2017. Of 103165 patients with cirrhosis, 16550 had ascites (16%)[61]. A further Korean study using a nationally representative database yielded a real-world burden of complications in patients with decompensated cirrhosis from 2016 to 2018, with ascites being the most common decompensated event (54.8%), followed by GEV bleeding, HE and hepatorenal syndrome (HRS)[62]. However, recent epidemiological information related to ascites in cirrhosis is relatively scarce.

Ascites infection is a frequent concurrent event in patients with cirrhosis and ascites, such as the prevalent spontaneous bacterial peritonitis (SBP) and, less commonly, fungal infections[63,64]. SBP is defined as spontaneous ascites infection in the absence of other causes of secondary peritoneal infection [65]. The diagnosis is based on the presence of > 250 polymorphonuclear cells/mm^3 in the ascites fluid as the high negative culture rate (up to 60% has been reported)[66,67]. Admissions for ascites infections increased by 76% in Queensland, Australia, from 2008 to 2017[61]. Another recent retrospective study included 1035 patients with cirrhosis from a single center in Israel between 1996 and 2020. A total of 173 (16.7%) of the patients developed SBP, and positive ascites fluid cultures were demonstrated in 47.4% of the SBP cases[68]. A recent meta-analysis including 99 studies comprising 5861142 patients with cirrhosis summarized the prevalence, resistance, and outcomes of SBP in cirrhosis worldwide[69]. The pooled global prevalence of SBP was 17.12% (95%CI 13.63%-21.30%), with Africa having the highest prevalence (68.20%) and North America having the lowest (10.81%). The prevalence of community-acquired SBP was 6.05% (95%CI 4.32%-8.40%) compared with 11.11% (95%CI 5.84%-20.11%) for health care-related SBP. The prevalence of antibiotic-resistant microorganisms in SBP was 11.77% (95%CI 7.63%-17.73%), with methicillin-resistant Staphylococcus aureus (6.23%), broad-spectrum β-lactamase-producing microorganisms (6.19%) and vancomycin-resistant enterococci (1.91%) being predominant. The incidence of SBP in outpatient paracentesis among patients with asymptomatic cirrhosis was estimated at 2% (95%CI 1%-3%) in a recent meta-analysis that included 16 studies with 1532 patients [70]. The global pooled mortality rate for SBP was 30.61% (23.30%-39.06%), with in-hospital, 30-d and 90-d mortality rates of 23.38%, 25.64% and 37.64%, respectively[69].

Variceal bleeding

Varices can be observed in up to two-thirds of patients with cirrhosis, while the annual incidence rate is 8%-10% and the rate of progression to large varices is 10%-12% annually[71]. Variceal bleeding is a common complication in cirrhosis associated with high mortality, with portal hypertension being the major driver. The common forms of variceal bleeding are esophageal and gastric variceal bleeding and, less commonly, rectal variceal bleeding. GEV bleeding can be present in 25%-35% of patients, which can develop in 40% of compensated cirrhosis patients and 85% of decompensated cirrhosis patients[72]. The six-week mortality rate for acute variceal bleeding ranges from 15% to 25%[73]. In a study that included 1902 children younger than 18 years who suffered esophageal variceal bleeding in 50 hospitals in the US from 2004-2019, the mortality rate for variceal bleeding was 7.3% (increasing to 8.8% after 6 wk) and 20.1% for any cause[74]. A retrospective study enrolled all patients in the NIS from 2016-2019 who were discharged with a diagnosis of esophageal variceal bleeding (166760 cases, of which 32.7% were

women), and found that males were associated with a higher mortality rate than females (9.91% compared to 8.31%, *P* value 0.008 after adjusting for confounders)[75]. However, there are relatively few relevant recent epidemiological reports.

HE

HE is a neuropsychiatric disorder in cirrhosis that is strongly associated with prognosis, and its clinical course can be divided into covert hepatic encephalopathy (CHE), which includes minimal hepatic encephalopathy (MHE) (cognitive deficits found on psychological tests) and Grade I HE, and overt hepatic encephalopathy (OHE), where clinically significant symptoms develop[76,77]. The median survival of patients with cirrhosis is significantly shorter at 0.95 years in those over 65 years after the diagnosis of HE was established[78].

The prevalence of CHE has been reported to be very high in patients with cirrhosis, but estimates vary considerably among studies depending on, for example, the diagnostic method and the severity of cirrhosis[79]. In a prospective multicenter study, the prevalence of MHE under the combined diagnostic criteria based on the critical flicker frequency (CFF) and Psychometric Hepatic Encephalopathy Score (PHES) was 18.2%, with 12.1% of patients having compensated cirrhosis and 22.5% of patients showing decompensated cirrhosis[80]. Another multicenter study validated the ability of the EncephalApp in diagnosing MHE. The prevalence of MHE was 51% for the norm-based EncephalApp, 37% for the PHES-based EncephalApp, and 54% for the inhibitory control test (ICT)-based EncephalApp[81]. In a recent study conducted in Turkey, the prevalence of MHE in compensated cirrhosis patients based on the PHES, CFF, and a combination of both was 29.8%, 27.4%, and 16.0%, respectively[82]. An attempt was made to examine the effect of single and combined diagnostic modalities in CHE. The prevalence of CHE varied among the different diagnostic sets, with rates of 18%, 25%, 29%, 35%, 37% and 54% for the PHES + ICT, ICT + Stroop EncephAlapp (StE), PHES + StE, ICT, PHES, and StE, respectively[83]. In addition, the underestimation of the burden of HE and other factors that may be regionally variable, such as smoking, diabetes, and alcohol intake, can impact the diagnosis of CHE[79], all contributing to the significant variability in the prevalence of CHE.

The incidence of OHE has also been described recently. A prospective study included 294 patients with Child A-B cirrhosis without previous HE from July 2016 to August 2018, with the incidence of OHE at one year being 14% in all patients, 10% in Child A patients, and increased to 25% in Child B patients[84]. A large population-based study included a randomized 20% of Medicare participants with cirrhosis and Part D prescription coverage from 2008-2014, with a total OHE incidence of 11.6 per 100 patient-years over a 5.25-year follow-up of 166,192 patients with cirrhosis (median age 65 years). Alcoholic cirrhosis and portal hypertension are key players in the development of OHE, and drug use, such as proton pump inhibitors (PPIs), benzodiazepines, gamma-aminobutyric acid and opioids, is also potentially relevant[85]. These findings indicate that other components may also be associated with the development of HE and influence the incidence. In fact, several factors, such as transjugular intrahepatic portosystemic shunts (TIPSs)[86], PPIs[87], albumin[88], sustained virological response (SVR) in HCV infection[89], and others, can contribute to the development of HE.

HE imposes a heavy burden on patients with cirrhosis, including increased hospitalization, costs, and readmissions, impairment of health-related quality of life (HRQOL), and decreased socioeconomic status[90]. During 2010-2014, data from the NIS show a 24.4% increase in the number of hospital admissions for HE and a 46.0% increase in the total cost of admissions (which reached $11.9 billion in the United States in 2014)[91]. HE-related 90-d readmissions comprised approximately 23.7% of patients with cirrhosis[92] and were significantly associated with readmission in patients with decompensated cirrhosis[93]. In a large multistate population-based study on the causes and rates of readmission in cirrhosis, HE was significantly correlated with both 30-d readmission and 90-d readmission, with adjusted ORs of 3.23 (95%CI 2.97-3.52) and 3.07 (2.86-3.30), respectively[94]. Moreover, HE is associated with an increased risk of falls and can cause serious outcomes leading to high comorbidity and mortality[95]. In socioeconomic terms, cognitive impairment due to HE has been shown to have a multilevel association with adverse outcomes of employment/income, driving ability, and HRQOL[79].

Acute kidney injury and HRS

Renal dysfunction is a common complication in patients with cirrhosis and ascites[96]. In 2015, the revised consensus of the International Club of Ascites defined acute kidney injury (AKI) in cirrhosis as an increase in serum creatinine (sCr) of 0.3 mg/dL in < 48 h or a 50% increase in sCr from baseline within the last 3 mo[97]. AKI comprises a variety of phenotypes, including functional AKI and structural AKI. Functional AKI includes volume-responsive prerenal azotemia (PRA) and HRS-AKI, while structural AKI presents with structural changes such as acute tubular necrosis (ATN). HRS-AKI (previously known as HRS-1) is defined as at least stage 2 or above AKI in patients with cirrhosis and ascites, while excluding other causes such as PRA and ATN[97]. HRS can thus be divided into HRS-AKI and HRS-non-AKI (previous HRS-2)[98].

In a prospective study of 405 patients with cirrhosis enrolled in 2016-2017, the prevalence of AKI was 19.3%, and survival was lower at 30 d and 90 d compared to that of non-AKI patients[99]. The prevalence of AKI ranges from 18.5% to 40.6% in some other regions[100-102]. A meta-analysis revealed that the prevalence of AKI in acute-on-chronic liver failure (ACLF) could be significantly increased to

41%[103]. The overall prevalence rates of PRA and ATN in patients with cirrhosis are 15%-45% and 15%-60%, respectively, which are higher than the 10-40% rate of HRS[104]. The prevalence of HRS in patients with decompensated cirrhosis was 3.6%, while the median LOS for HRS was 4 wk per year in a large representative Korean database from 2016-2018, significantly higher than that for patients with ascites (19 d) or GEV bleeding (13 d)[62]. A recent study that included patients with a primary diagnosis of HRS in the NIS from 2008-2018 found a notable increase in the number of HRS hospitalizations from 22864 in 2008 to 42985 in 2018; however, there was a decreasing trend in inpatient mortality (36.2% in 2008 to 25.7% in 2018)[105].

Infection

In addition to SBP, patients with cirrhosis are at substantially increased risk of developing infections, commonly urinary tract infections (UTIs), pneumonia, and soft tissue infections[106]. The most frequent types of infections in a study that included 877 hospitalized cirrhotic patients from 2011-2016 were UTI (33%), pneumonia (23%), SBP (14%), and bacteremia (11%)[107]. Using the Nationwide Readmissions Database from 2011-2014, the overall prevalence of infections was 29.2% in 1798830 admissions, including UTI (13.7%), pneumonia (8.9%), cellulitis (5.2%), *Clostridioides difficile* infection (CDI) (2.8%), and SBP (2.0%). Pneumonia, SBP, and CDI had notably higher mortality than cellulitis and UTI, and sepsis and organ failure were also more common. Pneumonia had the highest mortality in the multivariate regression analysis (OR 2.73, 95%CI 2.68-2.80) and caused multiple organ failure (OR 3.59, 95%CI 3.50-3.68)[108]. The prevalence of CDI in cirrhosis has shown an increasing trend at approximately 2.7% in 2014, while the mortality of CDI is on the decline, and in local hospitals, the incidence of CDI ranges from 4.9% to 18.8%[109]. In recent years, infections caused by multidrug-resistant organisms (MDRO) have posed a serious challenge in cirrhosis[110]. In a study conducted in Europe that prospectively included two series of cohorts of patients with decompensated cirrhosis in 2011 and 2017-2018, the prevalence of MDRO in culture-positive infections increased from 29.2% in 2011 to 38.0% in 2017-2018[111]. Another worldwide study enrolled 1302 patients with cirrhosis and infections at 46 centers (15 in Asia, 15 in Europe, 11 in South America, and 5 in North America) in 2015-2016 and found a 34% prevalence of MDROs with geographic variability (highest in Asia)[112].

EPIDEMIOLOGY OF HCC IN LIVER CIRRHOSIS

Primary liver cancer was the sixth most common and the third most deadly cancer in 2020, with HCC being the predominant phenotype[113]. According to the GBD 2019, the global age-standardized incidence rate, age-standardized mortality rate and age-standardized DALYs for liver cancer in 2019 were 6.51, 5.95, and 151.08 per 100000, respectively[114]. NASH is the fastest growing cause of liver cancer and is projected to continue to increase in the future[115]. In 2019, the most common contributing factor for liver cancer was hepatitis B (41%), followed by hepatitis C (28.5%), alcohol use (18.4%), NASH (6.8%) and other etiologies (5.3%)[115,116]. Cirrhosis is a precancerous lesion that predisposes patients to progressing to HCC. However, HCC can develop directly without the presence of cirrhosis in a proportion of individuals. In a large US multicenter study, 11.7% of 5,144 included HCC patients showed the absence of cirrhosis, with NAFLD (26.3%), HCV (12.1%) and HBV (10%) being the most common causes[117]. A recent meta-analysis concluded that 37% (95%CI 28%-46%) of patients with NAFLD-related HCC presented without cirrhosis[118]. The prevalence of NAFLD-related HCC was significantly higher in patients with cirrhosis than in those without (374.4/10000 *vs* 4.6/10000 persons)[119].

The epidemiology of HCC in patients with cirrhosis has recently been studied and is etiologically variable (Table 3). In a recent Swedish nationwide population-based cohort study, the incidence of HCC in the cirrhotic population was 23 per 1000 person-years (lowest in ALD at 15 per 1000 person-years and highest in viral hepatitis at 41 per 1000 person-years)[120]. The cumulative incidence of HCC in patients with cirrhosis at 5 and 10 years was 8.3% and 12.2%, respectively. At 10 years, the cumulative incidence was lowest in women with alcoholic cirrhosis (4.3%) and highest in men with viral hepatitis (26.6%)[120]. A study included two US prospective multiethnic contemporary cohorts of patients with cirrhosis, with a total enrolled population of 2733 patients with cirrhosis (19.0% had active HCV, 23.3% had cured HCV, 16.1% had ALD, and 30.1% had NAFLD). After 7,406 person-years of follow-up, the annual HCC incidence rate was 1.82%. The annual HCC incidence in patients with cured HCV, ALD and NAFLD was 1.71%, 1.32%, and 1.24%, respectively. The risk of developing HCC in patients with cured HCV cirrhosis was two-fold higher than that in patients with NAFLD (HR 2.04, 95%CI 1.24-3.35)[121]. Data on the mortality and public health burden of HCC in patients with cirrhosis are relatively scarce.

In a recent meta-analysis of patients with cured HCV, the incidence of HCC was 2.1 per 100 person-years and declined over time after the patients was cured[122]. A prospective study yielded a cumulative incidence of HCC of 7.4% at 5 years in patients with HBV cirrhosis receiving antiviral therapy[123], and partial virological response after two years of entecavir treatment was associated with an increased risk of HCC[124].

Table 3 Epidemiology of hepatocellular carcinoma in patients with cirrhosis

Ref.	Country	Study population	Study period	Study design	Epidemiology
[119]	US	392800 NAFLD patients from 26 major integrated US healthcare systems	2015-2020	Retrospective cohort study	Prevalence: 374.4/10000 persons
[120]	Sweden	15215 individuals with cirrhosis in the National Outpatient Register	2001-2016	Nationwide population-based cohort study	Incidence rate: 23 per 1000 person-years; cumulative incidence: 8.3% at 5 years and 12.2% (4.3% in women with alcoholic cirrhosis and 26.6% in men with viral hepatitis) at 10 years
[121]	US	2733 patients with cirrhosis in two contemporary prospective multiethnic cohorts	2016-2020 (with follow-up until June 30, 2021)	Prospective multiethnic cohort study	Annual incidence: 1.82% (1.71%, 1.32%, and 1.24% in cured HCV, ALD and NAFLD, respectively)
[122]	NA	29444 patients with HCV cure	NA	Meta-analysis	Incidence: 2.1 per 100 person-years
[123]	China	937 treatment-naïve adults with compensated HBV-induced cirrhosis	2012-2015 (with follow-up until June 30, 2019)	Prospective cohort study	Cumulative incidence: 7.4% at 5 years
[124]	Korea	359 patients with HBV-associated cirrhosis who were treated with ETV for at least 2 years	2007-2012 (median follow-up of 82 mo)	Retrospective cohort study	Cumulative incidence: 4.7%, 15.9%, 21.8% and 32.9% at 3, 5, 7 and 9 years, respectively
[125]	US	501 veterans with PBC and compensated cirrhosis	2008-2016 (with follow-up until December 31, 2019)	Retrospective cohort study	Incidence: 0.6 and 0.7 person-years in UDCA responders and UDCA partial responders, respectively
[126]	US	532 patients with PBC and compensated cirrhosis	2008-2016 (with follow-up until June 30, 2020)	Retrospective cohort study	Incidence: 0.9 and 0.3 person-years in males and females, respectively
[129]	NA	148333 patients with alcoholic cirrhosis	NA	Meta-analysis	Cumulative incidence: 1%, 2%, 3%, and 9% at 1, 3, 5, and 10 years, respectively
[131]	China	1095 patients with decompensated cirrhosis	2014-2019	Retrospective cohort study	Incidence: 3.92% in alcoholic cirrhosis
[132]	China	1515 patients with cirrhosis with alcoholism or/and HBV infection	2005-2020 (with follow-up until June 30, 2021)	Retrospective cohort study	Annual incidence: 3.5% (5.9%, 3.6%, and 2.9% in HBV plus alcoholism, HBV only and alcoholism only patients, respectively)

HCC: Hepatocellular carcinoma; US: United States; NAFLD: Nonalcoholic fatty liver disease; HCV: Hepatitis C virus; ALD: Alcoholic liver disease; NA: Not available; HBV: Hepatitis B virus; ETV: Entecavir; PBC: Primary biliary cholangitis; UDCA: Ursodeoxycholic acid.

In a retrospective study that included 501 patients with primary biliary cholangitis and compensated cirrhosis, a total of 22 cases of HCC occurred during the study period (4.39%)[125], which is similar to the findings of another study (4.51%)[126]. In patients with primary sclerosing cholangitis and cirrhosis, the risk of HCC development is very low, although the risk of gallbladder cancer and cholangiocarcinoma is high[127].

The absolute risk of developing HCC in alcoholic cirrhosis seems to be lower than in viral hepatitis (annual incidence of approximately 2%-5%)[128]. A recent meta-analysis that included 18 studies outlined the incidence of HCC in alcoholic cirrhosis. After accounting for the competing risk of death without HCC, the cumulative incidence of HCC at 1, 3, 5, and 10 years was 1%, 2%, 3%, and 9%, respectively. The overall incidence of HCC in alcoholic cirrhosis was 8.29 (95%CI 4.77-14.39) per 1000 person-years[129]. However, the prognosis for HCC due to alcoholic cirrhosis appears to be worse[130]. Furthermore, alcohol consumption increases the incidence of HCC in HBV-related cirrhosis[131-133], while abstinence from alcohol significantly reduces the risk of developing HCC[133].

In a nationwide survey conducted in Japan, HCV-associated cirrhosis was the leading cause of HCC (60.3% of cases). The proportion of HCC from 2008 to 2016 due to hepatitis virus-related cirrhosis decreased, while HCC due to NASH and ALD-related cirrhosis increased from 1.5 to 7.2% and 8.5 to 18.6%, respectively[134].

The value of HCC surveillance in patients with cirrhosis remains to be addressed given the lack of sufficient randomized controlled trials to confirm the overall benefits and harms[135]. However, recent studies have provided robust evidence of the significance of HCC screening. A recent meta-analysis that included 59 cohort studies concluded that HCC surveillance was associated with improved early detection, curative treatment receipts and survival, although few studies weighed the benefits against the harms[136]. In another prospective cohort of patients with cirrhosis, HCC surveillance improved early detection, with physical damage observed in 8.8% of patients and mostly mild[137]. Furthermore, a survey performed in patients with cirrhosis found that patients were more concerned about early

HCC detection than about potential surveillance harm[138]. A survey conducted in the United States showed that gastroenterology and hepatology providers also prefer HCC surveillance when the risk of HCC is below the threshold recommended for surveillance by professional societies[139].

Ultrasound with or without alpha-fetoprotein (AFP) is recommended for HCC surveillance, and the addition of AFP to ultrasound significantly increases the sensitivity of early HCC detection[140]. Clinical HCC surveillance is still underused in patients with cirrhosis. A meta-analysis noted that only 24% of patients were screened, and this underutilization occurred particularly in patients with alcohol- or NASH-related cirrhosis and those not followed in subspecialty gastroenterology clinics[141]. In a United States nationwide cohort of patients with cirrhosis, only 8.78% of patients were under surveillance for HCC[142]. A retrospective multicenter cohort study found that the main reason for barriers to surveillance was lack of surveillance orders or nonadherence[143]. Another United States survey identified patient-reported barriers to surveillance as knowledge deficits about HCC surveillance, cost, difficulty scheduling and transportation[144]. Individualized predictive modeling for risk stratification in patients with cirrhosis can facilitate and improve the cost-effectiveness of surveillance[145,146].

FUTURE DIRECTIONS

In the early 2020s, the outbreak and subsequent epidemic of coronavirus disease 2019 (COVID-19) imposed heavy and multifaceted consequences globally[147]. The effect of COVID-19 on cirrhosis has also been extensively researched. COVID-19 infection is associated with significantly higher morbidity and mortality in patients with liver cirrhosis[148-151]. The COVID-19 epidemic may also have implications for the etiology of cirrhosis. The prevalence of COVID-19 promotes alcohol consumption and is associated with liver disease[152-155] and metabolic disorders[156]. Therefore, the newer epidemiology of cirrhosis may change due to the COVID-19 epidemic.

Alcohol consumption and NAFLD-induced liver disease are growing rapidly. A nationwide study in the United States showed that the charges of alcoholic cirrhosis exceeded the cost of other causes of cirrhosis combined[157]. NAFLD and ALD-related cirrhosis will account for almost all newly diagnosed cases in Canada by 2040[158]. Alcohol intake can influence cirrhosis of any etiology[133,159-161]. Therefore, effective measures to prevent and reduce the associated contributing factors will likely help mitigate the epidemic. One study found that alcohol control policies can have a significant and immediate effect on mortality from cirrhosis[162]. Alcohol abstinence reduced HCC due to alcoholic cirrhosis, although only in patients without previous decompensated disease[163]. NAFLD is emerging as another major epidemic due to the prevalence of metabolic disorders such as obesity and diabetes, and there is currently no effective treatment for NAFLD. Cirrhosis due to NAFLD is expected to be a major component in the future, representing a shift in the associated epidemiology. Therefore, utilization of available interventions such as weight loss and available medications to minimize the progression of NAFLD and the detection of early liver fibrosis using effective and accurate tools will be instrumental in mitigating the risk of cirrhosis.

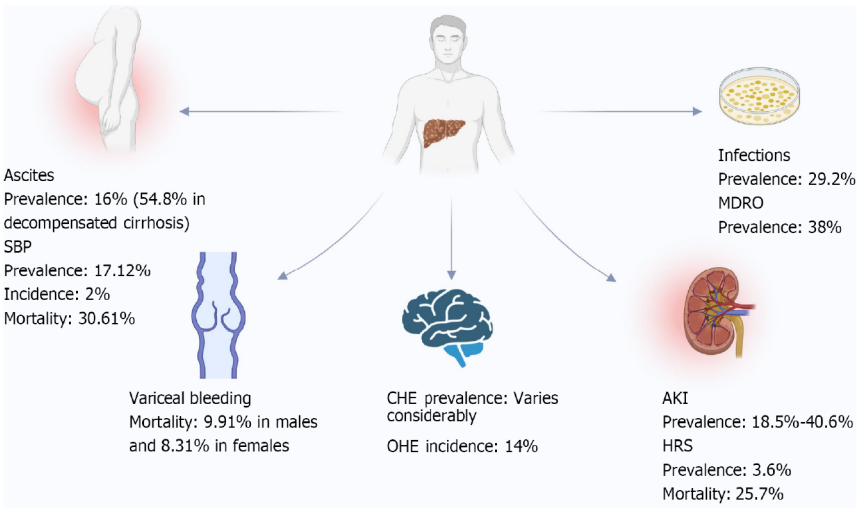
CONCLUSION

The latest epidemiological data revealed the heavy burden of cirrhosis globally (Table 4). In 2017, the age-standardized global prevalence of compensated cirrhosis was 1395.0 per 100000, compared to 132.5 per 100000 for decompensated cirrhosis. In 2019, cirrhosis caused 1.48 million deaths worldwide, an increase of 8.1% compared to 2017. In 2019, liver cirrhosis ranked 16th among all diseases for DALYs. The burden of cirrhosis due to HBV and HCV is declining, while the burden of NAFLD and alcohol consumption is mounting. Furthermore, there is currently a changing epidemiology of the major complications of cirrhosis (Figure 1). The burden of HCC in patients with cirrhosis is etiologically variable, and HCC due to NASH and alcohol intake is increasing.

Table 4 Latest global epidemiological features of cirrhosis

Ref.	Epidemiological figures	Latest research	Type or etiology of cirrhosis	Reported data
Prevalence				
[6]	Age-standardized prevalence	GBD 2017	Cirrhosis	Compensated cirrhosis: 1395.0 (1323.5-1470.5); decompensated cirrhosis: 132.5 (128.6-136.2) per 100000
[6]	Age-standardized prevalence	GBD 2017	HBV-related cirrhosis	Compensated cirrhosis: 451.9 (420.0-485.9); decompensated cirrhosis: 36.6 (34.7-38.4)
[6]	Age-standardized prevalence	GBD 2017	HCV-related cirrhosis	Compensated cirrhosis: 341.1 (314.1-368.7); decompensated cirrhosis: 32.5 (30.6-34.5)
[6]	Age-standardized prevalence	GBD 2017	Alcohol-related cirrhosis	Compensated cirrhosis: 288.1 (267.5-311.3); decompensated cirrhosis: 30.0 (28.2-31.8)
[6]	Age-standardized prevalence	GBD 2017	NASH-related cirrhosis	Compensated cirrhosis: 115.5 (105.0-126.5); decompensated cirrhosis: 11.3 (10.4-12.1)
Incidence				
[24]	Age-standardized incidence	GBD 2017	NASH-related cirrhosis	4.81 (4.38-5.28)
[26]	Age-standardized incidence	GBD 2019	HBV-related cirrhosis	4.91 (3.50-6.50)
[26]	Age-standardized incidence	GBD 2019	HCV-related cirrhosis	6.7 (5.0-8.6)
Mortality				
[26]	Age-standardized Mortality	GBD 2019	HBV-related cirrhosis	4.03 (3.39-4.76)
[26]	Age-standardized Mortality	GBD 2019	HCV-related cirrhosis	4.82 (4.09-5.57)
[25]	Age-standardized mortality	GBD 2017	NASH-related cirrhosis	1.5 (1.3-1.6)
Public health burden				
[52]	DALYs	GBD 2019	HBV-related cirrhosis	129.8 (95%CI 108.3-153.0)
[52]	DALYs	GBD 2019	HCV-related cirrhosis	146.2 (124.4-169.8)

GBD: Global burden of diseases, injuries, and risk factors study; HBV: Hepatitis B virus; HCV: Hepatitis C virus; UI: Uncertainty interval; CI: Confidence interval; NASH: Nonalcoholic steatohepatitis; DALYs: Disability-adjusted life-years.



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Figure 1 The latest epidemiological data on the major complications of liver cirrhosis. The prevalence of covert hepatic encephalopathy depends on the means of diagnosis, the stage of cirrhosis, the underestimation of HE, and the presence of other factors affecting the prevalence. For the prevalence of infections, these data were obtained from the Nationwide Readmissions Database; therefore, the total population included readmissions. SBP: Spontaneous bacterial peritonitis; CHE: Covert hepatic encephalopathy; OHE: Overt hepatic encephalopathy; AKI: Acute kidney injury; HRS: Hepatorenal syndrome; MDRO: Multidrug-resistant organisms.

FOOTNOTES

Author contributions: Liu YB and Chen MK proposed the idea for the article; Liu YB carried out the literature search, wrote the manuscript, and prepared the language refinement; Chen MK revised the manuscript as the corresponding author and provided comments; all authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Enhanced segmentation of gastrointestinal polyps from capsule endoscopy images with artifacts using ensemble learning

Jun-Xiao Zhou, Zhan Yang, Ding-Hao Xi, Shou-Jun Dai, Zhi-Qiang Feng, Jun-Yan Li, Wei Xu, Hong Wang

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Abstract

BACKGROUND

Endoscopy artifacts are widespread in real capsule endoscopy (CE) images but not in high-quality standard datasets.

AIM

To improve the segmentation performance of polyps from CE images with artifacts based on ensemble learning.

METHODS

We collected 277 polyp images with CE artifacts from 5760 h of videos from 480 patients at Guangzhou First People's Hospital from January 2016 to December 2019. Two public high-quality standard external datasets were retrieved and used for the comparison experiments. For each dataset, we randomly segmented the data into training, validation, and testing sets for model training, selection, and testing. We compared the performance of the base models and the ensemble model in segmenting polyps from images with artifacts.

RESULTS

The performance of the semantic segmentation model was affected by artifacts in the sample images, which also affected the results of polyp detection by CE using a single model. The evaluation based on real datasets with artifacts and standard datasets showed that the ensemble model of all state-of-the-art models performed better than the best corresponding base learner on the real dataset with artifacts. Compared with the corresponding optimal base learners, the intersection over union (IoU) and dice of the ensemble learning model increased to different degrees, ranging from 0.08% to 7.01% and 0.61% to 4.93%, respectively. Moreover,

in the standard datasets without artifacts, most of the ensemble models were slightly better than the base learner, as demonstrated by the IoU and dice increases ranging from -0.28% to 1.20% and -0.61% to 0.76%, respectively.

CONCLUSION

Ensemble learning can improve the segmentation accuracy of polyps from CE images with artifacts. Our results demonstrated an improvement in the detection rate of polyps with interference from artifacts.

Key Words: Artifacts; Capsule endoscopy; Polyps; Ensemble learning; Segmentation; Robustness

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Core Tip: Artificial intelligence has been widely used in capsule endoscopy to detect gastrointestinal polyps; however, it is often impaired by artifacts in clinical practice. At present, clear and high-quality images without artifacts are usually selected for research, which has not yet produced practical assistance regarding artifact interference. In this study, we demonstrated that ensemble learning can improve the segmentation performance of polyps under the interference of artifacts, which has a significant auxiliary role in the detection of polyps in clinical practice.

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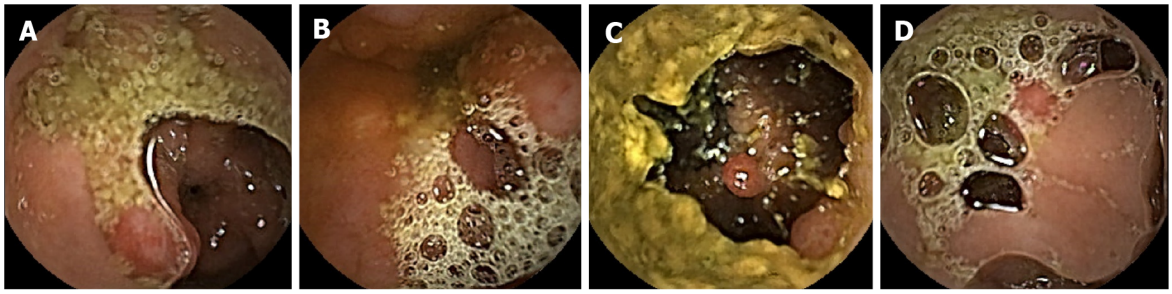
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INTRODUCTION

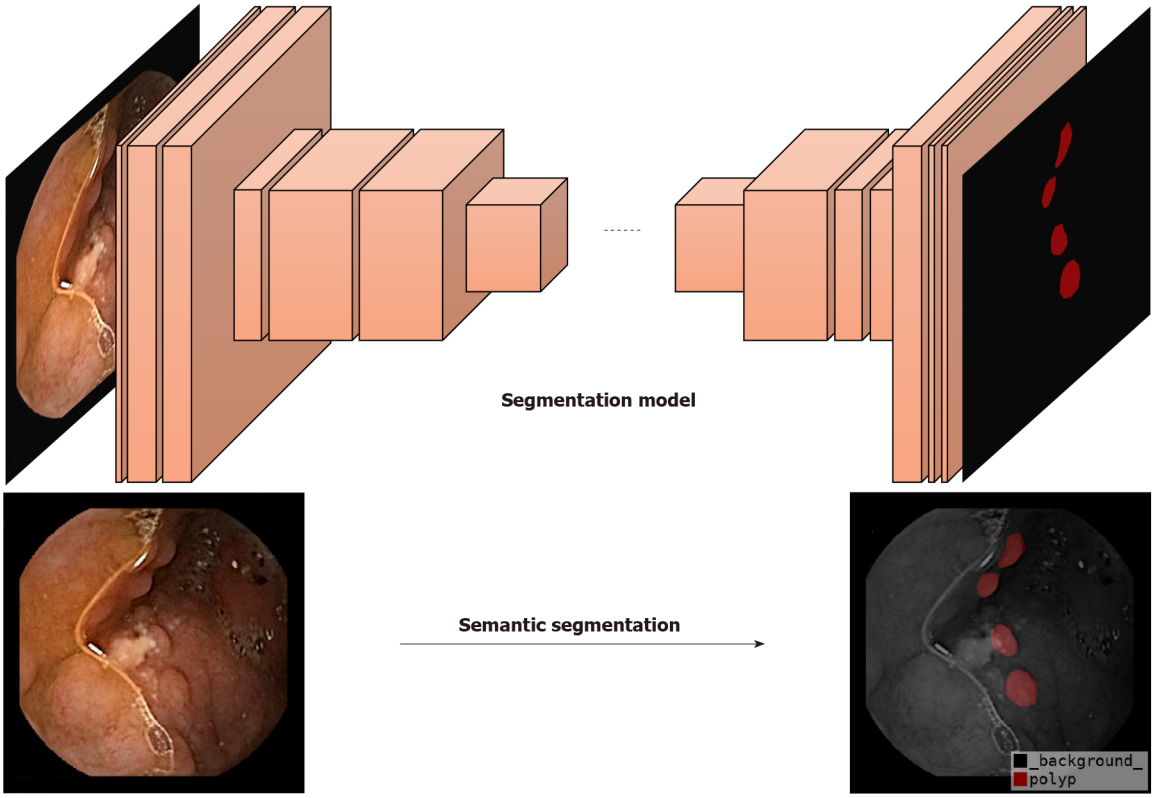
Colorectal cancer (CRC) is the second leading cause of death in the United States[1]. In China, an estimated 1101653 new cancer cases and 709529 cancer deaths from gastric cancer and CRC will occur in 2022, placing China first worldwide because of its large population[1]. Although other gastrointestinal lesions, such as erosions and ulcers, can also develop into cancers, most gastrointestinal cancers arise from precancerous polyps, which are the most common lesions found on endoscopy[2]. Therefore, early detection and removal of gastrointestinal polyps under endoscopy are critical for preventing gastrointestinal cancers[3-6]. Traditional gastroenteroscopy is widely used for the clinical assessment of gastrointestinal lesions. However, there are still some deficiencies, such as invasiveness and incomplete inspection of the site[7]. Additionally, some patients with small bowel diseases who have contraindications or are averse to undergoing gastroenteroscopy are more likely to use safer and non-invasive capsule endoscopy (CE) for visual examination of the digestive tract[8,9]. CE usually takes 8-12 h, which is not only time-consuming but also highly operator-dependent[10,11]. Otherwise, deep learning (DL) has greatly improved the sensitivity and specificity of CE for polyp detection while saving time[12]. Studies have indicated that for every 1% increase in the detection rate of colorectal adenoma, the risk of CRC can decrease by 3%[4]. However, inadequate intestinal cleansing can produce various artifacts, such as motion blur, specular reflections, bubbles, and debris (Figure 1), which can interfere with image reading, reduce the detection rate of polyps, cause patients to miss treatment, and increase the risk of tumor development[13,14]. In addition, high-quality and clear standard datasets that rarely appear in clinical practice are often used in these studies[15-17], and the intestinal lumen is often fully dilated in these images. This is significantly different from CE images with natural contraction of the intestinal lumen, which can present various artifacts (Figure 1). Therefore, these methods are often less effective in clinical practice. Hence, identifying gastrointestinal polyps and other lesions to the maximum extent when the gastrointestinal tract is insufficiently cleansed and dilated with interference factors, such as fecal residue, cloudy liquid, and bubbles in the lumen, is one of the biggest challenges in the application of artificial intelligence (AI) for CE in clinical practice and is of great concern to clinicians.

Currently, DL is a popular topic in the field of AI. It is based on the construction of computational models by simulating the neural network structure of the human brain[18]. Semantic segmentation is a part of DL algorithms that segments different objects according to each marked pixel in an image[19] (Figure 2). Some studies have proposed semantic segmentation models for medical images, such as SegNet[20], U-Net[21], Attention-UNet[22], Resnet-UNet[23], and HarDMSEG[24]. These studies have shown the significant superiority of various types of medical image semantic segmentation, as well as the feasibility of these models in tests with standard datasets. To improve the robustness of these



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Figure 1 Examples of artifact-infested endoscopy images used in our current study. A: Presence of cloudy liquid and specular reflections; B: Presence of bubbles and low contrast between lesion tissue and normal tissues; C: Presence of fecal residue; D: Presence of bubbles and specular reflections.



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Figure 2 Semantic segmentation problem in the field of computer vision.

models, researchers have begun to apply ensemble learning to medical image segmentation, not through a single model, but by combining several basic models to ensure the best prediction performance[25-27]. However, AI currently has limited ability to identify intestinal lesions with insufficient cleansing. For example, the detection rate of polyps in CE with a clean intestinal tract is significantly higher than that in CE with a dirty intestinal tract. In clinical practice, intestinal cleansing is not always performed well, and may not generate a clean image. Additionally, each patient has factors that can affect the identification by AI, such as insufficient intestinal distension, intestinal fecal residues, liquid residues, and air bubbles, resulting in the insufficient actual use of AI in clinical studies and low reliability.

In the present study, we combined semantic segmentation and ensemble learning methods for the first time to analyze CE images with artifacts. We then compared the performance of the ensemble and single models to further improve the detection rate of polyps. Our results demonstrate that ensemble learning can be used to reduce the influence of artifacts, which has a significant auxiliary role in the detection of polyps in clinical practice. To the best of our knowledge, this is the first study to propose the use of ensemble learning and semantic segmentation to reduce the negative impact of artifacts on model performance in clinical practice. Overall, our current findings have instructive significance for improving the analysis of medical images with artifacts in clinics.

MATERIALS AND METHODS

This retrospective study was approved by the Ethics Committee of Guangzhou First People's Hospital. All images were collected from videos of Ankon. This study has no conflicts of interest and did not receive any funding.

Data preparation

We collected 277 polyp CE images with artifacts selected from 5760 h of videos from 480 patients suffering from gastrointestinal disorders who received CE at Guangzhou First People's Hospital from January 2016 to December 2019. The selection criteria for the experimental images were as follows: (1) The lumen on the picture was in a natural contraction state; (2) Images of the digestive tract with polyps; and (3) Artifacts in the lumen, such as feces, motion blur, specular reflections, bubbles, and debris. The polyps in these experimental images were verified for authenticity by using a large number of clear videos and photos containing the polyps or double-balloon enteroscopy. Additionally, to ensure the accuracy and rigor of the data annotation, the image data were obtained by an experienced gastroenterologist who watched the video recordings, extracted the frames where the polyps were captured through ES Navi, and annotated the pixel points of the polyp lesions using Labelme. Next, the annotated polyp profiles were carefully reviewed by two other experienced gastroenterologists. The processing time for each patient's video was approximately 4-5 h. Before applying the dataset in the experiments, we cut off the black boxes of the images that typeset the patient's name and other information to obtain 512 × 512 images.

The other class of data comprehended publicly available high-quality datasets with images that rarely have artifacts and included the CVC_Colon[16] dataset (created by the Computer Vision Center and Computer Science Department, Universitat Autònoma de Barcelona) and the CVC_Clinic[17] dataset (captured by the Hospital Clinic, Barcelona, Spain and labeled by the Computer Vision Center, Barcelona, Spain). CVC_Colon provided 380 colonoscopy images containing polyps with a frame size of 500 × 574 pixels. Similarly, the CVC-Clinic contained 612 still images with a size of 288 × 384 from 29 different sequences. Both datasets are frequently used in gastrointestinal endoscopic computer-assisted polyp detection studies, and several representative studies have used these datasets in their experiments.

When using these datasets, we cropped or padded the edges of the images for two reasons. First, black edges or information, such as patient and time on the edges of the images, have no effect on the polyp region segmentation. Second and the main reason is that when we cross-sectionally compared various base learners in our experiments, the convolution and pooling designs of some of them were found to be more suitable for images whose length and width were both divisible by powers of two. Therefore, to minimize the changes in the hyperparameters of these base learners, we cropped or padded the input images to match the model hyperparameter design. Finally, the GZ_Capcam dataset contains 277 images of size 512 × 512, the CVC_Clinic contains 612 images of size 288 × 384, and the CVC_Colon dataset contains 380 images of size 512 × 576; all images are eight-bit three-channel color images[17]. All images used in this study contained at least one polyp class, including the standard datasets.

Snapshot ensemble method

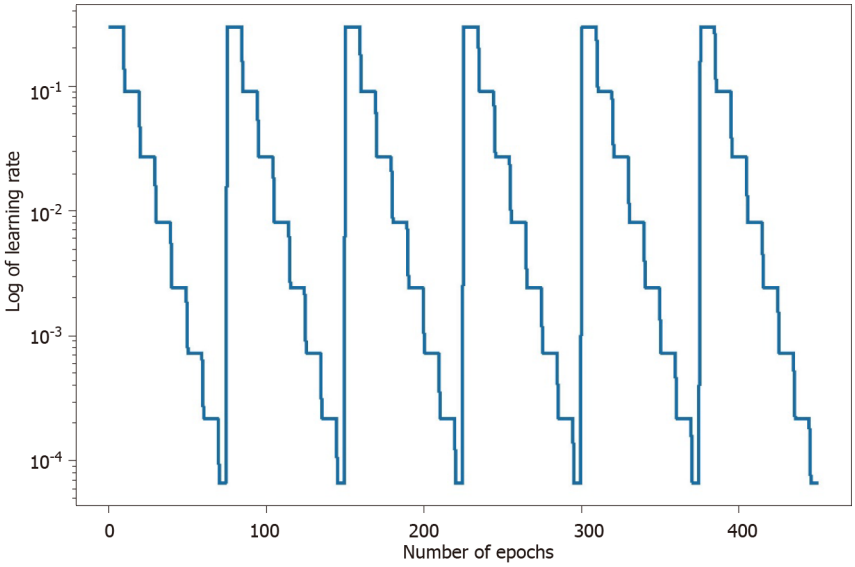
In supervised learning problems, we always expect to obtain models that perform well and are stable in all aspects; however, owing to the presence of randomness, the trained models are not always ideal, and the models obtained always have prediction preferences. The main goal of ensemble learning is to combine weak models to build a more integrated and comprehensive model that integrates the strengths of weak models. The snapshot ensemble method is a type of ensemble learning for DL models and was used in the present study[28].

In the DL method, the model parameters are adjusted according to the gradient of the objective function, as shown in Formula 1:

$$\theta_t := \theta_{t-1} - \alpha \frac{\partial}{\partial \theta_{t-1}} J(\theta_{t-1})$$

The parameters of the model take a step in the direction of the gradient descent at each iteration, and the size of the step depends on both the size of the current gradient and the learning rate, as shown in Formula 1, where θ_t denotes the model parameters in time step t and α denotes the learning rate. Usually, to speed up convergence and prevent DL models from repeatedly jumping at different local optima during training, the learning rate decays as the number of iterations increases, eventually causing the model to fall into a certain local optimum and not jump out. The core idea of the snapshot ensemble method is to restart the learning rate when it decays to less than a certain threshold so that the model jumps out of the current local optimum and finds a new local optimum nearby and converges, and Formula 2 and Figure 3 show the specific changes in the learning rate:

$$\alpha(t) = \alpha_0 \gamma^{\left\lfloor \frac{mod(t-1,T)}{M} \right\rfloor}$$



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Figure 3 Learning rate for each epoch in the snapshot ensemble learning, with γ and α_0 set for 0.3, M for 10, and T for 75.

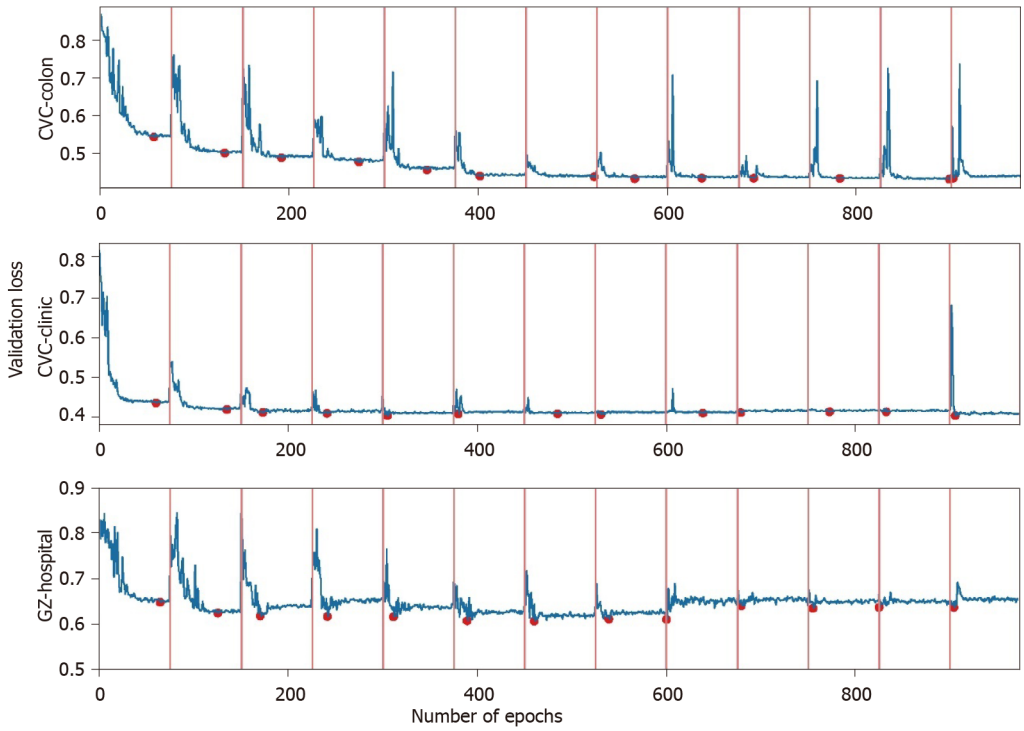
Where α_0, γ, M, and T represent the initial learning rate, learning rate decay rate, number of epochs per learning rate decay, and number of epochs per learning rate restart cycle, respectively. In the snapshot ensemble method, the model that is at the local optimum before each restart learning rate is recorded as a weak model, and in the end, the prediction results of multiple weak models are integrated by ensemble voting. In the process of training, we set the number of learning rate restart cycles to 13, the learning rate decay rate to 0.3, and the number of epochs per learning rate decay to 10 and perform a total of 75 epochs in each cycle, *i.e.*, 0.3 for γ, 10 for M, and 75 for T in Formula 2. In other words, the learning rate is reduced to 0.3 of the previous value every 10 epochs of training and reverts to the initial learning rate setting of 0.3 after 75 epochs. The model parameters that perform best on the validation set are retained in these 75 epochs as the parameters of the weak model. The entire training process lasted for 13 cycles, that is, we ended up with 13 weak learners. In the integration phase of weak models, we selected three, five, and seven weak learners with the best performance on the validation set and obtained the prediction results of the ensemble model by vote ensemble. All computational processes, including data pre-processing, model training, validation, and testing, were performed through Python programming. We built the model using PyTorch, and all experiments were based on an NVIDIA Titan V GPU. Figure 4 shows the change in validation loss in the experiment with the UNet model on the CVC_Colon dataset. The light pink line indicates the epochs of the restart learning rate, and the red points indicate the epochs of preserving the weak models.

State-of-the-art segmentation models

To show that the ensemble classification is effective in improving the segmentation in comparison with the single model when dealing with medical images with artifacts, and to illustrate the generality of its enhancement effect, we used five existing state-of-the-art (SOTA) segmentation models as base learners; SegNet[20], which is proposed to solve the deep network model of image semantic segmentation for autonomous driving or intelligent robots, and is mainly based on full convolutional networks; U-Net [21], which performs well on neuron structure segmentation datasets with only a small number of annotations, and is a basic solution for medical image analysis of small datasets; Attention-UNet[22], which is an improved model based on U-Net, and achieves performance beyond that of the U-Net model for semantic segmentation of human organs on abdominal three-dimensional computed tomography scans; ResNet-UNet[23], which is also an improved version of U-Net, and gets outstanding performance on the public challenge of identifying pneumothorax diseases on chest X-rays; and HarDMSEG[24], which is an efficient image segmentation model, and achieves SOTA level in terms of both computational efficiency and analytical accuracy, in comparison experiments to illustrate that ensemble learning method improves their analysis capability in the face of images with artifacts.

Setup of comparison experiments

First, we randomly divided the experimental and public data into training (195 images), validation (41 images), and testing (41 images) sets. The model was trained using the training set, and the best model was selected for the final test on the validation set to ensure that the model did not overfit the final test data. Finally, we tested the model using the testing set. For each model and dataset pair, multiple cycles of the learning rate restart were performed during the training phase. The best-performing model,



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Figure 4 Validation loss in training.

evaluated using the validation set data in each restart learning rate cycle, was retained as a weak model. Finally, every weak model and the strong model comprehending several of the best weak models were evaluated using the validation set and tested using the testing set. Figure 5 shows the overall experimental design.

Outcome measures

The intersection over union (IoU) and dice coefficients (Figure 6) are the most widely used metrics for semantic segmentation problems[29-31]. Both metrics measure the similarity between the sets of real and predicted regions. The calculation process is illustrated in Figure 6, where the area of intersection denotes the number of pixels in the intersection between the prediction area and ground truth, and the area of the union denotes that of the union. These two metrics were used to assess the performance of the segmentation models.

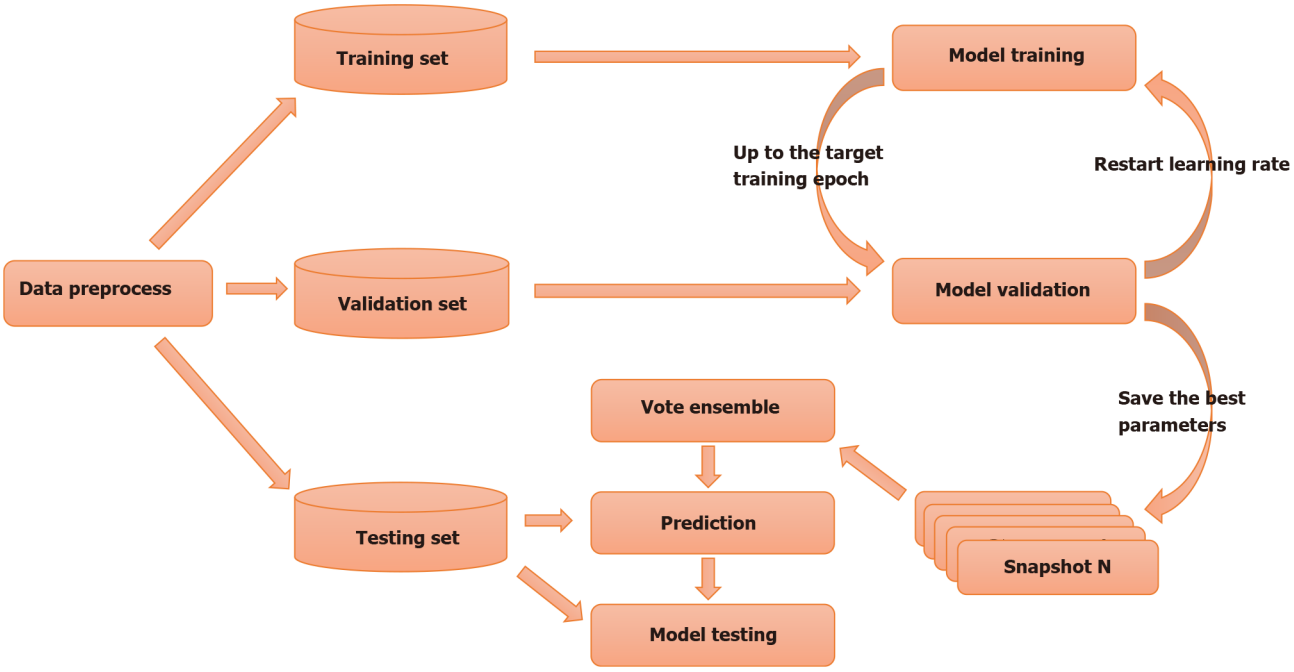
RESULTS

In summary, we performed two sets of comparison experiments using SOTA base models for the two types of datasets. In the first experiment, we compared the performance of the ensemble learning model with that of single models on a dataset with artifacts. In the second experiment, we compared the performance of the single models with that of the ensemble model on high-quality datasets without artifacts. Finally, we compared the differences between the improvements of the ensemble learning method for datasets with and without artifacts.

Comparison between the ensemble model and single models

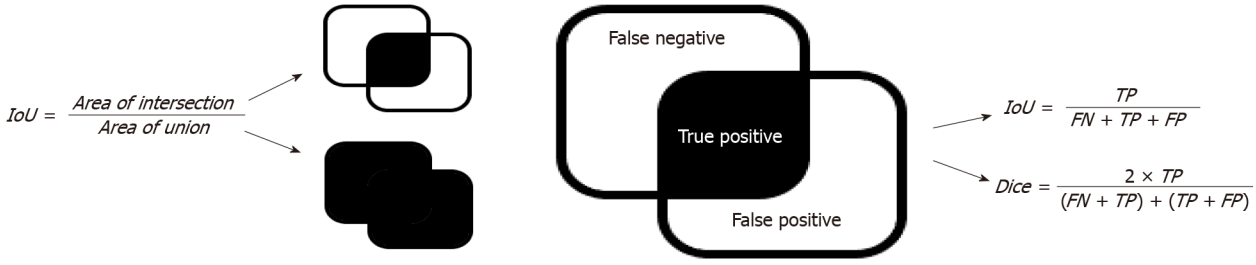
First, we compared the performance of the ensemble learning model with that of single models on CE images for all the five aforementioned base learners. A total of 41 images from the test dataset of GZ_Capcam were used for the final test. These test images were used only in the final testing phase to avoid data leakage and the consequent erroneous evaluation of the models. To illustrate that the ensemble learning model improves the performance of the single model on the artifact-infected dataset, we replicated all base learners mentioned in the previous section to illustrate the robustness of the conclusions in this study.

For U-Net, three test samples in the GZ-Hospital dataset were selected to compare the performances of the single and ensemble models (Figure 7). We can see that the semantic segmentation model was affected by different noises, such as stains, blurs, and light-dark variations in the sample images, leading to results that were not always clear. However, the performance of the ensemble model often met or



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Figure 5 Train-test workflow of the proposed method.



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Figure 6 Illustration of intersection over union and dice metrics. IoU: Intersection over union; FN: False negative; TP: True positive; FP: False positive.

exceeded the best results of a single model, indicating that a model constructed based on ensemble learning can effectively mitigate the effects of artifacts on the performance of the semantic segmentation model.

The results for the GZ_Capcam dataset are presented in Table 1, which includes images rich in artifacts. The IoU and dice metrics were calculated, as previously described. The performances of the single and ensemble models on the test set are presented in Supplementary Table 1. The results for all five basic learners on the CE dataset showed that the ensemble model outperformed the single models. Compared with single models, specifically, on the dataset with artifacts, the ensemble learning models with SegNet, U-Net, Attention-UNet, Resnet-UNet, and HarDMSEG as the base learners improved the detection by 0.08%, 7.01%, 3.88%, 5.13%, and 2.22%, respectively, using the IoU metric, and 1.71%, 4.93%, 1.40%, 2.86%, and 0.61%, respectively, using the dice metric. Overall, the ensemble model outperformed the single models. The performance of a truly single model, that is, a model obtained from a single training validation, was consistently worse than that of the ensemble model, as shown in the results for the weak models excluding the best one.

Comparisons using datasets without artifacts

Similarly, we checked the performance of the single and ensemble models using standard datasets (Figure 8, Tables 2 and 3). The performances of the single and ensemble models on the test set are presented in Supplementary Tables 2 and 3. By comparing the results presented in Figures 7 and 8, we found that the ensemble learning method can improve the robustness of the semantic segmentation model when the dataset is affected by artifacts.

Table 1 Comparison between the single and ensemble models on GZ_Capcam (artifact-affected) datasets

Model	SegNet		U-Net		Attention-UNet		ResNet-UNet		HarDMSEG	
Metric	IoU	Dice	IoU	Dice	IoU	Dice	IoU	Dice	IoU	Dice
Snap_max	0.341	0.454	0.379	0.507	0.361	0.502	0.414	0.522	0.538	0.649
Ens_max	0.341	0.462	0.406	0.532	0.375	0.509	0.435	0.537	0.550	0.653
Improve	0.000	0.008	0.027	0.025	0.014	0.007	0.021	0.015	0.012	0.004
Improve (%)	0.08	1.71	7.01	4.93	3.88	1.40	5.13	2.86	2.22	0.61

Snap_max and Ens_max denote the performance of the best performing single model and ensemble model, respectively, and the last two rows denote the improvement of the ensemble learning model compared to a single model. GZ_Capcam: The test set of experimental images from Guangzhou First People's Hospital. IoU: Intersection over union.

Table 2 Comparison between the single and ensemble models on CVC_Colon (clear) datasets

Model	SegNet		UNet		Attention-UNet		ResNet-UNet		HarDMSEG	
	IoU	Dice	IoU	Dice	IoU	Dice	IoU	Dice	IoU	Dice
Snap_max	0.700	0.780	0.713	0.788	0.754	0.830	0.747	0.819	0.840	0.901
Ens_max	0.702	0.779	0.711	0.783	0.752	0.829	0.750	0.816	0.840	0.901
Improve	0.002	-0.001	-0.002	-0.005	-0.002	-0.002	0.003	-0.002	0.000	0.000
Improve (%)	0.30	-0.08	-0.28	-0.61	-0.22	-0.22	0.42	-0.26	0.03	-0.01

Snap_max and Ens_max denote the performance of the best performing single model and ensemble model, respectively, and the last two rows denote the improvement of the ensemble learning model compared to a single model. IoU: Intersection over union.

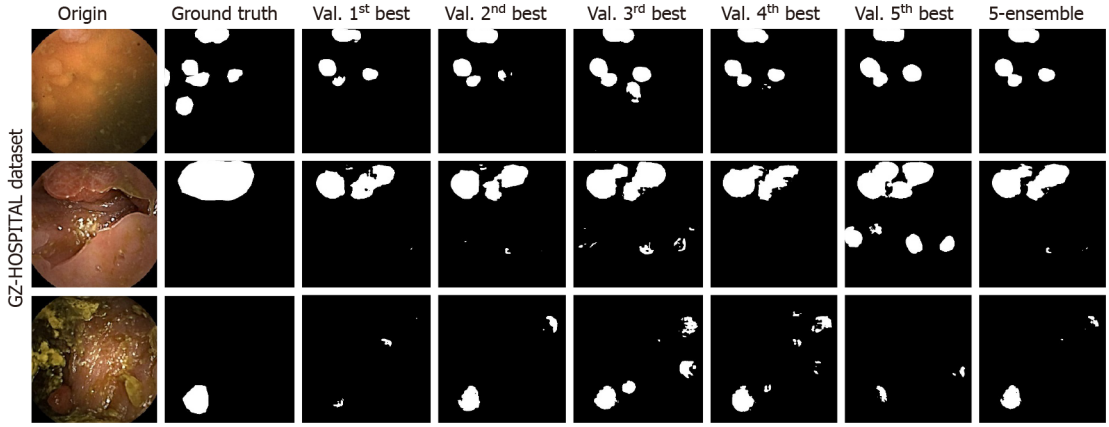
Table 3 Comparison between the single and ensemble models on CVC_Clinic (clear) datasets

Model	SegNet		UNet		Attention-UNet		ResNet-UNet		HarDMSEG	
	IoU	Dice	IoU	Dice	IoU	Dice	IoU	Dice	IoU	Dice
Snap_max	0.814	0.890	0.816	0.884	0.836	0.900	0.823	0.884	0.845	0.893
Ens_max	0.815	0.890	0.826	0.891	0.838	0.898	0.824	0.884	0.844	0.892
Improve	0.001	0.001	0.010	0.007	0.002	-0.001	0.001	0.000	-0.001	-0.001
Improve (%)	0.13	0.07	1.20	0.76	0.23	-0.14	0.08	0.02	-0.08	-0.06

Snap_max and Ens_max denote the performance of the best performing single model and ensemble model, respectively, and the last two rows denote the improvement of the ensemble learning model compared to a single model. IoU: Intersection over union.

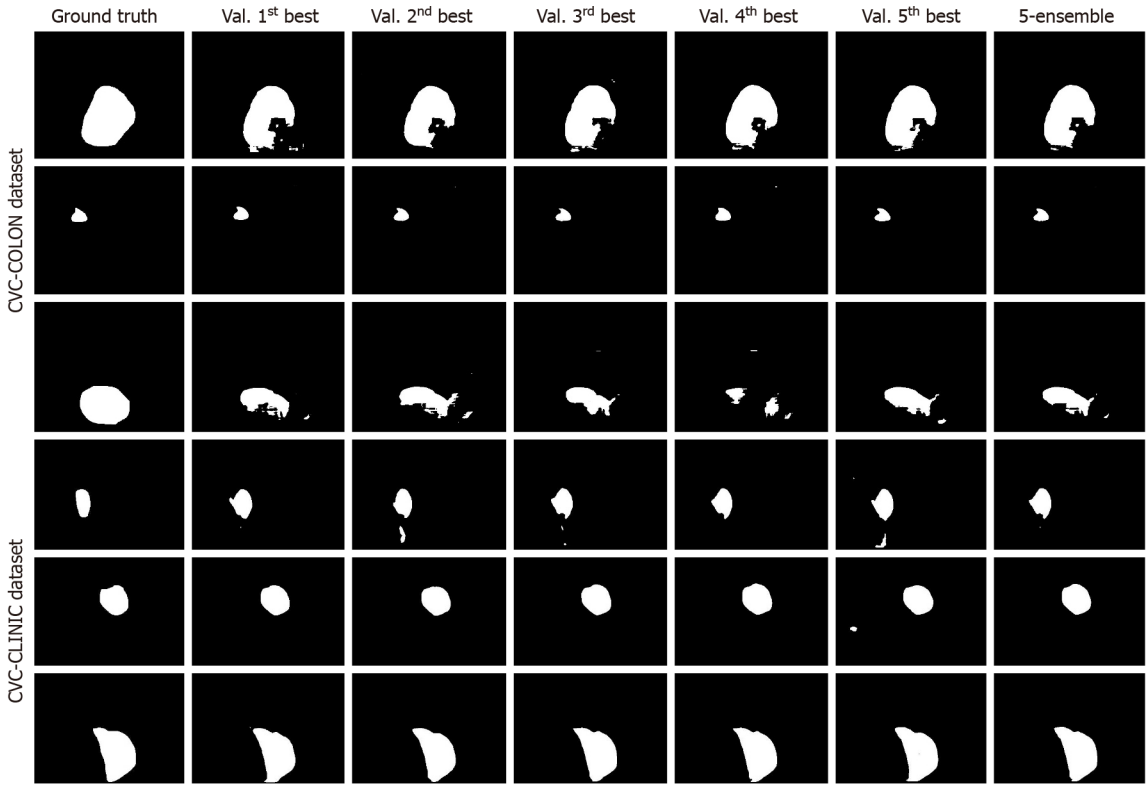
DISCUSSION

In the present study, we demonstrated that current computer-aided medical image analysis methods performed poorly in the presence of artifacts that were previously ignored. Nevertheless, almost every patient presents with insufficient intestinal cleansing. Thus, we used ensemble learning to improve the existing AI models and enhance their robustness in dealing with images with artifacts. Previous studies have extensively analyzed and concluded that integrated learning methods improve the robustness of medical image classification in a credible manner[32]. By improving the segmentation performance of the model, we can separate polyps more accurately from surrounding tissues, which can improve the detection probability of polyps and aid in monitoring the size of polyps in patients with unresectable polyps[33-36]. Semantic segmentation provides pixel-level classification and clearer polyp boundaries, which are also crucial in surgical procedures or radiofrequency ablation, and is expected to be used for real-time detection of polyp boundaries in surgical resection under gastroenteroscopy to assist polyp resection[33,36]. More in-depth studies have shown that the noise immunity of single models is weaker than that of integrated learning models[37], and clinical images, such as the CE images used in this study, are not always perfect in terms of image quality.



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Figure 7 Comparison between the ensemble and single models on artifact-affected images. Origin represents the images taken by the endoscope. Ground truth represents the anomalous locations marked by experts based on the original images. Val. 1st best to Val. 5th best represents the results of the five best-performing single models evaluated on the validation set. 5-ensemble represents the results of the ensemble model based on these single models.



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Figure 8 Comparison between the ensemble and single models on standard (clear) images. Ground truth represents the anomalous locations marked by experts based on the original images. Val. 1st best to Val. 5th best represents the results of the five best-performing single models evaluated on the validation set. 5-ensemble represents the results of the ensemble model based on these single models.

We used CE image datasets as samples, mainly because CE is an increasingly widely used and safe form of endoscopy but also has many artifacts[15,38]. The ensemble learning approach was tested for 15 pairs, consisting of three datasets and five SOTA segmentation models. The results showed that for CE images with various artifacts, ensemble learning improved the analytical performance of AI models. Herein, we demonstrated that ensemble learning can reduce the influence of artifacts on the semantic segmentation of CE images, which might also apply to other medical images.

In general, artifacts are prevalent in medical images and seriously challenge the performance of existing computer-aided diagnosis (CAD) models; therefore, this study discusses the enhancement of ensemble learning methods for CAD models to analyze images with artifacts, mainly using CE images as an example in the experiments. In addition, our experiments did not involve the injection of a priori knowledge of gastroenterology; in other words, the use of the ensemble learning approach mentioned in

this paper does not imply any additional workload or workflow reordering. The only additional cost associated with the method is the computational resources. Additionally, from the perspective of DL, better model performance often relies on more model parameters and computational resources. Methods that already use DL models can easily apply ensemble learning methods to improve model performance without the need for additional workflow tuning. It is worth mentioning that, although the ensemble learning approach can improve the robustness of CAD image analysis models, misuse may lead to a less-than-expected improvement in the model's effectiveness, mainly because the essence of ensemble learning is to reduce model variance, and when the variance of a single model is already very low, the improvement brought by ensemble learning may be very limited.

From the experimental results, although the ensemble learning approach improves the performance of the segmentation model on the dataset with artifacts, there are still false-positive and false-negative cases. On the one hand, the main reason for false-negative cases was that the model confuses normal-color polyps with normal gastrointestinal folds or confuses abnormal-color polyps with artifacts, such as yellow bubbles. However, the main cause of false-positive cases was that some artifacts or normal folds had a high similarity with polyps in the image, which led the model to misidentify them as polyps. Overall, the main reason for segmentation errors is that the color and texture are highly confusing, and we will further attempt to improve the ability of the model to distinguish polyps, normal tissues, and artifacts in a subsequent study.

In clinical practice, video frames can be completely infested with artifacts, making the content of the image simply unrecognizable. Therefore, the appearance of these frames is inevitable in clinical practice. In the present study, we confirmed the authenticity of polyps in pictures with artifacts by using more images, videos, and other inspection methods. Thus, we solved the dilemma of applying AI to these medical images. However, our study has some limitations. For example, the images were insufficient and did not involve lesions other than polyps.

We believe that the direction of feature AI for CE imaging research lies in making existing computer models better serve clinical diagnosis in a practical sense rather than letting these methods stay in the laboratory. CE is commonly used to examine digestive diseases. In addition to polyps, many digestive diseases can be detected using CE. Thus, AI for CE imaging can be considered to enrich the diagnosis, localization, and grading of more forms of the disease, such as ulcers and erosions, to assist doctors in more refined disease research and diagnosis. In the future, we will validate the ensemble learning method in clinical practice to demonstrate that it can improve the detection rate of polyps in CE in the clinic and evaluate the potential of this method for other types of medical images or lesions[39].

CONCLUSION

Ensemble learning can improve the semantic segmentation performance of AI models on CE images with artifacts.

ARTICLE HIGHLIGHTS

Research background

Artificial intelligence (AI)-assisted capsule endoscopy (CE) can improve the detection rate of gastrointestinal polyps and reduce the incidence of gastrointestinal cancer.

Research motivation

Most previous studies ignored the serious impact of the existence of a large number of artifacts in the real world on the detection ability of existing AI models for polyps in CE images.

Research objectives

In this study, semantic segmentation and ensemble learning methods were combined to analyze polyp images of CE with artifacts, proving that ensemble learning methods can better solve the impact of artifacts in the real world.

Research methods

This study retrospectively analyzed CE images of patients at our research center from January 2016 to December 2019. Polyp images with artifacts were selected and randomly divided into a training set (195 images), a validation set (41 images), and a test set (41 images). Further validation was performed on two public datasets with good background quality.

Research results

Compared with the corresponding optimal base model, intersection over union and dice are improved

by 0.08%-7.01% and 0.61%-4.93%, respectively. For public datasets with good background quality, the segmentation performance of most ensemble learning models was better than that of a single model.

Research conclusions

The ensemble learning method can improve the performance of semantic segmentation of polyps in CE images with artifacts.

Research perspectives

We will validate other digestive tract lesions and other medical images and perform real-time detection during endoscopic and surgical procedures.

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FOOTNOTES

Author contributions: Zhou JX collected and compiled the data; Yang Z and Xi DH were responsible for coding the model; Zhou JX, Yang Z, and Li J drafted the manuscript; Dai SJ, Feng ZQ, and Wang H performed the capsule endoscopy examinations and reviewed the images; Xu W and Wang H contributed to the study design and manuscript writing.

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ORIGINAL ARTICLE

Retrospective Study

Transjugular intrahepatic portosystemic shunt *vs* conservative treatment for recurrent ascites: A propensity score matched comparison

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Abstract

BACKGROUND

Transjugular intrahepatic portosystemic shunt (TIPS) placement is an effective intervention for recurrent tense ascites. Some studies show an increased risk of acute on chronic liver failure (ACLF) associated with TIPS placement. It is not clear whether ACLF in this context is a consequence of TIPS or of the pre-existing liver disease.

AIM

To better understand the risks of TIPS in this challenging setting and to compare them with those of conservative therapy.

METHODS

Two hundred and fourteen patients undergoing their first TIPS placement for recurrent tense ascites at our tertiary-care center between 2007 and 2017 were identified (TIPS group). Three hundred and ninety-eight patients of the same time interval with liver cirrhosis and recurrent tense ascites not undergoing TIPS placement (No TIPS group) were analyzed as a control group. TIPS indication, diagnosis of recurrent ascites, further diagnoses and clinical findings were obtained from a database search and patient records. The in-hospital mortality and ACLF incidence of both groups were compared using 1:1 propensity score

matching and multivariate logistic regressions.

RESULTS

After propensity score matching, the TIPS and No TIPS groups were comparable in terms of laboratory values and ACLF incidence at hospital admission. There was no detectable difference in mortality (TIPS: 11/214, No TIPS 13/214). During the hospital stay, ACLF occurred more frequently in the TIPS group than in the No TIPS group (TIPS: 70/214, No TIPS: 57/214, $P = 0.04$). This effect was confined to patients with severely impaired liver function at hospital admission as indicated by a significant interaction term of Child score and TIPS placement in multivariate logistic regression. The TIPS group had a lower ACLF incidence at Child scores < 8 points and a higher ACLF incidence at ≥ 11 points. No significant difference was found between groups in patients with Child scores of 8 to 10 points.

CONCLUSION

TIPS placement for recurrent tense ascites is associated with an increased rate of ACLF in patients with severely impaired liver function but does not result in higher in-hospital mortality.

Key Words: Liver cirrhosis; Ascites; Transjugular intrahepatic portosystemic shunt; Acute on chronic liver failure; Mortality; Propensity score

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Core Tip: Transjugular intrahepatic portosystemic shunt (TIPS) is an effective therapy for recurrent tense ascites, but there are concerns about further deterioration of liver function in patients with advanced cirrhosis. We retrospectively analyzed 214 patients receiving TIPS for ascites and compared their outcomes to matched conservatively treated patients. We found that TIPS can trigger acute on chronic liver failure (ACLF) in patients with severely impaired liver function. However, no increased mortality was found compared to conservatively treated patients. Despite an increased risk of ACLF, TIPS is a viable option for patients with ascites and hepatic impairment.

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INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) is an effective therapy for complications of portal hypertension, such as ascites or esophageal variceal bleeding. Although TIPS placement is effective against ascites, early studies showed no survival benefit after TIPS placement compared to repeated paracentesis and albumin substitution[1-3]. More recent studies have shown more promising results, such as survival benefit[4-7], improved renal function[8,9] and better quality of life[10,11]. TIPS placement is therefore recommended as the treatment of choice[12,13].

Nevertheless, TIPS placement is an invasive procedure with considerable risks. In addition to hepatic encephalopathy and bleeding complications due to the placement procedure, sudden worsening of liver function is a serious complication. It has been observed after 5% to 10% of TIPS procedures and has a serious prognosis[14,15]. Such an acute deterioration of liver function accompanied by single- or multi-organ-failure is a common complication of advanced liver cirrhosis. This clinical syndrome has been described as acute on chronic liver failure (ACLF)[16]. Due to the risk of liver failure, TIPS placement for ascites is often limited to patients with good liver function and most randomized controlled trials have been conducted in patients with good liver function. It is still unclear how often ACLF occurs after TIPS placement and whether it is due to the TIPS procedure or rather to the severity of the underlying liver disease[17]. Recent recommendations argue against strict cut-off values for MELD, Child or other scoring systems. Instead, they recommend individual decision-making[18]. To better address the risk of ACLF in this challenging clinical situation the aim of this study was: (1) To determine whether ACLF occurs more often in patients with recurrent tense ascites treated with TIPS than in patients receiving conservative therapy; (2) to compare the outcome of ACLF associated with TIPS placement with the outcome of ACLF in patients receiving conservative therapy; and (3) to evaluate whether the risk of ACLF and death associated with TIPS placement increases disproportionately in patients with marginal

liver function.

MATERIALS AND METHODS

Selection of patients

A database was constructed containing ICD and OPS codes as well as laboratory values of all inpatients of the Division of Gastroenterology of the Rostock University Medical Center. Patients who were treated for liver cirrhosis between 2007 and 2017 were identified based on their discharge diagnosis using ICD10 codes K70.3, K70.4, K71.7, K74.6 and K76.6 (2197 cases of 1404 patients). Patients who received TIPS were identified using OPS codes 8-839*. Only cases of patients receiving their first TIPS for recurrent tense ascites were selected. Therefore there was only one case per patient in the TIPS group. Cases of patients who had liver cirrhosis and tense ascites requiring paracentesis, but did not undergo TIPS placement were selected for comparison (No TIPS group). If several cases were available for the same patient in the No TIPS group (*e.g.*, because of multiple hospital admissions), the latest case was selected. TIPS indication, diagnosis of recurrent tense ascites, further diagnoses and clinical findings were obtained from ICD codes and from patient files. Laboratory values were obtained from the data base. Cases with missing data on relevant clinical or laboratory findings were removed (43 cases). Cases with pre-existing renal insufficiency requiring dialysis (30 cases) or with malignant tumors (471 cases) were also excluded. Patient selection resulted in 398 patients in the No TIPS group and 214 patients in the TIPS group. After data collection was completed, all patient data were pseudonymized. Patient selection criteria and reasons for exclusion from data analysis are depicted in Figure 1. The study was approved by the local ethics committee of the Rostock University Medical Center (A2018-0127).

The MELD-score and ACLF grade as defined by Moreau *et al*[16] at hospital admission and the highest ACLF grade achieved during hospital stay were determined for each patient. Furthermore, the in-hospital mortality of both groups was determined. Multivariate logistic regressions revealed that bilirubin, creatinine, INR, CRP, sodium, white blood cell count, albumin and age were predictive either for survival or for group membership in TIPS *vs* No TIPS group or for both. Therefore these covariates were chosen for the propensity score matching procedure. The matching (1:1 greedy matching, nearest neighbor, without replacement) resulted in a matched sample of 428 patients (214 patients in the No TIPS and 214 in the TIPS group).

Statistical analysis

Statistical evaluation and matching were carried out using R (R version 3.6.3[19] and the R Package MatchIt, Version 4.1.0[20]). The distribution of most of the continuous data had significant positive skew, therefore non-parametric test methods were used. Continuous variables were compared using the Mann-Whitney *U* test and categorical variables using the chi-square or Fisher's exact test. Data on an ordinal scale (ACLF, hepatic encephalopathy) were treated as continuous. To account for the loss of statistical independence due to the matching procedure[21,22], comparisons between the matched groups were carried out using the Wilcoxon signed rank test or McNemar test. Additional multivariate logistic regressions were performed as sensitivity analysis and for further insights into effects of liver function, TIPS placement and their interaction on ACLF incidence and in-hospital mortality. The statistical methods of this study were reviewed by Henrik Rudolf from Rostock University Medical Center, Institute for Biostatistics and Informatics in Medicine and Ageing Research.

RESULTS

Patient characteristics and matching

Patient demographics and liver disease characteristics of the unmatched cohort are summarized in Table 1. Continuous values are given as median and range, categorical values as total number and percentage. Patients receiving TIPS had better liver function as assessed by MELD and Child score, bilirubin, INR, albumin and severity of hepatic encephalopathy. In addition, CRP, platelets and leukocytes differed significantly. Creatinine did not differ significantly. After propensity score matching all covariates were balanced in both groups (Table 2) and all variables used for matching did no longer predict group membership in the matched patients.

From 2007 to 2017, both covered and uncovered stents were used for TIPS at our institution. Uncovered stents were placed in 42% and covered stents in 58% of cases. Stents were mostly dilated to 7-8 mm. Smaller or larger diameters were rarely chosen (6mm in 2 patients, 9 or 10 mm in 15 patients). No effect of stent type or stent diameter on any of our endpoints was found in either univariate or multivariate analyses (data not shown).

Table 1 Baseline characteristics at hospital admission (all patients)

Characteristics	No TIPS	TIPS	*P* value
Patients	398	214	
Male	269 (68%)	153 (71%)	0.320
Age (yr)	59.5 (26.4-93.4)	59.1 (29.9-80.7)	0.190
Cause of cirrhosis			0.130
-Alcohol	305 (77%)	179 (84%)	
-Viral hepatitis	11 (3%)	4 (2%)	
-Other	82 (21%)	31 (14%)	
Child points (min-max)	10 (7-15)	9 (7-14)	< 0.001
Hepatic encephalopathy (West-Haven)			0.040
-None	276 (69%)	165 (77%)	
-Grade 1-2	73 (18%)	30 (14%)	
-Grade 3-4	49 (12%)	19 (9%)	
ACLF grade at hospital admission			0.020
-No ACLF	294 (74%)	173 (81%)	
-ACLF grade 1	64 (16%)	39 (18%)	
-ACLF grade 2	32 (8%)	1 (0.5%)	
-ACLF grade 3	8 (2%)	1 (0.5%)	
Laboratory findings			
-Meld	18 (7-40)	14 (7-40)	< 0.001
-Total bilirubin (μmol/L)	49.7 (5.9-668.0)	28.1 (6.1-688.5)	< 0.001
-Creatinine (μmol/L)	100.5 (23.1-781.0)	107.0 (42.3-783.5)	0.180
-INR	1.45 (0.92-9.2)	1.28 (0.97-2.5)	< 0.001
-Sodium (μmol/L)	134 (106-149)	133 (115-146)	0.760
-Albumin (g/L)	22.6 (7.9-48.7); NA: 55	26.1 (11.0-39.6); NA: 23	< 0.001
-CRP (mg/L)	25.6 (1.0-283.0)	18.0 (2.0-181.0)	< 0.001
-Hemoglobin (mmol/L)	6.8 (2.0-9.8)	6.7 (3.4-10.0)	0.310
-Platelets (Gpt/L)	134.5 (22.0-715.0)	153.5 (13.0-668.0)	0.002
-Leucocytes (Gpt/L)	8.76 (1.34-44.90)	7.34 (2.72-33.20)	< 0.001

Continuous variables are given as median and range, categorical variables as total number and percentage. Continuous variables were compared using the Mann-Whitney-*U*-test and categorical variables using the chi-square test. NA: Not available; ACLF: Acute on chronic liver failure; TIPS: Transjugular intrahepatic portosystemic shunt.

Incidence of ACLF and in-hospital mortality

Table 3 shows the incidence of ACLF as well as the in-hospital mortality of the matched patients. Patients receiving TIPS more often had ACLF of any grade (TIPS: 70/214 patients *vs* No TIPS 57/214 patients) and achieved higher ACLF grades ($P = 0.04$). An increase in ACLF grade (as compared to the ACLF grade at hospital admission) was more common in the TIPS group than in the No TIPS group (in 38/214 patients *vs* 23/214 patients). The hospital stay was longer in the TIPS group. The majority of patients in both groups had ACLF 1, which was due to renal failure. Organ systems affected in patients with ACLF > 1 were brain (hepatic encephalopathy grade 3-4) and/or liver function based on bilirubin in addition to renal failure. ACLF > 1 was mostly due to acute infections.

There was no difference in terms of in-hospital mortality. In the TIPS group 11 of 214 patients died, in the No TIPS group 13 of 214 patients died. The mortality increased with the ACLF grade in both groups. Multivariate logistic regressions were performed as a sensitivity analysis and confirmed that TIPS was a risk factor for ACLF but not for in-hospital mortality (Table 4). Mortality in any ACLF stratum except ACLF 2 was comparable in both groups. For patients with ACLF 2, we found a lower mortality in the

Table 2 Baseline characteristics at hospital admission (matched groups)

Characteristics	No TIPS	TIPS	*P* value
Patients	214	214	
Male	142 (66%)	153 (71%)	0.30
Age (yr)	59.4 (26.4-93.4)	59.1 (29.9-80.7)	0.14
Cause of cirrhosis			0.26
-Alcohol	163 (76%)	179 (84%)	
-Viral hepatitis	8 (4%)	4 (2%)	
-Other	43 (20%)	31 (14%)	
Child points (min-max)	9 (7-14)	9 (7-14)	0.76
Hepatic encephalopathy (west-haven)			0.65
-None	173 (81%)	165 (77%)	
-Grade 1-2	22 (10%)	30 (14%)	
-Grade 3-4	19 (9%)	19 (9%)	
ACLF grade at hospital admission			0.37
-No ACLF	176 (82%)	173 (81%)	
-AACLF 1	36 (17%)	39 (18%)	
-ACLF 2	2 (1%)	1 (0.5%)	
-ACLF 3	0 (0%)	1 (0.5%)	
Laboratory findings			
-Meld	14 (7-36)	14 (7-40)	0.97
-Total bilirubin (μmol/L)	29.7 (5.9-261.0)	28.1 (6.1-688.5)	0.10
-Creatinine (μmol/L)	90.9 (23.1-781.0)	107.0 (42.3-783.5)	0.08
-INR	1.32 (0.92-2.41)	1.28 (0.97-2.50)	0.28
-Sodium (mmol/L)	135 (106-144)	133 (115-146)	0.10
-Albumin (g/L)	24.3 (7.9-48.7); NA: 27	26.1 (11.0-39.6); NA: 23	0.61
-CRP (mg/L)	18.7 (1.0-283.0)	18.0 (2.0-181.0)	0.10
-Hemoglobin (mmol/L)	6.8 (2.1-9.8)	6.7 (3.4-10.0)	0.43
-Platelets (Gpt/L)	141.0 (23.0-715.0)	154.0 (13.0-668.0)	0.08
-Leucocytes (Gpt/L)	7.58 (1.34-44.90)	7.34 (2.72-33.20)	0.98

Continuous variables are given as median and range, categorical variables as total number and percentage. Continuous variables were compared using Wilcoxon signed-rank test and categorical variables using McNemar-Test. NA: Not available; ACLF: Acute on chronic liver failure; TIPS: Transjugular intrahepatic portosystemic shunt.

TIPS group compared to the No TIPS group (OR 0.09, 95%CI 0.01-0.87). The mortality of TIPS patients who increased in ACLF by 2 or 3 grades after TIPS placement was high (4/10 died). This also applies to the No TIPS group with an even higher mortality (4/5 patients with an increase of 2 or 3 ACLF grades compared to ACLF grade at hospital admission died).

Most patients in both groups (No TIPS 89%, TIPS 82%) without ACLF at admission did not develop any ACLF during hospital stay. Many patients who developed an ACLF grade 2 or 3 already had ACLF at hospital admission (5/10 patients in the No TIPS group and 11/20 patients in the TIPS group). Three patients in the TIPS group developed ACLF during the period between hospital admission and TIPS placement, *i.e.* before TIPS was implanted. Many of the pre-TIPS ACLFs resolved after TIPS placement. When comparing the highest ACLF grade before TIPS to the ACLF grade at hospital discharge (assuming ACLF 3 for patients who died), 32 patients (15%) improved their ACLF grade after TIPS placement while only 21 patients (10%) had a worse ACLF grade at discharge than at the time of TIPS placement.

Table 3 Changes of acute on chronic liver failure grade during hospital stay and in-hospital mortality (matched groups)

Event	No TIPS	TIPS	*P* value/OR (95%CI)
Hospital stay (d)	10 (1-78)	14 (3-64)	*P* < 0.001
Highest ACLF grade			*P* = 0.041
-No ACLF	157 (73%)	144 (67%)	
-ACLF 1	47 (22%)	50 (23%)	
-ACLF 2	8 (4%)	15 (7%)	
-ACLF 3	2 (1%)	5 (2%)	
-Any ACLF	57 (27%)	70 (33%)	
Mortality by ACLF			
-Over all	13/214 (6.1%)	11/214 (5.1%)	OR: 0.84 (0.33 -2.08)
-No ACLF	3/157 (1.9%)	0/144 (0%)	OR: 0 (0.00 -2.63)
-ACLF 1	3/47 (6.4%)	4/50 (8%)	OR: 1.27 (0.20 -9.18)
-ACLF 2	6/8 (75%)	3/15 (20%)	OR: 0.09 (0.01 -0.87)
-ACLF 3	1/2 (50%)	4/5 (80%)	OR: 3.16 (0.03 -389.17)
-Any ACLF	10/57 (17.5%)	11/70 (15.7%)	OR: 0.88 (0.31-2.52)
Increase in ACLF grade			*P* = 0.03
-No increase	191 (89.3%)	176 (82.2%)	
-1 grade	18 (8.4%)	28 (13.1%)	
-2 grades	4 (1.9%)	7 (3.3%)	
-3 grades	1 (0.5%)	3 (1.4%)	
Mortality by ACLF increase			
-No increase	5/191 (2.6%)	2/176 (1.1%)	OR: 0.43 (0.04-2.66)
-1 grade	4/18 (22.2%)	5/28 (17.9%)	OR: 0.77 (0.14-4.55)
-2 grades	3/4 (75.0%)	2/7 (20.0%)	OR: 0.16 (0.003-3.50)
-3 grades	1/1 (100%)	2/3 (66.7%)	OR: 0 (0.00-116.8)
-Any increase	8/23 (34.8%)	9/38 (23.7%)	OR: 0.58 (0.16-2.14)

OR: Odds ratio; ACLF: Acute on chronic liver failure; TIPS: Transjugular intrahepatic portosystemic shunt.

Estimated in-hospital mortality and risk of ACLF

Using multivariate logistic regression models based on the MELD or Child scores at admission, the probabilities of death in-hospital and of an increase in ACLF grade were estimated for the TIPS and the No TIPS group (Figure 2). The likelihood of death increases with the severity of the disease at admission; independent of whether this is assessed by MELD or by Child scores (Figure 2A and B). The regression curves for mortality are almost parallel, indicating that mortality depends only on liver function, but not on TIPS placement or an interaction between TIPS placement and the liver function. However, the regression curves for an increase in ACLF grade differ clearly between TIPS and No TIPS (Figure 2C and D). The probability of an ACLF in the TIPS group is lower than in the No TIPS group at low to moderate MELD-and Child-levels, but it is higher than in the No TIPS group at high MELD and Child scores. The intersection of the regression curves suggests an interaction between MELD/Child score and TIPS placement. In fact, the multivariate logistic regression shows a statistically significant interaction term for Child-score and TIPS (P = 0.03; Table 5). In our model the TIPS group has a lower ACLF incidence at Child scores lower than 8 points and a higher ACLF incidence at 11 points and higher. Between 8 and 11 points the standard errors of both groups overlap, indicating that there is no relevant difference between both groups. The same effect can be observed when using the MELD score instead of the Child score. However, the interaction is weaker and not statistically significant (P = 0.19).

Table 4 Sensitivity analysis: Multivariate regressions (main effects only)

Variable	Estimate	SE	*P* value	Estimate	SE	*P* value
	Complete model			Best model		
Mortality						
Intercept	3.63	3.27	0.268	4.39	3.22	0.173
Creatinine	1.59×10^{-3}	1.37×10^{-3}	0.246	1.99×10^{-3}	1.32×10^{-3}	0.132
Bilirubin	7.26×10^{-4}	1.10×10^{-3}	0.505	-	-	-
INR	3.49×10^{-1}	2.15×10^{-1}	0.104	3.47×10^{-1}	2.12×10^{-1}	0.101
CRP	3.27×10^{-3}	3.10×10^{-3}	0.292	-	-	-
Leucocytes	7.01×10^{-2}	2.64×10^{-2}	0.008	7.84×10^{-2}	2.53×10^{-2}	0.002
HE 1-2	-2.88×10^{-1}	4.28×10^{-1}	0.500	-2.67×10^{-1}	4.1×10^{-1}	0.523
HE 3-4	2.24	3.56×10^{-1}	< 0.001	2.26	3.50×10^{-1}	< 0.001
Albumin	-9.61×10^{-2}	2.76×10^{-2}	< 0.001	-1.02×10^{-1}	2.72×10^{-2}	< 0.001
Sodium	-6.21×10^{-2}	2.45×10^{-2}	0.011	-6.51×10^{-2}	2.45×10^{-2}	0.004
Age	1.22×10^{-4}	4.24×10^{-5}	0.004	1.16×10^{-4}	4.04×10^{-5}	0.004
TIPS	-7.29×10^{-1}	4.12×10^{-1}	0.077	-8.22×10^{-1}	4.01×10^{-1}	0.040
ACLF						
Intercept	-1.822	2.713	0.502	-2.70	8.51×10^{-1}	0.002
Creatinine	-1.06×10^{-3}	1.21×10^{-3}	0.384	-	-	-
Bilirubin	2.81×10^{-3}	9.50×10^{-4}	0.003	2.99×10^{-3}	8.69×10^{-4}	0.001
INR	2.16×10^{-1}	2.01×10^{-1}	0.281	-	-	-
CRP	5.21×10^{-3}	2.58×10^{-3}	0.043	4.67×10^{-3}	2.39×10^{-3}	0.050
Leucocytes	6.28×10^{-3}	2.43×10^{-2}	0.780	-	-	-
HE 1-2	1.22×10^{-1}	3.06×10^{-1}	0.690	1.47×10^{-1}	3.02×10^{-1}	0.627
HE 3-4	1.62	3.01×10^{-1}	< 0.001	1.63	2.94×10^{-1}	< 0.001
Albumin	-3.80×10^{-2}	2.00×10^{-2}	0.058	-3.98×10^{-2}	1.99×10^{-2}	0.046
Sodium	-1.03×10^{-2}	1.98×10^{-2}	0.603	-	-	-
Age	6.39×10^{-5}	3.11×10^{-5}	0.039	5.63×10^{-5}	2.96×10^{-5}	0.057
TIPS	5.17×10^{-1}	2.64×10^{-1}	0.050	4.39×10^{-1}	2.55×10^{-1}	0.085

Dependent variables were in-hospital mortality (upper panel) and any increase in acute on chronic liver failure grade (lower panel). The full models (left side) included all parameters used for propensity score matching as covariates. After stepwise backward elimination by Akaike information criterion, a model (best model, right side) was selected for each dependent variable. ACLF: Acute on chronic liver failure; TIPS: Transjugular intrahepatic portosystemic shunt.

DISCUSSION

Most of the randomized controlled trials (RCT) have been performed in patients with good liver function. This applies in particular to the RCTs that showed a survival benefit. In these studies the mean MELD was 9.6[6] to 12.1[7]). Therefore many patients with refractory ascites receive no TIPS due to impaired liver function. Others have considered MELD scores ≥ 18[13,23,24] to ≥ 24[25,26] and bilirubin levels ≥ 51.3 to ≥ 85.5 μmol/L[13,27] as contraindications for TIPS. Our TIPS patients had a comparatively poor liver function at hospital admission (MELD median 14, mean 15.2), allowing to describe mortality and morbidity in this high-risk group.

In our cohort of patients with significantly impaired liver function ACLF incidence and in-hospital mortality was within the range observed in other studies on ACLF[16,28,29]. The in-hospital mortality was neither positively nor negatively influenced by TIPS placement despite the comparatively poor liver function of our patients. In the matched cohorts ACLF occurred more frequently in the TIPS group than in conservatively treated patients. The results of the multivariate logistic regressions suggest that this effect depends on the extent of the pre-existing liver damage. In patients with good liver function

Table 5 Multivariate logistic regressions with interaction terms

Model	Dependent variable	Parameters	Estimate	SE	*z* value	*P* value
A		Intercept	-3.8600	0.4340	-8.887	$< 2 \times 10^{-16}$
	In-hospital	MELD-Score	0.0990	0.0180	5.628	1.82×10^{-8}
	Mortality (y/n)	TIPS	-1.3570	1.0590	-1.281	0.200
		MELD: TIPS	0.0330	0.0500	0.668	0.504
B		Intercept	-7.1320	0.9810	-7.271	3.56×10^{-13}
	In-hospital	Child (points)	0.4880	0.0840	5.814	6.09×10^{-9}
	Mortality (y/n)	TIPS	-1.6760	2.2350	-0.750	0.453
		Child: TIPS	0.0934	0.2020	0.463	0.643
C		Intercept	-3.1860	0.3610	-8.824	$< 2 \times 10^{-16}$
	Increase in	MELD	0.1020	0.0160	6.461	1.04×10^{-10}
	ACLF grade	TIPS	-0.7780	0.7120	-1.092	0.275
	(y/n)	MELD: TIPS	0.0480	0.0368	1.318	0.187
D		Intercept	-5.2640	0.7480	-7.040	1.93×10^{-12}
	Increase in	Child (points)	0.3880	0.0670	5.807	6.37×10^{-9}
	ACLF grade	TIPS	-3.1980	1.5300	-2.090	0.0366
	(y/n)	Child: TIPS	0.3190	0.1145	2.191	0.0285

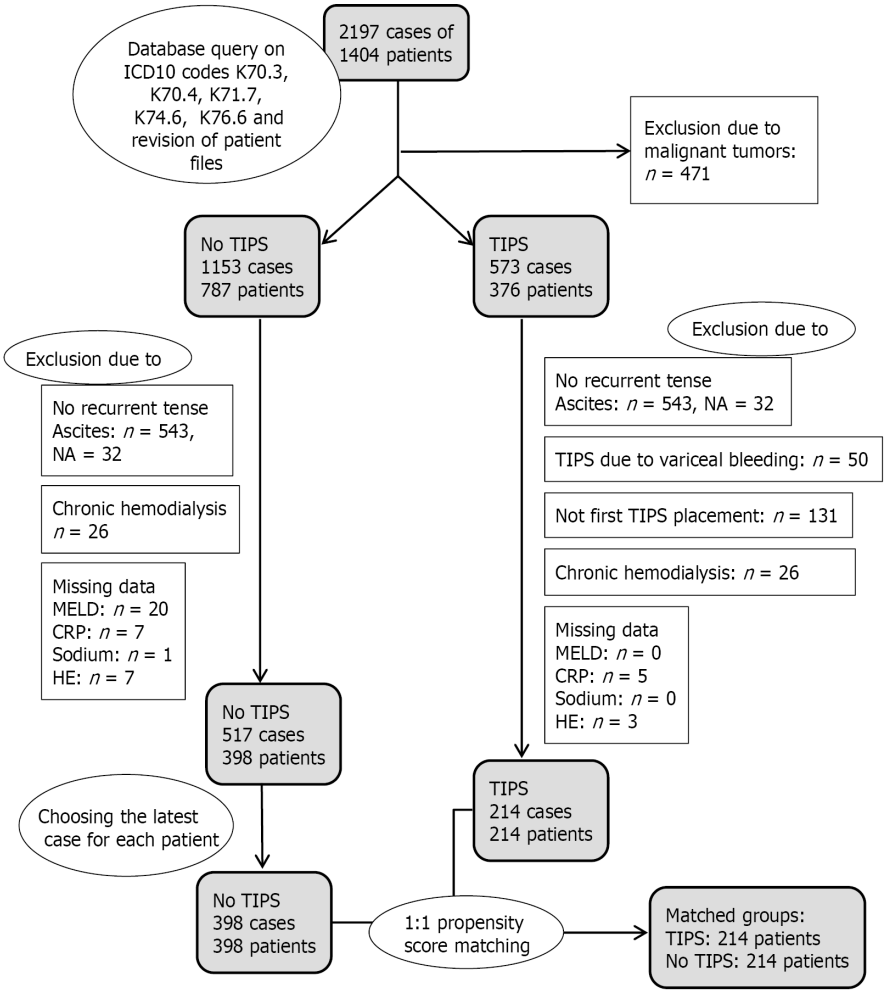
For models C and D death was treated as an increase in acute on chronic liver failure. Models A and B show an effect of only the MELD/Child scores on mortality. Transjugular intrahepatic portosystemic shunt (TIPS) and the interaction of TIPS and MELD/Child scores (MELD: TIPS, Child: TIPS) have no significant influence on mortality (A and B). In model D a significant interaction term Child:TIPS exists. In model C the interaction term MELD: TIPS is not significant, indicating a weaker interaction than in model D. ACLF: Acute on chronic liver failure; TIPS: Transjugular intrahepatic portosystemic shunt.

(Child ≤ 8) an ACLF occurs less frequently in the TIPS group. However, at higher scores (Child ≥ 11), the probability of developing an ACLF is higher in the TIPS group than in the No TIPS group. This interaction blurs the effect of TIPS on ACLF incidence in univariate analyses.

Not all ACLFs in the TIPS group can be attributed to TIPS. The majority of the ACLFs occurred already before TIPS placement and many patients already had at least an ACLF grade 1 on hospital admission. ACLFs grade 1 were almost exclusively due to renal failure. This was to be expected in patients with recurrent tense ascites. Patients whose ACLF increased by 2 or 3 grades during hospital stay had a particularly poor outcome in both groups. A serious deterioration of liver function after TIPS placement is often attributed to TIPS placement. In our patients such events occurred in both groups when we considered the entire hospital stay (No TIPS group 5/214 patients, TIPS group 10/214 patients). Some of the ACLFs after TIPS placement are likely due to other causes than TIPS, such as bacterial infections or gastrointestinal bleeding. Such events precede most ACLFs and can occur with and without TIPS placement[29]. In line with that, TIPS was not a precipitant of ACLF in a recently published study on acute decompensation and ACLF[28]. Furthermore, the majority of pre-TIPS ACLFs resolved after TIPS placement, suggesting that TIPS is more capable to overcome an ACLF than causing it. We have studied patients with recurrent tense ascites. The most common cause of ACLF within this group was kidney failure. It is plausible that a TIPS can improve such an ACLF, *e.g.*, since dose of diuretics can be lowered or diuretics can be discontinued altogether.

We did not include an analysis of the effect of TIPS on ascites resolution since it typically takes up to several months after TIPS placement for the underlying circulatory, renal and neurohumoral dysfunction to normalize[27]. Therefore, the effect of TIPS placement on ascites cannot be reliably assessed during hospital stay.

When interpreting these results, the limitations of a retrospective analysis have to be considered. Since this is a retrospective study, many patients in the No TIPS group lack data on the further course after hospital discharge. For the selected endpoints (highest ACLF during inpatient stay, death during inpatient stay), complete data are available in both groups. Therefore, we had to limit the analysis to inpatient stay. In this study propensity score matching was used prior to comparing the TIPS and No TIPS group. However, even with propensity score matching, a similar distribution of unknown confounders cannot be guaranteed. We only evaluated the short-term outcome during hospital stay. It is well known that the positive impact of a TIPS only takes effect after a few weeks to months[23,27]. In fact, some studies have observed an increased mortality after TIPS placement during the first few weeks



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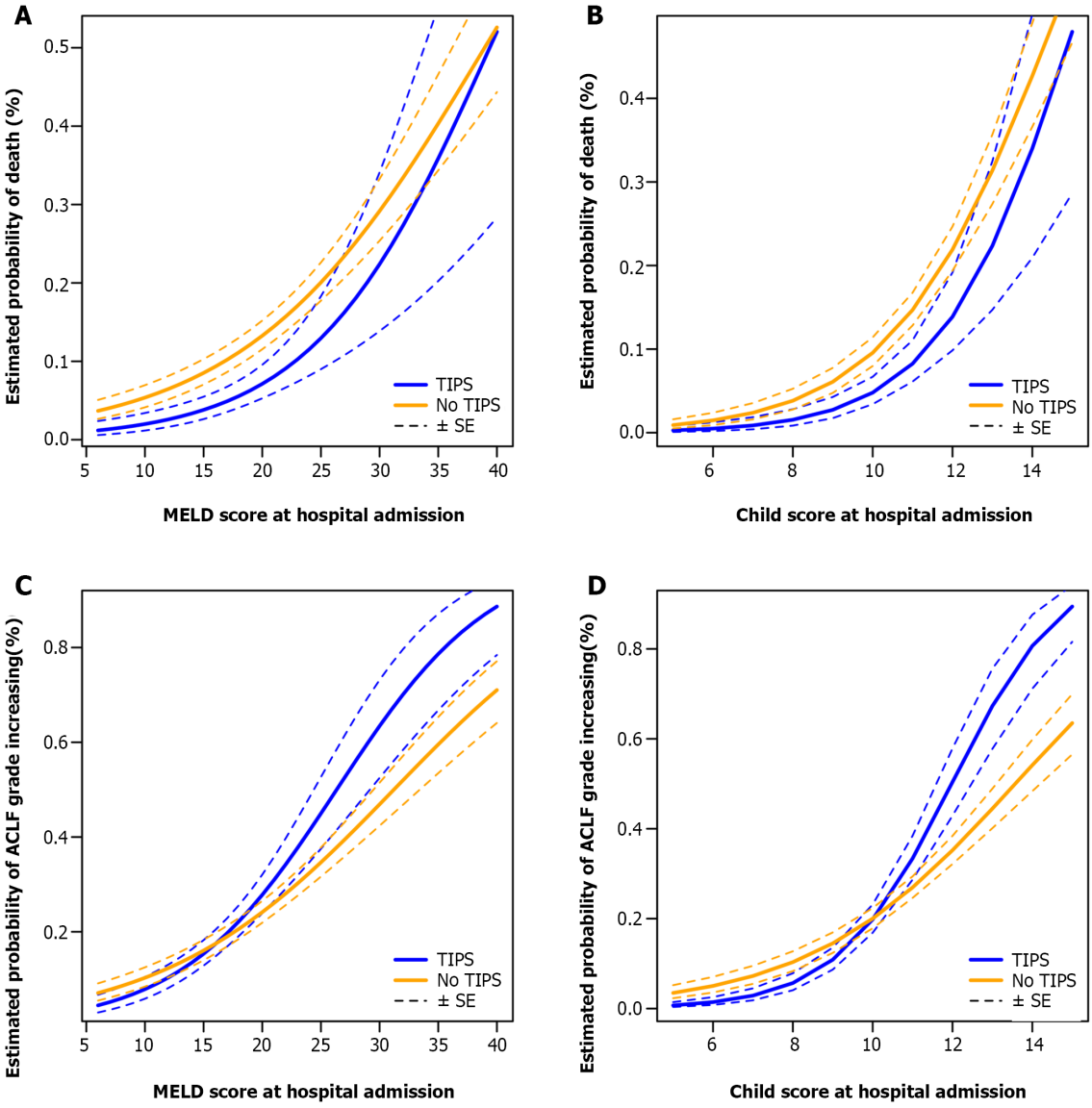
Figure 1 Flow diagram showing the study population and reasons for exclusion from data analysis. HE: Hepatic encephalopathy; NA: Not available; TIPS: Transjugular intrahepatic portosystemic shunt.

[24,30]. Therefore, positive effects of TIPS on survival might be underestimated. On the other hand, our results were confirmed and extended by the multivariate logistic regressions (Table 5). The multivariate logistic regression also provided insight into the complex interactions between liver function and TIPS as seen in Figure 2.

Some ACLFs were already present on admission, some occurred before TIPS, and some ACLFs improved after TIPS. The fact that some patients already had ACLF prior to TIPS complicates the interpretation of the relationship between TIPS and ACLF. As in all retrospective studies, conclusions about the causal relationship between ACLF and TIPS are impossible. Furthermore, we cannot analyze systematically why TIPS was chosen in some patients and not in others. We can only compare the clinical outcome of both groups after very careful propensity score matching.

Our TIPS patients had a comparatively poor liver function, but a bilirubin of 85.5 μmol/L or a MELD of 24 points was rarely exceeded (approx. 8% and 6% of patients). In addition, in patients with very high MELD scores on hospital admission, TIPS placement was performed only after initial stabilization and after MELD had improved. Since the number of observations in our study is limited for this situation, a decision for TIPS placement should be made with caution in such patients. Nevertheless, as shown in Figure 2 and in accordance with other studies the mortality in the TIPS group is not higher than in the No TIPS group even at the highest MELD and Child scores[17,31-33].

Our data show an increased risk of ACLF in the TIPS group in patients with severely impaired liver function (Child ≥ 11 points), but not in patients with good or moderately impaired liver function. These findings may explain why TIPS is often considered a risky intervention with potentially unfavorable outcomes in patients with high MELD or Child scores. Nevertheless, we did not find such a negative effect of TIPS placement on in-hospital mortality in patients with high to very high MELD and Child scores. We found that many ACLFs in the TIPS group occurred before TIPS placement and often resolved after TIPS placement. Unlike several previous RCTs we did not find a positive effect of TIPS on mortality. Possible reasons are the comparatively short follow-up and the significantly worse liver function of our TIPS patients compared to the patients in the RCTs. In the presence of moderately to



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Figure 2 Estimated in-hospital mortality and risk of acute on chronic liver failure depending on liver function. A and B: Estimated probability of dying in hospital depending on liver function at hospital admission; C and D: Estimated probability of acute on chronic liver failure (ACLF) occurring or existing ACLF worsening, depending on liver function at hospital admission. All probabilities were estimated using a multivariate logistic regression model based on the MELD and Child scores at hospital admission. TIPS: Transjugular intrahepatic portosystemic shunt.

severely impair liver function recurrent tense ascites may be a dominant symptom. TIPS is the most effective therapy for recurrent tense ascites. Therefore, we conclude that TIPS is a viable option not only for patients with good liver function but also for patients with high Child scores after carefully weighing the increased risk of ACLF against the expected benefits.

CONCLUSION

TIPS placement for recurrent tense ascites is associated with an increased incidence of ACLF. This effect occurs only in patients with severely impaired liver function (Child score ≥ 11) and does not lead to a higher in-hospital mortality compared with conservative treatment.

ARTICLE HIGHLIGHTS

Research background

Transjugular intrahepatic portosystemic shunt (TIPS) is an effective treatment for recurrent tense ascites.

Acute on chronic liver failure (ACLF) of various severities is a serious complication usually causally attributed to TIPS placement. But the potential of TIPS to improve ACLF grade 1 and 2, which is mostly related to acute kidney injury in these patients, may be underestimated.

Research motivation

TIPS placement for recurrent tense ascites may be beneficial even in patients with severely impaired liver and kidney function. But the exact medical limits need further clarification.

Research objectives

To retrospectively evaluate the in-hospital mortality of patients with recurrent tense ascites and reduced liver function-including severely reduced liver function-undergoing TIPS placement (TIPS group) and to compare these data to a carefully matched cohort with recurrent tense ascites receiving conservative treatment (No TIPS group). To better address the clinical scenario not only the time after TIPS placement but the entire hospital stays was analyzed.

Research methods

Two hundred and twenty-four patients undergoing TIPS placement for recurrent tense ascites were retrospectively compared to an equal number of propensity score matched, conservatively treated patients. Primary objectives were in-hospital mortality and the development or worsening or improvement of ACLF. Additional multivariate logistic regressions were performed as sensitivity analysis and for further insights into effects of liver function, TIPS placement and their interaction on ACLF incidence and in-hospital mortality.

Research results

TIPS placement did not result in an increased in-hospital mortality compared to the matched cohort. ACLF incidence in the TIPS group depended on liver function: At Child-Pugh-Scores < 8 TIPS reduced the risk of ALCF development, at scores of 8 to 10 ACLF risk did not differ between TIPS and No TIPS, and at scores ≥ 11 TIPS increased the risk of ALCF. Many preexisting ACLFs grade 1 resolved after TIPS placement. The relevant prognostic parameters for this need further elucidation. The data point to a biologic interaction of liver function and TIPS placement with regard to the development of ACLF, which needs further evaluation.

Research conclusions

In selected patients with severely impaired liver function TIPS placement does not result in an increased in-hospital mortality compared to conservatively treated patients. TIPS was associated with ALCF only in patients with severely impaired liver function (Child > 11 points).

Research perspectives

The medical limits of TIPS placement for recurrent tense ascites should be evaluated in prospective studies which need to address the indications, contraindications and the associated complex decision making.

FOOTNOTES

Author contributions: Philipp M, Blattmann T, Bienert J, Fischer K, and Hausberg L designed the research study and acquired the data; Philipp M and Lamprecht G analyzed the data and wrote the manuscript; Kröger JC, Heller T, and Weber MA performed transjugular intrahepatic portosystemic shunt placement and critically revised the manuscript; all authors have read and have approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Rostock University Medical Center (Approval No. A2018-0127).

Informed consent statement: The requirement for informed consent was waived by the Institutional Review Board considering the retrospective design of the study. Nevertheless, informed consent was obtained from all available patients.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: De-identified data and statistical code used in this study are available from the corresponding author upon reasonable request.

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ORIGINAL ARTICLE

Retrospective Study

Feasibility of same-day discharge following endoscopic submucosal dissection for esophageal or gastric early cancer

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Abstract

BACKGROUND

Endoscopic submucosal dissection (ESD) is an established technique for the treatment of early gastrointestinal neoplasia. Generally, multi-day (M-D) admission is required for patients undergoing ESD due to potential complications.

AIM

To evaluate the feasibility of a same-day (S-D) discharge strategy for ESD of the esophagus or stomach.

METHODS

The data of patients who underwent esophageal or gastric ESD were retrospectively collected from January 2018 to December 2021 at Peking University Cancer Hospital. The propensity score matching (PSM) method was applied to balance the unevenly distributed patient baseline characteristics between the S-D and M-D groups. Intraoperative and postoperative parameters were compared between the matched groups.

RESULTS

Among the 479 patients reviewed, 470 patients, including 91 in the S-D group and 379 in the M-D group, fulfilled the inclusion and exclusion criteria. Following

PSM, 78 patients in each group were paired using the 1:1 nearest available score match algorithm. No significant difference was found between groups with respect to intraoperative and postprocedural major adverse events (AEs). Tumor size, complete resection rate, and procedural duration were comparable between the groups. The S-D group demonstrated a significantly shorter length of hospital stay ($P < 0.001$) and lower overall medical expenses ($P < 0.001$) compared with the M-D group.

CONCLUSION

The S-D discharge strategy may be feasible and effective for esophagogastric ESD, and the procedural-related AEs can be managed successfully.

Key Words: Endoscopic submucosal dissection; Early esophageal cancer; Early gastric cancer; Same-day surgery; Adverse event

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Core Tip: Generally, multi-day (M-D) admission is required for patients with early gastrointestinal neoplasia undergoing endoscopic submucosal dissection (ESD) due to potential complications. We evaluated the feasibility of a same-day (S-D) discharge strategy for ESD of the esophagus or stomach. No significant difference was found between the S-D and M-D groups with respect to intraoperative and postprocedural major adverse events. However, the S-D group demonstrated a significantly shorter length of hospital stay ($P < 0.001$) and lower overall medical expenses ($P < 0.001$) compared to the M-D group. The S-D discharge strategy may be feasible and effective for esophagogastric ESD.

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INTRODUCTION

Endoscopic submucosal dissection (ESD) has been advocated as an effective treatment approach for early esophageal cancer and early gastric cancer[1-3]. ESD is safer, more cost-effective, has greater efficacy, and has a positive impact on health-related quality of life compared with surgery[4,5]. As ESD is associated with complications, including intraprocedural perforation rates between 2.2% and 4.5%[6-8] and postprocedural bleeding rates between 1% and 5.1%[6-9], a multi-day (M-D) hospital admission of 5 d to 7 d is generally required in daily practice[10]. Reducing the length of hospital stay can decrease medical expenses, and some studies have attempted to shorten the duration of postprocedural hospitalization after esophageal[11], gastric[12], and colorectal[13] ESD. However, data on the feasibility of same-day (S-D) discharge after esophagogastric ESD remain limited. Based on our previous studies with relatively low complications in ESD[14-16], our department has applied the S-D strategy to selected patients since 2020. In this study, we describe our preliminary experience with the S-D discharge strategy following ESD of the esophagus or stomach compared with conventional M-D hospital admission.

MATERIALS AND METHODS

Patients

We retrospectively reviewed clinical data from a prospectively maintained database of ESD for consecutive patients at Peking University Cancer Hospital between January 2018 and December 2021. The inclusion criteria were receipt of esophageal or gastric ESD and malignant final diagnosis. The exclusion criteria were receipt of laparoscopic endoscopic collaborative surgery, recurrent lesions, multiple lesions, or a history of esophagectomy or gastrectomy. The present study was approved by the Ethics Committee of Peking University Cancer Hospital (2022KT13) in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients or their families.

Outcome measures

According to the length of hospitalization, patients were divided into S-D and M-D groups. Patients in the S-D group were admitted to the ambulatory care unit on the morning of the ESD procedure day, whereas patients in the M-D group were admitted to the hospital ward the day before ESD. After the ESD procedure, patients in the S-D group were discharged from the ambulatory care unit on the S-D, while the M-D group patients returned to the hospital ward for at least one night before discharge. Patients in the S-D group were informed that they might be transferred to a hospital ward for hospital stay after the procedure if there was an intraprocedural perforation, unsatisfactory postanesthesia recovery, or other serious unexpected adverse events (AEs). Concerning patients who received antithrombotic therapy, after consultation with a cardiologist, agents were discontinued 5-7 d before ESD and resumed on day 7 after the procedure.

The following demographic and clinical information were collected: Age, sex, American Society of Anesthesiologists (ASA) physical status classification, comorbidities, history of antithrombic agent use, duration of ESD procedure, length of hospital stay, cost of hospitalization, pathological evaluation of specimen, and AEs during or after the procedure.

In this study, the primary endpoint was the presence of ESD-related major AEs (MAEs) within 30 d of the procedure. MAEs included bleeding and perforation. Bleeding was defined as active or oozing bleeding of the ESD wound requiring hemostasis during scheduled second-look endoscopy (SSLE), with or without a decrease in hemoglobin level of ≥ 2 g/dL. Perforation was defined as a muscle layer defect, allowing the observation of mesenteric fat or intraabdominal space during the procedure or free air found on a radiograph in symptomatic patients after the ESD procedure. AEs were categorized as intraprocedural and postprocedural according to the time point in which they emerged.

The secondary endpoints were the rates of *en bloc* resection and complete resection, length of hospital stay, and medical expenses. The tumor location was divided into the esophagus, and the upper, middle, and lower stomach. The upper stomach consists of the cardia and upper part of the gastric body, the middle stomach consists of the angle and middle body, and the lower stomach consists of the pylorus, antrum, and lower body. The macroscopic classification was divided into elevated (0-I), flat (0-II), and depressed (0-III) types according to the Paris classification of superficial neoplastic lesions in the digestive tract[17]. *En bloc* resection was defined as resection of the lesion in a single piece, and complete resection was defined as resection of a tumor without histological evidence of tumor cell involvement on the lateral and vertical resection margins[18].

ESD procedures and postoperative management

All ESD patients in our department followed the M-D strategy before 2020. Patients with an estimated specimen size < 4 cm or who lived nearby were selected as S-D strategy candidates since 2020; they were assigned to the S-D or M-D group based on the anesthesiologist's recommendation and the patient's intention after full consultation. All ESD procedures were performed under general anesthesia with tracheal intubation and propofol administration. A single-channel upper gastrointestinal endoscope (GIF Q260J; Olympus Co., Tokyo, Japan) was used in all ESD procedures. A premixed sterilized solution of glycerol (10% glycerol and 5% fructose; Cisen Pharmaceutical, Co., Ltd., Shandong, China) with indigo carmine was injected into the submucosal layer. A single-use electrosurgical knife with water injection function (Micro-Tech Co., Nanjing, China) was used for lesion marking, incision, and dissection with an electrosurgical unit (VIO 200S; ERBE Elektromedizin GmbH, Tübingen, Germany). The ENDO CUT Q mode (parameter setting effect 3, cutting duration 2, and cutting interval 4) was applied for both mucosal incision and submucosal dissection. Hemostasis was achieved with the FORCED COAG E2 mode, and the power was set to 40 W in the esophagus and 50 W in the stomach. If perforation occurred during the procedure, suturing was performed using hemoclips (Micro-Tech Co.). All ESD wounds were sprayed with porcine fibrin sealant (5 mL kit; Guangzhou Bioseal Biotechnology Co., Ltd., Guangzhou, China) after the lesions were resected.

All ESD procedures were performed by the same endoscopist. For patients without intraprocedural perforation, nasogastric tube was not placed. For all patients, water drinking was initiated 2 h after anesthesia recovery. All patients also underwent an SSLE the next day to identify possible bleeding, even if they had been discharged on the same day of ESD. If no bleeding was discovered in the SSLE, oral enteral nutritional suspension was prescribed for 1 wk, followed by soft diet for 1 wk before the full diet resumption. For patients with intraprocedural perforation or postprocedural bleeding, the oral diet was postponed depending on recovery. For all patients, proton pump inhibitor therapy (standard dosing) was administered intravenously until the patient was discharged, followed by oral administration for 4 wk. Follow-up consisted of telephonic contact, and AEs reported after discharge were recorded by a physician associate.

Statistical analyses

To minimize the effect of selection bias, the propensity score matching (PSM) method was applied to balance the unevenly distributed patient baseline characteristics in this non-randomized trial. Individual propensity scores were generated through a logistic regression model that included the following covariates: Age, sex, ASA physical status, comorbidities, use of antithrombotic agent, tumor location,

macroscopic appearance, tumor differentiation, depth of invasion, and specimen size. Subsequently, patients in the S-D and M-D groups were paired using a 1:1 nearest available score match algorithm with a match tolerance of 0.02.

Further statistical analyses were conducted to compare the differences between the two groups based on the matched data. Quantitative data with normal distribution are presented as the means ± SD, and categorical data are presented as frequencies. Differences between groups were examined using the student's *t*-test, χ^2 test, or Fisher's exact test where appropriate. Logistic regression was used to identify the risk factors for AEs. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using SPSS statistical software (version 22.0; IBM Corp., Armonk, NY, United States).

RESULTS

Patient characteristics

A total of 479 patients who underwent 482 esophageal or gastric ESD procedures were reviewed retrospectively (Figure 1). Among these, 3 patients who underwent laparoscopic endoscopic collaborative surgery, 3 patients with multiple lesions, 2 patients with history of esophagectomy or gastrectomy, and 1 patient with a recurrent lesion were excluded. Therefore, 470 patients were enrolled in the study. The clinicopathological characteristics of the patients are presented in Table 1. Before PSM, there were 91 patients in the S-D group and 379 patients in the M-D group. There were significant differences in ASA score ($P = 0.039$), tumor differentiation ($P = 0.004$), depth of invasion ($P = 0.022$), and specimen size ($P < 0.001$) between the two groups. After PSM, there were 78 patients in each group, and all baseline parameters were balanced between the two groups.

Clinical outcomes of ESD

As shown in Table 2, after PSM, no significant difference was found between the groups across pathological parameters including tumor size, rate of free vertical margin, and complete resection. As shown in Table 3, the ESD procedural time was comparable between the two groups after PSM (60.5 ± 34.9 min in the S-D group *vs* 65.8 ± 43.0 min in the M-D group; $P = 0.397$). In addition, the duration of hospitalization was significantly shorter in the S-D group than in the M-D group (1 d *vs* 4.9 ± 2.5 d, respectively; $P < 0.001$). In this study, the total medical expense was determined by categorizing the costs of the procedure, medical devices, medication, diagnostic tests, and administration. The overall medical expenses and the subitem costs were lower in the S-D group.

Safety of the ESD procedure

Thirty-five MAEs occurred in 35 (7.4%) patients, including 14 intraprocedural perforations (1 in the esophagus), 18 cases of oozing bleeding (1 in the esophagus) without hemoglobin decreased, and 3 cases of active bleeding (all in the stomach) with hemoglobin decreased 2 g/dL to 2.5 g/dL during SSLE. All MAEs were managed endoscopically. There was no recurrent bleeding that occurred after SSLE, and no rehospitalization was needed within 7 d of discharge in either group. Both before and after PSM, no significant differences were found between the groups with respect to intraprocedural and postprocedural MAEs (Table 4). Factors associated with postprocedural bleeding and intraprocedural perforation were also investigated. Following multivariate analysis, lesions located in the middle and lower thirds of the stomach were significantly associated with postprocedural bleeding (odds ratio: 5.3, 95% confidence interval: 1.3-22.2; $P = 0.023$) (Table 5), whereas no risk factor was identified for intraoperative perforation.

DISCUSSION

In China, ESD has developed rapidly over the recent years due to the popularization of digestive endoscopic screening and the improved detection rate of early neoplastic lesions. Generally, an M-D admission is required for patients undergoing ESD because of the known potential complications[10]. Based on our previous experience, the risk of AEs is relatively low and generally can be managed conservatively or endoscopically in esophagogastric ESD, with reported intraprocedural perforation rates being between 1.9% and 2.6% and postprocedural bleeding rates between 1.4% and 8.7%[14-16]. Our department has performed the S-D discharge strategy since 2020, and this study demonstrates the feasibility and efficacy of S-D discharge procedures in selected esophagogastric ESD patients.

It was gratifying that we did not find any significant differences in the incidence of MAEs between the groups both before and after PSM in this study. Postprocedural bleeding is the most common complication in upper gastrointestinal ESD, with a reported incidence of 1% in the esophagus and 5.1% in the stomach[6-9]. Tumor in the lower third of stomach is an independent risk factor for post-ESD bleeding[19], and active antral peristalsis as well as bile reflux might lead to a higher incidence of post-ESD bleeding[8]. In our series, a slightly higher incidence of postprocedural bleeding (6.0%) was noted

Table 1 Comparison of clinicopathological features of the same-day discharge and multi-day discharge groups

Characteristics	Overall sample, *n* = 470		SMD value	*P* value	Matched sample, *n* = 156		SMD value	*P* value
	S-D, *n* = 91	M-D, *n* = 379			S-D, *n* = 78	M-D, *n* = 78		
Age in yr			-0.052	0.657			0.025	0.402
≤ 60	33 (36.3)	147 (38.8)			30 (38.5)	25 (32.1)		
> 60	58 (63.7)	232 (61.2)			48 (61.5)	53 (67.9)		
Sex			0.069	0.550			-0.057	1.000
Female	21 (23.1)	99 (26.1)			16 (20.5)	16 (20.5)		
Male	70 (76.9)	280 (73.9)			62 (79.5)	62 (79.5)		
ASA physical status			0.224	0.039			0.000	1.000
≤ 2	89 (97.8)	347 (91.6)			76 (97.4)	75 (96.2)		
≥ 2	2 (2.2)	32 (8.4)			2 (2.6)	3 (3.8)		
Comorbidities			-0.087	0.457			-0.050	0.423
No	45 (49.5)	171 (45.1)			37 (47.4)	42 (53.8)		
Yes	46 (50.5)	208 (54.9)			41 (52.6)	36 (46.2)		
Antithrombotic agents use			-0.164	0.141			-0.041	1.000
Yes	5 (5.5)	40 (10.6)			4 (5.1)	5 (6.4)		
No	86 (94.5)	339 (89.4)			74 (94.9)	73 (93.6)		
Location of lesion			-0.197	0.168			0.176	0.357
Esophagus	23 (25.3)	115 (30.3)			22 (28.2)	27 (34.6)		
Upper 1/3 of the stomach	18 (19.8)	97 (25.6)			14 (17.9)	20 (25.6)		
Middle 1/3 of the stomach	22 (24.2)	89 (23.5)			20 (25.6)	15 (19.2)		
Lower 1/3 of the stomach	28 (30.7)	78 (20.6)			22 (28.3)	16 (20.6)		
Macroscopic appearance, type			0.184	0.092			0.000	1.000
0-II	89 (97.8)	383 (93.1)			76 (97.4)	75 (96.2)		
0-I and 0-III	2 (2.2)	26 (6.9)			2 (2.6)	3 (3.8)		
Tumor differentiation			0.316	0.004			-0.064	0.442
Differentiated	85 (93.4)	307 (81.0)			73 (93.6)	76 (97.4)		
Undifferentiated	6 (6.6)	72 (19.0)			5 (6.4)	2 (2.6)		
Depth of invasion			0.258	0.022			0.030	1.000
Intramucosal	81 (89.0)	297 (78.4)			69 (88.5)	69 (88.5)		
Submucosal	10 (11.0)	82 (21.6)			9 (11.5)	9 (11.5)		
Specimen size in mm, mean ± SD	31.2 ± 12.3	43.9 ± 17.0	0.749	0.000	33.3 ± 11.9	33.6 ± 15.5	0.031	0.913

ASA: American Society of Anesthesiologists; M-D: Multi-day; S-D: Same-day; SD: Standard deviation; SMD: Standardized mean difference.

in the stomach, whereas mid- to lower location in the stomach was identified as the only risk factor for postprocedural bleeding, suggesting that we should not only pay attention to the lesions in the antrum but also those in the angle and gastric body to minimize the risk of postprocedural bleeding. In addition, male sex, antithrombotic drugs, tumor size > 20 mm, resected specimen size ≥ 40 mm, and flat/depressed lesion types are also risk factors for postprocedural bleeding[9,20], but none were identified in our study, possibly because we expanded the definition of postprocedural bleeding. We not only included patients with massive bleeding but also patients with active or oozing bleeding that necessitated hemostasis during SSLE without an overt hemoglobin decrease, which might maximize the safety of the S-D strategy in patients.

Although routine use of SSLE is not advocated as it does not reduce the risk of delayed bleeding[21,22], this technique has been carried out in many studies[23-25]. The purpose of SSLE in our study was to detect oozing and active bleeding and perform hemostasis. We did not perform prophylactic

Table 2 Pathological characteristics of the specimen in the same-day discharge and multi-day discharge groups

Characteristics	Matched sample, *n* = 156		*P* value
	S-D, *n* = 78	M-D, *n* = 78	
Tumor size in mm	17.8 ± 11.6	17.3 ± 10.4	0.778
En bloc resection	77 (98.7)	76 (94.7)	1.000
Free horizontal margin	78 (100.0)	77 (98.7)	1.000
Free vertical margin	76 (97.4)	76 (97.4)	1.000
Complete resection	75 (96.2)	73 (93.6)	0.719

Table 3 Comparison of procedural time, hospitalization, and cost in the same-day discharge and multi-day discharge groups

Characteristics	Matched sample, *n* = 156		*P* value
	S-D, *n* = 78	M-D, *n* = 78	
Procedure time in min	60.5 ± 34.9	65.8 ± 43.0	0.397
Hospitalization in d			
Total	1	4.6 ± 2.0	0.000
Postprocedural	0	3.0 ± 1.8	0.000
Medical expenses in CNY	25749.0 ± 4389.3	37000.8 ± 8510.7	0.000
Procedure: ESD, anesthesia, other procedures	3616.1 ± 942.8	6079.3 ± 1646.5	0.000
Medical devices	13112.0 ± 1884.5	17956.7 ± 4977.2	0.000
Medication	6390.7 ± 3866.4	7759.9 ± 2241.8	0.008
Diagnostic test: Endoscopy, laboratory, radiology, pathology	3625.5 ± 1133.9	4025.9 ± 1561.5	0.069
Administration: Hospitalization, nursing	260.0 ± 232.7	1178.9 ± 1506.7	0.000

CNY: Chinese Yuan; ESD: Endoscopic submucosal dissection.

Table 4 Major adverse events of same-day discharge and multi-day discharge groups

Characteristics	Overall sample, *n* = 470		*P* value	Matched sample, *n* = 156		*P* value
	S-D, *n* = 91	M-D, *n* = 379		S-D, *n* = 78	M-D, *n* = 78	
Total major adverse events	3 (3.3)	32 (8.4)	0.093	3 (3.8)	6 (7.7)	0.495
Intraprocedural perforation	0	14 (3.7)	0.083	0	3 (3.8)	0.245
Postprocedural bleeding during SSLE	3 (3.3)	18 (4.7)	0.778	3 (3.8)	3 (3.8)	1.000
Oozing bleeding	2 (2.2)	16 (4.2)	0.546	2 (2.6)	3 (3.8)	1.000
Active bleeding	1 (1.1)	2 (0.5)	0.476	1 (1.3)	0	1.000

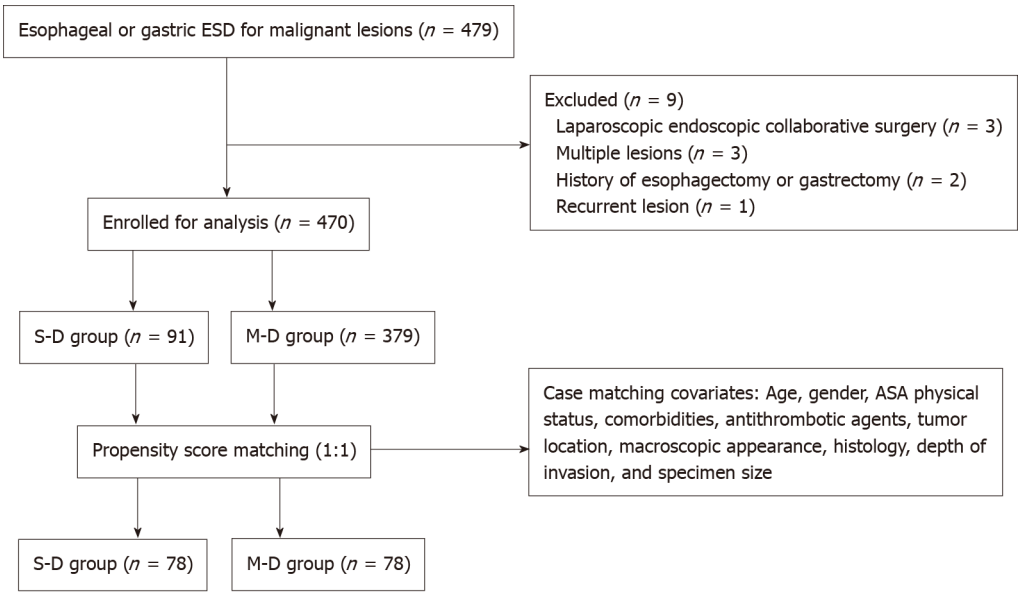
M-D: Multi-day; S-D: Same-day; SSLE: Scheduled second-look endoscopy.

coagulation on nonbleeding visible vessels smaller than 0.3 mm in the post-ESD ulcer. Our previous study showed that a wound microvessel-protective hemostatic technique followed by porcine fibrin sealant can promote ESD-induced ulcer healing without increasing delayed bleeding events[15]. Prophylactic hemostasis-induced tissue damage or necrosis may lead to the exposure of arteries on the base of the ulcer, which in turn would contribute to delayed episodes of bleeding[21]. Although the inconvenience of SSLE might limit benefit of the S-D discharge strategy, it does provide help in the early detection of postprocedural bleeding, especially as a nasogastric tube is not routinely deployed in our department. Meanwhile, a fairly short distance to the hospital would allow for the patients to obtain timely treatments in the case of MAE development[11].

Table 5 Factors affecting endoscopic submucosal dissection procedure-related postprocedural bleeding, *n* = 470

Variable	Total, *n*	PB, *n* (%)	OR (95%CI)	*P* value
Male sex	350	17 (4.9)	3.0 (0.8-11.1)	0.105
Age ≤ 60 yr	180	10 (5.6)	1.5 (0.6-4.2)	0.392
ASA score ≤ 2	436	19 (4.4)	2.7 (0.2-38.3)	0.457
ATA usage	45	2 (4.4)	1.7 (0.2-12.7)	0.610
Multi-day discharge group	379	17 (4.5)	1.9 (0.5-7.4)	0.361
Non-flat appearance	28	2 (7.1)	1.4 (0.3-7.6)	0.710
Located in the lower 1/3 of the stomach	106	11 (10.4)	2.1 (0.7-6.2)	0.163
Located in the lower 2/3 of the stomach	211	17 (7.7)	5.3 (1.3-22.2)	0.023
Differentiated type	392	17 (4.3)	1.2 (0.3-5.2)	0.799
Submucosal invasion	92	4 (4.3)	1.3 (0.3-4.7)	0.723
Lesion ≥ 2 cm	236	11 (4.7)	1.2 (0.4-3.8)	0.818
Specimen ≥ 4 cm	234	10 (4.3)	1.1 (0.3-3.7)	0.879

ASA: American Society of Anesthesiologists; ATA: Antithrombotic agents; CI: Confidence interval; OR: Odds ratio; PB: Postprocedural bleeding.



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Figure 1 Flow chart of patient enrollment for this study. A total of 479 patients received endoscopic submucosal dissection for the esophagus or stomach, and 470 cases met the inclusion and exclusion criteria. After propensity score matching, there were 78 patients in each group for further analysis. ESD: Endoscopic submucosal dissection; ASA: American Society of Anesthesiologists; M-D: Multi-day; S-D: Same-day.

As a relatively rare complication, intraprocedural perforation can be treated endoscopically in most cases, with a reported incidence of 2.2% in the esophagus and 4.5% in the stomach[6-8]. Larger tumor size (> 2 cm) and longer procedure time (> 2 h) are risk factors for perforation[26,27]. In this study, the rate of intraoperative perforation was 0.7% in the esophagus and 3.9% in the stomach. All perforations were sutured by hemoclips successfully, with no delayed perforation occurring. To avoid intraoperative perforation, it is important to obtain a good intraoperative field of view and to reliably discern the muscularis propria. The traction method is useful in many such cases[28], but we did not perform it routinely in our procedure. Greater experience and more delicate operation techniques might also reduce the risk of intraoperative perforation.

Achieving tumor-free margins is essential for the efficacy of ESD in early gastrointestinal malignancies. In this study, we obtained a similar complete resection rate of 96.2% and 93.6% in the S-D and M-D groups, respectively, which are comparable with previous studies[7,29,30]. Larger specimen sizes correlate with longer procedural duration[11], which is an independent risk factor for pulmonary

risk during anesthesia[31], and specimen size ≥ 4 cm is associated with delayed bleeding[20]. So when we started the S-D strategy in 2020, patients with estimated specimens smaller than 4 cm were selected as the S-D discharge candidates to minimize the associated risk above. Tumor differentiation should be noticed in specimen size estimation. In undifferentiated lesions, it is difficult to delineate the cancerous areas and easily obtain a positive lateral margin. Therefore, a further distance from the estimated border is usually needed to establish complete resection[32,33].

ASA physical status classification can reflect the severity of a patient's comorbidities, and those with an ASA score of 1 or 2 could be considered suitable for S-D discharge or outpatient ESD[11,34]. The results of the present study supported this data, as the proportion of patients with ASA score of 1 or 2 in the S-D group was more than that in the M-D group, but those patients experienced a similar profile of MAEs before and after PSM. Although the Charlson Comorbidity Index can provide a more detailed risk evaluation for patients with multiple comorbidities[35,36], the ASA score system is considered easier to apply in clinical settings.

ESD can greatly reduce the medical care costs associated with gastric cancer[37]. In Japan, ESD patients are usually admitted for 5-7 d, and in Europe for 2-4 d following ESD[10]. A reduction of hospitalization stay length or practice in an outpatient setting would minimize the medical expenses further[34]. A benchmark cost estimate for ESD treatment including 4 d of postoperative hospitalization in China is reportedly approximately 5400 United States Dollars[38], which is similar to our M-D group. Labor costs for doctors and nurses remain low in many East Asian countries, whereas medication and medical devices account for most of the total cost of ESD. A significant reduction in total cost could be established if ESD was performed with S-D discharge, as applied in our study. This is very important for Western countries, as their medical expenses increase with length of hospitalization. Using proper selection criteria, S-D discharge ESD could be a cost-effective strategy for esophagogastric early malignancies.

Our study had several limitations. First, all of the procedures were performed by a single skilled endoscopist with 14 years' experience in gastrointestinal ESD, and our experience reflected that of a high-volume center with a specialized endoscopist to perform ESD. Thus, our results might not be applicable to other centers. A further investigation involving more endoscopists, with varying degree of experience, from more centers, with diverse structure, is being designed and planned, and we hope to provide more conclusive findings in the future. Second, as a retrospective study, selection bias could not be ignored, although the PSM method was used to balance the characteristics of the patients in both groups. As an oncology-specific territory center, we lack specific experience in handling complex comorbidities. Most of the included patients had an ASA score of 1 or 2. Thus, we cannot generalize these results to patients with ASA scores of 3 or more. Third, we had implemented a S-D discharge policy for only 2 years. Due to a relatively small patient number, we were unable to identify a more detailed selection criterion other than an estimated specimen size of less than 4 cm for S-D discharge ESD to avoid potential complications during and after the procedure, and further investigation is needed.

CONCLUSION

In conclusion, this study, the first retrospective propensity score-matched study evaluating S-D discharge procedures for esophagogastric ESD in China, demonstrates that this strategy may be feasible and effective, and that the AEs related to ESD could be managed successfully. Additional prospective studies are warranted to establish more detailed standards to select patients for S-D discharge ESD.

ARTICLE HIGHLIGHTS

Research background

Endoscopic submucosal dissection (ESD) is an established technique for the treatment of early gastrointestinal neoplasia. Generally, a multi-day (M-D) admission is required for patients undergoing ESD due to potential complications. This retrospective study demonstrates that the same-day (S-D) discharge procedures for esophagogastric ESD may be feasible and effective.

Research motivation

ESD is safer, more cost-effective, has greater efficacy, and exhibits a positive impact on health-related quality of life in comparison with surgery. Reducing the length of hospital stay can decrease medical expenses, and some studies have attempted to shorten the duration of postprocedural hospitalization after esophageal, gastric, and colorectal ESD. However, data on the feasibility of S-D discharge after esophagogastric ESD remain limited.

Research objectives

In this study, we describe our preliminary experience with the S-D discharge strategy following ESD of the esophagus or stomach compared with conventional M-D hospital admission.

Research methods

To minimize the effect of selection bias, the propensity score matching method was applied to balance the unevenly distributed patient baseline characteristics in this non-randomized trial. Subsequently, patients in the S-D and M-D groups were paired using the 1:1 nearest available score match algorithm with a match tolerance of 0.02. Further statistical analyses were conducted to compare the differences between the two groups based on the matched data.

Research results

No significant difference was found between the groups with respect to intraoperative and postprocedural major adverse events (AEs). The tumor size, complete resection rate, and procedural duration were comparable between the groups. The S-D group demonstrated a significantly shorter length of hospital stay ($P < 0.001$) and lower overall medical expenses ($P < 0.001$) compared to the M-D group.

Research conclusions

This is the first retrospective study evaluating S-D discharge procedures for esophagogastric ESD in China. The result demonstrated the S-D discharge strategy may be feasible and effective for esophagogastric ESD, and the procedural-related AEs can be managed successfully.

Research perspectives

This first retrospective study evaluating S-D discharge procedures for esophagogastric ESD in China demonstrates that this strategy may be feasible and effective, and that the AEs related to ESD could be managed successfully. Additional prospective studies are warranted to establish more detailed standards to select patients for S-D discharge ESD.

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FOOTNOTES

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Data sharing statement: Contact wangjing_pku@bjmu.edu.cn to obtain the anonymized dataset.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic analysis of patients with combined hepatocellular-cholangiocarcinoma after radical resection: A retrospective multicenter cohort study

Ge Zhang, Bo-Wen Chen, Xiao-Bo Yang, Huai-Yuan Wang, Xu Yang, Fu-Cun Xie, Xiang-Qi Chen, Ling-Xiang Yu, Jie Shi, Yin-Ying Lu, Hai-Tao Zhao

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Abstract

BACKGROUND

Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a form of rare primary liver cancer that combines intrahepatic cholangiocarcinoma (ICC) and hepatocellular carcinoma.

AIM

To investigate overall survival (OS) and recurrence-free survival (RFS) after radical resection in patients with cHCC-CCA, and the clinicopathological factors affecting prognosis in two center hospitals of China.

METHODS

We reviewed consecutive patients with cHCC-CCA who received radical rese-

ction between January 2005 and September 2021 at Peking Union Medical College and the 5th Medical Center of the PLA General Hospital retrospectively. Regular follow-up and clinicopathological characteristics were systematic collected for baseline and prognostic analysis.

RESULTS

Our study included 95 patients who received radical resection. The majority of these patients were male and 82.7% of these patients were infected with HBV. The mean tumor size was 4.5 cm, and approximately 40% of patients had more than one lesion. The median OS was 26.8 (95%CI: 18.5-43.0) mo, and the median RFS was 7.27 (95%CI: 5.83-10.3) mo. Independent predictors of OS were CA19-9 ≥ 37 U/mL (HR = 8.68, P = 0.002), Child-Pugh score > 5 (HR = 5.52, P = 0.027), tumor number > 1 (HR = 30.85, P = 0.002), tumor size and transarterial chemoembolization (TACE) after surgery (HR = 0.2, P = 0.005).

CONCLUSION

The overall postoperative survival of cHCC-CCA patients is poor, and most patients experience relapse within a short period of time after surgery. Preoperative tumor biomarker (CA19-9, alpha-fetoprotein) levels, tumor size, and Child-Pugh score can significantly affect OS. Adjuvant TACE after surgery prolongs RFS, suggesting that TACE is a possible option for postoperative adjuvant therapy in patients with cHCC-CCA.

Key Words: Combined hepatocellular-cholangiocarcinoma; Radical resection; Clinicopathological factor; Integrated nomogram; Multicenter cohort

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Core Tip: Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a relatively rare type of primary liver cancer. Hepatectomy combined with lymph node dissection is the only possible cure. In our study, we found that the prognosis for this group of patients is poor, with a 2-year survival rate of approximately 50% after radical resection. Preoperative CA19-9 Level, tumor number, tumor size and whether or not to receive tumor size and transarterial chemoembolization (TACE) after surgery were independent factors affecting overall survival. Therefore, we recommend that patients with cHCC-CCA actively receive adjuvant TACE therapy after surgery.

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INTRODUCTION

Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a relatively rare primary liver cancer (PLC) and accounts for 0.4% to 14.2% of the incidence of PLC[1-4]. The definition of cHCC-CCA has been updated because of unclear understanding. In 2019, the WHO updated the cHCC-CCA classification[5], and in conventional histopathology of hematoxylin and eosin (H&E) staining, cHCC-CCA shows two different degrees of differentiation, hepatocellular and cholangiocarcinoma, within the same lesion. In contrast to the well-established management pathways for hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC), treatment remains a gray area for cHCC-CCA currently. The overall prognosis of patients with cHCC-CCA is worse than that of patients with HCC, and the prognosis is similar to that of patients with ICC. Vascular invasion actually seems to occur more frequently in cHCC-CCA than in HCC. In addition, lymph node metastases exhibit similar characteristics[6]. The treatment of cHCC-CCA has not been standardized in comparison to HCC and ICC, and a number of therapy strategies have been suggested. Radical tumor resection and lymph node dissection are the only curative options for patients with cHCC-CCA[7,8]. Nonetheless, the 5-year survival rate does not reach 30%, and the tumor recurrence rate is considerable (up to 80% after 5 years) in most studies[9-11].

In our research, we retrospectively analyzed cHCC-CCA patients who received surgical resection at two institutions to explore clinical case information for this rare tumor on prognosis, looking for factors affecting recurrence and long-term survival. All patients underwent rigorous organizational path-

ological confirmation to ensure cohort consistency.

MATERIALS AND METHODS

Patients

Among the patients who received hepatectomy for PLC in Peking Union Medical College Hospital and The 5th Medical Center of the PLA General Hospital from January 2005 to September 2021, 95 patients were pathologically diagnosed with cHCC-CCA based on the latest WHO criteria in 2019. Among these patients, 61 were treated in Peking Union Medical College Hospital, and 34 were treated in The 5th Medical Center of the PLA General Hospital. The inclusion criteria for these patients are described below: (1) Patients who received radical liver resection; (2) patients were pathologically diagnosed with cHCC-CCA; and (3) patients with complete clinical information and at least 2 follow-up visits after surgery. The exclusion criteria are described below: (1) Non-radical resection; (2) separated HCC and ICC; (3) incomplete clinical information, or irregular follow-up after surgery; and (4) history of other malignancies.

Based on regular medical records and telephone follow-up records, we determined how these patients were treated after surgery, whether they survived, and whether they experienced recurrence. Two patients had HCC and ICC at the same time, but the growth was dissociative, so they were excluded. Due to lost follow-up or too short follow-up time, another three patients were only used for baseline information statistics and not for prognosis analysis (Figure 1).

The study was approved by the Ethics Committee of Peking Union Medical College Hospital (Reg. numbers JS-3390) and The 5th Medical Center of the PLA General Hospital (Reg. number KY-2022-4-23-1), and the study protocol conforms to the ethical guidelines of the Declaration of Helsinki. All participants signed written informed consent.

Data collection

Through a search of the patients' medical records, we collected the following clinical information: Age, sex, background of liver disease, Eastern Cooperative Oncology Group (ECOG) score, gallstones, CA19-9 Level, alpha-fetoprotein (AFP) level, carcinoembryonic antigen (CEA) level, total bilirubin (TBil) level, direct bilirubin (DBil) level, albumin, ascites, and cirrhosis before surgery. The preserved liver functional was evaluated using the Child-Pugh (C-P) scoring system[12].

By reviewing the radiological reports, pathology reports and pathology sections of patients, we collected the following pathological information: tumor size, tumor number, macrovascular invasion (Macro VI), microvascular invasion (Micro VI), lymph node metastasis, distance to section, Ki-67, cytokeratin 7 (CK7), cytokeratin 19 (CK19), Hepatocyte paraffin 1 (HepPar-1), Glypican-3 (GPC-3), HCC differentiation, HCC percent, ICC differentiation, and ICC percent. HepPar-1 and GPC-3 were used as HCC markers, and CK7 and CK19 were used as biliary epithelial markers. Due to the absence of an optimal staging system for cHCC-CCA, we applied the American Joint Committee on Cancer (AJCC) staging manual (8th edition) to cHCC-CCA[13].

Overall survival (OS, defined as the time interval from the date of surgery to death or the last follow-up, depend on which came first) and recurrence-free survival (RFS, defined as the time interval from the date of surgery to recurrence, death, or the last follow-up, depend on which came first) were the primary measures for this study.

Statistical analysis

Normality tests for continuous variables were performed by the Shapiro-Wilk test[14]. Normal continuous variables were compared between patients in the two centers by analysis of variance. To compare nonnormal continuous variables, the Kruskal-Wallis test was utilized[15]. Categorical variable data were compared by Fisher's exact test[16]. Normal continuous variables were shown as the mean ± SD. Nonnormal continuous variables are shown as the median and IQR. Categorical variable data were displayed as numbers and percentages. The survival rate was determined using the Kaplan-Meier method. Univariate and multivariate analysis were performed using the log-rank test and Cox proportional hazards regression model, respectively. To identify independent prognostic factors, variables with P values < 0.15 in univariate analysis were incorporated into the Cox proportional hazards model. A P value with two tails < 0.05 was regarded as statistically significant. All analysis were performed using R 4.1.0.

RESULTS

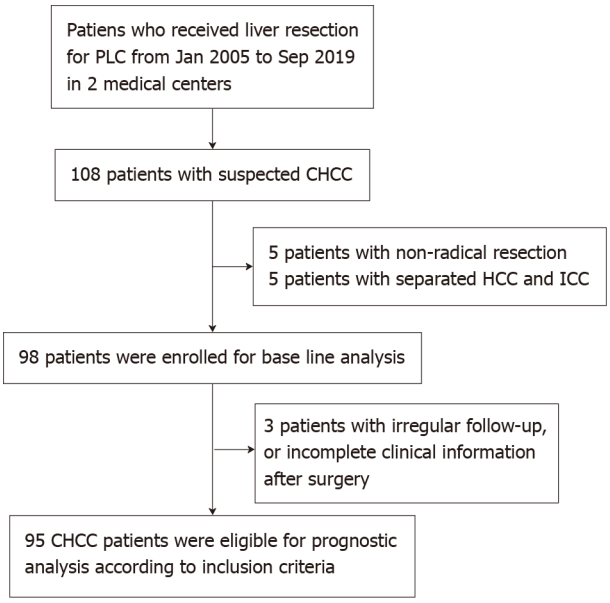
Clinical characteristics of patients

In our research, we analyzed the preoperative clinical data of 98 (95 plus 3) patients (Table 1). Of the 98 patients, 86 (87.8%) were male. The mean age was 55.3 ± 10.4 years. The majority of patients had well-

Table 1 Demographic characteristics of patients with combined hepatocellular-cholangiocarcinoma before radical resection

	Overall	The 5th Medical Center of the PLA General Hospital	Peking Union Medical College Hospital	*P* value
Number	98	34	64	
Age, mean ± SD	55.3 (10.4)	53.5 (10.4)	56.3 (10.3)	0.219
Sex				
Male	86 (87.8)	32 (88.2)	56 (87.5)	1 (Fisher)
Female (%)	12 (12.2)	4 (11.8)	8 (12.5)	
ECOG (%)				0.009 (Fisher)
0	84 (85.7)	26 (76.5)	58 (90.6)	
1	11 (11.2)	8 (23.5)	3 (4.7)	
NA	3 (3.1)	0 (0)	3 (4.7)	
Child-Pugh class				0.435 (Fisher)
A	86 (87.8)	32 (94.1)	54 (84.4)	
B	6 (6.1)	1 (2.9)	5 (7.8)	
NA	6 (6.1)	1 (2.9)	5 (7.8)	
Liver disease (%)				0.823 (Fisher)
NA	4 (4.1)	1 (2.9)	3 (4.7)	
HBV	81 (82.7)	28 (82.4)	53 (82.8)	
HCV	4 (4.1)	2 (5.9)	2 (3.1)	
Fatty liver	2 (2.0)	0 (0.0)	2 (3.1)	
Alcohol	7 (7.1)	3 (8.8)	4 (6.2)	
Gallstones (%)	13 (13.3)	3 (8.8)	10 (15.6)	0.533 (Fisher)
CA19-9 (U/mL)	26.5 [13.1, 56.2]	29.7 [15.1, 46.5]	23.6 [12.4, 56.4]	0.775 (non-norm)
< 37	58 (59.2)	21 (61.8)	37 (57.8)	0.813 (Fisher)
≥ 37	31 (31.6)	11 (32.4)	20 (31.2)	
NA	9 (9.2)	2 (5.9)	7 (10.9)	
AFP (ng/mL)	44.1 [7.0, 338.4]	43.4 [5.8, 294.7]	44.1 [7.8, 724.3]	0.389 (non-norm)
< 200	61 (62.2)	24 (70.6)	37 (57.8)	0.122 (Fisher)
≥ 200	30 (30.6)	10 (29.4)	20 (31.2)	
NA	7 (7.1)	0 (0.0)	7 (10.9)	
CEA (ng/mL)	2.7 [1.6, 4.4]	2.5 [1.5, 3.5]	2.7 [1.7, 4.8]	0.173 (non-norm)
< 6	80 (81.6)	32 (94.1)	48 (75.0)	0.038 (Fisher)
≥ 6	9 (9.2)	0 (0.0)	9 (14.1)	
NA	9 (9.2)	2 (5.9)	7 (10.9)	
TBil (μmol/L)	12.6 [10.4, 16.4]	12.2 [10.4, 14.0]	12.9 [10.7, 17.8]	0.260 (non-norm)
DBil (μmol/L)	4.3 [3.8, 5.7]	4.2 [3.8, 5.0]	4.5 [3.8, 5.8]	0.334 (non-norm)
Albumin (g/L)	41.0 [39.0, 43.5]	40.0 [38.0, 42.0]	41.0 [39.0, 44.0]	0.055 (non-norm)
Ascites (%)				0.094 (Fisher)
No	75 (76.5)	30 (88.2)	45 (70.3)	
Yes	18 (18.4)	4 (11.8)	14 (21.9)	
NA	5 (5.1)	0 (0.0)	5 (7.8)	
Liver cirrhosis (%)	82 (83.7)	32 (94.1)	50 (78.1)	0.143 (Fisher)

ECOG: Eastern Cooperative Oncology Group; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Alpha fetoprotein; CEA: Carcinoembryonic antigen; TBil: Total bilirubin; DBil: Direct bilirubin; NA: Not available.



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Figure 1 Research framework of this study.

preserved liver function (Child-Pugh class A or B), the vast majority had an ECOG score of 0-1 (96.9%), and the majority had HBV infection (82.7%).

Most patients had well-preserved liver function (C-P class A or B), and most (96.9%) had an ECOG score of 0-1. HBV infection was present in 82.7% of the patients. Preoperative level of CA19-9 was higher than normal in 31 patients (31.6%) (≥ 37 U/mL), preoperative level of AFP was higher than normal in 51 patients (52.0%) (20 ng/mL, not listed), of which 30 patients (31.6%) had levels higher than 200 ng/mL, and preoperative CEA levels were higher than normal in 9 patients (9.2%) (≥ 6 ng/mL). Ascites and liver fibrosis were present in 18 patients (18.4%) and 82 patients (83.7%), respectively.

Pathological characteristics of patients

Table 2 demonstrated the pathological features of our two-center cohorts. In more than half (56.1%) of the patients, the number of lesions was more than one. The mean tumor size was 4.5 cm [range (2.9, 6.5)], and 62 patients (63.2%) had tumors smaller than 5 cm. Surgical margin did not exceed 1 cm in more than half (55.1%) of the cases. The proportions of macrovascular and microvascular invasion were 24.5% and 63.3%, respectively. Lymph node metastases were found in 12.2% of these patients. Using the AJCC staging system, we evaluated the TNM stage in 98 patients. 18 (18.3%) patients were stage I (17 IA, 1 IB), 59 (60.2%) patients were stage II, 19 (19.4%) patients were stage III (3 IIIA, 16 IIIB), and 2 patients could not be evaluated.

Survival and recurrence

Ninety-five patients with follow-up longer than 1 mo were used in survival and recurrence analysis. The median follow-up time was 34.2 mo (95%CI: 28.0-43.3), and the median OS was 26.8 mo (95%CI: 18.5-43.0) (Figure 2A). The estimated cumulative survival rates at 1, 2, 3, and 5 years were 73.9%, 51.7%, 38.2%, and 23.6%, respectively. The median RFS was 7.27 mo (95%CI: 5.83-10.3) (Figure 2B), and the estimated cumulative RFS rates at 6 mo, 1 year, and 2 years were 58.4%, 33.6%, and 30.4%, respectively. Most patients experienced relapse within 1 year after surgery. In addition, we further staged the patients using the AJCC Staging Manual (8th edition), and the results the results revealed a substantial difference in the median OS between stage I/II patients and stage III patients.

Prognostic factors of OS

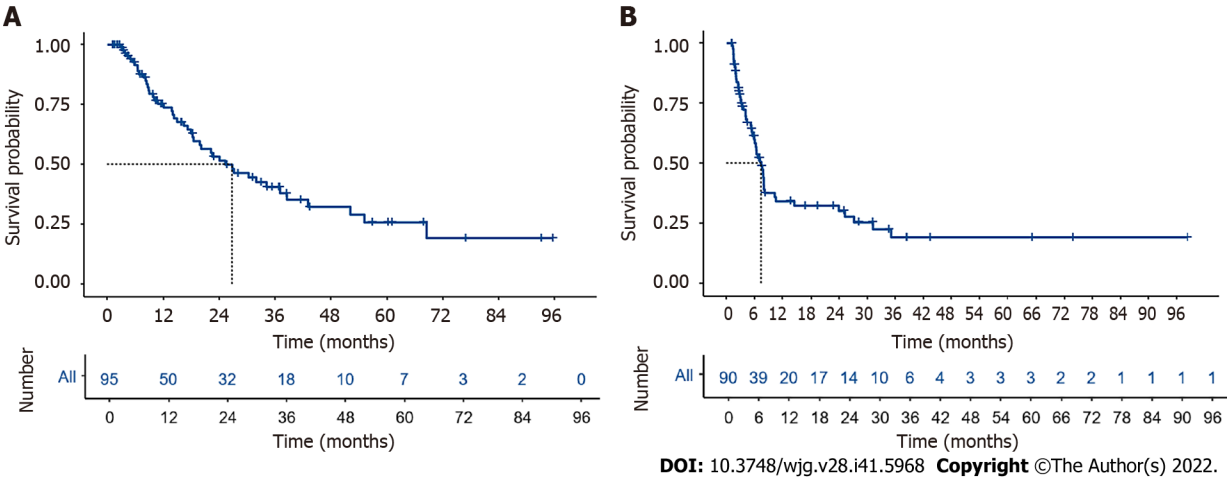
Subgroup analysis showed that preoperative liver function grading (C-P score 5 *vs* > 5) remarkably affected prognosis, and patients with a preoperative C-P score of 5 had a significantly better survive than those with a preoperative C-P score greater than 5 (Figure 3A). The median OS was considerably lower for patients with baseline CA19-9 Levels over 37 U/mL than it was for those with levels below 37 U/mL (Figure 3B); however, subgrouping for AFP levels did not yield similar results (Supplemen-

Table 2 Clinicopathological characteristics of patients with combined hepatocellular cholangiocarcinoma

Item	Patients (*n* = 98)
Tumor number	
Solitary	55 (56.1)
Multiple	39 (39.8)
NA	4 (4.1)
Tumor size, median [IQR]	4.5 [2.9, 6.5]
≤ 3cm (%)	26 (26.5)
3-5 cm (%)	36 (36.7)
> 5 cm (%)	34 (34.7)
NA	2 (2.0)
Resection margin (%)	
≤ 1cm (%)	54 (55.1)
> 1cm (%)	21 (21.4)
NA	23 (23.5)
Macro VI (%)	24 (24.5)
Micro VI (%)	62 (63.3)
Lymph node metastasis (%)	12 (12.2)
TNM Stage (AJCC 8th) (%)	
I	18 (18.4)
II	59 (60.2)
III	19 (19.4)
NA	2 (2.0)
Ki-67 (%)	
≤ 50%	36 (55.4)
> 50%	29 (44.6)
CK7 (%)	
Negative	9 (11.1)
Weak positive	29 (35.8)
Strong Positive	43 (53.1)
CK19 (%)	
Negative	9 (10.8)
Weak positive	27 (32.5)
Strong Positive	47 (56.6)
HepPar-1 (%)	
Negative	29 (34.1)
Weak positive	23 (27.1)
Strong Positive	33 (38.8)
GPC-3 (%)	
Negative	16 (28.6)
Weak positive	13 (23.2)
Strong Positive	27 (48.2)
HCC differentiation (%)	

Poorly differentiated	19 (41.3)
Well or moderate differentiated	27 (58.7)
ICC differentiation (%)	
Poorly differentiated	30 (65.2)
Well or moderate differentiated	16 (34.8)
ICC percent (%)	
≤ 50%	11 (30.7)
> 50%	16 (59.3)

Macro VI: Macrovascular invasion; Micro VI: Microvascular invasion; AJCC: American Joint Committee on Cancer; CK7: Cytokeratin 7; CK19: Cytokeratin 19; HepPar-1: Hepatocyte paraffin 1; GPC-3: Glypican-3; HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma; NA: Not available.



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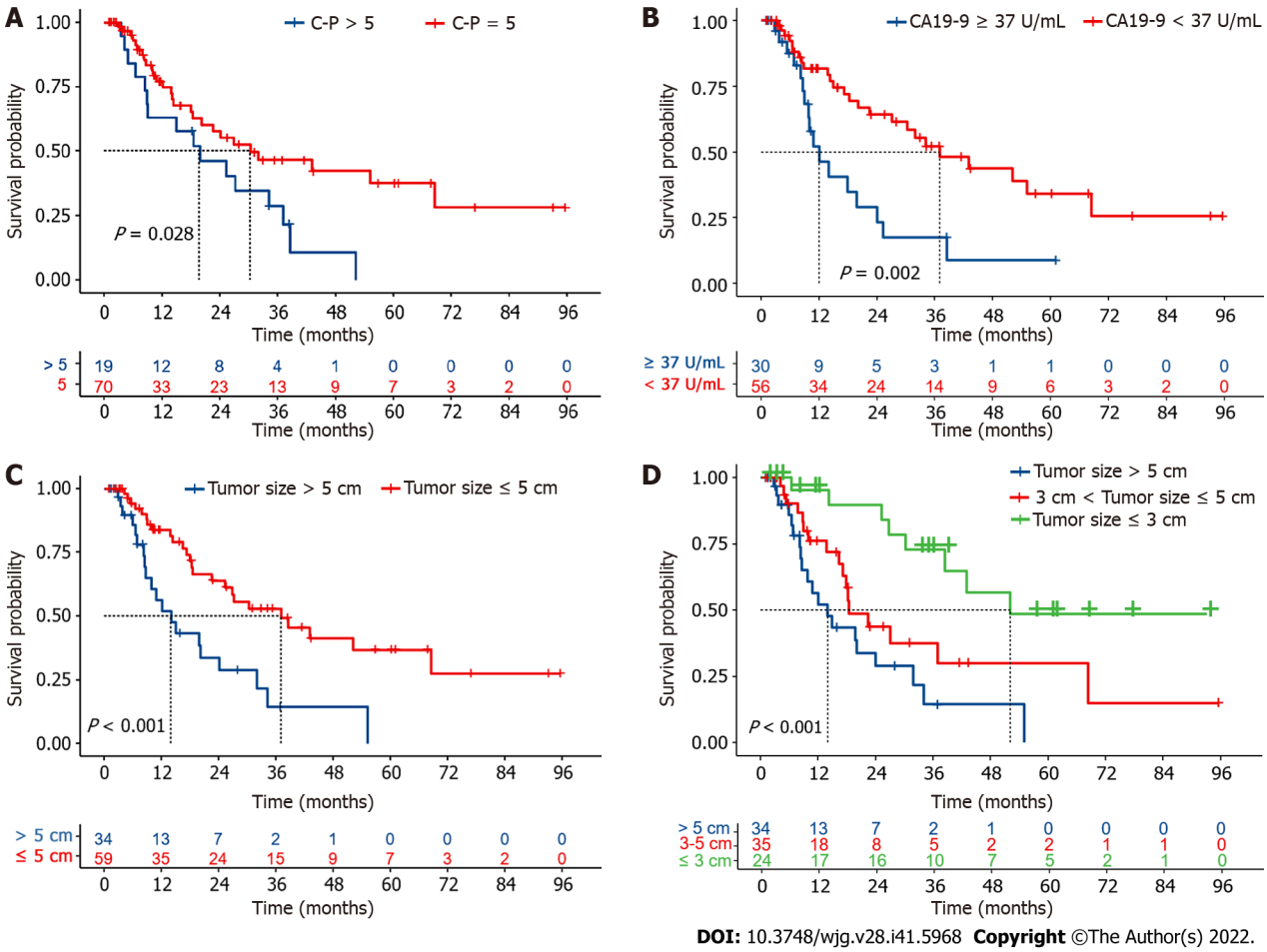
Figure 2 Survival and recurrence in patients after radical resection. A and B: Overall survival (A) and recurrence-free survival (B) curves of patients with combined hepatocellular-cholangiocarcinoma from two medical centers.

tary Figure 1A). Additionally, when a lesion size of 5 cm was set as the threshold, subgroup analysis for pathological features revealed notably differences in OS between these two subgroups (Figure 3C). Further subgroup analysis among patients with a tumor size < 5 cm displayed that patient with a tumor size of less than 3 cm had a considerably better survive than those with a lesion size of between 3 cm and 5 cm (Figure 3D). The 3-year OS rates for these two subgroups were 67.1% and 30.9%, respectively. However, analysis for the number of lesions showed that patients with a single lesion did not show a significantly improved prognosis compared to patients with multiple lesions (Supplementary Figure 1B). Macrovascular invasion did not significantly affect prognosis (P = 0.07) (Supplementary Figure 1C), but showed a similar trend. The Micro VI grouping (with or without) did not demonstrate a meaningful predictive difference (Supplementary Figure 1D).

The results of univariate analysis indicated that the factors that prominently influenced OS were CA19-9 Level (≥ 37 U/mL *vs* < 37 U/mL), C-P score (> 5 *vs* 5), tumor size, and postoperative transarterial chemoembolization (TACE) intervention. The background of liver disease, macrovascular invasion, GPC-3 expression, and HCC differentiation showed similar effects (0.05 < P < 0.10). In contrast, age, gender, AFP level (≥ 200 ng/mL *vs* < 200 ng/mL), number of lesions, cut margins, and Micro VI were not associated with OS (Supplementary Figure 2). Further multivariate analysis revealed CA19-9 ≥ 37 U/mL (HR = 8.68, P = 0.002), C-P score > 5 (HR = 5.52, P = 0.027), tumor number > 1 (HR = 30.85, P = 0.002), tumor size, and postoperative TACE intervention (HR = 0.2, P = 0.005) as independent prognostic factors affecting OS (Figure 4A).

Prognostic factors of RFS

The similar subgroup analysis was carried out to further evaluate the variables impacting patient recurrence as patients with cHCC-CCA typically suffered recurrence within a short period of time. The results showed that patients with a preoperative C-P score of 5 had an actually longer RFS than patients with a C-P score greater than 5 (Supplementary Figure 3A). In addition, RFS was also significantly shorter in patients with multiple lesions (Supplementary Figure 3B), with patients with a tumor size ≤ 3 cm having a significantly longer RFS than those with tumors larger than 3 cm (Supplementary Figure 3).



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Figure 3 Prognostic analysis between different subgroups. A-D: Overall survival between patients with different Child-Pugh (C-P) score (> 5 *vs* 5) (A), CA19-9 Level (≥ 37 U/mL *vs* < 37 U/mL) (B), tumor size (> 5 cm *vs* ≤ 5 cm) (C) and tumor size (≤ 3 cm *vs* 3-5 cm *vs* > 5 cm) (D).

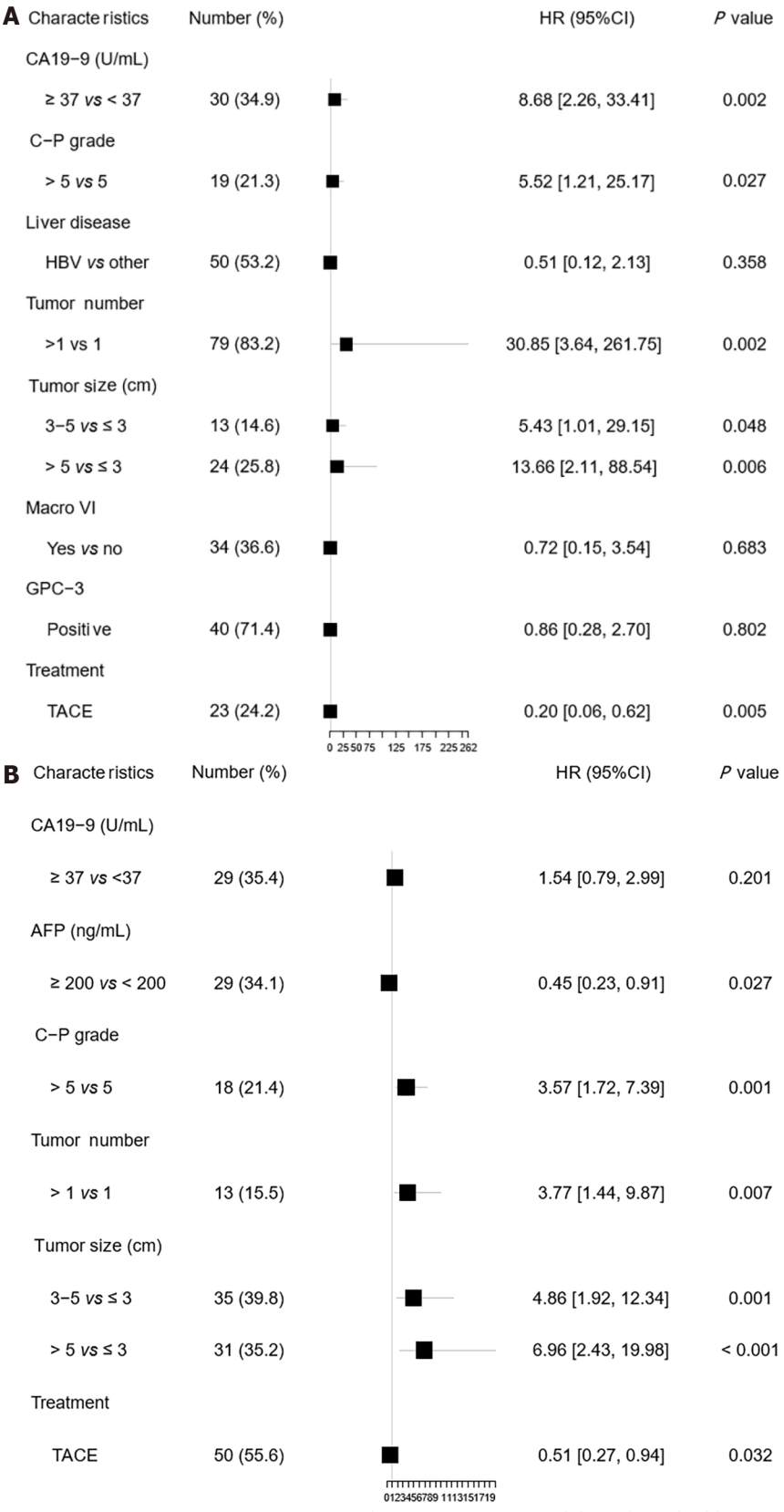
The univariate analysis results were consistent with the subgroup analysis. Factors that significantly affected RFS were the C-P score, tumor number, tumor size and ICC differentiation ($P < 0.05$). In addition, postoperative TACE intervention was effective in prolonging patients' RFS (Supplementary Figure 4). Further multivariate analysis showed that the C-P score > 5 (HR = 3.57, $P = 0.001$), AFP ≥ 200 ng/mL (HR = 0.45, $P = 0.027$), tumor number (HR = 3.77, $P = 0.007$), tumor size, and TACE intervention before recurrence (HR = 0.51, $P = 0.032$) were independent prognostic factors affecting RFS. AFP ≥ 200 ng/mL and postoperative TACE treatment were protective factors for RFS (Figure 4B).

According to the results of the multivariate analysis, we constructed a nomogram which integrated the important factors for predicting OS and RFS in patients with cHCC-CCA. For predicting OS, Harrell's concordance index (C-index) was 0.767 (Figure 5A), and this value was 0.737 when predicting RFS (Figure 5B).

DISCUSSION

As a rare kind of PLC, the percentage of cHCC-CCA varies in different studies, with the vast majority of studies concluding that its incidence is less than 15%[3,17-19]. Previous definitions of cHCC-CCA have also been changing, from the Allen and Lisa class proposed in 1949[18]; to the Goodman type proposed in 1985[19], the 2010 WHO classification (4th edition) and the 2019 WHO classification (5th edition)[1]. Currently, the pathological definition of cHCC-CCA has been refined; however, its clinical features, treatment and prognosis are still controversial, with some studies suggesting that cHCC-CCA is more comparable to HCC, and some suggesting that it is analogous to ICC[20-22], and the latest AJCC Staging Manual also suggests applying the ICC staging system to cHCC-CCA[13].

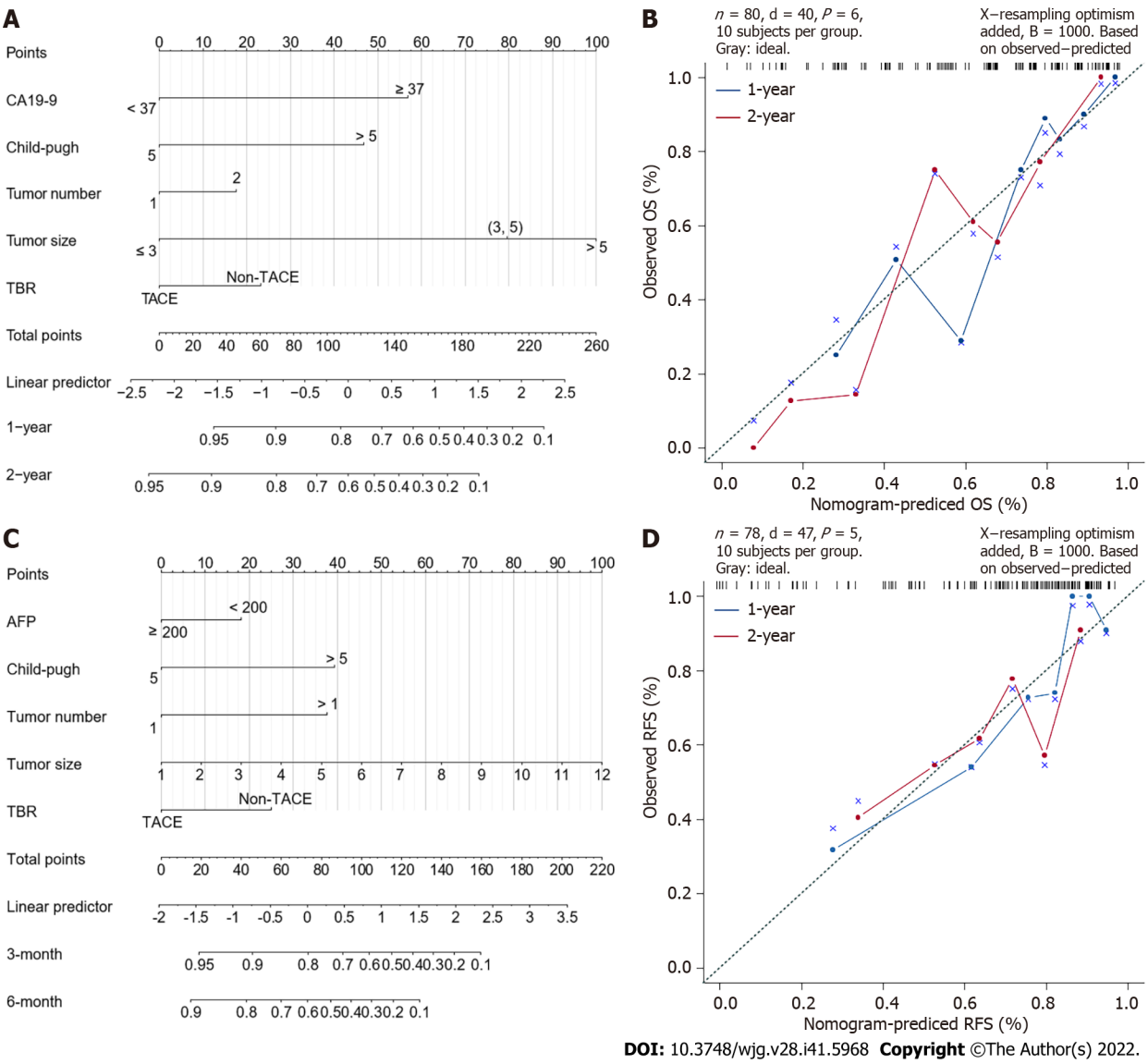
The comparison of prognosis between cHCC-CCA, HCC, and ICC has long been contentious. In present research, the median OS of cHCC-CCA patients was 26.8 mo. In previous studies, most studies concluded that the long-term survival of cHCC-CCA was worse than HCC and better than ICC[23-25], and some researchers concluded that the prognosis of cHCC-CCA was comparable to ICC[26]. However, many recent studies using propensity score matching have found no significant differences



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Figure 4 Multivariate analysis of all patients on overall survival and recurrence-free survival. A: Overall survival; B: Recurrence-free survival. C-P: Child-Pugh; Micro VI: Microvascular invasion; GPC-3: Glypican-3; TACE: Transarterial chemoembolization.

between the prognosis of cHCC-CCA and HCC or ICC when appropriate matching conditions were used[25,27], suggesting that the poorer prognosis of cHCC-CCA may be related to the behavior of the tumor.



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Figure 5 Nomogram for overall survival and recurrence-free survival. A: Overall survival (OS) nomogram for patients with combined hepatocellular-cholangiocarcinoma (cHCC-CCA); B: Calibration curve of overall survival for 1- and 2-year OS; C: Recurrence-free survival (RFS) nomogram for patients with cHCC-CCA; D: Calibration curve of recurrence-free survival for 3-mo and 6-mo RFS. OS: Overall survival; RFS: Recurrence-free survival; TBR: Treatment before recurrence; AFP: Alpha-fetoprotein.

In terms of predictive factors of cHCC-CCA in our cohort, multivariate analysis showed that CA19-9 was an important factor influencing the survive after radical surgery, and patients with high CA19-9 had a significantly worse prognosis. This is consistent with previous studies[7,28], suggesting that the ICC component may be a key factor affecting the prognosis of cHCC-CCA. Notably, AFP ≥ 200 ng/mL was a protective factor for prognosis, although in another study, there was no significant correlation between AFP and prognosis[6]. Overall, few researches have stated the connection between AFP and cHCC-CCA prognosis, and more studies are needed to investigate it.

In addition to tumor biomarkers, tumor size was an important factor affecting prognosis in our study. The median OS for patients with tumors > 5 cm was only 14 mo, and the prognosis was significantly worse in this subgroup patients ($P < 0.001$). And this result is in line with the findings of several prior investigations[28-30]. Based on the latest AJCC Staging Manual, ICC staging system is also applicable to cHCC-CCA, and in this TNM staging system, 5 cm is also used as a basis for differentiating between stages IA and IB. However, considering that a variable proportion of cHCC-CCA also has an HCC component, a further stratified analysis was performed for these patients. This analysis showed that patients with tumors up to 3 cm in size had a significantly better prognosis than those with tumors 3-5 cm in size (median OS: 52.1 mo *vs* 18.5 mo, $P < 0.001$), whereas patients in the 3-5 cm subgroup did not have a significantly better prognosis than those in the > 5 cm subgroup (median OS: 18.5 mo *vs* 14.0 mo), a phenomenon that suggests the need for more precise differentiation of cHCC-CCA patients with a tumor size ≤ 5 cm. However, in a previously conducted study of small HCC[31], the three-year OS rate after surgical resection was 91.4%, and in another similar study enrolling small HCC patients (≤ 3 cm)

without vascular invasion, the 3-year survival rate after surgical resection was 96%[32]. In addition, in a recent retrospective study of ICC, the 5-year OS rate was 52.6% in 53 patients with small ICC (≤ 3 cm) [33]. In contrast, in another study, the 5-year OS rate was 40% in 44 patients with ICC, although the mean tumor size in that study was 5.5 cm[34]. These results imply that patients with cHCC-CCA have a considerably poorer prognosis than those with HCC of the same size, and their prognosis is even inferior to that of patients with ICC of the same size, suggesting that cHCC-CCA is a distinct entity of PLC that should be treated separately.

Due to the lack of accepted treatment protocols for cHCC-CCA, there are many discussions on postoperative adjuvant treatment choices for patients after resectable cHCC-CCA[22]. In our study, the univariate and multivariate results showed that postoperative TACE therapy significantly prolonged OS and RFS. TACE is a common adjuvant therapy after HCC, and previous studies have shown that TACE prolongs OS and RFS in HCC patients[35], which is based on the rationale of hindering the rich blood supply of HCC, thus promoting tumor necrosis[36]. TACE treatment has also been linked to improved survival in patients with cHCC-CCA following radical surgery, according to recent researches[24,25]. Studies including patients with unresectable cHCC-CCA have also shown that cHCC-CCA lesions with a rich blood supply have a higher response rate and better treatment outcomes for TACE[37]. These phenomena suggest that TACE might be an efficient postoperative adjuvant therapy modality for some patients with cHCC-CCA, and more studies are needed to further identify appropriate postoperative adjuvant treatment options.

Our study has some limitations. First, although our data were derived from multiple centers, selective bias in some of the data as a retrospective study and irregularities in postoperative follow-up are unavoidable. Second, our cohort was predominantly HBV-infected cHCC-CCA patients, and the applicability of these findings to non-HBV-infected cHCC-CCA patients remains to be further validated. Third, among patients with tumors ≤ 5 cm, our study found that the prognosis was significantly better for patients with tumors ≤ 3 cm, but further investigation with bigger samples is still required for this subgroup of patients. Fourth, there is still a large gap in postoperative adjuvant therapy for cHCC-CCA. In addition to TACE therapy, the role of targeted therapy and immunotherapy in preventing recurrence needs more research.

CONCLUSION

Herein, we discuss the clinical situation and prognostic features of resectable cHCC-CCA, using data from two centers. Overall, the prognosis of these patients is poor, with most patients recurring rapidly. TACE is an effective postoperative adjuvant therapy that may prolong RFS and improve patient prognosis.

ARTICLE HIGHLIGHTS

Research background

Combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a relatively rare type of primary liver cancer. For patients who undergo radical resection, despite being able to undergo surgery, the overall postoperative prognosis is poor and the factors affecting postoperative recurrence and survival are unknown.

Research motivation

The motivation for this study was the poor prognosis of patients with cHCC-CCA who underwent radical surgery. Factors affecting postoperative survival remain controversial. There is a lack of clear guidelines for the choice of postoperative adjuvant therapy.

Research objectives

To explore the factors affecting postoperative recurrence and survival in patients with cHCC-CCA who underwent radical resection, leading to better risk stratification of patients and to investigate the impact of postoperative adjuvant therapy on prognosis.

Research methods

This study is a multicenter retrospective study focusing on rare cancer types. Ninety-five patients who underwent radical resection and had surgical pathology confirmed cHCC-CCA were included. Clinical information was collected and follow-up was performed for these patients. The number of patients enrolled in this study was large and the follow-up was adequate.

Research results

For patients with cHCC-CCA undergoing radical resection, most patients recur within 1 year after surgery, with a median survival of approximately 2 years. The 5-year survival rate does not exceed 30%. In addition to the biological characteristics of the tumor, postoperative transarterial chemoembolization (TACE) can significantly affect the prognosis. This finding helps to assist physicians and patients in the selection of postoperative adjuvant therapy.

Research conclusions

Most patients with cHCC-CCA experience recurrence within a short period of time after surgery. Postoperative adjuvant TACE prolongs RFS and is a possible option for postoperative adjuvant therapy.

Research perspectives

The main direction of future research is to explore appropriate preoperative diagnostic methods as well as postoperative adjuvant treatment options.

FOOTNOTES

Author contributions: Zhao HT, Lu YY, and Shi J led the entire project, and all authors participated in the discussion and interpretation of the data and results; Zhang G, Chen BW, and Yang XB performed the data collection, main analysis, and wrote the original manuscript; Wang HY, Xie FC, and Yu LX were participated in data collection and generation of figures and tables; Yang X and Shi J were involved in pathology review.

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ORIGINAL ARTICLE

Retrospective Study

High incidence combination of multiple primary malignant tumors of the digestive system

Xiao-Bo Yang, Long-Hao Zhang, Jing-Nan Xue, Yun-Chao Wang, Xu Yang, Nan Zhang, Dan Liu, Yan-Yu Wang, Zi-Yu Xun, Yi-Ran Li, Hui-Shan Sun, Li-Jin Zhao, Hai-Tao Zhao

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Abstract

BACKGROUND

Clinical reports of multiple primary malignant tumors (MPMTs) in the digestive system are increasing. In China, although the survival rate of patients with MPMTs is increasing, the quality of life is very low. Many patients have reached the advanced stage when the second primary tumor is found, resulting in no early intervention and treatment. This is due to the misunderstanding of MPMTs by clinicians, who treat such tumors as metastases. Therefore, before a patient has a second primary tumor, doctors should understand some common combinations of digestive system MPMTs to provide clinical guidance to the patient.

AIM

To explore the high incidence combination of digestive system MPMTs under heterochronism and synchronization.

METHODS

A total of 1902 patients with MPMTs at Peking Union Medical College Hospital were analyzed retrospectively. They were divided into metachronous MPMT and synchronous MPMT groups, and then the high incidence combinations of the first primary cancer and the second primary cancer in metachronous cancer and synchronous cancer were sorted. Sex and age differences between metachronous and synchronous tumors were tested by the chi square test and *t* test, respectively.

A P value < 0.05 was considered as statistically significant, and SPSS version 26.0 (SPSS Inc., Chicago, Illinois, United States) was used for statistical analysis.

RESULTS

Among the 1902 patients with MPMTs confirmed by pathology, 1811 (95.2%) cases were secondary primary cancers, 89 (4.7%) cases were tertiary primary cancers, and 2 (0.1%) cases were quaternary primary cancers. Most (88.2%) of the secondary primary cancers were identified as metachronous multiple primary cancers six months after diagnosis of the first primary cancer. The top ten most common MPMTs in the first primary cancer group ranged from high to low as follows: Breast cancer, thyroid cancer, nonuterine cancer, lung cancer, colon cancer, kidney cancer, uterine cancer, bladder cancer, rectal cancer, and gastric cancer. The highest incidence rate of the first primary cancer in male metachronous cancer was lung cancer (11.6%), the highest incidence rate of the second primary cancer was still lung cancer (24.9%), the highest incidence rate of the first primary cancer in female metachronous cancer was breast cancer (32.7%), and the highest incidence rate of the second primary cancer was lung cancer (20.8%). Among them, breast cancer, nonuterine cancer and uterine cancer were female-specific malignant tumor types, and thyroid cancer also accounted for 79.6% of female patients. The top five metachronous cancer combinations, independent of female-specific malignant tumor types and thyroid cancer, were colon cancer and lung cancer (26 cases), kidney cancer and lung cancer (25 cases), rectal cancer and lung cancer (20 cases), gastric cancer and lung cancer (17 cases), and bladder cancer and lung cancer (17 cases). The most common synchronous cancer combination was colon cancer and rectal cancer (15 cases).

CONCLUSION

Screening for lung cancer should be performed six months after the detection of colon cancer while rectal cancer screening should be performed within six months.

Key Words: Multiple primary malignant tumors; Colon cancer; Rectal cancer; Metachronous carcinoma; High incidence combinations; First primary carcinoma

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Core Tip: This is a retrospective study to explore the high incidence combination of multiple primary malignant tumors (MPMTs). Among the 1902 patients with MPMTs confirmed by pathology, after excluding the effect of male-female specific malignancies, it was found that digestive system malignancies were very common as the first primary cancer. Therefore, the common combination of second primary cancers should be followed up at the limit of 6 mo after the detection of digestive system malignancies. Without excluding the influence of male-female specific malignancies, it was found that the combination of breast cancer and nonuterine cancer was the most common in metachronous multiple primary malignancies, and the combination of colon cancer and rectal cancer was the most common in synchronous multiple primary malignancies.

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INTRODUCTION

Multiple primary malignant tumors (MPMTs) are defined as the simultaneous or successive occurrence of two or more primary malignant tumors in the same individual, which can be derived from the same organ, paired organs, different parts of the same system or different organs of different systems[1]. The diagnostic criteria were as follows: (1) Each tumor must have definite malignant histopathological changes; (2) Each tumor must have an independent pathological type; and (3) The possibility of invasion and metastasis of the second cancer as the first primary cancer must be excluded. MPMTs were divided into metachronous cancer and synchronous cancer. Synchronous MPMTs are defined as the second primary cancer that occurs within 6 mo of the first primary cancer, and metachronous MPMTs are the opposite of synchronous cancer.

Table 1 Clinical characteristics of multiple primary cancers patients. Clinical characteristics of patients with metachronous and synchronous multiple primary cancers

Clinical variable	Metachronous multiple primary cancers (*n* = 1687, 88.2%)	Synchronous multiple primary cancers (*n* = 224, 11.8%)	Total (*n* = 1902)	*P* value
Sex				< 0.001[1]
Male	562 (33.5%)	119 (53.1%)	681 (35.8%)	
Female	1116 (66.5%)	105 (46.9%)	1221 (64.2%)	
Age				0.377[2]
Mean (SD)	60.5 (11.7)	59.8 (11.8)	60.5 (11.7)	
Rang	22.0-90.0	24.0-88.0	22.0-90.0	

[1]chi-squared test.
[2]*t*-test.

In recent years, as many as two to five primary cancers have been reported in a single case[2-5]. However, double primary MPMTs are more common[6,7]. This study investigated the combined association between second primary cancer and first primary cancer. With the development of cancer nanotechnology[8], cancer patients can be clearly diagnosed and properly treated, and the survival rate of cancer patients has been greatly improved[9-11]. Due to the long-term side effects of chemotherapy and/or radiotherapy[12,13], the improvement of diagnostic sensitivity and the continuous influence of genetic and behavioral risk factors, cancer patients have an increased risk of developing a second cancer due to the improvement of survival rate[14], which can threaten their health. Due to the complex pathogenesis of cancer and the influence of the tumor microenvironment[15], it is difficult to find a perfect anticancer therapy that can resist the growth of malignant tumors without increasing toxicity and causing adverse pharmacological interactions. Predicting the occurrence of the second cancer among cancer survivors is conducive to the diagnosis and treatment of cancer patients, and some MPMTs seem to have a correlation with one another, as they appear in specific combinations. Understanding common cancer combinations is of significance for the clinical diagnosis and treatment of cancer patients.

MATERIALS AND METHODS

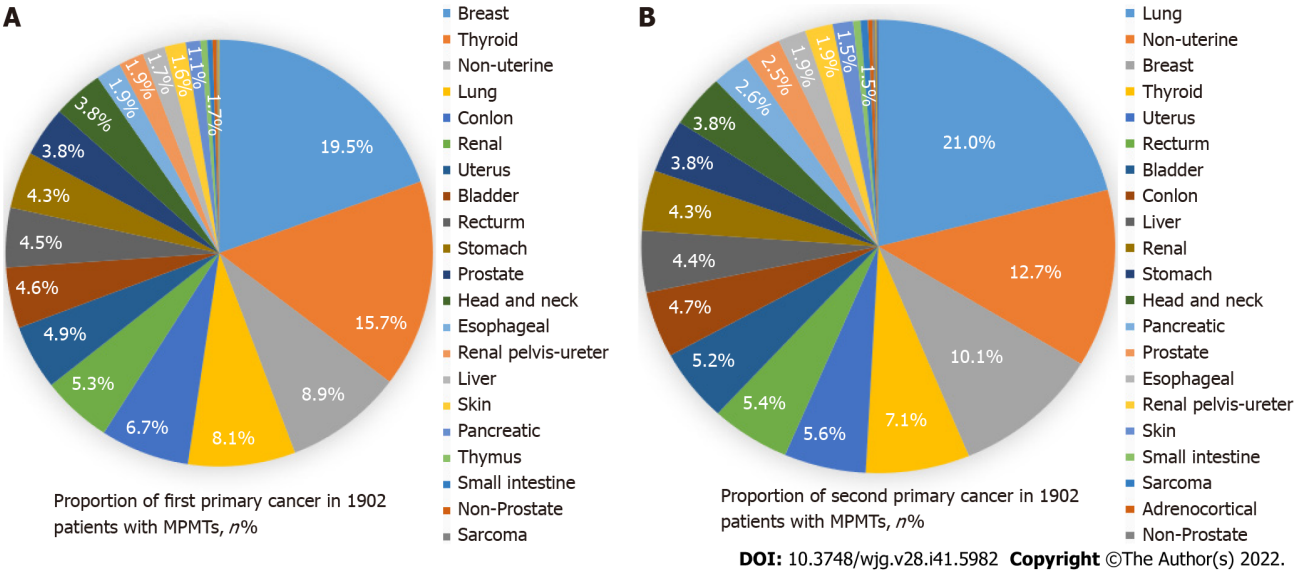
A total of 1902 patients were diagnosed with MPMTs at Peking Union Medical College Hospital from January 1, 2000, to June 1, 2021. According to the 3rd Edition description and definition[16], the primary cancer site is divided into 23 main types of solid cancer. The types are breast cancer; thyroid cancer; nonuterine cancer; lung cancer; colon cancer; rectal cancer; renal cancer; uterine cancer; bladder cancer; gastric cancer; head and neck cancer; prostate cancer; renal pelvis and ureter; oesophageal cancer; liver cancer; skin cancer; pancreatic cancer; thymus cancer; non-prostate cancer; small intestine cancer; adrenal cortex; sarcoma; bone and chondroma. Male genital cancer is divided into prostate cancer and non-prostate cancer. Nonuterine cancer includes cervical, vaginal, ovarian and fallopian tube cancers. Sex and age differences between metachronous and synchronous tumors were tested by the chi square test and *t* test, respectively. A *P* value < 0.05 was considered as statistically significant, and SPSS version 26.0 (SPSS Inc., Chicago, Illinois, United States) was used for statistical analysis.

RESULTS

The top ten, first-diagnosed cancers of metachronous and synchronous multiple primary cancers. Among 1902 patients with multiple primary cancers, including 681 males and 1221 females (1:1.79), 1678 cases (88.2%) were metachronous multiple primary cancers, and 224 cases (11.8%) were simultaneous multiple primary cancers. The majority of patients with multiple primary cancers were women (64.2%). There was a significant difference in the distribution of metachronous and simultaneous cancers between gender groups ($^tP < 0.001$). The average age at diagnosis of the first metachronous and simultaneous cancers was 61 and 60 years, respectively (Table 1).

The first and second primary cancers in the top ten MPMTs

We found that the first primary cancer types of the top ten MPMTs were as follows (Figure 1A): Breast cancer (19.5%); thyroid cancer (15.7%); nonuterine cancer (8.9%); lung cancer (8.1%); colon cancer (6.7%);



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Figure 1 Distribution of first and second primary cancers of multiple primary cancers. A: The first primary cancer; B: The second primary cancer.

renal cell carcinoma (5.3%); uterine cancer (4.9%); bladder cancer (4.6%); rectal cancer (4.5%); gastric cancer (4.3%). The second primary cancer types of the top ten MPMTs were as follows (Figure 1B): Lung cancer (21.0%); nonuterine cancer (12.7%); breast cancer (10.1%); thyroid cancer (7.1%); uterine cancer (5.6%); rectal cancer (5.4%); bladder cancer (5.2%); colon cancer (4.7%); liver cancer (4.4%); and renal cell carcinoma (4.3%).

The proportion of male to female first primary cancer and second primary cancer metachronous cases

The incidence rate of lung cancer in metachronous MPMTs as the first primary cancer (Figure 2A) was the highest in men (11.6%), and the second primary cancer in male metachronous MPMTs was still the highest in lung cancer incidence rate (Figure 2B) (24.9%). The incidence rate of breast cancer as the first primary cancer in metachronous MPMTs (Figure 2C) was the highest in women (32.7%), and the incidence rate of lung cancer (Figure 2D) was the highest (20.8%) in female metachronous MPMTs. It should also be noted that most second primary cancers occur more than 6 mo after the diagnosis of the first primary cancer.

The proportion of male and female cancers of the first primary cancer and the second primary cancer in synchronous cancer

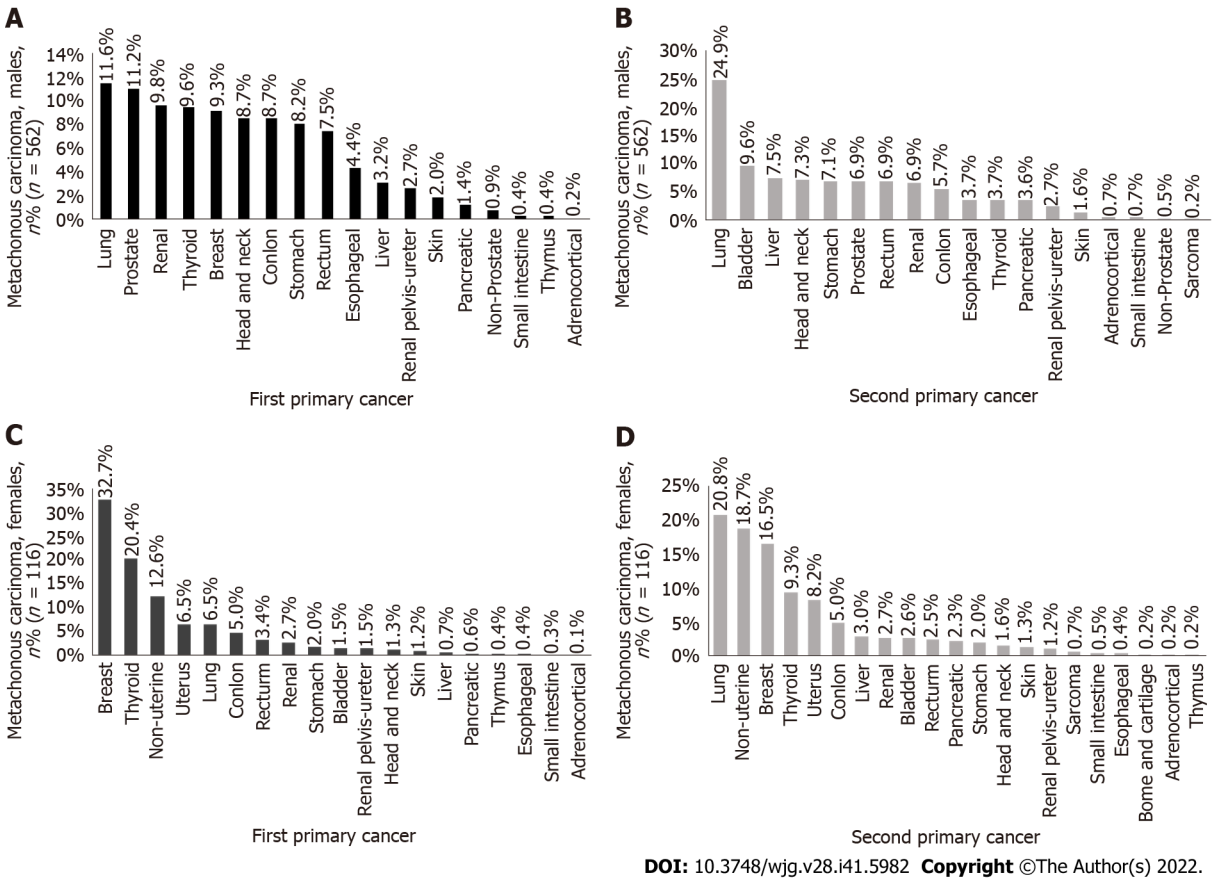
Among synchronous cancers, the incidence rate of colon cancer (Figure 3A) was the highest among the first primary cancers in men (15.1%), the incidence rate of rectal cancer (Figure 3B) was the highest among the second primary cancers (21.0%), the incidence rate of nonuterine cancer (Figure 3C) was the highest among the first primary cancers in women (26.7%), and the incidence rate of nonuterine cancer (Figure 3D) was the highest among the second primary cancers (30.5%).

After excluding the influence of male-female specific malignant tumors, the proportion of male and female digestive system malignant tumors

After excluding male-female specific cancers, 749 patients (440 males and 309 females) were found to have metachronous cancers, of which 43.2% were digestive system malignancies in males and 44.7% were digestive system malignancies in females (Figure 4A). There were 143 patients (102 males and 41 females) with synchronous cancer. Among them, 49.9% of male and 34.0% of female digestive system malignancies were digestive system malignancies (Figure 4B).

The combination of the first primary cancer and the metachronous and synchronous second primary cancer in multiple primary cancers

The combination of the first primary cancer and the second primary cancer in the top ten metachronous multiple primary cancers was as follows (Figure 5A): Breast cancer and nonuterine cancer (105 cases); thyroid cancer and lung cancer (85 cases); breast cancer and lung cancer (79 cases); thyroid cancer and breast cancer (63 cases); breast cancer and thyroid cancer (52 cases); thyroid cancer and nonuterine cancer (41 cases); nonuterine cancer and breast cancer (34 cases); breast cancer and uterine cancer (34 cases); colon cancer and lung cancer (26 cases); and renal cancer and lung cancer (25 cases). Breast or



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Figure 2 The proportion of male to female first primary cancer and second primary cancer metachronous cases. A: The first primary cancer is distributed in men with metachronous cancer; B: The second primary cancer is distributed in men with metachronous cancer; C: The first primary cancer is distributed in women with metachronous cancer; D: The second primary cancer is distributed in women with metachronous cancer.

thyroid cancer may become the most prevalent second primary cancer among multiple primary cancers. The top five synchronous multiple primary cancers, the combination of the first primary cancer and the second primary cancer, were as follows (Figure 5B): Colon cancer and rectal cancer (15 cases); uterine cancer and nonuterine cancer (14 cases); nonuterine cancer and nonuterine cancer (13 cases); nonuterine cancer and uterine cancer (9 cases); and there were 8 cases of thyroid carcinoma.

Relationship between nonuterine cancer and nonuterine cancer in synchronous cancer

In the combination of nonuterine cancer and nonuterine cancer (Table 2), the results were as follows: Cervical cancer and ovarian cancer (5 cases); vaginal carcinoma and cervical carcinoma (3 cases); cervical vaginal cancer (2); fallopian tube carcinoma and cervical carcinoma (1 case); and ovarian cancer and cervical cancer (column 1). Nonuterine cancer and nonuterine cancer are not duplicates, as the term refers to any cancer occurring in the female reproductive system. Therefore, when a patient has one of the nonuterine cancers, doctors should check whether the other nonuterine organs have lesions at the time of diagnosis to avoid a missed diagnosis.

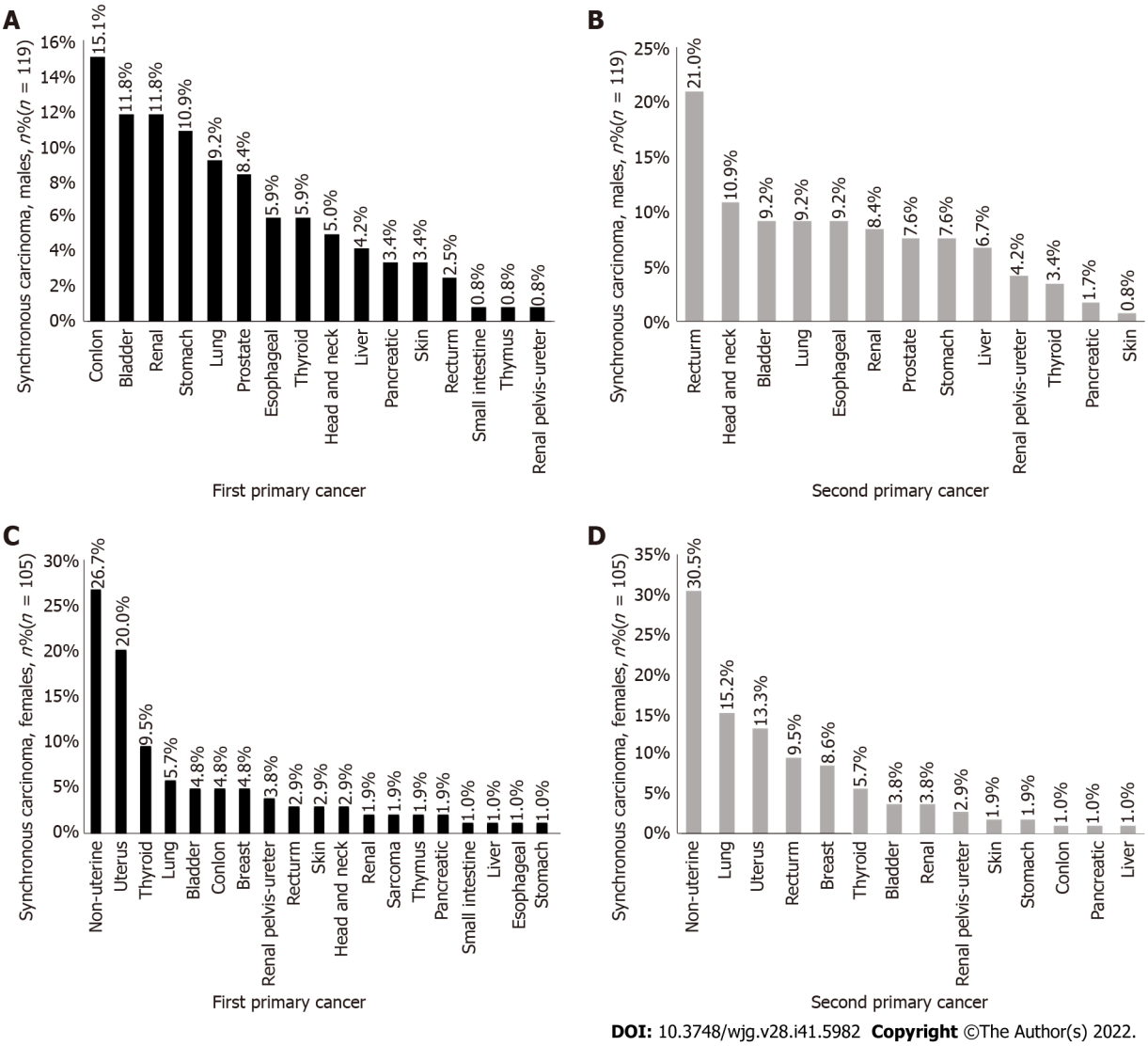
DISCUSSION

In our study, female-specific malignant tumors accounted for the vast majority. Therefore, to balance the influence caused by the excess of certain malignant tumors between male and female malignant tumors, we tried to distinguish the malignant tumors specific to male and female patients from the malignant tumors likely to affect both sexes for discussion and found that the combination of colon cancer and lung cancer (26 cases) was the most common in metachronous cancer, followed by renal and lung cancer (25 cases), rectal and lung cancer (20 cases), gastric and lung cancer (17 cases), and bladder and lung cancer (17 cases). The combination of colon cancer and rectal cancer (15 cases) was the most common in synchronous cancer. There is evidence that breast-cancer susceptibility gene 1 (BRCA1)/BRCA2 and MMR genes are indeed closely related to the occurrence of first and second colon cancer[17]. For the combination of colon cancer and rectal cancer, it is hoped that future genetic testing will be performed. Colon cancer is more likely to appear as the first primary cancer in the combination of synchronous

Table 2 Combination of non-uterine cancer and non-uterine cancer

	Cervix	Vagina	Ovary
Cervix	0	2	5
Vagina	3	0	0
Fallopian tube	1	0	1
Ovary	1	0	0

The row represents the first primary cancer and the column represents the second primary cancer.

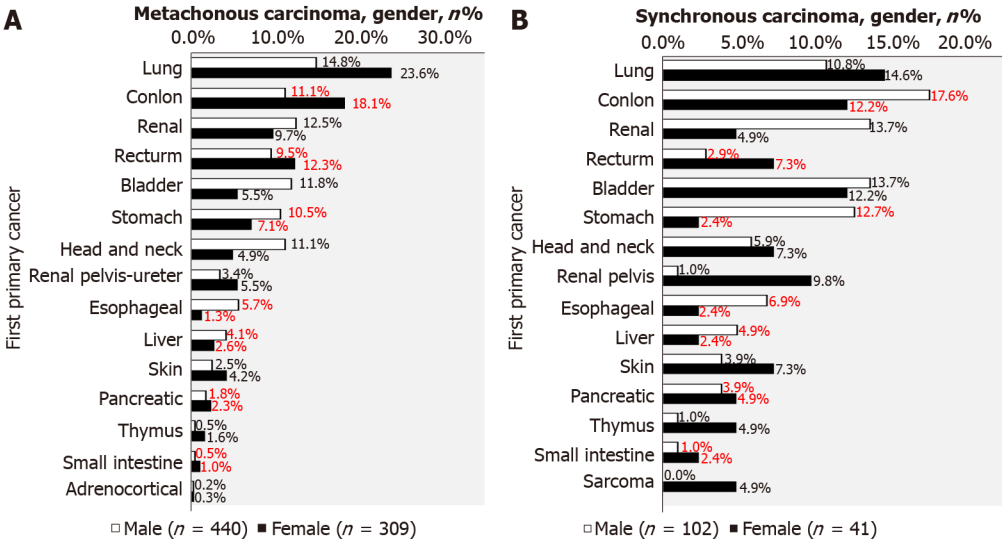


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Figure 3 The proportion of male and female cancers of the first primary cancer and the second primary cancer in synchronous cancer. A: The distribution of the first primary cancer in men with synchronous cancer; B: The second primary cancer in men with synchronous cancer; C: The distribution of the first primary cancer in women with metachronous cancer; D: The second primary cancer in women with metachronous cancer.

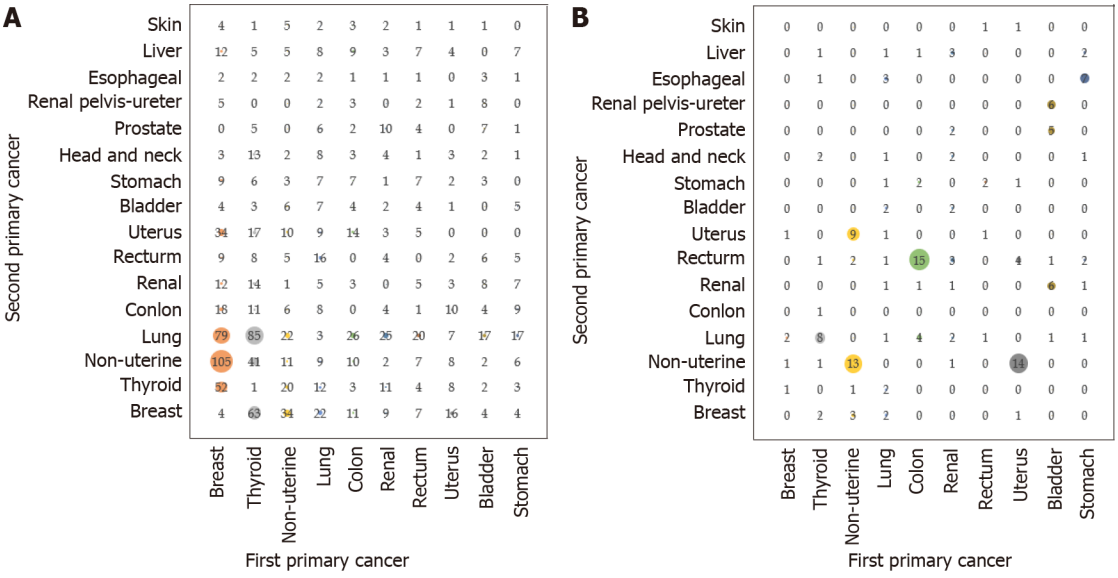
cancer and metachronous cancer. Since the majority of tumors occur in the digestive system, regular follow-up of the lungs should be performed 6 mo after the discovery of colon, rectal, or gastric cancer, and the rectum should be followed up and screened within 6 mo to prevent the occurrence of the second colorectal cancer[18-20].

We found that metachronous MPMTs are more common than synchronous MPMTs, which is consistent with a study conducted in Thailand last year[21]. However, Thailand has the highest incidence rate of liver cancer among metachronous cancer types, which is related to their eating habits. They like to eat sashimi, which leads to infection caused by parasitic liver flukes, resulting in a higher



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Figure 4 After excluding the influence of male-female specific malignant tumors, the proportion of male and female digestive system malignant tumors. A: The proportion of male and female primary malignant tumors after excluding the influence of male-female specific cancers, the proportion of male and female primary tumors of metachronous cancer; B: The proportion of male and female primary malignant tumors of synchronous cancer. The red numbers represent the percentage of digestive tumors.



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Figure 5 The top ten combined cases of first primary cancer and subsequent second primary cancer of metachronous and simultaneous multiple primary cancer. A: The combination of first primary cancer and second primary cancer of metachronous cancer; B: The combination of first primary cancer and second primary cancer of synchronous cancer.

incidence of liver cancer than other cancer types[22]. On the other hand, we have less data on liver cancer because there are more cases of metastasis of other cancer species in the liver, so it was not included in the study. It is noteworthy that China has carried out systematic management of patients with hepatobiliary tumors in recent years, including clinical treatment and exploration methods (extended period), and proposed an ideal model (three-dimensional period) for future use[23]. Moreover, in recent years, China has made some progress in the treatment of hepatobiliary tumors, including the objective remission rate of lenvatinib and pembrolizumab in refractory biliary tract cancer reaching 25% and the disease control rate reaching 78.1%[24]. Stereotactic therapy is a feasible transformation therapy that can make patients with hepatocellular carcinoma with extrahepatic metastasis resectable[25]. In this new era for cancer treatment, targeted drugs, targeted immune checkpoint inhibitors or their combination bring new hope for the conversion of hepatocellular carcinoma to surgery and adjuvant therapy[26].

In this study, the number of single primary malignant tumors was not counted, so the incidence rate of MPMTs was not calculated, but this did not affect our study on the combination of the first primary cancer and the second primary cancer in MPMTs. Of all the statistics, we found that breast cancer is the most common primary cancer, which is also related to the high incidence of breast cancer in China[27]. The second most common primary malignant tumor is lung cancer, which has a high incidence in China [28]. Recent studies in the United States have shown that bladder cancer is the most common primary cancer, and lung cancer is the second most common primary cancer[29]. Compared with the first primary cancer, the cancer types in China and the United States are different. The reasons for this difference are as follows: (1) In China, there are more women than men with MPMTs, and the most common cancer among females is breast cancer. In contrast, in the United States, the incidence rate of bladder cancer is highest; (2) This difference is because the research subjects are different: We study Chinese people, mainly from some cities in northern China, while the main research subjects in Europe and America are Caucasian and Black people[29]; and (3) Different levels of development lead to different exposure factors, such as living habits, air pollution, occupational exposure, viruses, bacterial infection and other carcinogenic factors.

The incidence rate of breast cancer is the highest among the metachronous cancer types in China. Moreover, the male to female incidence rate of MPMTs is 1:1.79, which is different from the previously reported male to female incidence rate of cancer in China. The reason for this difference is that there are great differences in the natural ecological environment, lifestyle, and disease risk factors in the eastern, central and western regions of China, and there are regional differences in the incidence rate of malignant tumors between men and women[30]. This study is limited by the sample size, so it will have some impact on the study. It is worth noting that to facilitate the study, we regard each segment of the colon as a primary cancer because colon cancer seems to be segmented, but their pathogenesis is similar. Studies have shown that noncoding RNAs play a key role in the carcinogenesis and progression of the colon cancer[31].

In this study, for female patients, breast cancer and the second primary cancer nonuterine cancer had the most heterochronous combinations (105 cases). Gene analysis showed that the occurrence of breast cancer and ovarian cancer was related to the loss of BRCA1 or BRCA2 gene function leading to homologous recombination defects[32]. The common combinations of primary breast cancer and secondary breast cancer may be caused by chemotherapy and radiotherapy of primary tumors, genetic variation linking the two diseases, hormone signals from oestrogen, lifestyle and environmental factors [33], and the thyroid gland, whose cancer has a similar incidence pattern to breast cancer. The combination of the first or second primary cancer and thyroid cancer may be caused by thyroid hormone signals[33]. Therefore, when it is first discovered that a female patient has breast cancer, their vagina, cervix, fallopian tubes, ovaries and thyroid gland should be checked for lesions after 6 mo to achieve early detection and treatment[34-36]. Thyroid cancer was the second most common type of cancer in this study, and there were many combinations where the second primary cancer was lung cancer in metachronous MPMTs (85 cases). There is evidence that abnormalities in the oncogene rearrangement during transfection are the cause of lung cancer[37].

The metachronous combination of breast cancer and lung cancer (79 cases) should not be ignored. Lung cancer often appears in metachronous cancer in the form of a second primary cancer. There is evidence that the risk of primary lung cancer after treatment of breast cancer increases because smoking habits, age and the disease stage of breast cancer may affect the risk of secondary primary lung cancer in breast cancer patients[38]. Therefore, the regular follow-up of patients, including monitoring the lungs after 6 mo, should be carried out while breast cancer is treated. If lung cancer is found, it should be treated quickly, with both surgical treatment and adjuvant treatment[39,40].

We studied the high incidence combination of MPMTs, but there were some limitations. First, the population of this study is very limited. Most patients came from northern China, not all of China, and the basic data of the above foreign studies are from the Surveillance, Epidemiology and End Results program of the domestic population-based cancer registry or the data of many long-standing and highly reliable cancer centres on cancer diagnosis[41-42]. We recognize that this difference may also introduce bias to this study. Second, we have less synchronous cancer data, which is not enough to explain the strong association between synchronous cancer combinations. Third, we did not carry out genetic examination on patients, so the pathogenesis and aetiology of MPMTs were not discussed. Fourth, this study is a cross-sectional study involving 23 types of malignant tumors and describes the incidence of malignant tumors between men and women. It does not involve therapy, immune chemistry, next-generation sequencing, *etc.* Despite the limitations mentioned above, our results are informative. For patients treated with primary malignant tumors, attention should be given to the high incidence combination of the first and second primary cancers. In the absence of male-female specific cancer effects, attention should be given to the digestive system as a combination of primary malignancy and lung cancer. Without distinguishing between male-female specific malignancies, the possible metachronous cancer combination of breast cancer and nonuterine cancer and the synchronous cancer combination of colon cancer and rectal cancer should be given. After distinguishing synchronous cancer from metachronous cancer, the high incidence cancer combination should be followed up regularly for 6 mo. Through clinical experience and examination, some common cancers can be detected. If they can be removed surgically, they should be removed at the earliest stage possible. If the tumors cannot be

removed, they should also be treated with the best adjuvant treatment, such as radiotherapy and chemotherapy, targeted therapy, and endocrine therapy, among others, to achieve the maximum therapeutic benefits[43,44].

CONCLUSION

In conclusion, after excluding the effect of cancers specific to men and women, screening for lung cancer should be performed 6 mo after detection of colon cancer and for rectal cancer within 6 mo.

ARTICLE HIGHLIGHTS

Research background

Multiple primary malignant tumors (MPMTs) of the digestive system are common clinically, usually presenting as a metachronous combination of colon cancer and lung cancer and a synchronous combination of colon cancer and rectal cancer.

Research motivation

Understanding some common combinations of multiple primary malignancies can help in providing clinical guidance to patients.

Research objectives

This study aimed to classify MPMTs of the digestive system and explore the combination of high incidence with the second primary malignant tumors.

Research methods

This retrospective study analyzed patients diagnosed with multiple primary malignancies in our centre over a 20-year period, classified the tumors, and further explored the high incidence of digestive system malignancies in the combination of multiple primary malignancies.

Research results

The most common metachronous combination pairs of multiple primary malignancies of the digestive system were colon cancer and lung cancer (26 cases), and synchronous combination pairs were colon cancer and rectal cancer (15 cases).

Research conclusions

Through our retrospective study, we found that when patients were diagnosed with colon cancer, they should be screened separately for lung cancer and rectal cancer at the limit of 6 mo.

Research perspectives

To provide clinical guidance to patients based on the combination of common multiple primary malignancies.

FOOTNOTES

Author contributions: Yang XB, Zhang LH, Xue JN contributed equally to this work; Wang YC, Yang X, Liu D, Wang YY and Xun ZY performed the radiological diagnosis; Zhang LH, Xue JN performed pathological diagnosis; Yang XB, Zhang LH designed the research study; Xue JN, Zhang N, Li YR and Sun HS performed the primary literature and data extraction; Yang XB and Zhang LH analysed the data and wrote the manuscript; Zhao HT and Zhao LJ were responsible for revising the manuscript for important knowledge content and contributed equally to this work, and all authors read and approved the final version.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Peking Union Medical College (Approval No. K22C0171).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data. The ethical Committee of Peking Union Medical College Hospital agreed to waive the

informed consent.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

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CASE REPORT

Collagenous gastritis in a young Chinese woman: A case report

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Abstract

BACKGROUND

Collagenous gastritis (CG) is a rare condition whose pathogenesis may be related to immune abnormalities. We report a case of CG from China.

CASE SUMMARY

A 24-year-old woman presented with recurrent abdominal distension and discomfort for 3 mo. Upper gastrointestinal endoscopy found diffuse nodular elevation-depression changes in the mucosa of the entire gastric corpus. Endoscopic ultrasound showed predominant involvement of the lamina propria and submucosa, and computed tomography imaging showed mild enhancement of the gastric wall. Pathological histology revealed that the thickness of the subepithelial collagen band was about 40 μm, and the Masson trichrome staining result was positive and the Congo red staining result was negative. This case is consistent with the child-adolescent type of CG.

CONCLUSION

Serum pepsinogen I, pepsinogen II, pepsinogen I/II ratio, and gastrin-17 may be potential non-invasive monitoring markers. Currently, treatments for CG vary, and the likely prognosis is unknown. Individual cases of gastric cancer in patients with CG have been reported.

Key Words: Endoscopy; Endoscopic ultrasound; Collagenous gastritis; Hematoxylin and eosin staining; Masson's trichrome staining; Case report

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Core Tip: We report a case of collagenous gastritis. We introduce the clinical features, findings of esophagogastroduodenoscopy and endoscopic ultrasound, and response to treatment in this young female patient.

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INTRODUCTION

Collagenous gastritis (CG) is a rare condition characterized histopathologically by the deposition of collagenous bands under the mucosal epithelium and infiltration of inflammatory cells within the lamina propria of the mucosa. It is more prevalent in children and adolescents. The pathogenesis and clinical outcome remain unclear.

The first case of CG was reported in 1989 by Colletti and Trainer in the United States[1]. Since then, the literature on CG has predominantly included case reports. A search of PubMed using the keyword "collagenous gastritis" revealed < 100 reported cases. The first and second cases of CG in China were respectively reported in 2010[2] and 2018[3]. We now report the third Chinese case of CG diagnosed in 2020 at the Affiliated Hospital of Guilin Medical University.

CASE PRESENTATION

Chief complaints

A 24-year-old female patient presented to our outpatient service in June 2020 with the complaints of recurrent abdominal distension and discomfort for 3 mo.

History of present illness

The patient reported feeling epigastric distension after eating that could last anywhere from 30 min to 12 h, with no other significant discomfort.

Personal and family history

Following hospital admission, the patient denied a history of allergies, asthma, and pet exposure. Both her father and mother were living and healthy.

Physical examination

The physical examination on admission revealed stable vital signs, no yellow staining of the skin or sclera, and no enlargement of superficial lymph nodes. Cardiopulmonary and abdominal examinations showed no abnormalities.

Laboratory examinations

Laboratory tests revealed a normal blood count, liver function, and renal function and normal carcinoembryonic antigen, cancer antigen 125, cancer antigen 19-9, and alpha-fetoprotein values. Her liver fibrosis test was normal, and she had normal levels of immunoglobulins immunoglobulin (Ig) A, IgM, and IgG, as well as serum type III procollagen, type IV collagen, laminin, and hyaluronic acid. Her serum pepsinogen (PG) I value was 133.41 μm/L, her PG II value was elevated at 25.99 μm/L, her pepsinogen I/II ratio (PGR) value was reduced at 5.13, and her gastrin-17 value was 54.01 pmol/L. The ^{13}C urea breath test negative, the allergen assay was negative, and her extractable nuclear antigen antibodies were negative.

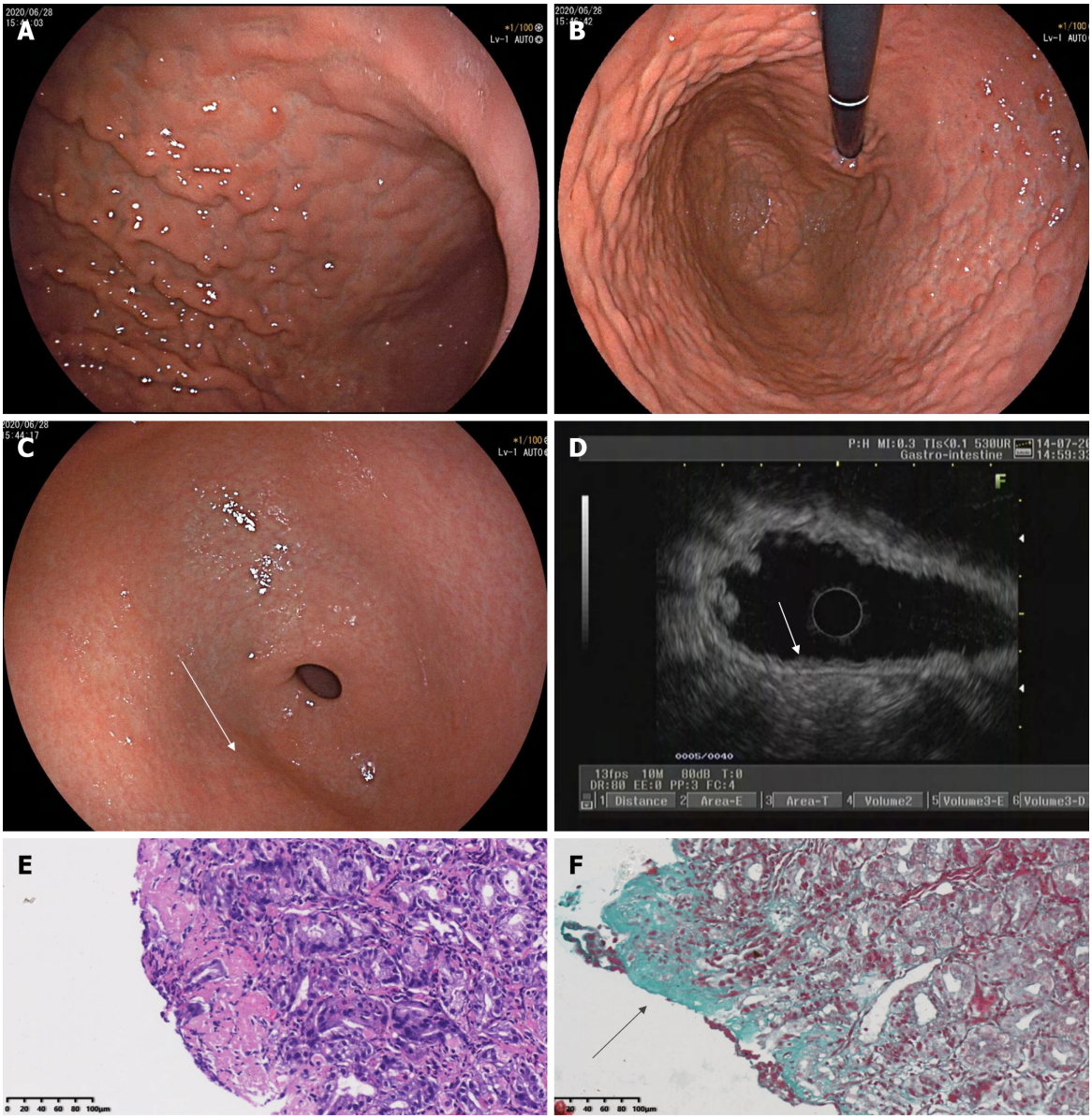
Imaging examinations

Eesophagogastroduodenoscopy (EGD) performed on June 28, 2020 revealed diffuse nodular elevation-depression changes in the mucosa of the entire gastric corpus (Figure 1A and B).

Nodular reddening-like changes were seen in the anterior wall of the gastric antrum (Figure 1C). Then, endoscopic ultrasound (EUS) completed in July 2020 suggested an intact 5-layer structure of the gastric corpus wall with hypoechoic changes in the mucosal layer (Figure 1D). Histopathology reported moderate chronic inflammation with erosion and mild activity (Figure 1E).

In May 10, 2021, EGD confirmed that no mass or ulcer could be found in the esophagus or duodenum. The mucosa of the angulus was smooth, while the mucosa of the gastric corpus and fundus appeared uneven, with elevation-depression changes (Figure 2A).

Magnifying endoscopy with blue laser imaging (ME-BLI) showed clear borders of the elevation, regular arrangement of the marginal crypt epithelium (MCE), and widening of the microvascular vessels. The MCE in the depression was indistinct, and the irregular microvascular pattern showed dendritic changes with a constant ductal diameter, and the microvessels here were thinner than those of



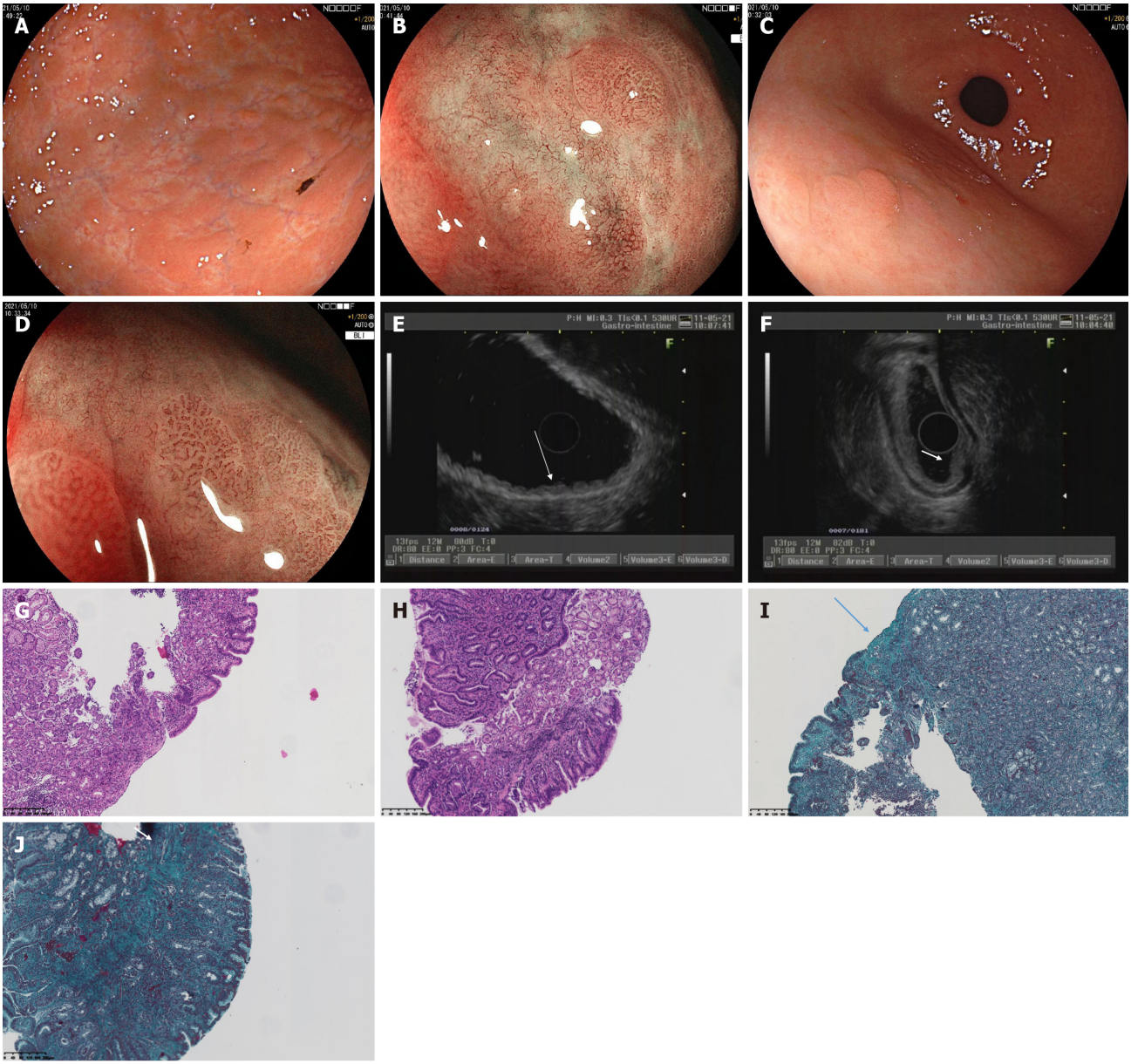
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Figure 1 Findings of white light endoscopy, endoscopic ultrasound, and histopathology in 2020. A: Esophagogastroduodenoscopy (EGD) in 2020 in a forward endoscopic view showed diffuse nodular elevation-depression changes in the mucosa of the entire gastric corpus; B: EGD in 2020 (in a retroflexed endoscopic view) showed diffuse nodular elevation-depression changes in the mucosa of the entire gastric corpus; C: EGD in 2020 showed nodular reddening-like changes in the anterior wall of the gastric antrum; D: Endoscopic ultrasound in 2020 showed an intact five-layer structure of the gastric corpus wall with hypoechoic changes in the mucosal layer, which is different from the echoes of normal mucosal layer; E: A biopsy of the gastric corpus in 2020 reported moderate chronic inflammation with erosion and mild activity; F: Masson staining of a gastric corpus specimen in 2020 showed collagen bands (blue areas).

the elevated area of the gastric antrum (Figure 2B). Nodular reddening-like changes were seen in the anterior wall of the gastric antrum (Figure 2C). The findings of ME-BLI were similar to those from the gastric corpus (Figure 2D). EUS showed that the mucosal layer of the gastric corpus was thickened, appearing slightly hypoechoic, with no thickening of its posterior submucosal layer (Figure 2E).

The normal structures of the mucosa, muscularis mucosa, and submucosa at the elevation of the gastric antrum had disappeared and were replaced by inhomogeneous hypoechoic changes, with the muscularis propria and serosa still intact (Figure 2F). Colonoscopy confirmed that the mucosa of the whole colon, rectum, and terminal ileum was smooth, and no mass or ulcer was visible. Computed tomography showed a relative thickening of the gastric wall in the gastric corpus and antrum, and mild enhancement was seen in the arterial phase (Figure 3A and B).

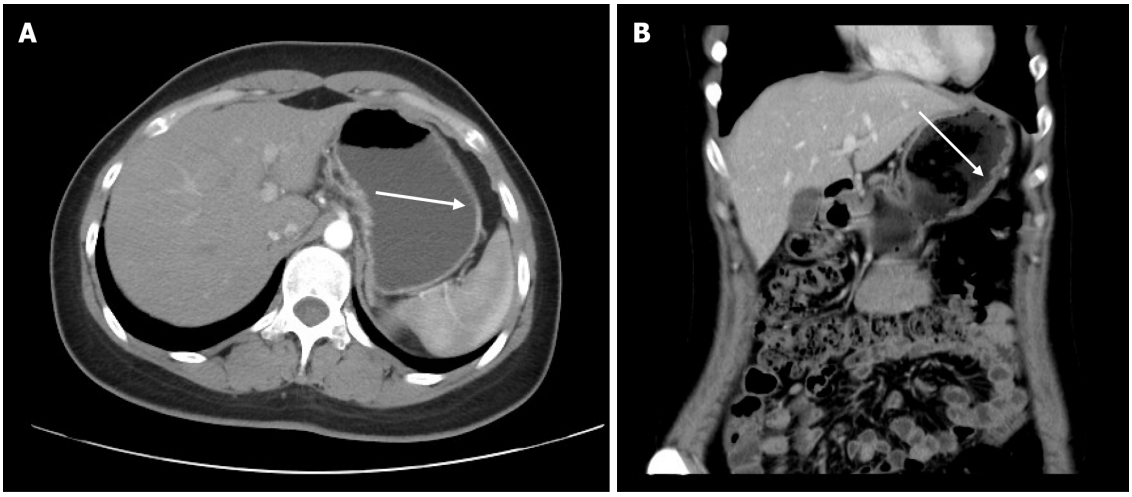
Histopathological findings of the gastric fundus, body, and antrum biopsies included moderate chronic inflammation with erosion, mild activity, a higher number of lymphocytes, a few neutrophils and eosinophils [20-30/high-powered field (HPF)] infiltrating the interstitium, multifocal lymphoid tissue hyperplasia, and negative Congo red staining (Figure 2G and H). Collagenous bands deposited under the mucosal epithelium (about 40 μm) were seen (Figure 2I and J). Angulus biopsy revealed moderate chronic inflammation with erosion and positive Masson's trichome staining. The bulbous,



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Figure 2 Findings of white light endoscopy, magnifying endoscopy, endoscopic ultrasound, and histopathology in 2021. A: Esophagogastroduodenoscopy (EGD) in 2021 showed that the mucosa of the angulus was smooth, while the mucosa of the gastric corpus and fundus appeared uneven, with elevation-depression changes; B: Magnifying endoscopy with blue laser imaging (ME-BLI) in 2021 showed regular arrangement of the marginal crypt epithelium (MCE) and widened microvessels in the elevated area of the corpus, and dendritic irregular microvascular pattern and unremarkable MCE in the depressed area of the corpus; C: EGD in 2021 showed nodular reddening-like changes in the anterior wall of the gastric antrum, which is similar to those in 2020; D: ME-BLI in 2021 showed similar findings in the gastric antrum to those in the corpus; E: Endoscopic ultrasound (EUS) in 2021 showed that the mucosal layer of the gastric corpus was thickened, exhibiting slightly hypoechoic and wavy changes; F: EUS in 2021 showed that the normal five-layer structures in the gastric antrum were replaced by inhomogeneous hypoechoic changes; G: Histopathological findings of the gastric corpus in 2021 (corresponding to Figure 2A and B) included moderate chronic inflammation with erosion, mild activity, a higher number of lymphocytes, and a few neutrophils and eosinophils (20-30/high-powered fields); H: Histopathological findings of the gastric antrum in 2021 (corresponding to Figure 2C and D) showed moderate chronic inflammation with erosion and mild activity; I: Masson staining of a gastric corpus specimen obtained in 2021 (corresponding to Figure 2G). The arrow indicates the collagen band, which had a thickness of about 40 μm; J: Masson staining of a gastric antrum specimen obtained in 2021 (corresponding to Figure 2H). The arrow also indicates the collagen band.

terminal ileum, and ascending colon biopsies showed moderate mucosal chronic inflammation with erosion, mild activity, focal lymphocytosis, and positive Masson's trichome staining. The rectal biopsy showed moderate mucosal chronic inflammation with erosion and positive Masson's trichome staining. Furthermore, complementary Masson staining of the patient's gastric corpus biopsy specimen from June 28, 2020 revealed the presence of collagenous band deposited under the epithelium (approximately 40 μm) (Figure 1F).



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Figure 3 Computed tomography findings in 2021. A: Computed tomography (CT) in 2021 showed a relative thickening of the gastric wall in the gastric corpus and antrum, and mild enhancement was seen in the arterial phase; B: Coronal CT in 2021 showed thickening of the gastric wall of the corpus, as indicated by the arrow.

FINAL DIAGNOSIS

The patient was diagnosed as having CG.

TREATMENT

Esomeprazole (20 mg every day, taken 30 min before breakfast) and mosapride (5 mg three times a day, *i.e.*, once before each of all 3 meals) were given orally for 2 wk, and the patient's abdominal distension was subsequently relieved. However, the patient continued to experience intermittent abdominal distension for > 6 mo thereafter, with episodes lasting for the same duration as before, while taking esomeprazole and mosapride irregularly. The patient was then hospitalized at the Affiliated Hospital of Guilin Medical University from May 9 to May 12, 2021.

Treatment with oral glucocorticoids was initiated for the patient in June 2021. Thirty milligrams daily of prednisone acetate was given for 2 wk, and then reduced by 5 mg every 2 wk thereafter and finally discontinued when the dose reached 5 mg daily and was maintained as such for 2 wk. During the 8 wk of treatment, the patient continued to present with intermittent abdominal distention, lasting 12 h in severe episodes, and she was given 5 mg of oral mosapride three times daily 30 min before meals. A re-examination showed that her PG I value was 105.21 μm/L, her PG II value was elevated to 24.3 μm/L, her PGR value was decreased to 4.33, and her gastrin-17 value was 46.99 pmol/L.

OUTCOME AND FOLLOW-UP

After treatment, the patient's abdominal distension was relieved for 2-3 d every week. Laboratory tests performed in October 2021 revealed that her PG I value was 118 μm/L, her PG II value was elevated to 33.74 μm/L, her PGR value was decreased to 3.5, and her gastrin-17 value was elevated to 39.69 pmol/L. As of November 2021, the patient was still undergoing follow-up (Table 1).

DISCUSSION

Pathology and pathogenesis

The pathology is characterized by a deposited collagen band with a thickness of > 10 μm under the epithelium, with an average thickness of 30 μm and a maximum thickness of up to 120 μm[4]. Inflammatory cell infiltration is seen in the lamina propria of the mucosa. Infiltrating inflammatory cells include lymphocytes, plasma cells, monocytes, and eosinophils. Collagenous gastritis can be classified as eosinophilic, lymphocytic, and atrophic according to the type of infiltrated cells. The main criteria for these types are as follows: Eosinophils in the lamina propria > 30/HPF (eosinophilic type); lymphocytes > 25/HPF (lymphocytic type); and a reduction in glands, a reduction in specialized cells such as parietal

Table 1 Timeline

Date	Description
June 2020	First visit, chief complaints: Recurrent abdominal distension and discomfort for 3 mo
June 2020	1st EGD + pathological histology
July 2020	1st EUS
July 2020	Esomeprazole and mosapride for 2 wk; abdominal distension subsequently relieved
Late 2020 to first half of 2021	Continued intermittent abdominal distension for > 6 mo
May 9 to May 12, 2021	Hospitalized; PG I, PG II, and PGR tested; 2nd EGD + pathological histology; ME-BLI; 2nd EUS
June 2021	Oral glucocorticoids for 8 wk and tapered; PG I, PG II, and PGR retested
October 2021	PG I, PG II, and PGR retested

EGD: Esophagogastroduodenoscopy; EUS: Endoscopic ultrasound; ME-BLI: Magnifying endoscopy with blue laser imaging; PGR: Pepsinogen I/II ratio.

cells and chief cells, pyloric gland metaplasia, and hyperplasia of smooth muscle in the lamina propria of the mucosa (atrophic type). The same case may contain just one or all of these types. Considering special staining in addition to Masson's trichome staining, tenascin positivity in immunohistochemistry may also be a characteristic indicator[5]. In addition to the stomach, the disease may include collagenous stomatitis and collagenous enteritis, depending on the site of onset, and these three locations are currently considered to be different sites of the same disease.

The pathogenesis of CG is unclear. Some pathological findings suggest an association with immune abnormalities. For example, signs of local immune activation were detected in some specimens, such as overexpression of human leucocyte antigen DR in epithelial cells, increased $CD3^+$ intradermal lymphocytes, and $CD25^+$ cells found in the lamina propria[6], as well as a large number of IgG4-positive plasma cells that failed to confirm an association with IgG4-related disease[5]. The histological changes in collagenous gastritis may be caused by a local immune response. In a few patient specimens, collagen bands can be isolated from type III and type IV collagen fibers. During the repair process, type III collagen is released by subepithelial fibroblasts[5]. Specimens were also found to be positive for tenascin, a marker suggestive of cell proliferation and migration[5]. Therefore, collagen synthesis in collagenous gastritis is a reparative response. In the pathogenesis of collagenous gastritis, an association with reduced serum IgA levels has also been reported[7].

Clinical features

Most of the CG studies reported so far are single case reports and retrospective analyses, lacking large sample data. Cases of CG have been documented mainly in Europe, the United States, and Japan. Women are more commonly afflicted than men, and the age of patients ranges from 7-85 years[8,9]. Clinical symptoms include abdominal pain, abdominal distension, diarrhea, nausea, vomiting, gastrointestinal bleeding, weight loss, anemia, fatigue, retrosternal pain, dyspepsia, perforation[10], dysphagia[11], and constipation.

Lagorce-Pages *et al*[4] classified CG into child-adolescent type and adult type. The child-adolescent type occurs mainly in early adolescence, where inflammation is usually limited to the stomach and anemia and abdominal pain are the main symptoms. The adult type often combines with collagenous enteritis, which is characterized by chronic watery diarrhea. CG and collagenous colitis exist on a clinical spectrum, with the difference being the site of involvement of the GI tract.

The typical endoscopic feature of collagen gastritis is the presence of mucosal nodules. These nodules vary in size and are often diffuse in the gastric corpus and antrum, with their size and number depending upon the severity of the inflammation. Endoscopic manifestations also include mucosal erythema, erosion, and exudation. The endoscopic presentation differs between pediatric and adult patients. Pediatric and adolescent patients usually present with gastric nodules, whereas adult patients often present with mucosal erythema, atrophy, and relatively uncommon nodules[9]. In image-enhanced endoscopy, glandular duct structures are seen on the surface of the nodules under magnifying narrow-band imaging, and microvascular thinning and tortuosity are seen in the structureless area[12,13].

Patients can also have other co-morbidities, including *Helicobacter pylori* infection[9], human immunodeficiency virus infection combined with gastric Kaposi's sarcoma[14], and Sjögren's syndrome [15].

Our case is a young female patient who presented with abdominal distension as the main manifestation, without obvious symptoms of anemia, abdominal pain, and diarrhea. Pathological findings supported the idea that the inflammation was limited to her stomach only, and colonoscopy and biopsy showed no intestinal involvement, consistent with the child-adolescent type. The

pathological histology of this case in our hospital showed the presence of a higher number of eosinophilic infiltrates in the lamina propria, consistent with the eosinophilic type. Endoscopy showed a depressed lesion surrounding the elevation of the gastric corpus and fundus greater curvature, with a structureless area in the depression under image-enhanced endoscopy and thinning microvessels, similar to the findings observed by Kawasaki *et al*[12] using magnifying narrow-band imaging in the gastric corpus.

EGD at both our hospital and Hangzhou No. 1 Hospital[3] showed redness on the elevation of the gastric antrum, and biopsy suggested a collagen deposition band of > 10 μm. A routine biopsy of the gastric angulus with a smooth surface mucosa failed to find a band of collagen deposition at our hospital, and it was hypothesized that the CG inflammation might be multifocal and discontinuously distributed. The EUS findings in this case are not exactly the same as those reported previously in China [3]. The similarity between these cases is that the lesion of the gastric antrum is a hypoechoic replacement of normal structures; however, the difference is that a rough and swollen mucosa with nodular elevation of the gastric corpus was seen under white-light in the case at Hangzhou First Hospital[3], which corresponds to thickening of the muscularis mucosa layer to the submucosal layer with an unclear boundary and low echoes, while, using EGD at our hospital, we found depressed lesions surrounding the elevated ones in the gastric corpus, which corresponds to the presence of five layers of the gastric wall with clear boundaries, as seen by EUS. The mucosal elevation under white-light endoscopy in the present case showed slightly hypoechoic thickening changes under EUS with no thickening of the posterior submucosa.

The white-light endoscopic findings in 2020 and 2021 were similar, both in the gastric corpus and in the antrum. In 2021, the white-light endoscopic findings were different in the gastric corpus and antrum, with elevation-depression changes in the gastric corpus and nodular reddening-like changes in the gastric antrum. EUS findings were also different in the gastric corpus and the gastric antrum. Only the mucosa was involved in the gastric corpus, while the mucosa, muscularis mucosa, and submucosa were all involved in the gastric antrum.

Considering the white-light endoscopic and EUS images, it was presumed that the development of CG inflammation might be progressively from the mucosal layer to the submucosal layer, and the progression of lesions in the gastric corpus and antrum might not parallel each other. The fact that the lesion is hypoechoic might be a EUS change of CG.

Management, follow-up, and prognosis

There are a number of treatments available for CG, some of which are merely symptomatic. H2 receptor antagonists or proton pump inhibitors, aluminum, iron supplementation in patients with anemia, a gluten-free diet in patients with celiac disease[16], glucocorticoids (prednisone[2,8,17], budesonide), other drugs including salicylic acid preparations such as mesalazine and salazosulfapyridine, and parenteral nutrition[9] have been used with varying degrees of efficacy according to different reports.

We gave our patient esomeprazole and mosapride successively, but she had no relief from her symptoms. Then, she was given prednisone acetate for 8 wk according to the treatment protocol for eosinophilic gastritis, and there was no significant relief of her abdominal distension.

The prognosis of CG at follow-up varies significantly, with some patients having reduced or even no collagen deposition and some experiencing recurrent symptoms[9]. The first reported CG case had unremarkable changes in collagen deposition during 12 years of follow-up[18] and showed mild dysplasia. One case of gastric adenocarcinoma positive for Epstein-Barr virus was confirmed after EGD follow-up 8 years after the diagnosis of collagenous gastritis in a patient with IgM reduction[19]. The duration of the follow-up interval and the endpoint are currently unclear.

At present, most facilities use EGD as a follow-up tool, but EUS can clearly identify all layers of the gastric wall, which helps to determine the depth of lesion involvement and is superior to EGD. The hypoechoic lesion may be an EUS change of CG. However, the availability of other non-invasive tests as tools of follow-up has not been clinically reported. The biochemical indices of PG I, PG II, PGR, and gastrin-17 in this patient in our hospital were consistent with atrophic gastritis, but the blood eosinophil and lymphocyte counts, IgA, type III procollagen, and collagen type IV findings were not abnormal. The patient continued to have episodes of abdominal distension after treatment with glucocorticoids, and the results of PG I, PG II, PGR, and gastrin-17 on several retests were abnormal, suggesting that the values obtained by this test may parallel the condition. PG I, PG II, PGR, and gastrin-17 may be used as biochemical indicators for disease treatment and follow-up. As of November 2021, the patient is still being followed up with.

CONCLUSION

In addition to histopathology, diffuse nodular elevation-depression under white-light endoscopy, hypoechoic changes in the lamina propria and submucosa under EUS, and tests of serum pepsinogen I, pepsinogen II, PGR, and gastrin-17 may be helpful in the diagnosis of CG. There is a lack of specific treatment for CG.

FOOTNOTES

Author contributions: Zheng QH performed the Eesophagogastroduodenoscopy and endoscopic ultrasound, made the clinical diagnosis and treatment plan, and wrote the manuscript; Hu J performed the histopathological diagnosis; Yi XY collected the clinical data and performed the follow-up; Xiao XH completed the magnifying endoscopy; Zhou LN helped the endoscopists with the endoscopic procedures as a nurse; Li B supervised the diagnosis and treatment of this patient; Bo XT wrote the English version of the paper; all authors have read and approved the final manuscript.

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REVIEW

Role of radiomics in the diagnosis and treatment of gastrointestinal cancer

Qi Mao, Mao-Ting Zhou, Zhang-Ping Zhao, Ning Liu, Lin Yang, Xiao-Ming Zhang

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Abstract

Gastrointestinal cancer (GIC) has high morbidity and mortality as one of the main causes of cancer death. Preoperative risk stratification is critical to guide patient management, but traditional imaging studies have difficulty predicting its biological behavior. The emerging field of radiomics allows the conversion of potential pathophysiological information in existing medical images that cannot be visually recognized into high-dimensional quantitative image features. Tumor lesion characterization, therapeutic response evaluation, and survival prediction can be achieved by analyzing the relationships between these features and clinical and genetic data. In recent years, the clinical application of radiomics to GIC has increased dramatically. In this editorial, we describe the latest progress in the application of radiomics to GIC and discuss the value of its potential clinical applications, as well as its limitations and future directions.

Key Words: Gastrointestinal cancer; Diagnosis; Treatment; Radiomics; Therapeutic response; Hepatocellular carcinoma

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Core Tip: In this editorial, we summarize the latest advances of radiomics in the field of gastrointestinal cancer diagnosis and treatment. Radiomics has great potential in precision treatment decision-making for gastrointestinal cancer. However, radiomics studies have had relatively marked heterogeneity in their workflow. In the future, it will be necessary to establish and promote an imaging data acquisition protocol, standardize the research workflow, and conduct multicenter prospective studies on quality control.

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INTRODUCTION

Gastrointestinal cancer (GIC) has high morbidity and mortality rates[1]. It causes approximately 5000000 new cases and 3540000 deaths worldwide each year, making it one of the main causes of cancer death[1]. Because of the high heterogeneity of these tumors, it is difficult to implement precision treatment[2]. Lambin *et al*[3] proposed the concept of radiomics in 2012. The emerging field of radiomics can convert potential pathophysiological information in existing medical images that cannot be recognized by the human eye into high-dimensional quantitative image features[2-4]. By analyzing the relationships between these features and clinical and genetic data, we can characterize tumor lesions, evaluate therapeutic responses, and predict survival. In recent years, research on the application of radiomics to GIC has grown dramatically. With this editorial, we aim to describe the latest advances of radiomics in the assessment of GIC and to explore the value of its potential clinical applications, its limitations, and its future directions.

RADIOMICS WORKFLOW

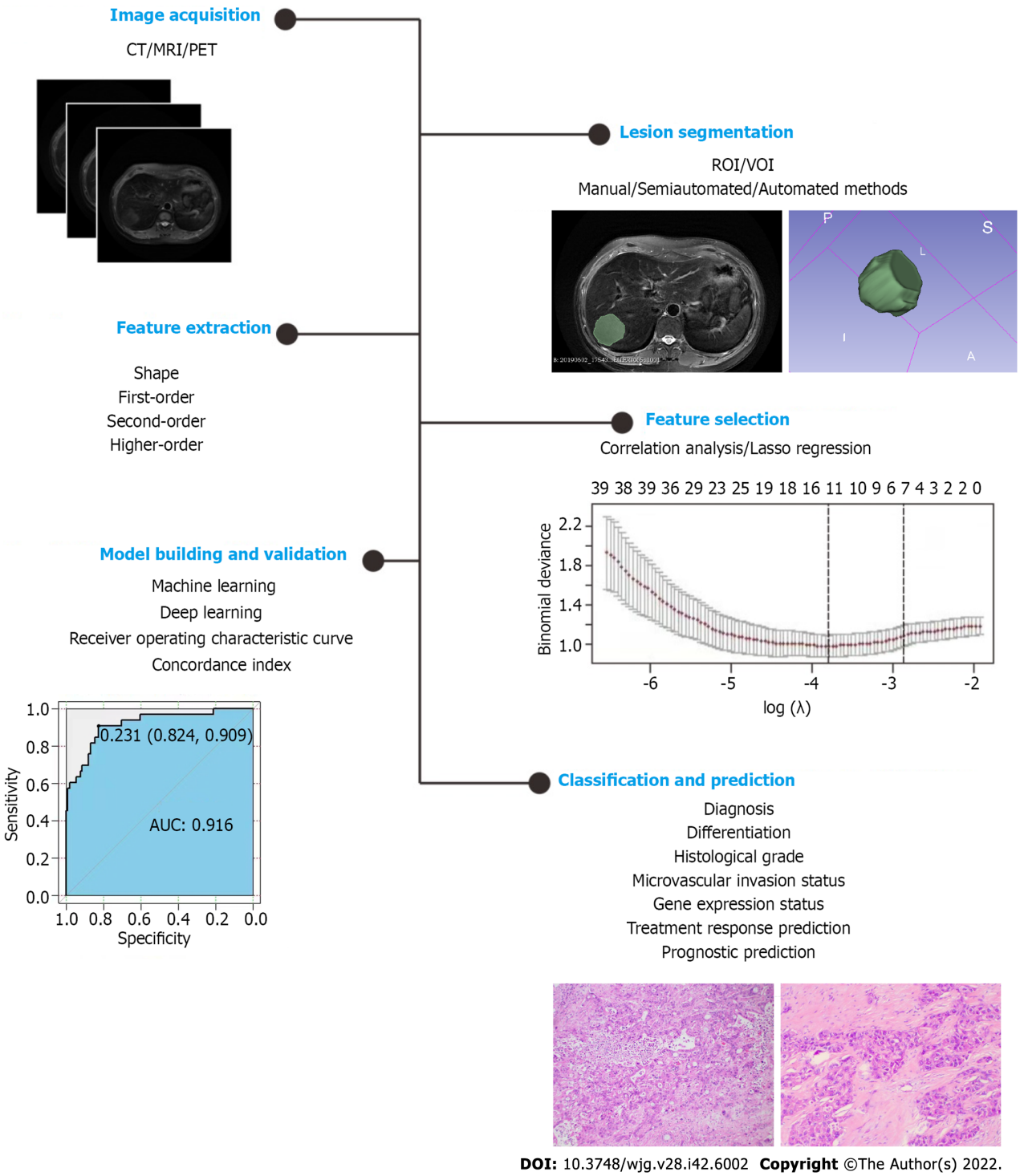
Imaging modalities that can be used for radiomics analysis include computed tomography (CT), magnetic resonance imaging (MRI), and positron-emission tomography (PET). Since CT is the most commonly used staging method for esophageal cancer (EC) and gastric cancer (GC), most radiomics studies on EC and GC are based on CT images[5-9]. In contrast, as MRI is widely used for colorectal cancer (CRC) staging, most radiomics studies on CRC are based on MRI features[10-13].

The workflow of radiomics usually includes image acquisition, lesion segmentation, feature extraction and selection, model building, and validation[14]. Lesion segmentation and feature extraction are the most essential steps. Manual, automatic, and semiautomatic segmentation methods are often used to segment the region of interest (ROI) or volume OI (VOI) (2D or 3D) in a target lesion, and manual segmentation is the most commonly used method (gold standard)[15]. After lesion segmentation, hundreds of radiomic features (shape, first-order, second-order, and higher-order radiomic features) can be extracted from the acquired image. Using all radiomic features to analyze an image will lead to overfitting; thus, feature selection is performed to reduce the number of features that are redundant and irrelevant. The best radiomic features can be selected by dimensionality reduction to improve model efficiency. After feature selection, a radiomics model must be generated. Most published studies use machine learning (ML) and deep learning (DL) methods to build classification and prediction models. Finally, the radiomics model can be validated in internal and external cohorts such that the model can be further optimized and the prediction performance can be maximized. The receiver operating characteristic (ROC) curve is the most commonly used method to evaluate model performance (Figure 1).

EC

Published studies have mainly investigated the predictive ability of radiomics in the staging, therapy response, and postoperative recurrence of EC[16-19].

Radiomic characteristics based on CT have good predictive potential for EC staging[20,21]. Yang *et al*[19] reported that CT radiomic characteristics were significantly correlated with the tumor (T) stage and tumor length of EC and showed good predictive performance for both; the area under the ROC curve (AUC), sensitivity, and specificitywere 0.86, 0.77 and 0.87, respectively, and 0.95, 0.92 and 0.91. Radiomic features also have good efficacy in predicting EC lymphatic metastasis[7,22-24]. Liu *et al*[20] suggested that baseline CT texture is a biomarker for the preoperative assessment of T, lymph node (N), and overall staging of esophageal squamous cell carcinoma (ESCC). Wu *et al*[25] established a model based on the radiomic characteristics of the late arterial phase of CT, which well distinguished early (I-



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Figure 1 The framework of the proposed liver lesion classification. ROI: Region of interest; VOI: Volume of interest.

II) and late (III-IV) ESCC, and the model's efficacy was better than that of tumor volume.

Locally advanced EC often requires neoadjuvant chemoradiotherapy (NACRT)[26], whose treatment outcome is associated with tumor heterogeneity[27,28]. Radiomics can extract tumor heterogeneity data and has good application potential in improving the treatment stratification of patients. Radiomic characteristics are helpful for evaluating the response of EC to NAC or NACRT, distinguishing responders from nonresponders, for which it performs better than traditional parameters[29-32]. A prospective multicenter study[33] developed and validated a three-dimensional DL model applied to preprocessed CT images to predict the response of patients with locally advanced thoracic esophageal squamous cell carcinoma (TESCC) to concurrent chemoradiotherapy. The three-dimensional DL model achieved good predictive performance, with an AUC in the training cohort of 0.897 [95% confidence interval (CI): 0.840-0.959] and an AUC in the validation cohort of 0.833 (95%CI: 0.654-1.000). It is also feasible to use radiomics to predict the pathological complete response (pCR) of EC[34,35]. Patients with

a pCR after NACRT have a higher overall survival (OS) rate[36,37], but nonresponders will not benefit from this therapy[38]. This information can provide guidance for personalized treatment of EC patients [28]. A CT-based radiomics study showed that a model that combined the intratumoral and peritumoral radiological characteristics could improve the predictive performance of the pCR of EC NACRT. In the test set, the AUC was 0.852 (95%CI: 0.753-0.951), the accuracy was 84.3%, the sensitivity was 90.3%, and the specificity was 79.5%[35]. Several studies of radiomics based on MRI or ^{18}F-fluorodeoxyglucose (^{18}F-FDG) PET also showed its efficacy in predicting the response to EC treatment[39-42]. The application of radiomics to immunotherapy has also achieved good response prediction value[43].

Radiomics has also made progress in predicting the recurrence and prognosis of EC patients[44-47]. Tang *et al*[48] predicted the early recurrence of locally advanced ESCC after trimodal therapy based on enhanced CT radiomics. The results showed that in the training cohort, the AUCs of the radiomics model, the clinical model, and the combined model were 0.754, 0.679, and 0.821, respectively, and they were 0.646, 0.658, and 0.809 in the validation cohort; the combined model was the best. Qiu *et al*[49] developed and validated a prediction model based on radiomic features extracted from contrast-enhanced CT images to estimate the recurrence-free survival (RFS) of patients who achieved pCR through NACRT and surgery. The results showed that the radiomic characteristics were significantly correlated with RFS. In the training cohort and the validation cohort, compared with the nomograms of the radiomic characteristics and of clinical risk factors, the nomogram combining the radiomic characteristics and clinical risk factors had optimal performance. Other studies have shown that combining the radiomic characteristics of primary tumors and regional lymph nodes with clinical-pathological factors can improve OS prediction[50].

Other studies showed that CT-based radiomics features also had good predictive performance for classifying patients according to histological differentiation[51-53], the expression of programmed death-ligand 1, and CD8$^+$ tumor-infiltrating lymphocytes of EC[5].

GC

In recent years, some researchers have also explored the value of radiomics to the diagnosis and treatment of GC[9,54,55]. The CT radiomics model has high application value in the identification of GC [54,56-58]. Feng *et al*[59] used a transfer learning radiomics nomogram (TLRN) with whole-slide images of GC as the source domain data to distinguish Borrmann type IV GC from primary gastric lymphoma before surgery. The TLRN that integrated transfer learning radiomics signatures (TLRS), clinical factors, and CT subjective findings was developed through multiple logistic regression (LR). The results showed that the TLRN performed better than the clinical model and the TLRS. The AUCs of the internal and two external validation cohorts were 0.958 (95%CI: 0.883-0.991), 0.867 (95%CI: 0.794-0.922), and 0.921 (95%CI: 0.860-0.960), respectively[59]. Wang *et al*[60] reported that a DL radiomics model based on CT images had a potential role in the T staging of GC. For distinguishing T2 from T3/4 tumors, the AUCs of the arterial phase-based radiomics model in the training group and the test group were 0.899 (95%CI: 0.812-0.955) and 0.825 (95%CI: 0.718-0.904), respectively. The AUC of the radiomics model based on the portal vein phase in the training and testing cohorts was 0.843 (95%CI: 0.746-0.914) and 0.818 (95%CI: 0.711-0.899), respectively[60]. An important factor in the failure of GC treatment is lymph node metastasis (LNM) and cancer spread in the peritoneal cavity[61]. In GC, the most common metastatic sites are the distant lymph nodes (56%), liver (53%), and peritoneum (51%)[62]. Accurate assessment of LNM and preoperative N staging is critical for the accurate treatment of GC patients. Most studies have shown that CT-based radiomics models have good accuracy in predicting early GC lymph node and peritoneal metastasis before surgery[63-66]. A ML model based on preoperative ^{18}F-FDG-PET/CT obtained similarly good results[67].

CT-based radiomic characteristics also perform well in predicting the response to NAC and radiotherapy in patients with advanced GC[68-71]. Jiang *et al*[72] showed that a DL CT signature could help to identify patients who might benefit from adjuvant chemotherapy for GC and improve prognostic prediction. A radiomics study based on ^{18}F-FDG-PET signatures obtained similar results[73]. In addition, radiomics can be used to predict the histological grade of GC before surgery[74] and is useful for GC classification[75,76].

CRC

The application of radiomics to CRC has mainly focused on the evaluation of stage, neoadjuvant therapy outcome, and gene mutations[77,78].

Radiomics models are helpful for CRC staging[79-81]. LNM is an independent risk factor affecting the prognosis of CRC patients. Radiomics models can effectively predict LNM in CRC patients before surgery[82-85]. Liu *et al*[84] found that multiregional-based MRI radiomics combined with clinical data could improve the efficacy of predicting LNM. He *et al*[85] developed and tested five ML models based on the radiomic features of F-18-FDG-PET/CT and PET for their preoperative prediction of LNM in the CRC region: LR, support vector machine, random forest (RF), neural network, and extreme gradient boosting. The results showed that the LR (AUC 0.866, 95%CI: 0.808-0.925) and extreme gradient boosting models (AUC 0.903, 95%CI: 0.855-0.951) performed the best, outperforming F-18-FDG-PET/CT on both the training set and the test set[85]. Other studies have also shown that radiomics has a good ability to predict metastasis to distant organs, such as the liver and lung, as well as vascular and

perineural invasion[86,87]. It is reported that the predictive power of CT-based radiomics for the preoperative staging of CRC. The results showed that the radiomic features were an independent predictor of CRC staging. CRC was successfully divided into stages I-II and III-IV in the training and validation datasets. The AUC in the training dataset was 0.792 (95%CI: 0.741-0.853), the sensitivity was 0.629, and the specificity was 0.874. The AUC in the validation dataset was 0.708 (95%CI: 0.698-0.718), the sensitivity was 0.611, and the specificity was 0.680[79].

Radiomics models have had excellent performance in noninvasively predicting the response to NAC and NACRT in patients with locally advanced CRC (including liver metastasis)[88-91]. They have also achieved good efficacy in predicting the response to CRC targeted therapy[77,92].

Mutations in the *KRAS*, *NRAS*, or *BRAF* gene indicate that CRC patients will lack a response to drugs targeting epidermal growth factor receptor. In 2016, the National Comprehensive Cancer Network guidelines recommended that all patients with suspected or confirmed metastatic CRC should be tested for *KRAS*/*NRAS*/*BRAF* mutations, but this requires pathological tissue specimens. It is gratifying that some radiogenomics studies have shown that the radiomic characteristics of CT and MRI may help to predict the genotype of CRC tumors before surgery[93-95]. Yang *et al*[96] reported that CT radiomic characteristics were associated with *KRAS*/*NRAS*/*BRAF* mutations. Another MRI radiomics study found a good correlation between quantitative features and gene mutations, while there was no correlation between qualitative features and gene mutations[97].

More recent studies have shown that radiomics can predict CRC histological grade before surgery[98,99].

LIVER CANCER

The application of radiomics to hepatocellular carcinoma (HCC) involves differential diagnosis, determination of microvascular invasion (MVI) status, histological grade, gene expression status, and treatment response, and prognostic prediction[100-104].

Because HCC has a typical enhancement mode, dynamic contrast-enhanced CT, MRI, and ultrasound have played major roles in the diagnosis and differentiation of HCC[105-107]. However, it is sometimes difficult to distinguish some small nodules from atypical lesions[108-111]. Radiomics can achieve quantitative analysis of tumor biological behavior and heterogeneity, helping identify liver nodules[112-114]. Yasaka *et al*[115] investigated the performance of a DL method to distinguish liver masses on dynamic enhanced CT. There are five types of these masses: Type A, classic HCC; type B, malignant liver tumors other than HCC; type C, indeterminate masses or mass-like lesions, plus rare benign liver masses other than hemangiomas and cysts; type D, hemangiomas; type E, cysts. The median accuracy of the mass identification on the test set was 0.84. The AUC that distinguished the types A-B from types C-E was 0.92. Hamm *et al*[116] used a DL method to classify common liver lesions with typical imaging characteristics on multiphasic MRI, including a total of 494 liver lesions from six categories, which were divided into training (n = 434) and test groups (n = 60). Their DL system had an accuracy of 92%, a sensitivity of 92%, and a specificity of 98%. For HCC classification, the true-positive rate and false-positive rate were 93.5% and 1.6%, respectively, and the AUC was 0.992[116]. Other studies have reached similar conclusions[108,110].

The 5-year recurrence rate of HCC resection can reach 70%[103]. Pathological features such as histological grade and MVI of HCC were significantly correlated with postoperative recurrence and prognosis[117-120]. Histological grade, MVI status[121-125], and gene expression[113,126,127] in HCC can be successfully predicted by radiomics models before surgery. An MRI-based radiomics study showed that the AUCs of the MVI nomogram in the validation cohort using the RF algorithm and LR analysis were 0.920 (95%CI: 0.861-0.979) and 0.879 (95%CI: 0.820-0.938), respectively[123].

Radiomics models based on contrast-enhanced CT and MRI can predict the response of middle- and late-stage HCC to local treatment and systemic treatment[10,128-130]and the early recurrence and the prognosis after HCC resection[101,102,131,132]. Zhang *et al*[133] evaluated the effectiveness of predicting OS after HCC resection based on contrast-enhanced MR imaging features. The results showed that preoperative clinical features and semantic imaging features were significantly correlated with the survival rate; the combined model had the best predictive performance[133].

Some studies using radiomics to predict the occurrence of CRC liver metastases are particularly interesting[134-137]. Rao *et al*[137] retrospectively analyzed the primary staging CT data of 29 CRC patients. The patients were divided into three groups: The non-liver-metastasis group, the simultaneous liver metastasis (LM) group, and the metachronous LM group within 18 mo. Whole-liver texture analysis was performed on the liver parenchyma that was clearly disease-free on the portal vein image. The results showed that compared with those in nonmetastatic patients, the mean entropy 1.5 (E1.5)and E2.5 values of the whole liver in patients with synchronous metastasis were significantly increased, and the uniformity 1.5 (U1.5)and U2.5 values were significantly decreased. The AUCs for the diagnosis of synchronous metastasis based on E1.5, E2.5, U1.5, and U2.5 were 0.73-0.78[137]. Beckers *et al*[138] conducted a similar retrospective multicenter study. They included a total of 165 cases of CRC, which were also divided into the nonmetastasis group, the synchronous metastasis group, and the metastasis

group (within 24 mo). Univariate analysis confirmed that U, sex, tumor site, nodal stage, and carcinoembryonic antigen (CEA) were potential predictive factors; multivariate analysis showed that U was still a factor predicting early metastasis; and none of the parameters could predict intermediate/late metastasis[138]. Other studies have shown no significant difference in CT texture parameters of liver parenchyma between CRC patients with and without liver metastasis[134,135]. The conclusions of these studies are inconsistent, so the prediction of LM of CRC based on the texture characteristics of the liver parenchyma requires further study. Recently, Liet al[139] investigated the efficacy of a radiomics model based on baseline CRC contrast-enhanced CT in predicting metachronous liver metastases in CRC patients. The AUC of the radiomics feature model was 0.78 ± 0.07, and the AUC of the clinical feature model was 0.79 ± 0.08. The model combining the two performed best, with AUCs of 0.79 ± 0.08 and 0.72 ± 0.07 in the internal and external validation cohorts, respectively. They believed that the radiomic characteristics of primary CRC lesions are often affected by fewer factors and are more stable; their radiomic characteristics have the potential to distinguish patients at risk of liver metastasis.

PANCREATIC CANCER (PC)

For PC, the application of radiomics mainly focuses on identification, treatment response prediction, and prognostic prediction[140-142]. Many studies have focused on the diagnosis and differentiation of pancreatic ductal adenocarcinoma (PDAC)[143-146]. Chu *et al*[146] investigated the utility of CT radiomics in distinguishing PDAC from normal pancreas. In their retrospective casecontrol study, 190 PDAC patients and 190 healthy potential renal donors were included. The overall accuracy of RF binary classification was 99.2%, with an AUC of 99.9%; all PDAC cases were correctly classified. Park *et al*[145] confirmed that CT-based ML of radiomics features was helpful to distinguish between autoimmune pancreatitis and PDAC, with an overall accuracy of 95.2%. The radiomics model based on PET/CT also showed good performance in distinguishing benign autoimmune pancreatitis from malignant PDAC lesions[143,144].

Other studies have shown that radiomics can better predict the treatment response and prognosis of PC[142,147]. Simpson *et al*[141] evaluated the potential of MRI-based radiomics to predict the response to PC treatment. A total of 20 patients with unresected nonmetastatic PDAC were enrolled, all of whom received NAC followed by five rounds of MR-guided stereotactic body radiotherapy. Half of the 20 patients were defined as having histopathological tumor regression or tumor response based on an enhanced CT. The AUC of the model based on the RF algorithm was 0.81 (95%CI: 0.594-1.000); the adaptive least absolute shrinkage and selection operator (LASSO) algorithm achieved AUC of 0.81 (95%CI: 0.596-1.000). Xie *et al*[148] used a CT-based radiomics nomogram to predict the survival of patients with resected PDAC. The radiomics score developed based on CT imaging features was significantly correlated with disease-free survival (DFS) and OS in patients with PDAC. The radiomics nomogram showed good discrimination in both the training cohort and the validation cohort, being superior to the clinical model and the TNM staging system for survival estimation. The model integrating the radiomics score and clinical data had the best predictive performance, but there was no correlation between the radiomics score and recurrence pattern. Similar results were seen by Healy *et al*[149].

LIMITATIONS AND FUTURE DIRECTIONS

In this editorial, we summarize the results of the application of radiomics to the field of GIC diagnosis and treatment. These results show that radiomics has great potential for decision-making about precision treatments for GIC. Moreover, these results have important reference value for studies of other systemic tumors.

However, some limitations to the clinical application of radiomics remain[150,151]. The first key challenge is the use of different imaging techniques by different institutions and/or scanners. To ensure that the academic community can obtain high-quality radiological data resources, it is necessary to establish and promote certain imaging acquisition protocols[149]. Second, the current research uses different software and different feature selection methods, focuses on different feature sets, and applies different statistical and bioinformatic methods for data analysis and interpretation, which limit the reproducibility of radiomics models[152,153]. Future research workflows need to be standardized. Third, many relevant radiomics studies employ single-center retrospective datasets. A quality-controlled multicenter prospective study plan is ideal. In addition, the evidence level rating reflects the feasibility of incorporating radiomics research into clinical practice. Recently published guidelines and checklists aiming to improve the quality of radiomics studies, including the radiomics quality score, modified Quality Assessment of Diagnostic Accuracy Studies tool, image biomarker standardization initiativeguideline, and Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis checklist, have been applied to radiomics evaluations[154-157]. These studies have shown that the current scientific and reporting quality of many radiomics studies is insufficient;

feature reproducibility, open science categories, and clinical utility analyses need to be improved; and study objectives, blinding, sample sizes, and missing data must be reported[154-157]. In the future, radiomics studies should adhere to these guidelines to facilitate the translation of radiomics research into clinical practice[156].

CONCLUSION

Radiomics has great potential in precision treatment decision-making for gastrointestinal cancer. However, radiomics studies have had relatively marked heterogeneity in their workflow. In the future, it will be necessary to establish and promote an imaging data acquisition protocol, standardize the research workflow, and conduct multicenter prospective studies on quality control. In addition, the combination of radiomics with multiomics may lead to a major breakthrough in individualized medical treatment of tumors.

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REVIEW

COVID-19 associated liver injury: A general review with special consideration of pregnancy and obstetric outcomes

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Abstract

Liver injury is an increasingly recognized extra-pulmonary manifestation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Coronavirus disease 2019 (COVID-19) associated liver injury (COVALI) is a clinical syndrome encompassing all patients with biochemical liver injury identified in the setting of SARS-CoV-2 infection. Despite profound clinical implications, its pathophysiology is poorly understood. Unfortunately, most information on COVALI is derived from the general population and may not be applicable to individuals under-represented in research, including pregnant individuals. This manuscript reviews: Clinical features of COVALI, leading theories of COVALI, and existing literature on COVALI during pregnancy, a topic not widely explored in the literature. Ultimately, we synthesized data from the general and perinatal populations that demonstrates COVALI to be a hepatocellular transaminitis that is likely induced by systemic inflammation and that is strongly associated with disease severity and poorer clinical outcome, and offered perspective on approaching transaminitis in the potentially COVID-19 positive patient in the obstetric setting.

Key Words: COVID-19 liver injury; Pregnancy; Perinatal liver disease; Systemic inflammation; Special populations

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Core Tip: Liver injury is an increasingly recognized extra-pulmonary manifestation of coronavirus disease 2019 (COVID-19). COVID-19 associated liver injury (COVALI) is a clinical syndrome encompassing all patients with COVID-19 infection and biochemical liver injury. Unfortunately, most information on COVALI is derived from the general population and may not be applicable to individuals under-represented in research, including pregnant individuals. In this review we summarize clinical features of COVALI and the leading theories of pathophysiology and present existing literature on COVALI during pregnancy, a topic not widely explored in the literature.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease pandemic (COVID-19) is responsible for and upwards of 6.3 million fatalities worldwide[1]. The SARS-CoV-2 virus is a member of the *Coronaviridae* family, a diverse family of single-stranded positive RNA viruses[2]. Coronaviruses are frequently implicated in mild upper respiratory infections and cause 15%-30% of cases of the "common cold"[3,4]. However, *Coronaviridae* viruses have also demonstrated an ability to infect the lower respiratory tract and cause severe lung disease associated with substantial mortality[5,6].

Mortality associated with COVID-19 is usually secondary to lung pathology that causes severe respiratory distress syndrome[7-9]. However, patients infected with SARS-CoV-2 often suffer other devastating end-organ injuries[10], suggesting the virus causes systemic infection and inflammation. These observations have prompted interest in the extra-pulmonary manifestations of COVID-19[11], including those in the heart[12,13], intestines[14,15], kidney[16], reproductive system[17,18], and the liver, where the effect of SARS-CoV-2 is poorly understood[19,20].

COVID-19 associated liver injury (COVALI) is a clinical entity encompassing any abnormal liver function test present in individuals positive for SARS-CoV-2[20]. Currently there are no specific or unique diagnostic criteria for COVALI relative to other causes of transaminitis[21] which complicates the process of synthesizing evidence from clinical studies. This is most salient when applying available data to those underrepresented in the literature, such as pregnant and birthing persons.

In the first section of this review, we will summarize clinical features of COVALI and the leading theories on the mechanism of liver damage in the general population. In the second section, we present existing literature on liver injury in SARS-CoV-2 positive pregnant persons, a topic not widely explored in the literature despite significant clinical relevance. Ultimately, we aim to synthesize data on COVALI in the general and perinatal populations and offer perspective on approaching this problem in the obstetric setting.

GENERAL POPULATION

Background

At the present time, COVALI is an umbrella term that applies to all patients with SARS-CoV-2 infection and transaminitis. Meta-analyses estimate that one in four patients with COVID-19 develop acute liver injury[22-24], but this figure is variable across studies and ranges from 14%-74%[25-27]. There seem to be no demographic factors to account for this variability, which may ultimately be due to differences in the study timeline or definition of liver injury[28]. Interestingly, only one of the three cited meta-analyses on COVALI included a study involving pregnant patients (n = 9)[29]. In the following sections we will review clinical and pathophysiologic considerations for COVALI in the general population.

Clinical Considerations

COVID-19 associated liver injury is a hepatocellular or mixed pattern liver injury with aspartate aminotransferase (AST) predominant transaminitis[28,30-33]. Most studies report mild liver injury with liver enzymes that peak at values less than five times the upper limit of normal[34-38]. Conversely, some reports suggest up to 25% of patients' aminotransaminases exceed this threshold[39,40] and there

is mounting evidence that liver enzymes can increase to the thousands (U/L) in patients with severe COVID-19[26,38,41-45]. The timeline of developing liver injury is not fully elucidated and has varied between studies[32,33].

Non-transaminase laboratory evidence of liver damage has also been identified, but is reported less consistently in the literature. Specifically, total bilirubin and alkaline phosphatase have been reported to be elevated in 1%-53%[46-48] and 0.3%-80.0%[48,49] of patients, respectively. This variability may be due to study timeline relative to the temporal course of laboratory changes in patients with COVALI. For example, it has been shown that alkaline phosphatase elevations begin and peak later in the disease course than aminotransaminases and may not be captured by studies that don't follow laboratory data for extended periods[50,51].

The interest in COVALI is rooted in its association with disease severity and negative patient outcomes. First, patients with elevated liver enzymes at presentation or at hospital admission are more likely to develop severe COVID-19 lung disease[5,52-54]. Additionally, a large study by Guan *et al*[55] reported laboratory data from patients at over 500 hospitals and found patients with severe COVID-19 were more likely to have transaminitis compared to patients with non-severe COVID-19. Going further, Bloom *et al*[31] studied trends in aminotransaminase levels from time of admission to peak in patients hospitalized for COVID-19 and found that in addition to higher mean AST and alanine aminotransferase (ALT), there was a greater change from baseline to peak transaminases in patients with severe compared to non-severe COVID-19. A small single center study found that elevated AST was observed more often in patients who required intensive level care compared to those who did not require intensive care[56]. Further, in a cohort of 1611 hospitalized patients across 11 Latin American countries, abnormal liver enzymes conferred a 2.6-fold risk for severe COVID-19 pneumonia and a 1.5-fold risk of death[37].

Within the umbrella of COVALI, AST has been shown to have specific prognostic value[29,37,57]. For example, the numeric value for serum AST has been incorporated in clinical calculators created to predict progression from mild or moderate to severe COVID-19 disease[57]. Moreover, elevated AST has been found to be independently associated with increased risk of death, apart from other markers of hepatic dysfunction[29,34,50,58,59]. In a study including 206 patients across 26 institutions in Brazil, AST level greater than twice the upper limit of normal significantly increased the risk of in-hospital mortality when adjusted for age and biologic sex[29]. However, is important to note that when elevated, bilirubin may be a stronger predictor of death than AST in some cohorts[34].

Given the association with poor patient outcomes, identifying potential risk factors for COVALI is imperative. We found one meta-analysis that sought to define predictors for the development of COVALI. In this study Harapan *et al*[60] pooled data from 16 studies (n = 6253) to assess whether any of the following were associated with development of severe liver injury in patients infected with SARS-CoV-2: Age, biologic sex, body mass index (BMI), diabetes mellitus, coronary artery disease, hypertension, underling liver disease, white blood cell count, lymphocyte count, and neutrophil count. They observed significant association between male sex, higher BMI, presence underlying liver disease, elevated white blood cell, and elevated lymphocyte counts with development of acute liver injury. After controlling for bias introduced by the meta-analysis, they concluded male sex and lymphocyte count were found to be independent risk factors for COVALI[60]. Not evaluated in this meta-analysis, inflammatory markers have also been shown to be a risk factor associated with liver injury[61-64]. For example, multiple studies have inflammatory markers directly correlate with liver enzymes[64] and that liver injury can be predicted using inflammatory markers such as ferritin and C-reactive protein[61,62].

Professional societies recommend clinically relevant work up for other causes of liver injury in patients who develop COVALI[21,65-68]. The American and Asian Pacific Association(s) for the Study of Liver Diseases suggest ruling out other causes of viral and toxin-mediated hepatitis in all COVID-19 patients with liver injury[21,66]. More nuanced suggestions include considering cytokine-syndrome, myositis, or cardiac injury in patients with disproportionally elevated AST, and primary sclerosing cholangitis in critically ill patients with cholestatic liver injury[21,65,66]. They advise trending liver enzymes of patients hospitalized with COVID-19, those with known chronic liver disease diagnosed with COVID-19 and of those receiving anti-retroviral medications for treatment of COVID-19 pneumonia[66]. Patients with chronic hepatitis B may be at particularly high risk both due to risk of severe infection and viral reactivation when receiving immunosuppressive therapy[66,69]. However, they do not recommend changing management and offer no specific intervention for liver injury in most cases of COVALI. They endorse targeting the viral illness in the acute setting is sufficient for liver injury and encourage work up for chronic liver disease when illness is resolved.

Pathophysiology

The underlying mechanism(s) of liver injury in COVID-19 are not fully understood. While there is increasing literature on this topic, the absence of explicit diagnostic criteria has resulted in heterogeneity in clinical studies and has impeded recognition of specific mechanisms of injury. There is consensus that COVALI is likely multifactorial and due to a combination of exacerbation of underlying liver disease, direct cytotoxicity, hypoxic liver injury, drug induced injury and systemic inflammation with immune dysregulation[28,70].

Early theories focused on exacerbation of underlying liver disease and toxicity from pharmacologic agents used to treat severe COVID-19 infection as a sources of liver injury. It is true that patients with cirrhosis are at risk for developing severe pneumonia and hepatic decompensation during SARS-CoV-2 infection[21]. Likewise, some antiviral medications used to treat COVID-19 have hepatotoxic properties and have been associated with abnormal liver function during the pandemic (*e.g.*, lopinavir/ritonavir)[27,40,61,71]. In a combination of these, SARS-CoV2 infection treated with corticosteroids or tocilizumab has been showed facilitate reactivation and accelerate liver injury in patients with chronic hepatitis B[72]. However, these two factors are unable to explain most of this phenomena as: (1) Over 90% of patients with COVALI have no evidence of underlying liver disease; and (2) transaminitis is often present at baseline prior to administration of medications[73]. While it is possible that liver injury during SARS-CoV-2 infection may be exacerbated by these factors, COVALI is likely a distinct clinical entity.

Diverse studies have demonstrated direct viral infection of the liver can occur during COVID-19 infection. In a study including 156 autopsy samples, postmortem hepatic tissue evaluation revealed typical coronavirus particles in hepatocyte cytoplasm with associated mitochondrial swelling and endoplasmic reticulum dilatation in patients who died with COVID-19[74]. Other reports have shown SARS-CoV-2 nuclear material in liver tissue, including RNA in hepatocytes of live patients who underwent liver biopsy during SARS-CoV-2 infection[75]. Some of the most convincing data comes from a recent paper by Wanner *et al*[76] who demonstrated SARS-CoV-2 can be detected in up to three-fourths of post-mortem liver biopsies using reverse transcriptase-polymerase chain reaction. Ultimately, there is irrefutable histologic evidence that SARS-CoV-2 directly infects hepatocytes, providing strong evidence that SARS-CoV-2-mediated cytopathy plays a role in COVALI. It is thought that angiotensin converting enzyme 2 and/or its receptor (ACE-2) may mediate cytopathy by enabling viral access to the liver[76,77]. However, the understanding of SARS-CoV-2 hepatotropism of is still evolving.

Epidemiology-based correlates support direct ACE-2 mediated entry into hepatocytes based on data that shows groups at increased risk of COVALI also have increased hepatic ACE-2 expression. For example, ACE-2 Levels are higher in males than females[78] and ACE-2 is upregulated in decompensated cirrhosis[79]. Interestingly, it has been shown that ACE-2 is dominantly expressed in cholangiocytes relative to hepatocytes and that infection of cholangiocytes may occur more often than infection of hepatocytes[80]. While this may seem to contradict direct cytotoxicity, it is possible that cholangiocyte infection can still result in direct viral access to the liver. In-vitro infection with SARS-CoV-2 has been associated with decreased expression of the cholangiocellular tight junctions that usually protect parenchymal liver cells from toxins in the biliary tree[81]. It has been further speculated that reduced barrier function of cholangiocytes during active COVID-19 infection may lead to hepatic injury through leakage of toxic bile into the adjacent liver parenchyma[81]. Lastly, it is known that ACE-2 can mediate viral entry into endothelial cells[82]. Viral infection of the portal systems and vascular cells in the liver may contribute to the endothelitis, microvascular changes, and intravascular thrombosis visualized in post-mortem examination of hepatic tissue in patients who died from COVID-19[83].

Reduced blood oxygen, which can negatively affect the liver, occurs in up to 50% of patients with COVID-19 infection[84]. However, only a small percentage of patients have transaminitis to the degree expected in ischemic hepatitis[34-38]. While ischemia from low blood oxygen seems to have a limited direct role in COVALI pathophysiology, the relationship between hypoxia and inflammatory pathways is significant. Specifically, hypoxia can trigger and amplify immune dysregulation *via* inflammatory pathways mediated by hypoxia inducible factor and tumor necrosis factor[85]. This may explain the link between severity of lung disease with liver injury and provide support for and transition to the inflammatory hypotheses of COVALI[86].

There is substantial data suggesting systemic inflammation and associated immune dysregulation, endotheliopathy and thrombosis are central to the pathophysiology of COVALI[87,88]. It is well established that severe COVID-19 infection induces systemic inflammation and that concentrations of several clinically evaluated inflammatory markers are increased in patients with COVID-19, such as D-dimer, C-reactive protein, procalcitonin, ferritin, and interleukin-6 (IL-6)[89-92]. Inflammatory markers are also higher in COVID-19 positive patients with biochemical liver derangements compared to COVID-19 positive patients without such derangements across, suggesting a link between liver injury and inflammation[61,62,92-94]. For example, a large retrospective analysis (n = 800) showed patients with COVID-19 complicated by COVALI had higher levels of C-reactive protein, procalcitonin, D-dimer, and serum ferritin compared to patients without COVALI[61]. In a unique study, Diaz-Louzao *et al*[86] used joint regression modeling to evaluate the temporal relationship between increases in markers of liver injury and inflammation. They found that elevation of inflammatory markers precedes elevation of liver enzymes. Ultimately they created a statistical model that implicates inflammation in causation of liver injury. The specific inflammatory markers increased during COVALI are known to be involved in *in vivo* endotheliopathy and hypercoagulability[95,96], as has been visualized in hepatic tissue of patients with liver injury secondary to COVID-19. Further, histologic findings of macrovesicular steatosis, mild acute hepatitis, portal inflammation and portal/sinusoidal thrombosis in hepatic tissue of patients who have direct viral infection of the liver support that even with direct cytopathy, inflammation may have a preceding role[83,97-100].

Interleukin-6 is an inflammatory cytokine associated with endotheliopathy and a hallmark indicator of severe COVID-19. It has been shown that IL-6 can activate platelets and precipitate endothelitis in multiple organs during systemic COVID-19 infection, particularly those with a predilection for intravascular clot formation (*e.g.*, the liver)[95]. Due to its association with biochemical liver injury[85,101-103] and known function[85,101,102], IL-6 has received interest as a likely active contributor to development of liver injury in COVID-19[103]. Recent work by McConnell *et al*[102] found a potential mechanism for this in that activating a soluble form of the IL-6 receptor triggers downstream pro-inflammatory and pro-coagulation pathways in the liver[102,104]. Further, that IL-6 signaling induces a hypercoagulable state in liver sinusoidal cells[85,104], which may contribute to the known endothelitis and thrombosis in hepatic tissue of patients with COVALI. Similarly, increased staining of a well-known platelet marker (CD-61) has been identified within dilated sinusoids in COVID-19 patients with elevated liver enzymes, suggesting activated platelets and endotheliopathy are critical in liver injury during COVID-19[85]. These findings are consistent with studies showing portal or sinusoidal vascular thrombosis is present in hepatic tissue of up to 50% of patients with COVID-19[83]. In context of literature on inflammatory markers in COVALI, Il-6 shows true mechanistic potential and bolsters the theory that inflammation, endotheliopathy and thrombosis are at the crux of this clinical syndrome.

OBSTETRIC POPULATION

Background

Liver injury is a rare and potentially serious complication of pregnancy that is estimated to affect 3%-5% of birthing persons[105]. The differential diagnosis for hepatic dysfunction in this population includes specific pregnancy related (perinatal) liver diseases[106], such as pre-eclampsia/eclampsia, hemolysis elevated liver enzymes and low platelet (HELLP) syndrome, acute fatty liver of pregnancy and intrahepatic cholestasis of pregnancy (obstetric cholestasis), and non-pregnancy related liver diseases, such as auto-immune hepatitis, viral hepatitis, non-alcoholic steatohepatitis, and now COVALI[107-109]. Perinatal liver diseases are associated with significant mortality and often require prompt delivery of the fetus for safety of the mother (summarized in Table 1). Because liver injury can strongly influence decisions regarding delivery[107], COVALI during pregnancy is of serious clinical significance.

Clinical considerations

General clinical course: Clinical characteristics of COVID-19 during pregnancy given current knowledge are well represented in the literature, but there is limited data specific to the course of liver injury. In the obstetric setting, COVALI is an AST-predominant transaminitis that affects 13%-42% of COVID-19 positive pregnant patients[108,110-112]. While these statistics are comparable to the general population, a meta-analysis that included pregnant patients reported key differences. They found (1) higher prevalence of COVALI in pregnant patients compared to non-pregnant patients; and (2) more severely elevated liver enzymes in pregnant patients with COVALI compared to non-pregnant patients with COVALI[113]. This was confirmed in a study that directly compared laboratory values of COVID-19 positive pregnant patients with non-pregnant counterparts and found COVALI was more common in pregnancy[110]. The authors of this study cautioned that many of their observations were likely related to physiological changes of pregnancy, but they concluded the rate of COVID-19 positive pregnant individuals with acute liver injury was out of proportion to expected physiologic changes. This may indicate that COVID-19 confers an increased risk of liver injury specific to pregnancy.

Clinical Cases: Obstetric providers are tasked with differentiating liver disease that necessitates urgent delivery for the health and safety of the pregnant person *vs* that which can be managed expectantly and will be stable or resolve without delivery. Multiple reports illustrate this dilemma through cases of pregnant patients with acute liver injury who are COVID-19 positive and have concurrent features of high-risk perinatal liver diseases[114-120]. We identified seven cases and classified them according to the pattern in which liver enzymes improved throughout the clinical course: A, improved without delivery; B, improved with delivery; C, other (no improvement within 72 h of delivery, no timeline of COVID-19 symptoms) (Table 2). We will discuss cases that improved without delivery and highlight features that favored COVALI relative to perinatal liver diseases.

In a case described by Azimi *et al*[115], a 27-year-old Gravida (G) 2 Para (P) 1 woman presented at 30-wk' gestation with a headache and was found to have abnormal liver enzymes, low platelets, increased inflammatory markers (LDH, ferritin D-dimer), and chest radiograph showing diffuse ground glass opacities, concerning for autoimmune disease *vs* HELLP *vs* systemic COVID-19. Pending extensive laboratory evaluation that was negative for autoantibodies and signs of hemolysis, the patient was noted to be improving with only supportive care. She was discharged at 33-wk' gestation and underwent normal delivery at 39-wk' gestation. The next case was that of a 35-year-old G2 P1 with prior obstetric cholestasis presenting at 28-wk' gestation with progressive fever and cough who was found to have high ALT and elevated serum bile acids[114]. The patient denied pruritis and had normal labs at her 20-wk appointment which reduced the likelihood of obstetric cholestasis; she subsequently tested

Table 1 Clinical characteristics and management of COVID-19 and perinatal liver diseases

	COVID-19	PEC/severe PEC	HELLP	ICHP	AFLP
Epidemiology	-	5.0%-7.5%	1%	0.3%-5.6%	0.005%-0.010%
Symptoms	Respiratory +/- GI symptoms	Variable: Headache, swelling, vision changes (or none)	Variable: Headache, nausea, vomiting, RUQ pain (or none)	Pruritis; starting at palms + soles (can be diffuse)	Nausea, vomiting, abdominal pain
Pathophysiology of liver disease	SARS-CoV-2 infection and systemic inflammation	Inflammation and imbalanced endothelial activity	Thrombotic micro-angiopathy	Hormonal cholangiopathy	Mitochondrial dysfunction + fatty acid accumulation in hepatocytes
Increased transaminases	13%-42%, 2-5 × ULN	Approximately 50%, > 2 × ULN	Typical, > 2 × ULN	Typical, > 2 × ULN	Always, < 10 × ULN
Jaundice	Rare (unclear%)	Rare	Rare (< 5%)	Uncommon (< 25%)	Mostly (> 70%)
Other findings	Radiographic lung disease	HTN, ↑ sFLT-1/PIGF	↓ PLC; ↓ haptoglobin; ↑ LDH ↑ D-dimer	↑ ALP; ↑ bile acids	Coagulopathy; hypoglycemia
Diagnosis	Viral antigen PCR or Nucleic acid amplification test (NAAT)	HTN ≥ 140/90 + organ dysfunction (proteinuria not required)	Tennessee or Mississippi classification	Bile acids (BA); > 10 umol/L	Swansea criteria; biopsy if unclear
Management	Anti-virals +/- Steroids Mono-clonal antibodies	HTN control; delivery if > 37 wk GA or > 34 wk if severe	Delivery after 34 wk GA	Ursodiol; delivery at 36 wk GA if BA > 100 or 36-39 wk if BA < 100	Prompt delivery
Complications	↑ Risk of post-partum hemorrhage; ↑ multi-systemic organ failure	↑ Complications; mortality: 1%-5%; ↑ Neonatal respiratory distress + mortality	↑ Complications Mortality: 1%-3%	↑ Neonatal complications	↑ Maternal + neonatal complications; morality: 20% (mother); 6%-77% (neonate)

Comparison of the clinical presentation and management of Coronavirus disease 2019 (COVID-19) complicated by COVID-19 associated liver injury (COVALI) in pregnant compared to liver diseases specific to pregnancy[105-107,134-138]. Many of the diseases present along a spectrum however, they each have features that can help narrow the differential diagnosis. Importantly, management of perinatal liver diseases includes early delivery in most cases based on American College of Obstetrics and Gynecology recommendations, whereas management of COVALI does not involve decisions about pregnancy delivery. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; PEC: Pre-ec clampsia; HELLP: Hemolysis, elevated liver enzymes, low platelets; Ahellp: atypical HELLP; AFLP: Acute fatty liver of pregnancy; ICHP: Intrahepatic cholestasis of pregnancy/obstetric cholestasis; LDH: Lactate dehydrogenase; PLC: Platelet count.

positive for COVID-19 which was then thought to be the source of her liver injury. The final case is that of a 39-year-old G5P1 presenting at 26 wk' gestation with progressive dry cough and dyspnea. She was found to have new hypertension (BP 152/132), severe transaminitis (AST 1154 U/L, ALT 864 U/L), and PCR proven COVID-19 infection, concerning for pre-eclampsia with severe features *vs* systemic COVID-19[116]. Based on high suspicion for pre-eclampsia, the patient received betamethasone and dexamethasone to assist fetal lung maturation. Surprisingly, the patient's blood pressure was noted to be improving, inconsistent with pre-eclampsia which requires delivery to return to normotension. To further evaluate this, serum maternal placental growth factor was tested and normal. Normal maternal placental growth factor effectively ruled out pre-eclampsia and favored a diagnosis of COVID-19 with COVALI. This patient went on to deliver a healthy full-term fetus. In each case hypertension and liver injury improved with conservative management for COVID-19 and did not require delivery as is the case with perinatal liver diseases.

Cases in the latter two sections demonstrate complicated cases that are difficult to parse out based on clinical course. For example, in the case by Arslan *et al*[118], the patient's proteinuria was concerning for pre-eclampsia and liver enzymes trended down as expected after cesarean delivery, though both maternal and neonatal outcomes were poor which complicates interpretation of the case. Similarly, in the case by Choudhary *et al*[111], hypoglycemia and elevated bilirubin were highly suspicious for AFLP, but liver enzymes remained elevated for multiple days after delivery.

Outcomes: COVID-19 associated liver injury correlates with worse clinical outcomes and increased mortality in the obstetric setting. A retrospective cohort study of 122 COVID-19 positive pregnant patients in Istanbul found acute liver injury conferred a 3.5-fold risk of becoming critically ill during hospitalization[112]. Maternal mortality is reportedly more common in pregnant patients who delivered while COVID-19 positive with acute liver injury than COVID-19 positive without liver injury[111].

The largest published study evaluating COVALI in pregnancy is a 249-patient prospective cohort study performed at large tertiary care hospital in eastern India[111]. Unlike in previous studies, patients with hypertensive disorders, diabetic disorders, or concern for intrahepatic cholestasis were not excluded. Overall, 107 (42.1%) had evidence of hepatic dysfunction, but liver injury was more common

Table 2 Summary of case reports

Ref.	Patient	Case information	Laboratory data		Clinical course
Improved without delivery					
Anness and Siddiqui [114], 2020	35 y/o G2P1, GA 28^{5}w, PMH: IHCP	CC: Progressive dyspnea and cough; vitals: HR 133, RR 42, O2 96%; chest CT: Patchy peri-hilar inflammatory changes; differential: ICHP *vs* ICHP + COVID-19 *vs* COVID-19	AST		Normal bile acids @ GA 20
			ALT	571	↑ Bile acids and NO itch
			Bilirubin	0.76	Conservative management
			PLC	135	LFTs resolved with COVID-10
			CRP	60	Discharged home
			LDH	194	Healthy delivery at GA 39^{1}
			Ferritin		
Azimi *et al*[115], 2021	27 y/o G2P1, GA 30 wk	CC: Headache and lower limb pain; vitals: BP 100/70, HR 90-100; chest CT: Peripheral GGO's + consolidation; differential: HELLP *vs* systemic lupus *vs* COVID-19	AST	126	No delivery
			ALT	89	LFTs resolved with COVID-19
			Bilirubin	2.3	Discharged at GA 33
			PLC	220	Healthy delivery at GA 39
			CRP	114	
			LDH	1036	
			Ferritin	1360	
Naeh *et al*[116], 2022	39 y/o G5P1, GA 26^{4} wk	CC: Dry cough and dyspnea; vitals: BP 152/132, HR 141, RR 20, SpO2 96%; chest CT: Patchy multi-focal GGO's; differential: PEC with severe features *vs* COVID-19	AST	1154	Evaluated for PEC with PIGF
			ALT	864	PIGF 158 (high)→No delivery
			Bilirubin		LFTs resolving with COVID-10
			PLC	WNL	Discharged HD13; AST 331
			CRP		Healthy delivery at GA 39^{2}
			LDH	1018	
			Ferritin		
Improved with delivery					
Ronnje *et al*[117], 2020	26 y/o, G2P1, GA 32^{1} wk	CC: Cough, fever. Dyspnea, abdominal pain; vitals: BP 116/71, HR 113, RR 22, SpO2 95; chest CT bilateral diffuse GGO; differential: aHELLP *vs* COVID-19	AST	1687	5 d earlier normal labs
			ALT	348	Delivery on HD2 @ GA 32^{6}
			Bilirubin	1.23	LFTs trend down after delivery
			PLC	122	
			CRP	136	
			LDH	2039	
			Ferritin	875	
Arslan *et al*[118], 2022	30 y/o G3P2, GA 32 wk	CC: 6 d of chills, cough, dyspnea; vitals: RR 26, SpO2 84%; chest CT bilateral GGO's + peripheral thickening; differential: HELLP *vs* PEC *vs* AFLP *vs* SLE *vs* COVID-19	AST	146	HD 2: BP 185/120, + *proteinuria*, intubated, IV nitroprusside
			ALT	102	HD 3: Cardiac injury, ↓ PLC, ↑ fetal distress→ Cesarean section
			Bilirubin	2.54	HD4: LFTs improved
			PLC	59	Patient + child died
			CRP	215	
			LDH	697	
			Ferritin		
Delivery without improvement in 24-72 h of delivery (or other)					
Madaan *et al*[119], 2022	26 y/o G1P0, GA 39w	CC: RUQ pain and headache; vitals: BP 160/100, HR 98, SpO2 95%; chest	AST	589	Suspicion of HELLP→ Cesarean section

		CT: Bilateral diffuse GGO's; differential: Not given	ALT	300	Improved over hospitalization and LFTs trended down (no timeline given)
			Bilirubin	9.4	
			PLC	90	
			CRP	78.5	
			LDH	3100	
			Ferritin	734	
Choudhary *et al*[120], 2021	27 y/o G1P0, GA 35 wk, di-di twins	CC: Cough, fever, abdominal pain; vitals: BP 142/94, HR 88, RR 20. SpO2 98%; chest X-ray: Bilateral basal opacities; differential: aHELLP *vs* PEC *vs* AFLP *vs* COVID 19	AST	728.5	Suspicion of aHELLP→Cesarean-section
			ALT	473.2	POD 0: Hypo-glycemia, altered mentation, ↑ bilirubin→AFLP
			Bilirubin	4.9	Transfer to ICU + IV labetalol
			PLC	162	POD 8 discharged, normal LFT's
			CRP	22	
			LDH	96.9	
			Ferritin	120	

Gestational age is noted as week[d]. Vitals reported as: BP: Blood pressure (mmHg); HR: Heart rate (beats per minute); RR: Respiratory rate (breaths per minute); Spo2: Oxygen saturation (%). Laboratory values are reported with the following standardized units: AST: Aspartate aminotransaminase (U/L); ALT: Alanine aminotransaminase (U/L); bilirubin (mg/dL); PLC: Platelet count (× 10^3/ mm); CRP: C-reactive protein (mg/dL); LDH: Lactate dehydrogenase (u/L); ferritin (ng/dL). PEC: Pre-ec clampsia; HELLP: Hemolysis, elevated liver enzymes, low platelets; Ahellp: Atypical HELLP; AFLP: Acute fatty liver of pregnancy; ICHP: Intrahepatic cholestasis of pregnancy/obstetric cholestasis; CT: Computed tomography; GGO: Ground glass opacities; GA: Gestational age; WNL: Within normal limits; LFTs: Liver function tests; HD: Hospital day; C-section: Cesarean section; POD: Post-operative day; ICU: Intensive care unit.

in patients with perinatal hypertensive, diabetic, or cholestatic disorders (47/87, 54%) compared to those without (60/162, 37%). Although no statistical metric of significance was provided by the study, it appears that COVID-19 may increase risk of or exacerbate underlying obstetric conditions associated with liver injury. The primary aim of the study was to evaluate the relationship between liver injury in COVID-19 and obstetric outcomes. While no associations between liver injury and mode of delivery or neonatal outcomes were identified, those with liver injury tended to deliver pre-term and/or require cesarean delivery more often, both of which increase morbidity. Their key finding was that obstetric complications were significantly higher in COVID-19 positive pregnant patients with liver injury, despite no differences in maternal or gestational age[111]. Specifically, pregnant persons with COVALI were less likely to have a normal vaginal delivery than those without liver injury (18.7% *vs* 30.3%). Further, postpartum hemorrhage, sepsis, and death were more common in those who delivered while COVID-19 positive with acute liver injury[111].

Pathophysiology

The pathophysiology COVID-19 is not well studied outside the general population and thus the pathophysiology of COVALI in pregnancy is not well understood. Studies comparing COVID-19 positive pregnant individuals with acute liver injury and COVID-19 positive pregnant individuals with normal liver enzymes are crucial to build understanding of disease mechanisms in this cohort. However, there are only a handful of published studies on this to date[111,112,121]. We first use these studies to establish that relationships relevant to pathophysiology in the general population also exist in the obstetric population.

Specifically: (1) What is the relationship between severe COVID-19 disease and COVALI in pregnant patients? Patients with severe COVID-19 are more likely to develop COVALI. A prospective cohort study found that 87.5% of pregnant patients with severe COVID-19 pneumonia during hospitalization developed abnormal liver enzymes after having normal liver enzymes at baseline[122]. A later study demonstrated pregnant patients with liver injury had more severe disease and two thirds of this cohort ultimately died due to COVID-19 lung disease[111]; (2) what is the relationship between COVALI and markers of inflammation in pregnant patients? COVALI during pregnancy has been associated with elevated markers of inflammation. COVID-19 positive pregnant patients with liver injury have higher serum ferritin than expected in normal pregnancy, where a state of physiologic anemia is to be expected [112]. Furthermore, a study by Deng *et al*[121] evaluating liver chemistries in 37 COVID-19 positive pregnant patients found those with liver injury had higher inflammatory markers, such as procalcitonin and IL-6; and (3) what is the relationship between COVALI and systemic inflammatory manifestations

of COVID-19? Research by Choudhary *et al*[111] showed that obstetric complications were found to be more common in patients with COVALI. Most of these complications were related to inflammation, endotheliopathy, and coagulopathy. For example, they found pregnant persons with liver injury had higher prothrombin time and were more likely to experience postpartum hemorrhage requiring blood transfusion. Further, systemic inflammation was more common in those who delivered while COVID-19 positive with acute liver injury, as evidence by increased risk of sepsis with multi-organ failure[111].

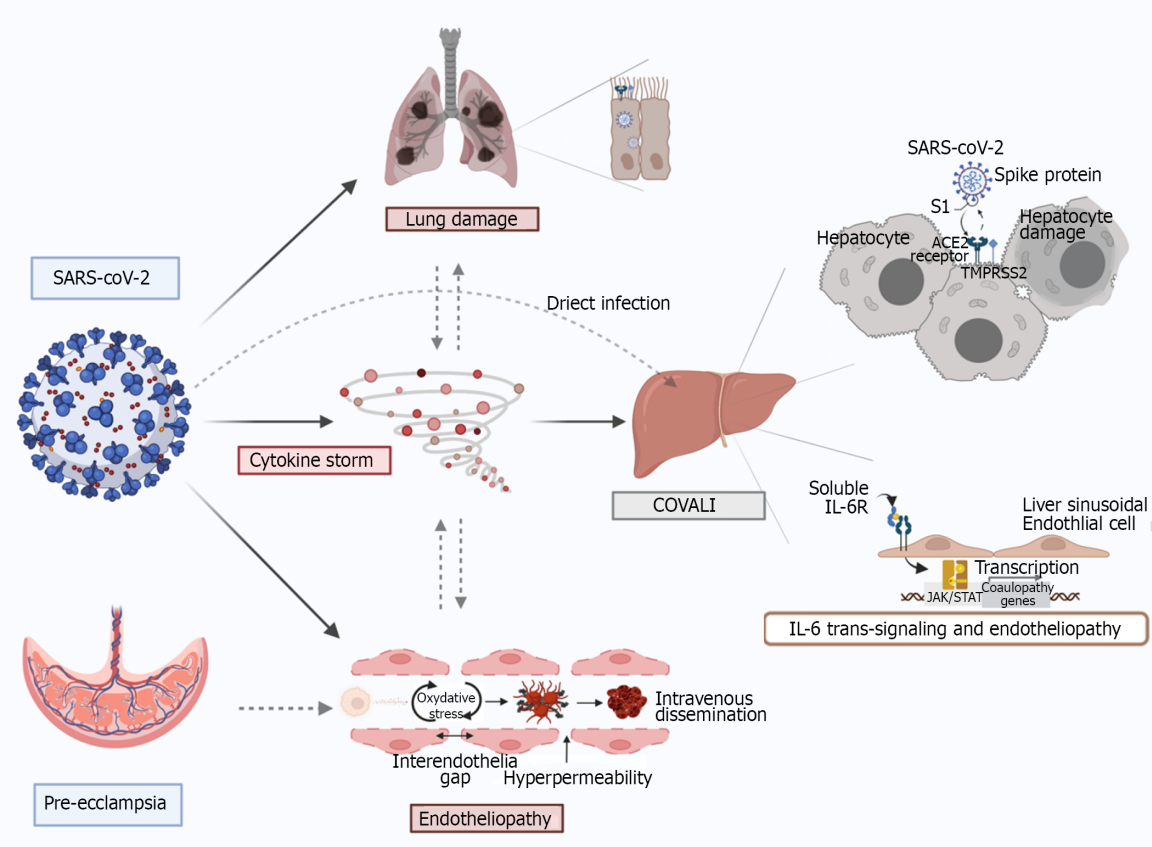
Overall, these studies suggest the relationships between liver injury and disease severity, patient outcomes, and inflammation identified in the general population persist in the obstetric population. While pathophysiology is likely stable across cohorts, considering the increased risk of COVALI during pregnancy could help further elucidate pathophysiology.

One potential link to the increased risk is the upregulation of ACE-2 to increased plasma levels above non-pregnant individuals, secondary to increase in estrogen production[123,124]. During pregnancy ACE-2 is highly expressed in the placenta and helps regulate blood pressure *via* systemic vascular resistance. This suggests there is increased activity of ACE-2 in the endothelium of pregnant patients [125] leading to the placenta as a potential target for COVID-19 infection. The interruption of the physiologic function of ACE-2 in pregnancy has been postulated to be a major contributing factor to the development of complications[126]. Lower levels of ACE-2 have been detected in the placentas from COVID-19 positive patients, suggesting that COVID-19 infection may alter ACE-2 expression and its biologic function in both the placenta and more widely in maternal circulation[124], potentially causing endothelial dysregulation as seen in COVALI.

Based on clinical manifestations, it is also reasonable to consider that pathophysiology of COVALI resembles or amplifies that of obstetric hepatobiliary pathology. This is exhibited in the case reports narrating the difficulty of differentiating COVALI from obstetric disorders that cause transaminitis in the clinical setting. Overall, the greatest overlap occurs between severe pre-eclampsia and the extra-pulmonary manifestations of COVID-19, and pre-eclampsia has been diagnosed more often in pregnant persons with COVID-19 compared to pregnant persons without COVID-19[127,128]. A potential link to the increased risk is alpha-1-antitrypsin, an enzyme that can inhibit SARS-CoV-2 infection and protects endothelial cells from oxidative stress during pregnancy, which is reduced in seen in pregnant patients with pre-eclampsia[129,130].

Work by Mendoza *et al*[122] sought to determine the prevalence of "pre-eclampsia findings" in 42 COVID-19 positive pregnant women. Eight women had severe pneumonia secondary to COVID-19 of which seven (87.5%) had elevated liver enzymes consistent with COVALI and five (62.5%) had hypertension meeting criteria for pre-eclampsia. However, sonographic evidence of placental hypoperfusion was only found in one patient who ultimately required delivery to prompt resolution of hypertension and liver injury. The remaining patients did not require delivery and instead, liver injury and hypertension improved in parallel with symptoms of pneumonia due to COVID-19. They measured ratio of soluble fms-like tyrosine kinase-1 (sFlt-1) ad serum placental growth factor (PIGF), which has been shown to be predictive of pre-eclampsia[131], and found sFlt-1/PIGF normal ratio in patients who did not require delivery compared to an elevated sFlt-1/PIGF ratio in the patient with evidence of placental hypoperfusion who required delivery. These findings suggest severe COVID-19 complicated by COVALI can mimic hypertensive disease of pregnancy and may represent shared disease mechanisms (Figure 1).

Literature that was published during the writing of this review directly compared the pathophysiology of pre-eclampsia and COVID-19. In this study, Palomo *et al*[132] compared endothelial inflammation and angiogenesis in pregnant patients with pre-eclampsia *vs* COVID-19 pneumonia *vs* normotensive controls. They measured circulating inflammatory markers in patient blood and found different biomarker profiles of coagulopathy, endothelial inflammation, and angiogenesis. Both COVID-19 and pre-eclampsia had increased vascular cell adhesion molecules expression relative to controls and increased markers of innate immunity. Fortunately, there were multiple factors helpful in differentiating pre-eclampsia and COVID-19: (1) COVID-19 had higher von Willebrand factor and soluble tumor necrosis factor-receptor but lower PIGF; and (2) Pre-eclampsia had higher soluble tumor necrosis factor-receptor and sFlt-1but lower von Willebrand factor. They observed altered sFlt-1 to PlGF ratio was predictive of pre-eclampsia, consistent with findings of Mendoza *et al*[122] In the latter part of their study they observed how sera from each patient cohort induced change when applied to human dermal microvascular cells. Despite different angiogenic and endothelial profiles, sera from both cohorts activated a common downstream pathway associated with endothelial inflammation, potentially indicating a shared end-pathway. While liver injury was not specifically evaluated in this study, these findings can be interpreted as evidence supporting endothelial dysfunction and inflammation as drivers of systemic manifestations of COVID-19 that are also present in pre-eclampsia, such as liver injury. Shared histologic findings in COVALI and pre-eclampsia including microvascular changes and signs of platelet activation, further support this theory[106,133].



DOI: 10.3748/wjg.v28.i42.6017 **Copyright** ©The Author(s) 2022.

Figure 1 Mechanisms of COVID-19-associated liver injury: Inter-organ crosstalk. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) enters host cells *via* interaction of its spike protein with the receptor angiotensin converting enzyme 2 in the presence of TMPRSS2 in many tissues. Proposed mechanisms for SARS-CoV-2-mediaded liver injury include: (1) Direct viral cytopathic effect; (2) IL-6 trans-signaling in liver sinusoidal endothelial cells which leads to endotheliopathy; (3) cytokine storm-induced damage; and (4) hypoxemic injury. There is also a lung-gut crosstalk which promotes an increased inflammatory state as well as dysbiosis which increases intestinal permeability, thus facilitating viral entry. Furthermore, direct viral injury to the vascular endothelium leads to increased cytokine release, enhanced reactive oxygen species production and thrombo-embolic events involving both micro and macro circulation. In a similar fashion, pre-eclampsia spectrum syndromes cause inflammation and endotheliopathy that pre-disposes to liver injury and can be synergistic coronavirus disease 2019 (COVID-19) and COVID-19 associated liver injury. Original figure was created with BioRender.com.

CONCLUSION

In this paper we reviewed COVID-19 associated liver injury with a special focus on pregnancy. We demonstrated COVALI to be an inflammatory mediated AST-predominant transaminitis associated with COVID-19 disease severity and poorer patient outcomes. Emerging research in the general and obstetric populations supports inflammation and endothelial dysfunction as central to pathophysiology in systemic COVID-19 and COVALI. Figure 1 summarizes proposed mechanisms of COVALI and illustrates how some physiologic changes in pregnancy can pre-dispose or exacerbate processes of liver injury during COVID-19. There is significant opportunity to improve understanding of COVALI during pregnancy. At present COVALI appears to be independently associated with worse post-partum outcomes, though this has not been fully parsed on in the literature. Further research should be done to elucidate the relationship between post-partum outcomes and COVALI, relevant to short and long-term outcomes. There is also data supporting the use of specific of circulating biomarkers to differentiate systemic COVID-19 from other causes of transaminitis in pregnancy, but further research is required to define criteria that can guide management.

FOOTNOTES

Author contributions: Cooper KM conceptualized this article, completed research collection, and lead the writing and editing of the manuscript; Colletta A assisted in conceptualizing the article, wrote portions of the manuscript, edited the initial, and revised manuscripts; Asirwatham AM assisted in conceptualizing portions of the article and provided expert feedback in the area of Obstetrics and Gynecology; Moore Simas TA reviewed content, edited the initial and revised manuscript, and provided expert feedback in the area of Obstetrics and Gynecology; Devuni D reviewed content, edited the initial and revised manuscript, and provided expert feedback in the area of Hepatology.

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MINIREVIEWS

Angiogenesis and immune checkpoint dual blockade: Opportunities and challenges for hepatocellular carcinoma therapy

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Abstract

The disease burden related to hepatocellular carcinoma (HCC) is increasing. Most HCC patients are diagnosed at the advanced stage and multikinase inhibitors have been the only treatment choice for them. Recently, the approval of immune checkpoint inhibitors (ICIs) has provided a new therapeutic strategy for HCC. It is noteworthy that the positive outcomes of the phase III clinical trial IMBrave150 [atezolizumab (anti-programmed cell death ligand 1 antibody) combined with bevacizumab (anti-vascular endothelial growth factor monoclonal antibody)], showed that overall survival and progression-free survival were significantly better with sorafenib. This combination therapy has become the new standard therapy for advanced HCC and has also attracted more attention in the treatment of HCC with anti-angiogenesis-immune combination therapy. Currently, the synergistic antitumor efficacy of this combination has been shown in many preclinical and clinical studies. In this review, we discuss the mechanism and clinical application of anti-angiogenics and immunotherapy in HCC, outline the relevant mechanism and rationality of the combined application of anti-angiogenics and ICIs, and point out the existing challenges of the combination therapy.

Key Words: Anti-angiogenesis; Immunotherapy; Combination therapy; Vascular endothelial growth factor; Immune checkpoint blockade; Hepatocellular carcinoma

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Core Tip: Anti-angiogenesis combined with immunotherapy in hepatocellular carcinoma (HCC) has attracted extensive attention. Although the mechanism of these combinations is largely understood, the biomarkers for predicting treatment response are still lacking. Thus, we outline the relevant mechanism and rationality of the combined application of anti-angiogenics and immune checkpoint inhibitors, and further explore the biomarkers that are associated with treatment response, which are critical in studies of HCC.

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INTRODUCTION

According to the statistics from the National Cancer Center of China, the incidence and mortality of hepatocellular carcinoma (HCC) are increasing annually[1]. The World Health Organization (WHO) estimates that, from 2015 to 2030, approximately 10 million people in China will die due to liver cirrhosis and HCC. Although early-stage disease can be cured by surgical removed, transplantation, or interventional therapy, most patients have unresectable disease at the time of diagnosis[2], and current treatments are insufficient to prevent the high metastasis and recurrence rates after HCC treatment.

Currently, immunotherapy is receiving a great deal of attention in the treatment of tumors. Among the immunotherapy options, immune checkpoint blockade (ICB)-based immunotherapy which reactivates dysfunctional or exhausted T cells has shown excellent efficacy in a variety of solid cancers and hematological tumors[3-7]. However, 50%-80% of cancer patients still do not benefit from immunotherapy, and many of them suffer serious adverse events (AEs) during treatment[8]. In fact, there is still no clear mechanism to explain the tolerance of many cancers to immune checkpoint inhibitors (ICIs). HCC is a solid tumor with complex pathophysiological barriers. It is difficult for external lymphocytes to penetrate and infiltrate into tumor tissue. In addition, the rapidly growing tumor cells release immunosuppressive factors, which result in HCC forming an immunosuppressive immune microenvironment, which greatly limits the efficacy of immunotherapy[9]. In addition, the rapidly growing tumor cells release several factors, which result in HCC forming an immunosuppressive immune microenvironment, that greatly limits the efficacy of immunotherapy[10,11]. Therefore, normalizing tumor vasculature and improving the tumor hypoxic microenvironment is expected to reverse the immunosuppressive microenvironment of HCC and promote HCC immunotherapy.

The TME is mainly composed of the vasculature, resident or infiltrating immune cells and various stromal cells. Previous studies have shown that abnormal tumor vasculature promotes the formation of an immunosuppressive TME[11]. Therefore, therapies that promote normalization of the vasculature are of great significance for enhancing immunotherapy of HCC. This review outlines measures to normalize the vasculature of HCC and the common immunotherapy regimens for HCC, and further describes and discusses how to normalize the tumor vasculature to improve the efficacy of immunotherapy in HCC (especially ICB). Additionally, we discuss the challenges associated with emerging combinations of vascular normalization therapy and immunotherapy for HCC.

ABNORMAL ANGIOGENESIS AND VASCULAR NORMALIZATION MEASURES FOR HCC

Abnormal angiogenesis and vascular endothelial growth factor/vascular endothelial growth factor receptor

The excessive growth and abnormal proliferation of tumor cells depend on rapid tumor angiogenesis. Tumor angiogenesis not only provides tumor cells with oxygen, nutrients and removes waste, but also serves as a channel for metastasis of tumor cells and immune cell infiltration[12,13]. However, compared with vessels in normal tissues, tumor neo-vessels have obvious aberrations in both structure and function[11]. Leakage is one of the most notable features of tumor vessels. On the one hand, this property can lead to tumor hypoxia and decreased intra-tumoral pH by impairing perfusion, and on the other hand, leakage will increase interstitial pressure in the TME[10]. Tumor cells overcome these harsh conditions through multiple mechanisms to gain a survival advantage[11]. Abnormal vessels limit the circulation of drugs and immune cells into the tumor, thereby limiting anti-tumor activity[11]. The hypoxia and pH reduction in the tumor caused by abnormal vessels will further lead to abnormal neovascularization, forming a vicious circle.

In the field of cancer research, most studies on angiogenesis has focused on the increased expression of angiogenesis factors [such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), angiopoietin, hepatocyte growth factor, endoglin (CD105, *etc*)] and decreased expression of anti-angiogenesis factors, such as angiostatin, endostatin, and thrombospondin 1, which is mainly caused by upregulation of the hypoxia-inducible factor protein. Among these, VEGF/VEGF receptor (VEGFR) axis are widely known to play a major role in vascular abnormalities and are crucial for the occurrence and progression of HCC[14-16]. The VEGF family consists of VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor (PlGF) 1-4, which are involved in tumor angiogenesis (VEGFA, PlGF), maintenance of new blood vessels (VEGFB), lymph-angiogenesis and angiogenesis (VEGFC/D), vascular permeability (VEGFA/C), chemotaxis (VEGFB), migration (VEGFA, PlGF), differentiation (VEGFD) and survival (VEGFA/B/C, PlGF)[17,18]. VEGFR mainly includes VEGFR1, VEGFR2, and VEGFR3[15]. Of these, VEGFR2 is the critical receptor of this family, which is expressed on almost all endothelial cells and is activated by binding to VEGFA, VEGFC or VEGFD, and VEGFA is its major ligand[19]. This binding results in the phosphorylation cascade that triggers downstream cellular pathways, ultimately leading to endothelial cell proliferation and migration, and the formation and branching of new tumor blood vessels[19]. These neo-vessels often manifest as abnormal leaky vasculature, resulting in high interstitial pressure and severe hypoxia or necrosis in tissue regions, further promoting the malignant potential of tumor cells[20]. Previous studies have shown that circulating VEGF levels are increased in HCC, and increased VEGFA has been shown to be associated with angiogenesis in HCC[21,22]. In addition, related studies also observed a positive association between increased local and circulating VEGF and high tumor micro-vessel density with rapid disease progression and decreased survival. These findings support the application of therapies that target the VEGF/VEGFR pathway in HCC[21,22].

Vascular normalization measures in HCC and targeting of the VEGF/VEGFR axis

Various molecular and physical mechanisms have been reported to contribute to tumor vascular dysfunction, the most prominent of which is the imbalance of angiogenic signaling mediated by pro- and anti-angiogenic molecules[11,23]. In normal tissue, this balance is precisely maintained to ensure normal vascular morphology and function[24]. However, during the process of carcinogenesis, this balance usually tends to angiogenesis, and the generated neo-vessels are immature abnormal vessels without complete structure[24]. In view of the key role of the VEGF/VEGFR axis in abnormal angiogenesis of HCC, rational targeting of this axis can promote the normalization of tissue vessels and limit the occurrence and development of HCC.

In the past few decades, the development of anti-angiogenesis therapy has mainly focused on blocking VEGF[17,18]. Several studies have also focused on blocking VEGF signaling by silencing VEGFA expression at the transcriptional and post-transcriptional levels. For example, Zou *et al*[25] identified emodin that could greatly increase seryl-tRNA synthetase expression in tripe-negative breast cancer (TNBC) cells, consequently reducing VEGFA transcription, and emodin potently inhibited vascular development of zebrafish and blocked tumor angiogenesis in TNBC-bearing mice, greatly improving the survival. Li *et al*[26] and Ding *et al*[27] raised that VEGF small interference RNA can precisely and efficiently silence VEGF expression and block VEGF signal pathway, leading to a significant decrease in tumor blood vessels and suppression of tumor growth and metastasis. However, these studies have only been tested in animals. Preclinical evidence suggests that monotherapy which blocks VEGF reduces micro-vessel density, inhibits tumor growth in many cancerous subcutaneous xenografts, and even inhibits tumor cell metastasis[28,29]. Ferrara *et al*[30] researched and developed the first anti-angiogenesis inhibitor (bevacizumab), a recombinant humanized monoclonal antibody that blocks VEGFA. Bevacizumab binds to VEGF in the bloodstream, thereby inhibiting the interaction between VEGF and VEGFR. In clinical trials of combination therapy for HCC, multiple lines of evidence suggest that bevacizumab has a potential therapeutic effect[31,32].

On the other hand, many anti-angiogenesis therapies in HCC focus on targeting VEGFR. Multikinase inhibitors (MKIs) and monoclonal antibodies (mAbs) were developed to inhibit VEGFR and its downstream targets to inhibit endothelial cell proliferation, thereby reducing the nutrient and oxygen supply required by tumors. Currently, the VEGFR-targeted MKIs and mAbs used in advanced HCC mainly include sorafenib, regorafenib, lenvatinib, cabozantinib, and ramucirumab (Table 1).

Sorafenib is an oral MKI that blocks VEGFR1-3, c-Kit, PDGF receptor (PDGFR)-b, and FMS-like tyrosine kinase-3 (FLT-3)[33]. The phase III clinical trial SHARP showed a 2.8-mo survival advantage for sorafenib over placebo in patients with advanced HCC. This recent study also showed that sorafenib can benefit patients with HCC regardless of etiology, and patients with hepatitis C appeared to experience greater benefit[34]. Treatment-related AEs were more common in the sorafenib group than in the placebo group (80% *vs* 52%), and the incidence of dose reductions and interruptions was high during treatment. The MKI regorafenib also targets VEGFR, RET, c-Kit, B-Raf, FGFR1 and PDGFR[33]. It is the first therapy to demonstrate a survival benefit in advanced HCC patients who have progressed on sorafenib[35]. Fatigue, hypertension, diarrhea and hand-foot skin reactions were common AEs in the regorafenib-treated group. Other analyses showed that the survival benefit of first line sorafenib and second line regorafenib was more than 24 mo[35]. The targets of lenvatinib include VEGFR1-3, FGFR1-4, PDGFRa, RET and c-Kit. Recently, a phase III study of lenvatinib *vs* sorafenib in patients with

Table 1 Currently approved anti-angiogenic therapy for advanced hepatocellular carcinoma

Therapy	Type	Line of therapy	Target	Study name	Primary outcome in the study (mo) (95%CI)	Adverse events in the study [affected/at risk (%)]
Anti-VEGFA						
Bevacizumab (combination with atezolizumab)	mAb	First line (plus atezolizumab)	VEGF-A	IMBrave150	OS at CCOD 30 mo: 19.2 (17.0-23.7); PFS: 6.8 (5.6-8.3)	Gastrointestinal perforation [1/329 (0.30%)], haemorrhage [40/329 (12.15%)], hypertension [2/329 (0.61%)] and proteinuria [100/329 (30.40%)], *etc.*
Anti-VEGFR						
Sorafenib	TKIs	First line	VEGFR1-3, c-Kit, PDGFR-b, and FLT-3	SHARP	OS: 10.8 (9.5-13.5); TTSP: 4.2 (3.5-4.2)	Diarrhea [166/297 (55.89%)], hand-foot skin reactions [2/297 (0.67%)], fatigue [145/297 (48.82%)], *etc.*
Lenvatinib	TKIs	First line	VEGFR1-3, FGFR1-4, PDGFRa, RET and c-Kit	REFLECT	OS: 13.6 (12.1–14.9); PFS: 7.4 (6.9–8.8)	Diarrhea [154/468 (33%)], decreased appetite [204/468 (44%)], *etc.*
Regorafenib	TKIs	Second line	VEGFR, RET, c-Kit, B-Raf, FGFR1 and PDGFR	RESORCE	OS: 10.7 (9.2-12.3); TTP:3.9 (2.9-4.26)	Toxicities were manageable this sorafenib-tolerant population and were similar to those observed with sorafenib, including diarrhea [163/374 (43.58%)], *etc.*
Cabozantinib	TKIs	Second and third line	VEGFR2, c-Kit, RET, FLT-3, Tie2, and Axl	CELESTIAL	OS: 10.2 (9.1-12.0)	Hypertension [137/467 (29.34%)]. Pneumonia [16/467 (3.43%)], *etc.*
Ramucirumab	mAb	Second line and AFP ≥ 400 ng/mL	VEGFR2	REACH-2	OS: 8.5 (7.0-10.6)	Hypertension [48/197 (24.37%)], hyponatremia [11/197 (5.58%)], *etc.*

TKIs: Tyrosine kinase inhibitor; CCOD: Clinical cut off date; OS: Overall survival; PFS: Progression free survival; TTSP: Time to symptomatic progression; TTP: Time to progression; CI: Confidence interval; AFP: Alpha fetoprotein; VEGFR: Vascular endothelial growth factor receptor; FLT: FMS related tyrosine kinase 1; FGFR: Fibroblast growth factor receptor; PDGFR: Platelet-derived growth factor receptor.

unresectable HCC showed that overall survival (OS) with lenvatinib was non-inferior to sorafenib[36]. The most common AEs in the lenvatinib group were diarrhea, fatigue, *etc*[36]. It should be noted that patients with tumors with more than 50% hepatic masses or involvement of branches of the main portal vein were excluded from the trial (NCT01761266); thus, further clinical trials should be conducted. Despite this problem, lenvatinib remains the only drug in first-line clinical trials that was positive against the proven active control, sorafenib. In addition to targeting VEGFR2, c-Kit, RET, FLT-3, Tie2, and Axl, cabozantinib has the unique property of inhibiting c-Met, and its potential activity was observed in phase II trials[37]. The subsequent phase III clinical trial, which compared cabozantinib to placebo in advanced HCC, met its primary endpoint of improved OS after up to two prior treatments, one of which included sorafenib[38]. Hypertension, pneumonia were common AEs in the cabozantinib group[38]. Ramucirumab, the mAb that antagonizes VEGFR2, improved OS in a phase III study in patients with sorafenib progression or intolerance with baseline alpha-fetoprotein (AFP) ≥ 400 ng/mL [39]. Hypertension and hyponatremia were the only over grade 3 AEs in patients of the test group[39]. Based on the results of the previous phase III study, patients can be selected for treatment based on baseline AFP values. A survival benefit was observed with ramucirumab in a subgroup of patients with higher baseline AFP (400 ng/mL), which is the first positive clinical trial in the biomarker-selected HCC population[39].

IMMUNOTHERAPY IN HCC

Driven by the success of ICB therapy in melanoma, ICB has been extensively studied in a variety of malignancies including HCC[40,41]. Long-term liver injury or chronic hepatitis infection often leaves the liver in a state of chronic inflammation[42]. Moderate inflammation can defend against pathogens and repair tissue damage, whereas persistent liver inflammation can disturb the microenvironment, thus favoring carcinogenesis. On the one hand, hepatic endocrine cytokines play a key role in tumorigenesis through regulating regulatory T cells (Tregs) that inhibit $CD8^+$ T cell activation[43]. It was previously reported that Tregs derived from hepatitis B virus-positive HCC tumors exhibited higher programmed cell death protein 1 (PD-1) expression and superior inhibitory capacity against $CD8^+$ T cells. On the other hand, cytotoxic immune populations frequently express markers of exhaustion such as PD-1, cytotoxic t-lymphocyte associated antigen 4 (CTLA-4) and lymphocyte activating gene 3[44].

Under normal conditions, these molecules inhibit T cell activation to maintain inflammatory homeostasis, protect tissue integrity, and prevent unnecessary autoimmunity[45]. However, in tumors, expression of these markers of exhaustion is inversely correlated with their function, making them a prime target for ICBs to revitalize and restore the cytotoxic capacity of $CD8^+$ T cells[43]. At the same time, the expression of PD-1 and its ligand (PD-L1) in tumor cells is upregulated, and when it binds to PD-1 expressed by T cells activated by tumor infiltration, it induces T cell exhaustion and suppresses the anti-tumor immune activity of these immune cells, thereby enabling tumor cells to evade immunity[46]. ICBs generate robust multitarget immune responses and can even induce long-lasting tumor remissions in some patients. Inhibition of the PD-1/PD-L1 interaction reverses the depleted state of these cytotoxic immune cells and reactivates their antitumor activity[46,47]. In HCC, the mAbs pembrolizumab and nivolumab that target PD-1, and nivolumab combined with ipilimumab (a mAb directed against CTLA-4), has been approved in the United States for sorafenib-treated patients, based on promising results from clinical trials[48-50]. A clinical trial confirmed the efficacy and safety of PD-1-targeting immunotherapy in HCC[51]. However, subsequent phase III trials of nivolumab *vs* sorafenib in first-line therapy failed to meet the primary survival endpoints[50,52]. The combination of phase III nivolumab and ipilimumab is currently under evaluation (NCT04039607).

In the current phase III clinical treatment of HCC, immune combination therapy has attracted considerable attention, especially the combination of ICIs and anti-angiogenic inhibitors (Table 2). Lenvatinib combined with pembrolizumab, bevacizumab combined with atezolizumab (PD-L1-targeting mAb) (T+A combination), and cabozantinib combined with atezolizumab have all obtained encouraging results in clinical studies[53-55]. Of these, the combination of bevacizumab and atezolizumab has been successful in phase III clinical trials of first-line treatment of HCC (IMbrave150). Compared with sorafenib, this combination improved the primary endpoints: OS and progression-free survival, and this combination was shown to be safe and improved quality of life[56]. Hypertension and proteinuria were typical side effects of bevacizumab and were the top two AEs in this test group. Upper gastrointestinal bleeding, another known side effect of bevacizumab, occurred in 7% of patients in this group, and was within the range of previous evaluations of bevacizumab AEs for the treatment of HCC[57,58]. Elevated transaminases and pruritus are common side effects of atezolizumab[32]. As this study applied the usual inclusion criteria in HCC clinical trials and included only Child-Pugh A patients, further clinical trials are pending[32].

Angiogenesis and immune checkpoint combination blockade in HCC

As shown above, the success of the phase III trial (IMbrave150) with the combination of atezolizumab and bevacizumab in advanced HCC is groundbreaking as it is the first treatment with a better survival rate than sorafenib since the approval of sorafenib in 2007 and is also the only successful first-line immunotherapy combination therapy for HCC in the world[32]. Antibodies targeting VEGF not only inhibit tumor growth but also reprogram the TME from immunosuppressive to immune activation[8,59]. Based on these findings, PD-1/PD-L1 inhibitors combined with VEGF/VEGFR inhibitors have attracted extensive attention in the treatment of HCC. Next, we will outline the mechanism and rationality of PD-1/PD-L1 inhibitors combined with VEGF/VEGFR inhibitor therapy, and the biomarkers of response to targeted immune combination therapy.

Mechanism and rationality

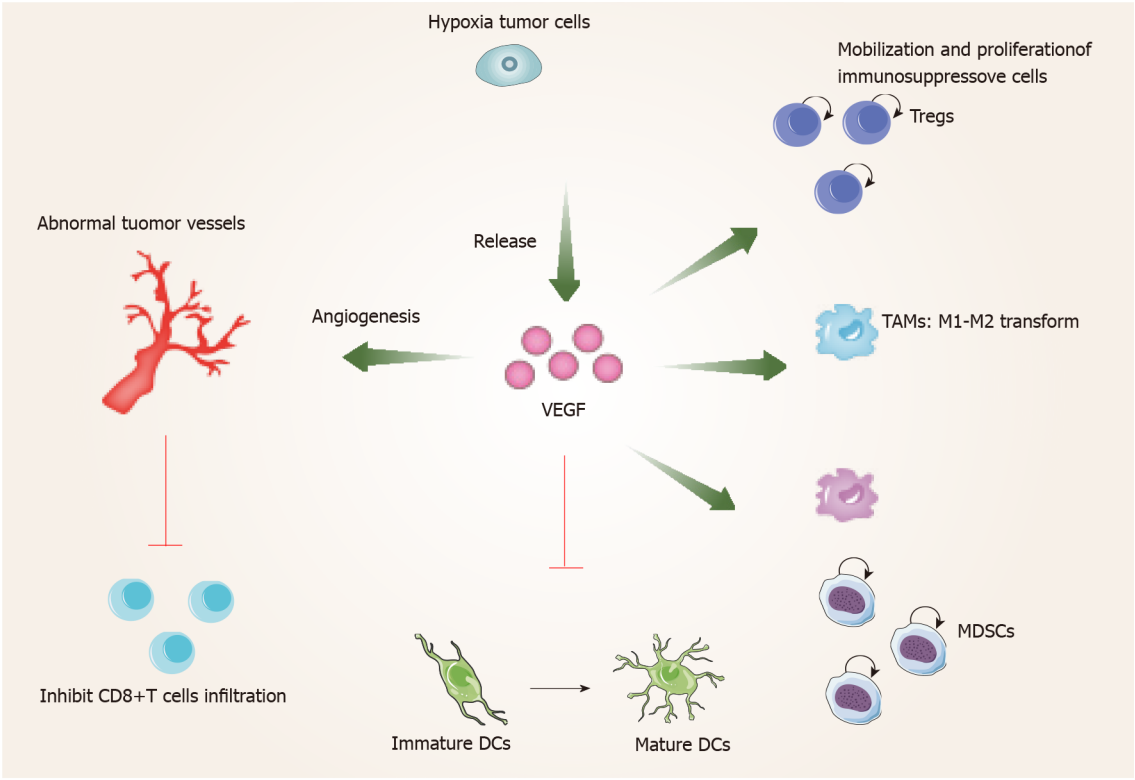
In the tumor area, VEGF released by hypoxic cancer cells promotes tumor cell growth and metastasis by angiogenesis[8]. On the other hand, VEGF can also promote the malignant progression of tumors by affecting the TME (Figure 1). The tumor immune cycle mainly includes seven steps, the release of tumor antigens, the uptake and presentation of tumor antigens by dendritic cells (DCs), the initiation and activation of T cells, the migration of T cells to tumors, the invasion of T cells against tumors, the recognition of tumor cells by T cells, and the attack of tumor cells by T cells[60]. VEGF is involved in almost every step of the tumor immune cycle and finally promotes tumor immune escape[61-64]. VEGF enhances the mobilization and proliferation of immunosuppressive cells, including Tregs, tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) *etc*, enhances the release of immunosuppressive cytokines[8,59], and promotes M1-TAMs to M2 type polarization. In addition, Tregs and TAMs release immunosuppressive factors, such as VEGF and angiopoietin 2, which form positive feedback to further promote tumor progression[65]. Furthermore, VEGF inhibits the maturation and antigen presentation of DCs. Thus, even in the presence of neoantigens, VEGF can reduce the proliferation and activation of naive CD8+ cells by inhibiting DCs[65]. VEGF prevents antigen activated CD8+ T cells from infiltrating tumor tissue by promoting the formation of abnormal tumor vessels. In addition, VEGF forms an immunosuppressive TME that inhibits the function of T cells in tumors during the effector phase of the immune response[65]. Therefore, inhibition of VEGF/VEGFR interaction not only normalizes vessels but also enhances antitumor immunity.

Previous studies have shown that inhibition of VEGF/VEGFR can enhance antitumor immunity. Gabrilovich *et al*[63] found that the targeted drugs which inhibit VEGF/VEGFR lead to an enhanced antigen-presenting capacity of DCs. In addition, studies also found that these drugs inhibit the production of Tregs, TAMs and MDSCs at tumor sites, and negatively regulate the expression of immunosuppressive cytokines such as transforming growth factor-beta and interleukin-10[66].

Table 2 Phase III clinical trials of combinations of anti-angiogenic inhibitors and immune checkpoint inhibitors in hepatocellular carcinoma

Trial name	Treatment	Setting	Primary endpoints	Current status	Enrollment, *n*
NCT03434379	Atezolizumab + bevacizumab	Advanced HCC; first line	OS, PFS	Active, not recruiting	558
NCT04770896	Atezolizumab + lenvatinib/sorafenib versus lenvatinib/sorafenib	Unresectable HCC; second line	OS	Recruiting	554
NCT03713593	Pembrolizumab + lenvatinib versus lenvatinib	Advanced HCC; first line	OS, PFS	Active, not recruiting	794
NCT03755791	Atezolizumab + cabozantinib versus sorafenib	Advanced HCC; first line	OS, PFS	Recruiting	740

OS: Overall survival; PFS: Progression free survival; HCC: Hepatocellular carcinoma.



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Figure 1 Vascular endothelial growth factor promotes the formation of an immune suppressive microenvironment. VEGF: Vascular endothelial growth factor; DCs: Dendritic cells; MDSCs: Myeloid-derived suppressor cells; TAMs: Tumor-associated macrophages; Tregs: Regulatory T cells.

Therefore, blocking VEGF/VEGFR reprograms the immunosuppressive TME[67]. At the same time, the combination of PD-1/PD-L1 antibody can further enhance the antitumor activity of T cells. First, by reversing the VEGF-mediated suppression of DCs maturation resulting in efficient priming and activation of T cells[67,68]; second, by normalizing tumor vasculature and promoting efficient T cell infiltration into tumors[69]; and third, VEGF/VEGFR inhibitors inhibit the activity of MDSCs, Tregs, and TAMs, leading to the reprogramming of the immunosuppressive microenvironment to immune activation[61]. Finally, PD-1/PD-L1 inhibitors enhance the ability of T cells to attack tumor cells. These four aspects can lead to effective antitumor immunity and tumor growth inhibition. As described above, the use of molecularly targeted drugs against VEGF/VEGFR reactivates the aberrant immunosuppressive TME caused by VEGF, and finally allows the tumor cells to be effectively attacked[60,62]. A recent study of T+A therapy showed that the improved outcome of the combination of bevacizumab and atezolizumab compared with atezolizumab alone was mainly related to higher Tregs expression, suggesting that bevacizumab inhibits VEGF-enhanced antitumor immunity mainly related to inhibiting the function of Tregs[70]. Notably, previous studies have shown that anti-PD-1 therapy can increase tumor blood perfusion by normalizing blood vessels in breast and colorectal cancer models, which is

closely related to its antitumor efficacy[71]. These studies form the rationale for the combination of VEGF/VEGFR inhibitors and PD-1/PD-L1 inhibitors.

Biomarkers to assess the response of targeted immunotherapy

A major factor limiting the benefit of angiogenesis and immune checkpoint dual blockade for HCC is the lack of biomarkers to predict patients who can benefit from treatment. PD-L1 expression in tumor specimens was not associated with prognosis in HCC patients treated with nivolumab[48]. This study demonstrated that further comprehensive tumor and stromal immune scores, or tumor gene signatures should be explored. A recent study showed that pre-existing immunity (high expression of CD274, T-effector signature and intra-tumoral CD8+ T cell density) was positively associated with better clinical outcomes with the T+A combination and reduced clinical benefit was associated with a high Treg to effector T cell (Teff) ratio and expression of oncofetal genes (GPC3, AFP)[70]. Hatanaka *et al*[72] reported that C-reactive protein and AFP could be useful for predicting therapeutic outcomes and treatment-related AEs in HCC patients treated with the T+A combination. These results indicate that the clinical studies of bevacizumab combined with atezolizumab, lenvatinib combined with pembrolizumab, cabozantinib combined with atezolizumab and other combination therapies are a valuable platform for the analysis of other potential biomarkers of response to targeted immunotherapy and offer several new possibilities for identifying subpopulations of patients who benefit from these treatments. In several cancers, tumor mutational burden (TMB) and microsatellite instability (MSI) are associated with longer OS after ICB therapy. Considering the low frequency of TMB and MSI in HCC, their predictive applications in HCC are limited. However, it is worth noting that studies have shown that HCC with high TMB and low MSI responded to nivolumab for more than 2 years[73]. Thus, much more research is needed to determine the biomarkers of targeted immunotherapy in HCC.

CONCLUSION

The global disease burden of HCC is increasing year by year. According to statistics, the annual incidence of HCC may exceed 1 million cases in the near future, and most patients are in advanced stages at diagnosis. Currently, only reasonable systemic treatment can effectively delay the progression of HCC. This article describes the characteristics and treatment strategies of abnormal angiogenesis in HCC, and briefly reviews the immunotherapy of HCC. The strategy and rationality of angiogenesis and immune checkpoint dual blockade are further discussed. Among these combinatorial strategies, the success of the IMBrave150 clinical trial demonstrated that bevacizumab altered the tumor immune microenvironment, enabling greater responses to ICB, successfully transforming the immunosuppressive TME to an immune-activated microenvironment. Therefore, the efficacy achieved by the combination of anti-PD-1/PD-L1 antibody and VEGF/VEGFR inhibitor may be due to normalization of the TME. In addition to the combination of atezolizumab and bevacizumab, other combination therapies targeting the same mechanism have also received attention. While the clinical development of VEGF/VEGFR-targeted drugs is due to their anti-angiogenesis inhibitory effects, the potential of this class of drugs is as immunomodulators in combination with immunotherapy.

In the post-sorafenib era of advanced HCC treatment, a great number of combination therapies are being studied. However, one of the biggest challenges with combination therapy is the discovery of predictive biomarkers to accurately identify patients most likely to respond to treatment. In the study of HCC, anti-angiogenesis therapy has been used for more than a decade and ICB has been approved for several years, but these two therapies still lack convincing biomarkers. Therefore, for combination therapy, a better understanding of the mechanism of synergistic therapeutic effect and the discovery of predictive biomarkers will help to design more precise treatment regimens.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Clinical value of predictive models based on liver stiffness measurement in predicting liver reserve function of compensated chronic liver disease

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Abstract

BACKGROUND

Assessment of liver reserve function (LRF) is essential for predicting the prognosis of patients with chronic liver disease (CLD) and determines the extent of liver resection in patients with hepatocellular carcinoma.

AIM

To establish noninvasive models for LRF assessment based on liver stiffness measurement (LSM) and to evaluate their clinical performance.

METHODS

A total of 360 patients with compensated CLD were retrospectively analyzed as the training cohort. The new predictive models were established through logistic regression analysis and were validated internally in a prospective cohort (132 patients).

RESULTS

Our study defined indocyanine green retention rate at 15 min (ICGR15) ≥ 10% as mildly impaired LRF and ICGR15 ≥ 20% as severely impaired LRF. We constructed predictive models of LRF, named the mLPaM and sLPaM, which involved only LSM, prothrombin time international normalized ratio to albumin ratio (PTAR), age and model for end-stage liver disease (MELD). The area under the curve of the mLPaM model (0.855, 0.872, respectively) and sLPaM model (0.869, 0.876, respectively) were higher than that of the methods for MELD, albumin-

bilirubin grade and PTAR in the two cohorts, and their sensitivity and negative predictive value were the highest among these methods in the training cohort. In addition, the new models showed good sensitivity and accuracy for the diagnosis of LRF impairment in the validation cohort.

CONCLUSION

The new models had a good predictive performance for LRF and could replace the indocyanine green (ICG) clearance test, especially in patients who are unable to undergo ICG testing.

Key Words: Liver stiffness measurement; Chronic liver disease; Liver reserve function; Indocyanine green clearance test; Predictive model

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Core Tip: This study aimed to establish predictive models of liver stiffness measurement (LSM) in patients with compensated chronic liver disease based on LSM and evaluate their clinical value. The results showed that the new models had a good predictive performance for liver reserve function (LRF). The area under the curve of the models was higher than that of the model for end-stage liver disease, albumin-bilirubin grade and prothrombin time international normalized ratio to albumin ratio. Moreover, the predictive performance of the new models was validated in a prospective cohort. We believe that these models could replace the indocyanine green (ICG) clearance test to assess LRF, especially in patients who are unable to undergo ICG testing.

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INTRODUCTION

The high prevalence of chronic liver disease (CLD) in China has become a severe public health problem. Cirrhosis, hepatocellular carcinoma (HCC), hepatic encephalopathy and other decompensated complications are the leading causes of mortality in CLD patients without treatment. Liver reserve function (LRF) is defined as the compensated ability of the liver to maintain normal physiological functions in the presence of injury, which mainly depends on the quality and quantity of hepatocytes in the remnant liver[1,2]. There are no obvious clinical symptoms in CLD patients in the early stage, but their LRF may be impaired. Early evaluation of LRF is of great help in identifying disease progression, timely implementation of interventions and appropriate treatment strategies in CLD patients. Several scoring systems, including the Child-Turcotte-Pugh (CTP), model for end-stage liver disease (MELD), albumin-bilirubin (ALBI) and APRI, can be used to evaluate LRF[1,3-5]. Although the CTP score is widely used to assess LRF, it includes subjective criteria, such as ascites and hepatic encephalopathy. The MELD score is initially used as a standard model to assess the prognosis of patients with decompensated cirrhosis, but its creatinine (Cr) value can be significantly affected by age and gender.

The indocyanine green (ICG) clearance test is commonly used for LRF assessment, which is considered the most valuable method for evaluating LRF. ICGR15 had become a standard dynamic preoperative instrument to evaluate the hepatic functional reserve before liver resection and predict post-hepatectomy liver failure[6,7]. However, the ICG clearance test process is tedious and requires a technical operator; thus, most of these tests can only be carried out in major hospitals. In addition, some patients are allergic to ICG, which can lead to failure of the test. Due to impossible implementation of the ICG clearance test in CLD patients, a new method to accurately assess LRF is needed.

Liver stiffness measurement (LSM) is commonly used to evaluate the degree of liver fibrosis, and due to its non-invasiveness, cost-efficiency and safety, it has been widely applied in clinical treatment. Previous studies have shown that LSM can predict the occurrence of liver failure after HCC resection[8-10]. Therefore, LSM has potential value in evaluating hepatic functional reserve.

The purpose of this study was to analyze the association between LSM and ICGR15 in evaluating LRF. We constructed the predictive models based on LSM and examined their clinical application value in evaluating LRF in compensated CLD patients.

MATERIALS AND METHODS

Research population

All patients with CLD (≥ 18 years old) consecutively observed in the inpatient department of the Hepatology Research Institute of the First Affiliated Hospital, Fujian Medical University, China, from March 2016 to June 2019 were retrospectively analyzed as the training cohort. From September 2019 to August 2020, patients with CLD were prospectively evaluated to validate the new models. Information regarding the patients' demographics, ICG clearance test, laboratory data and Fibro-scan examination was abstracted from the electronic medical record system of the First Affiliated Hospital of Fujian Medical University. Patients with the following conditions were excluded: (1) Decompensated cirrhosis with CTP grade B and C; (2) insufficient data; and (3) complicated with other tumors, or gestation. After exclusion, 492 patients were identified for study inclusion, comprising 389 chronic hepatitis B patients, 35 fatty liver disease patients, 21 autoimmune liver disease patients, 8 hepatitis C virus patients and 39 patients with other etiologies. All enrolled patients were divided into the training cohort (360 patients) and validation cohort (132 patients), including 105 HCC patients who met the diagnostic criteria in the guidelines for diagnosis and treatment of primary liver cancer in China (2019 edition)[11].

Clinical and laboratory parameters

The demographic data collected included age and gender. The clinical laboratory information included prothrombin time (PT), international normalized ratio (INR), total bilirubin (TBIL), aspartate aminotransferase, alanine aminotransferase, albumin (ALB), glomerular filtration rate, alkaline phosphatase, gamma-glutamyltransferase, cholinesterase, platelet count, and hemoglobin. The parameters were detected using an Olympus AU2700 automatic biochemical analyzer. The calculation of CTP score included five items, namely ALB, TBIL, PT, hepatic encephalopathy and ascites[12]. The CTP classifications were defined as grade A (5-6 points), grade B (7-9 points), and grade C (10-15 points). The MELD score was calculated by the formula 3.78 × ln[TBIL (mg/dL)] + 11.2 × ln (INR) + 9.57 × ln[Cr (mg/dL)] + 6.43 × etiology (0 for cholestasis and alcohol, and 1 for others)[13]. The prothrombin time international normalized ratio to albumin ratio (PTAR) score was calculated by the formula INR/ALB (g/dL)[14]. The ALBI score was calculated by the formula ln[TBIL (mol/L)] × 0.66 + ln[ALB (g/L)]-0.0852[15].

All patients received the ICG clearance test after overnight fasting, a dose of 0.5 mg/kg of ICG was rapidly injected into patients *via* a peripheral vein in the forearm. An optical probe attached to the patient's nose was used to monitor plasma ICG concentrations, and the value of ICGR15 was calculated by a Pulse Dye Densito-Graph Analyzer (DDG-3300K, Nihon Kohden, Tokyo, Japan)[16]. The LRF was defined as normal if ICGR15 < 10%, mild impairment if ICGR15 ≥ 10%, and severe impairment if ICGR15 ≥ 20%.

The Fibro-Scan 502 Touch (Echosens, Paris, France) was performed by the same trained operator according to the manufacturer's instructions. LSM was performed on the right lobe of the liver through the intercostal spaces. Ten successful acquisitions were performed for each patient. The success rate (≥ 60%) was calculated as the number of successful measurements divided by the total number of measurements recorded[17]. LSM was expressed as the median and IQR [in kilopascals (kPa)] of all valid measurements obtained. A LSM was considered reliable if 10 valid acquisitions were obtained. Patients with poorly reliable measurements (IQR/median ratio > 0.30 with a median LSM > 7.1 kPa) were excluded[18]. This retrospective study was approved by the ethics committee of the First Affiliated Hospital of Fujian Medical University, China.

Statistical analysis

Statistical analyses were performed using SPSS 23.0. The normally distributed continuous variables are presented as mean ± SD, which were further evaluated by Student's *t*-test in the different groups. Whereas, variables showing skewed distributions were evaluated by the Mann-Whitney *U* test, and are presented as median (interquartile range). Categorical variables are described using frequencies and proportions, and the Pearson's chi-squared test was used to compare categorical variables.

Multivariable analyses were conducted on variables that reached $P < 0.1$ at univariate analysis. Multivariate analysis was performed using the logistic regression analysis, and we established regression prediction models to predict the hepatic functional reserve. The continuous variables (cut-off value of LSM was 12.4 and PTAR was 0.280) were transformed into dichotomous variables. In order to avoid collinearity of some clinical indicators, stepwise forward regression was used in multivariate analysis. The optimal cut-off level of the model was determined by a receiver operator characteristic curve analysis. The areas under the curve (AUCs) were measured and compared to evaluate the discrimination ability of different models. The final predictive model was fitted on an internal validation dataset and on the entire prospective population. A two sided *P* value less than 0.05 was considered significant.

RESULTS

Summary of baseline clinical and demographic data of chronic liver disease patients

Overall, 492 patients were included in the study, including 103 patients with HCC (Table 1). 350 (71.14%) of 492 patients were male, the predominant etiology of liver disease was related to HBV (n = 389, 79.07%). Patients in the validation cohort were older than those in the training cohort (mean age, 54.84 ± 27.70 *vs* 48.71 ± 13.34, $P < 0.001$), and there was a statistically significant difference in ALB and TBIL levels. However, the two cohorts had a similar level of LSM and MELD ($P = 0.066$, $P = 0.241$, respectively).

Construction of the LRF predictive model based on LSM

With ICGR15 ≥ 10% and ICGR15 ≥ 20% as the predictive points, the new models of mildly impaired LRF (mLPaM) and severely impaired LRF (sLPaM) were constructed based on LSM. In the training cohort, 360 patient variables were included in the multivariate logistic stepwise regression analysis. LSM (OR = 4.357, 95%CI: 2.248-8.445), PTAR (OR = 3.544, 95%CI: 1.838-6.835), age (OR = 1.048, 95%CI: 1.024-1.073) and MELD score (OR = 1.340, 95%CI: 1.150-1.562) were independent influencing factors of ICGR15 ≥ 10% (Table 2). LSM (OR = 3.120, 95%CI: 1.125-8.656), PTAR (OR = 3.524, 95%CI: 1.267-9.801), age (OR = 1.059, 95%CI: 1.024-1.096) and MELD score (OR = 1.377, 95%CI: 1.146-1.655) were independent influencing factors of ICGR15 ≥ 20% (Table 3). The predictive models using the above 4 variables were constructed as follows: mLPaM = 1.472 LSM (LSM ≥ 12.4 = 2, LSM < 12.4 = 1) + 1.265 PTAR (PTAR ≥ 0.280 = 2, PTAR < 0.280 = 1) + 0.047 age (years) + 0.291 MELD-7.600 and sLPaM = 1.138 LSM (LSM ≥ 12.4 = 2, LSM < 12.4 = 1) + 1.260 PTAR (PTAR ≥ 0.280 = 2, PTAR < 0.280 = 1) + 0.058 age (years) + 0.320 MELD-9.750.

A comparison of the predictive performance of the constructed model and other methods in the training cohort

The AUC values of the mLPaM model (0.855) and sLPaM model (0.872) were greater than that of MELD score, PTAR and ALBI evaluation tools, and their sensitivity and negative predictive values were better than these evaluation methods (Table 4 and Figure 1).

Internal validation of the new predictive model in the validation cohort

132 CLD patients were prospectively considered for enrollment in the internal validation cohort. The performance of the various methods at predicting LRF is reported in Table 5. The AUC values of the mLPaM model (0.869) and sLPaM model (0.876) were greater than other LRF predictive methods. The mLPaM model showed good sensitivity (89.1%) and optimal accuracy (78.94%) for the diagnosis of mild LRF impairment, and the sLPaM model showed optimal sensitivity (92.9%) for the diagnosis of severe LRF impairment (Table 5 and Figure 2).

DISCUSSION

To date, accurate evaluation of LRF has been a hot topic in national and international research. As classic scoring systems, the CTP score and MELD score have been widely used in clinical practice. The CTP has introduced an element of bias into the scoring system due to the subjective nature of how clinical encephalopathy and ascites variables may be graded[19]. The MELD score is a continuous variable, and each indicator is given a corresponding weight through statistical analysis, which has further accuracy in evaluating LRF. In recent years, the ALBI and PTAR models have been gradually applied in clinical practice, which better evaluated LRF[20,21]. However, the ICG clearance test is currently considered the most valuable test in assessing LRF.

Although the ICG clearance test is a simple and helpful tool to assess individual LRF, it is an invasive and complex procedure, and the result is influenced by many factors (such as biliary excretion disorder and low proteinemia). In particular, the ICG clearance test is not applicable in pregnant women, patients with a history of iodine allergy or hyperthyroidism[22]. Transient elastography (TE) is a non-invasive and reproducible technique for assessing liver fibrosis, and is even a replacement for liver biopsy[23, 24]. The Baveno VII Consensus showed that TE was an accurate tool for the prediction of CSPH[25]. In a previous study, it was found that LSM could predict postoperative liver failure in patients with HCC [26]. Therefore, LSM is considered to have a strong relationship with liver function.

As liver function impairment is the primary determinant of the development of post-hepatectomy liver failure, the vast majority of candidates for liver resection had CTP grade A[27]. According to the CTP classification, the majority of patients with HCC were classified as grade A, but their liver function may vary significantly[15]. A previous study revealed that ICGR15 was more accurate than the CTP score and MELD score in predicting hepatic functional reserve before hepatectomy[3]. The study showed that ICGR15 > 15% was an accurate method of predicting postoperative hepatic decompensation in patients with CTP grade A[28]. In patients with an ICGR15 > 20%, a previous study

Table 1 Comparison of demographic and clinical characteristics of chronic liver disease patients in the training cohort and validation cohort

	Validation cohort, *n* = 132	Training cohort, *n* = 360	*P* value
Gender (male/female, *n*)	90/42	260/100	0.381
Age (yr)	54.84 ± 27.70	48.71 ± 13.34	0.001
Etiology			0.097
HBV	111	278	
Others	21	82	
HCC (*n*)	42	63	**0.001**
HB (g/dL)	14.28 ± 10.86	13.71 ± 2.16	0.346
PLT (× 10^9/L)	174.96 ± 83.93	170.95 ± 74.69	0.610
PT (s)	13.21 ± 1.66	12.84 ± 1.67	**0.030**
APTT (s)	33.28 ± 7.73	32.54 ± 6.14	0.273
INR	1.16 ± 0.16	1.09 ± 0.16	**0.000**
AST (U/L)	72.14 ± 78.19	86.16 ± 135.81	0.264
ALT (U/L)	106.35 ± 166.60	136.73 ± 247.37	0.192
ALB (g/L)	38.03 ± 5.22	40.03 ± 5.08	**0.000**
TBIL (μmol/L)	28.29 ± 31.05	19.94 ± 16.99	**0.000**
CHE (U/L)	6163.11 ± 2647.19	6748.44 ± 2127.44	**0.015**
ALP (U/L)	118.60 ± 95.93	179.64 ± 721.94	0.334
GGT (U/L)	128.91 ± 201.79	116.89 ± 188.75	0.539
GLO (g/L)	28.70 ± 4.81	29.02 ± 5.19	0.534
Cr (μmol/L)	65.36 ± 23.23	65.68 ± 15.38	0.860
MELD	8.14 ± 3.46	7.80 ± 2.56	0.241
ALBI	-2.36 ± 0.54	-2.60 ± 0.48	**0.000**
PTAR	0.31 ± 0.07	0.28 ± 0.07	**0.000**
ICGR15 (%)	11.55 ± 11.82	8.16 ± 8.56	**0.000**
LSM (kPa)	19.54 ± 18.28	16.34 ± 16.62	0.066

HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HB: Hemoglobin; PLT: Platelet count; PT: Prothrombin time; APTT: Active prothrombin time; INR: International normalized ratio; AST: Aspartate transaminase; ALT: Alanine aminotransaminase; ALB: Albumin; TBIL: Total bilirubin; CHE: Cholinesterase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyltransferase; GLO: Globulin; Cr: Creatinine; MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin grade; PTAR: Prothrombin time international normalized ratio to albumin ratio; ICGR15: Indocyanine green retention rate at 15 min; LSM: Liver stiffness measurement.

recommended non-anatomical resection rather than anatomical resection for the treatment of a solitary 2–5-cm-diameter HCC without macroscopic vascular invasion[29]. Therefore, it is essential to assess LRF before HCC hepatectomy, thereby assisting clinical decision-making.

Our research constructed new models for clinical prediction of LRF impairment based on LSM, and the models were superior to other existing methods for predicting LRF (Table 4 and Figure 1). Moreover, compared to the other four methods, the models also showed better performance for predicting LRF in the prospective validation cohort (Table 5 and Figure 2). Therefore, based on the analysis of the above research results, these models could be an alternative tool for LRF assessment, especially in evaluating a population almost entirely stratified as CTP grade A.

Limitations of the study

Despite the significant findings in this study, our research also had a few limitations. First, the study was limited by its single-center prospective cohort nature. The patients were recruited from the same medical facility, and not all patients with complete clinical data were obtained from a treatment database. Second, most of the patients in this study were Asians with B viral hepatitis. Therefore, the performance of the model in patients of other ethnicities (*e.g.*, Caucasians, Africans, *etc.*) still needs

Table 2 Multivariate logistic stepwise regression analysis of indocyanine green retention rate at 15 min ≥ 10% in the training cohort

Variable	*B*	SE	Wald	*P* value	OR (95%CI)
LSM (kPa)	1.472	0.338	19.008	< 0.001	4.357 (2.248-8.445)
PTAR	1.265	0.335	14.260	< 0.001	3.544 (1.838-6.835)
Age (yr)	0.047	0.012	15.329	< 0.001	1.048 (1.024-1.073)
MELD	0.291	0.078	13.844	< 0.001	1.337 (1.147-1.558)
Constant	-7.600	1.022	55.302	< 0.001	0.007

LSM: Liver stiffness measurement; PTAR: Prothrombin time international normalized ratio to albumin ratio; MELD: Model for end-stage liver disease.

Table 3 Multivariate logistic stepwise regression analysis of indocyanine green retention rate at 15 min ≥ 20% in the training cohort

Variable	*B*	SE	Wald	*P* value	OR (95%CI)
LSM (kPa)	1.138	0.520	4.778	0.029	3.120 (1.125-8.656)
PTAR	1.260	0.521	5.825	0.016	3.524 (1.267-9.801)
Age (yr)	0.058	0.017	11.226	0.001	1.059 (1.024-1.096)
MELD	0.320	0.094	11.652	0.001	1.377 (1.146-1.655)
Constant	-9.750	-1.454	44.963	< 0.001	0.001

LSM: Liver stiffness measurement; PTAR: Prothrombin time international normalized ratio to albumin ratio; MELD: Model for end-stage liver disease.

Table 4 Comparison of the predictive performance of the new constructed models (mLPaM and sLPaM) and other models in the assessment of impaired liver reserve function in the training cohort

	AUC (95%CI)	Optimal cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
mLPaM	0.855 (0.809-0.901)	0.135	91.3	66.4	36.09	97.35	70.68
MELD	0.752 (0.688-0.817)	7.662	80.0	61.4	31.25	93.33	54.75
ALBI	0.776 (0.717-0.835)	-2.557	76.3	67.9	37.67	91.85	69.90
PTAR	0.728 (0.664-0.791)	0.150	73.8	71.8	42.11	90.79	72.24
LSM (kPa)	0.733 (0.672-0.794)	1.50	78.8	67.9	37.67	92.86	70.05
sLPaM	0.872 (0.823-0.921)	0.046	96.8	64.6	14.83	99.69	66.53
MELD	0.786 (0.687-0.886)	9.380	71.0	85.2	35.45	96.25	83.74
ALBI	0.798 (0.706-0.890)	-2.220	64.5	87.4	39.81	95.01	84.78
PTAR	0.731 (0.644-0.818)	0.150	80.6	65.5	15.33	97.76	66.59
LSM (kPa)	0.706 (0.618-0.795)	1.50	80.6	60.6	12.79	97.76	61.94

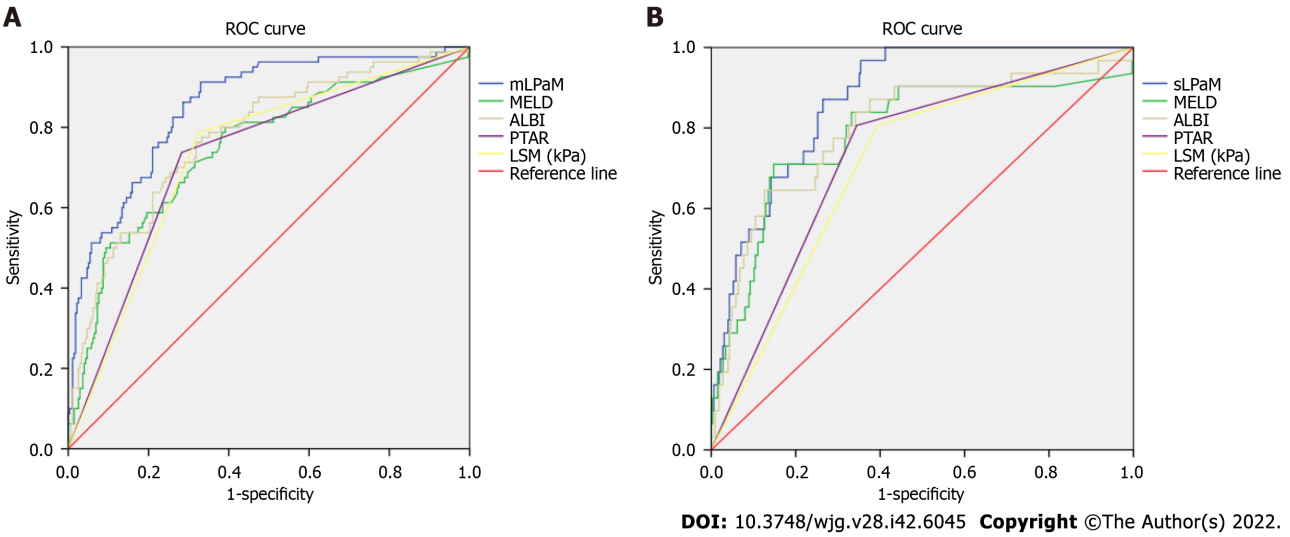
MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin grade; PTAR: Prothrombin time international normalized ratio to albumin ratio; LSM: Liver stiffness measurement; AUC: Area under the cure; CI: Confidence interval; NPV: Negative predictive value; PPV: Positive predictive value; mLPaM: Mildly impaired liver reserve function model; sLPaM: Severely impaired liver reserve function model.

further investigation. Third, the models were mainly used to evaluate LRF in patients with compensated CLD, and their predictive value in patients with decompensated stage needs further evaluation. Finally, although the formula for the models was relatively complex, a mobile app or web-based calculator could calculate the score easily and rapidly in the current high-tech era. Despite these limitations, this study provided the first accurate models for evaluating LRF based on LSM in China.

Table 5 Comparison of the predictive performance of the new constructed models (mLPaM and sLPaM) and other models in the assessment of impaired liver reserve function in the prospective validation cohort

	AUC (95%CI)	Optimal cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
mLPaM	0.869 (0.810-0.929)	0.240	89.1	74.4	60.85	93.86	78.94
MELD	0.729 (0.633-0.824)	9.743	43.5	96.5	93.65	59.01	67.74
ALBI	0.824 (0.749-0.900)	-2.315	78.3	76.7	63.78	87.09	77.25
PTAR	0.672 (0.580-0.765)	1.500	89.1	45.3	30.70	93.86	54.66
LSM (kPa)	0.782 (0.702-0.862)	1.500	91.3	65.1	49.93	95.15	72.33
sLPaM	0.876 (0.812-0.940)	0.073	92.9	68.3	49.94	95.15	72.33
MELD	0.803 (0.701-0.904)	9.187	64.3	85.6	61.54	87.00	79.98
ALBI	0.836 (0.743-0.929)	-1.897	71.4	88.5	67.44	90.27	84.22
PTAR	0.666 (0.566-0.767)	1.500	92.9	59.6	28.43	97.98	64.50
LSM (kPa)	0.743 (0.653-0.833)	1.500	92.9	55.8	25.37	97.98	60.96

MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin grade; PTAR: Prothrombin time international normalized ratio to albumin ratio; LSM: Liver stiffness measurement; AUC: Area under the cure; CI: Confidence interval; NPV: Negative predictive value; PPV: Positive predictive value; mLPaM: Mildly impaired liver reserve function model; sLPaM: Severely impaired liver reserve function model.

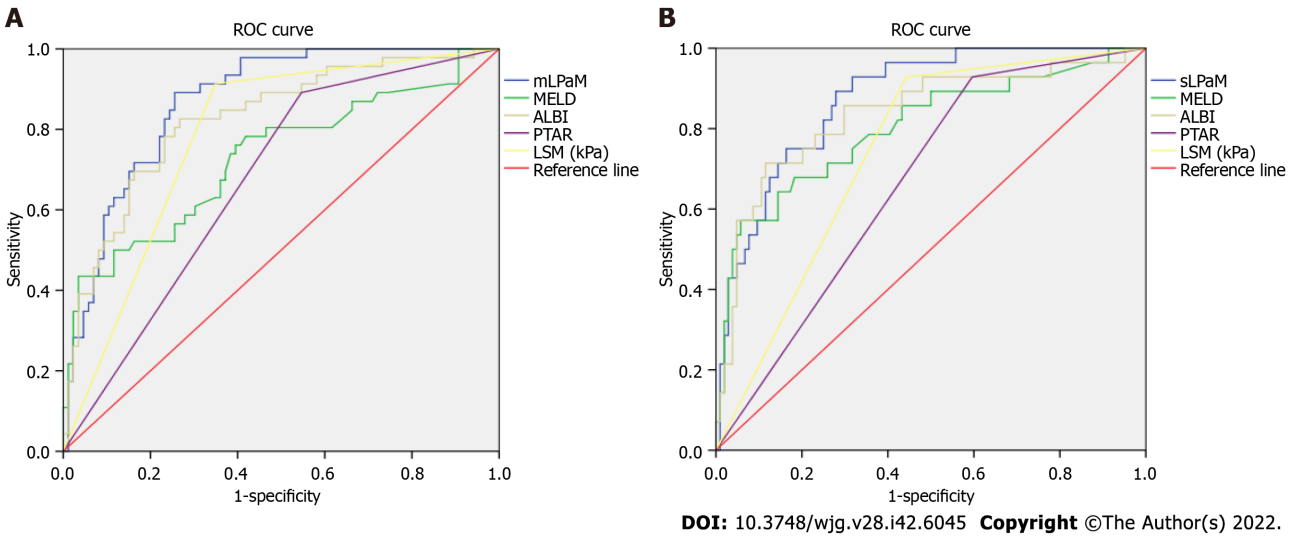


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Figure 1 Comparison of different liver reserve function assessment methods by receiver operator characteristic curves in the training cohort. A: mLPaM is indocyanine green retention rate at 15 min (ICGR15) ≥ 10%; B: sLPaM is ICGR15 ≥ 20%. ROC: Receiver operator characteristic; MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin grade; PTAR: Prothrombin time international normalized ratio to albumin ratio; LSM: Liver stiffness measurement; mLPaM: Mildly impaired liver reserve function model; sLPaM: Severely impaired liver reserve function model.

CONCLUSION

The first predicted models based on LSM could facilitate accurate, reliable and simple-to-use prediction of LRF irrespective of etiology. They are entirely objective based on routine clinical and laboratory parameters. These models would be a useful tool for realizing individualized LRF evaluation to improve the popularity of testing and avoid possible risks during the ICG clearance test, ultimately achieving a clinically feasible and safe LRF test.



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Figure 2 Comparison of different liver reserve function assessment methods by area under the curves in the validation cohort. A: mLPaM is indocyanine green retention rate at 15 min (ICGR15) ≥ 10%; B: sLPaM is ICGR15 ≥ 20%. ROC: Receiver operator characteristic; MELD: Model for end-stage liver disease; ALBI: Albumin-bilirubin grade; PTAR: Prothrombin time international normalized ratio to albumin ratio; LSM: Liver stiffness measurement; mLPaM: Mildly impaired liver reserve function model; sLPaM: Severely impaired liver reserve function model.

ARTICLE HIGHLIGHTS

Research background

There are no obvious clinical symptoms in chronic liver disease (CLD) patients at the early stage, but their liver reserve function (LRF) may be impaired. Early evaluation of LRF is of great help in identifying disease progression. Assessment of LRF is essential for predicting the prognosis of patients with CLD and determines the extent of liver resection in patients with hepatocellular carcinoma (HCC).

Research motivation

Liver function impairment is the primary determinant in the development of post-hepatectomy liver failure. There are no obvious clinical symptoms in CLD patients at Child-Turcotte-Pugh A stage, but their LRF may be impaired. Due to impossible implementation of the indocyanine green (ICG) clearance test in some CLD patients, a new method to accurately assess LRF is needed.

Research objectives

This study aimed to establish noninvasive models of LRF assessment based on LSM. The new predictive models were established through logistic regression analysis and were validated internally in a prospective cohort. The new models had a good predictive performance on LRF and could replace the ICG clearance test, especially in the patients who are unable to undergo ICG testing.

Research methods

Clinical data from 360 patients with compensated CLD were retrospectively collected and analyzed in the training cohort. The new predictive models were established through logistic regression analysis and were validated internally in a prospective cohort (132 patients). The areas under the ROC curve (AUCs) were measured and compared to evaluate the discrimination ability of different models.

Research results

Our study defined the indocyanine green retention rate at 15 min (ICGR15) ≥ 10% as mildly impaired LRF and ICGR15 ≥ 20% as severely impaired LRF. We constructed predictive models of LRF, named the mLPaM and sLPaM, which involved only LSM, prothrombin time international normalized ratio to albumin ratio, age and model for end-stage liver disease. The AUC of the mLPaM model (0.855, 0.872, respectively) and sLPaM model (0.869, 0.876, respectively) were higher than that of other methods in the two cohorts. In addition, the new models showed good sensitivity and accuracy for the diagnosis of LRF impairment in the validation cohort.

Research conclusions

Our study found that the new models had a good predictive performance for LRF and could replace the ICG clearance test, especially in patients who are unable to undergo ICG testing.

Research perspectives

This was not a multicenter study and most of the CLD patients in this study were Asians. Therefore, a multi-center prospective cohort study could further evaluate the performance of the predictive models, and the models in patients of other ethnicities need further investigation. The predictive value of the models in patients with a decompensated stage need further evaluation.

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FOOTNOTES

Author contributions: Lai RM and Zheng Q conceived and designed the study; Wang MM and Lin XY collected clinical data of the patients and contributed to the data analysis; Lai RM and Chen J wrote the manuscript; and all authors approved the final version of the manuscript.

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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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ORIGINAL ARTICLE

Retrospective Study

Novel management indications for conservative treatment of chylous ascites after gastric cancer surgery

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Abstract

BACKGROUND

Chylous ascites (CA) presents a challenge as a relatively common postoperative complication in gastric cancer (GC). Primary conservative therapy involved total parenteral nutrition, continuous low-pressure drainage, somatostatin, and a low-fat diet. Drainage tube (DT) clamping has been presented as a potential alternative conservative treatment for GC patients with CA.

AIM

To propose novel conservative treatment strategies for CA following GC surgery.

METHODS

The data of patients with CA after GC surgery performed at the Fudan University Shanghai Cancer Center between 2006 and 2021 were evaluated retrospectively.

RESULTS

53 patients underwent surgery for GC and exhibited postoperative CA during the study period. Postoperative hospitalization and time of DT removal showed a significant positive association (R^2 = 0.979, P < 0.001). We further observed that delayed DT removal significantly extended the total and postoperative hospitalization, antibiotic usage duration, and hospitalization cost (postoperative hospitalization: 25.8 d *vs* 15.5 d, P < 0.001; total hospitalization: 33.2 d *vs* 24.7 d, P < 0.01; antibiotic usage duration: 10.8 d *vs* 6.2 d, P < 0.01; hospitalization cost: ¥9.2 × 10^4 *vs* ¥6.5 × 10^4, P < 0.01). Multivariate analysis demonstrated that postoperative infection and antibiotic usage were independent factors for delayed DT removal. Furthermore, DT removal times were shorter in seven patients who underwent DT clamping (clamped DT *vs* normal group, 11.8 d *vs* 13.6 d, P = 0.047; clamped DT *vs* delayed group, 13.6 d *vs* 27.4 d, P < 0.001). In addition, our results indicated

that removal of the DT may be possible after three consecutive days of drainage volumes less than 300 mL in GC patients with CA.

CONCLUSION

Infection and antibiotic usage were vital independent factors that influenced delayed DT removal in patients with CA. Appropriate standards for DT removal can significantly reduce the duration of hospitalization. Furthermore, DT clamping might be a recommended option for conservative treatment of postoperative CA.

Key Words: Gastric cancer; Chylous ascites; Conservative treatment; Drainage tube

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Core Tip: Chylous ascites (CA) is one of uncommon postoperative complication in the patients received gastric cancer (GC) surgery. Previously, the primary treatment for CA was conservative therapy, which mainly involved total parenteral nutrition, continuous low-pressure drainage, somatostatin, and a low-fat diet. Therefore, we retrospectively analyzed the patients with CA after GC surgery in our center, aiming to explore the vital factors that influence CA treatment and recommend novel conservative treatment strategies for postoperative CA in GC.

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INTRODUCTION

Chylous ascites (CA) was first reported by Morton in 1691 and is defined as the leakage of milk-like fluid that contains high level of triglyceride (TG)[1,2]. Gastric cancer (GC) is one of the most common malignant tumors worldwide, and a standardized protocol for radical surgical resection has been widely accepted as a safe and effective treatment[3,4]. CA generally occurs following abdominal surgery, the incidence of postoperative CA ranges from 2.06% to 11.80% in GC patients[5-7], as a result of disturbance of the cisterna chyli or its major tributaries[8,9]. The increased incidence of CA is considered to be likely due to the increased number of cancer patients undergoing more aggressive surgical interventions in addition to laparoscopic surgery[10]. CA presents a challenge as a relatively common postoperative complication and impacts subsequent adjuvant treatments in GC. In addition, massive and prolonged CA may lead to infection, malnutrition and immunodeficiency[11].

To date, treatment options for CA have included dietary measures, use of pharmacological agents and surgical or percutaneous interventions. A high-protein and low-fat diet with medium-chain triglycerides is often recommended for patients with CA[12]. Patients who do not respond to dietary restriction should receive total parenteral nutrition (TPN), which bypasses the bowel and may thus reduce lymph flow[13]. Continuous low-pressure drainage and somatostatin also represent effective conservative treatment for postoperative CA[6,14]. CA can be cured by lymphangiography and adjunctive embolization techniques that include direct percutaneous injection of glue into the leakage site or into the surrounding lymphoid tissue[15]. Furthermore, the use of surgical measures to successfully treat CA has been reported in patients with cirrhosis and CA that is resistant to conservative therapy[1].

In this study, we retrospectively analyzed 53 patients with CA after GC surgery, aiming to explore the vital factors that influence CA treatment and recommend novel conservative treatment strategies for postoperative CA in GC.

MATERIALS AND METHODS

Patients

We retrospectively reviewed all patients with CA who had undergone surgery for GC at our institution from 1 March 2006 to 31 May 2021. Three investigators performed a thorough review of all available data from the Fudan University Shanghai Cancer Center (FUSCC) medical record system, using RED

Cap electronic data capture tools. In this cohort, 53 patients were admitted for gastric resection and lymphadenectomy: 2 underwent palliative resection and 51 underwent radical gastric resection with curative intent. This study was approved by the FUSCC review board in accordance with Chinese bioethical regulations, and all enrolled patients signed informed consent forms.

Definitions

CA was defined as the presence of milky or creamy peritoneal fluid in the drainage tubes, at a volume of ≥ 200 mL/d and a TG levels ≥ 110 mg/dL[1,11]. Additionally, the chyle test was routinely performed if the milky peritoneal fluid was suspected to be CA[16]. Clinical and pathological data, including the age, gender, AJCC (American Joint Committee on Cancer) stage, surgical procedure, lymph node dissection, drainage tube (DT) removal, time of oral feeding, time to CA onset, drainage duration, and hospitalization duration were collected and analyzed. All patients with CA were managed conservatively; the conservative treatments included TPN, continuous low-pressure drainage, somatostatin, DT clamping, and a low-fat diet. The time to CA onset was defined as the interval between the surgical procedure and the appearance of CA. Delay DT removal was defined as a DT removal time > 16 d after surgery for all patients or patients discharge with DT. Additionally, white blood cell counts, body temperature measurement, and germiculture were performed to diagnose CA combined with infection. DT clamping is defined as physical closing of the abdominal DT, with a daily open drainage time of about 2 h.

Statistical analysis

Categorical variable analysis was performed using the χ^2 test or Fisher's exact test, and continuous variables were compared using Student's t test. We used univariate logistic regression models to evaluate the risk factors of delayed DT removal in GC patients with postoperative CA, and a Cox regression model was used to perform multivariable analysis to calculate relative risk. All values were categorized into groups according to medians. All results were considered clinically significant at a P value < 0.05. Statistical analyses were performed using SPSS software version 19.0.

RESULTS

Clinical characteristics of gastric cancer patients with postoperative chylous ascites

Between 1 March 2006 and 31 May 2021, 16074 GC patients were hospitalized in our department and 7081 patients underwent gastrectomy and lymphadenectomy. Of these patients, 53 underwent surgical resection for GC and developed CA. The main characteristics and patient selection are shown in Table 1 and Figure 1. The patients had an average age of 61.0 ± 11.3 years, a high ratio of male and advanced stage of disease (Male *vs* female: 77.40% *vs* 22.60%, early stage *vs* advanced stage: 39.6% *vs* 60.4%), 51 underwent radical surgery, 43 underwent D2 lymph node dissection, and 13 were discharged with DT. The average oral feeding and CA onset times after surgery were 3.8 and 7.5 d, respectively. The average durations of DT drainage and postoperative hospitalization were 14.3 and 21.9 d, respectively.

Delayed drainage tube removal has an important influence on gastric cancer patients with postoperative chylous ascites

In our data, 40 patients (75%) had their DTs removed during the hospitalization period, and 13 patients (25%) were discharged with DT (Figure 2A). As shown in Figure 2B and Supplementary Figure 1, both postoperative (R^2 = 0.979, P < 0.001) and total hospitalization time (R^2 = 0.791, P < 0.001) had a significant positive association with DT removal time. Moreover, the median postoperative DT removal time of the patients discharged with or without DT was 30 and 16 d, respectively (Figure 2C and Supplementary Figure 2). We defined the patients' DT removal time > the median time (16 d) or the patients discharged with DT as delayed DT removal, and the patients were categorized into either the delayed DT removal or normal group (Figure 2D). Comparing the delayed and normal groups, delayed DT removal significantly extended the total and postoperative hospitalization times, duration of antibiotic usage, and hospitalization costs in the GC patients (postoperative hospitalization duration: 25.8 d *vs* 15.5 d, P < 0.001; total hospitalization duration: 33.2 d *vs* 24.7 d, P < 0.01; antibiotic usage: 10.8 d *vs* 6.2 d, P < 0.01; hospitalization cost: ¥9.2 × 10^4 *vs* ¥6.5 × 10^4, P < 0.01) (Figure 2E).

Characteristic differences between the normal and delayed drainage tube removal groups in gastric cancer patients with postoperative chylous ascites

We present the characteristic differences between the normal and delayed DT removal groups in Figure 3, Table 2 and Supplementary Table 1. First, we evaluated the clinical characteristics and detected that there were no differences between the two groups regarding gender, age, tumor size or location, lymphadenectomy, and AJCC stage. Second, the treatment-related features were further explored. Of note, the patients in the normal group were more likely to undergo DT clamping than the delayed DT removal group (35.0% *vs* 0%, P < 0.001). In addition, compared with the patients in the delayed group, a

Table 1 Clinical characteristics of gastric cancer patients with postoperative chylous ascites

Characteristics	Cases
Age, yr	61.0 ± 11.3
Gender, *n* (%)	
Male	41 (77.4)
Female	12 (22.6)
Tumor location, *n* (%)	
Upper	18 (40.0)
Middle	11 (20.6)
Bottom	24 (45.3)
AJCC 8th stage, *n* (%)	
I	21 (39.6)
II	11 (20.8)
III	18 (34.0)
IV	3 (5.7)
Type of surgery, *n* (%)	
Radical	51 (96.2)
Non-radical	2 (3.8)
LN dissection, *n* (%)	
D1	8 (15.1)
D2	43 (81.1)
D3	2 (3.8)
Discharged without DT, *n* (%)	
Yes	40 (75.5)
No	13 (24.5)
Postoperative time of oral feeding (d)	3.8 ± 1.0
Postoperative time of CA appearance (d)	7.5 ± 2.4
DT removal duration (d)	14.3 ± 12.6
Postoperative hospitalization duration (d)	21.9 ± 11.1

SD: Standard deviation; AJCC: American Joint Committee on Cancer; LN: Lymph node; DT: Drainage tube; CA: Chylous ascites.

shorter duration of low-fat diet were slightly shared in the normal group patients (40.0% *vs* 63.6%, P = 0.082). Third, we estimated the DT drainage variation between the two groups. Obviously, the delayed DT removal group generally had a longer duration of DT drainage than the normal group; however, the CA onset time and maximum drainage volumes were not significantly different between the two groups.

Infection and antibiotic usage were key independent factors influencing the delay of drainage tube removal

As shown in Table 3 and Figure 4, the univariate analysis revealed that early postoperative intake (RR: 2.22, 1.10–4.48, P = 0.031), postoperative infection (RR: 2.20, 1.21-4.61, P = 0.003), and antibiotic usage (RR: 0.45, 0.22–0.91, P = 0.009) were significantly associated with delayed DT removal in GC patients with CA. However, the baseline characteristics (age, gender, and AJCC stage), lymph node dissection, CA onset time, maximum drainage volume, postoperative albumin, postoperative hemoglobin, and DT clamping were not significantly associated with delayed DT removal (all P > 0.05). Furthermore, multivariate analysis demonstrated that postoperative infection (HR: 2.40, 1.63-4.14, P = 0.007) and antibiotic usage (HR: 0.86, 0.76-0.96, P = 0.009) were independent factors that influenced delayed DT removal in GC patients with postoperative CA.

Table 2 Clinical characteristics differences between the normal and delayed drainage tube removal groups

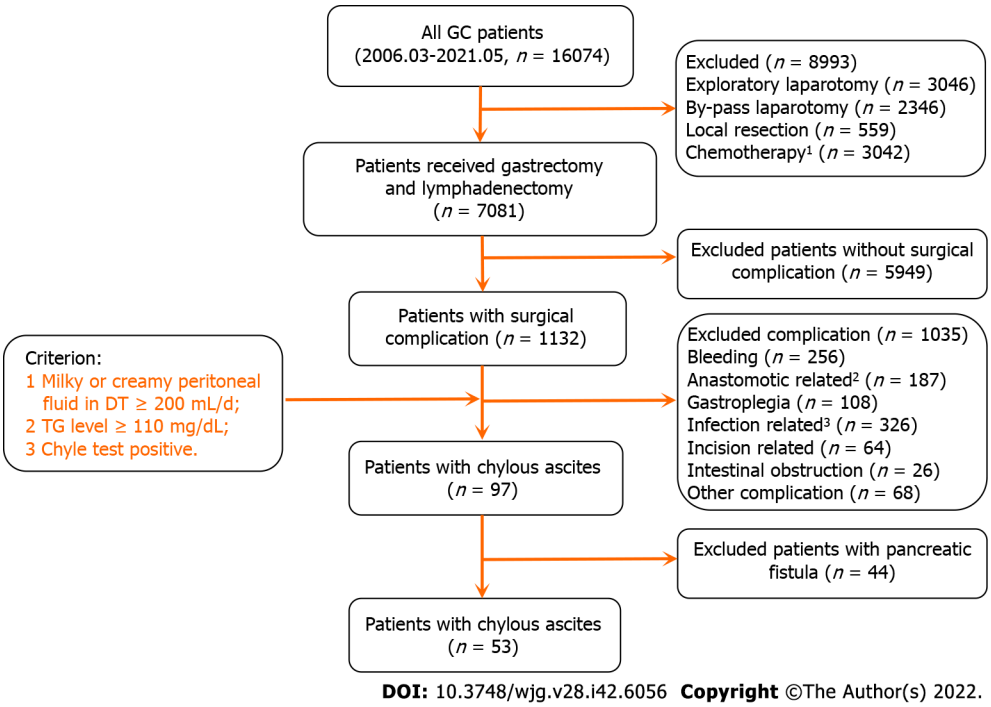
Subgroup	No. of patients	
	Normal (*n* = 20)	Delayed DT removal (*n* = 33)
Clamp DT		
Yes	7	0
No	13	0
Preoperative HGB, g/L		
≤ 130	12	15
> 130	8	18
Preoperative ALB, g/L		
≤ 41	9	17
> 41	11	16
Maximum drainage, mL		
≤ 540	13	13
> 540	7	20
Postoperative intake[1], d		
≤ 3	10	17
> 3	10	16
CA onset time, d		
≤ 7	15	16
> 7	5	17
Antibiotic usage, d		
≤ 5	12	16
> 5	8	17
Postoperative infection		
Yes	8	12
No	12	21
AJCC stage		
Early	8	13
Advanced	12	20
LN dissection		
D1	6	2
D2+	14	31
Age, yr		
≤ 60	8	15
> 60	12	18
Gender		
Male	15	26
Female	5	7

[1]Time of oral feeding after gastric surgery.
HGB: Hemoglobin; ALB: Albumin; AJCC: American Joint Committee on Cancer; LN: Lymph node; DT: Drainage tube; CA: Chylous ascites.

Table 3 Postoperative infection-related complications significantly affect gastric cancer patients with postoperative chylous ascites to remove abdominal drainage tubes in time

	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
DT clamping	0.39 (0.11-1.72)	0.281	-	-
Postoperative HGB	1.49 (0.72-3.08)	0.283	-	-
Postoperative ALB	1.82 (0.88-3.76)	0.303	1.09 (0.98-1.21)	0.127
Maximum drainage	0.98 (0.47-2.05)	0.367	-	-
Postoperative intake time	2.22 (1.10-4.48)	0.031	1.86 (0.94-4.21)	0.234
CA onset time	0.81 (0.40-1.62)	0.486	-	-
Duration of antibiotic usage	0.45 (0.22-0.91)	0.009	0.86 (0.76-0.96)	0.009
Postoperative infection	2.20 (1.21-4.61)	0.003	2.40 (1.63-4.14)	0.007
AJCC Stage	0.95 (0.75-1.21)	0.676	-	-
LN dissection	0.87 (0.53-1.42)	0.595	-	-
Age	1.34 (0.66-2.70)	0.471	-	-
Gender	2.02 (0.85-4.78)	0.141	3.13 (0.85-11.1)	0.187

HR: Hazard ratio; CI: Confidence interval; HGB: Hemoglobin; ALB: Albumin; AJCC: American Joint Committee on Cancer; LN: Lymph node; DT: Drainage tube; CA: Chylous ascites.

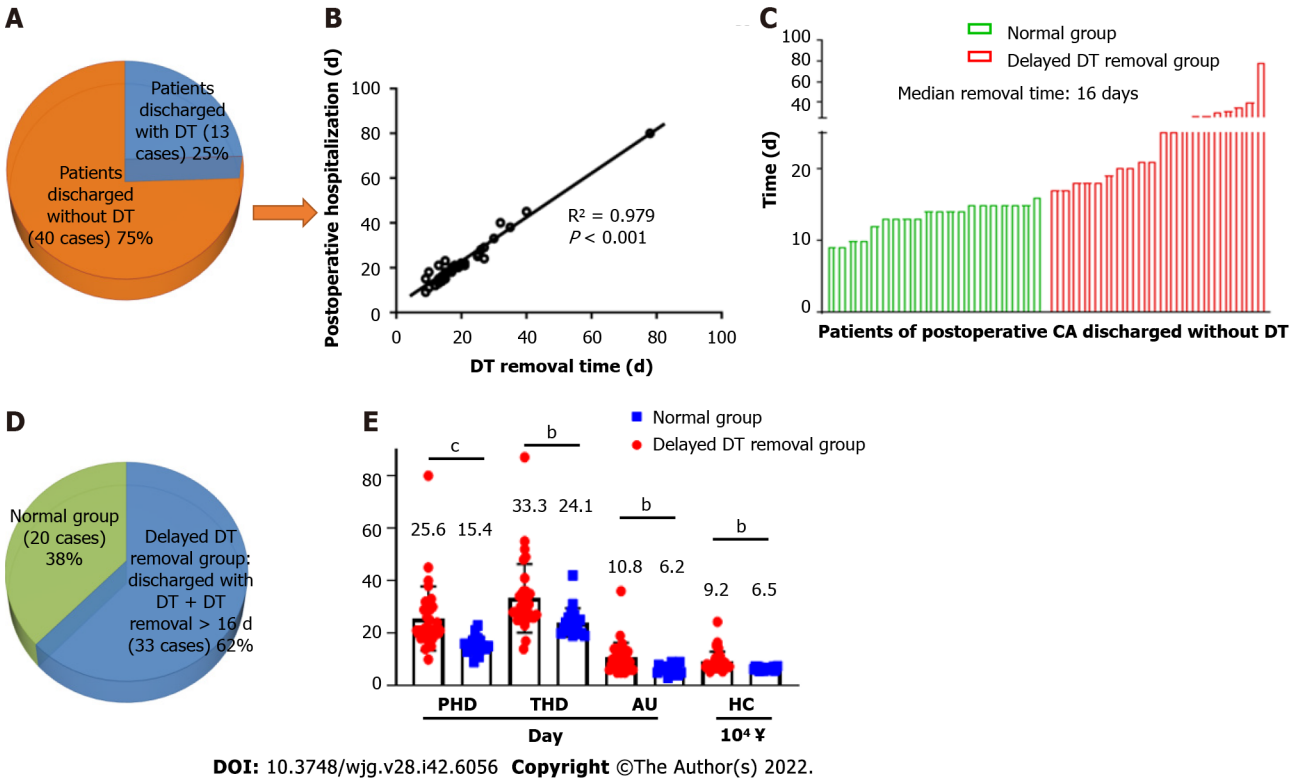


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Figure 1 Flowchart of study included patients. [1]Include the patients underwent neo-adjuvant, adjuvant, and palliative chemotherapy. [2]Include the patients with anastomotic stenosis and fistula, except for the patients with anastomotic bleeding. [3]The patients with all infection events except abdominal infection. GC: Gastric cancer; DT: Drainage tube; TG: Triglyceride.

Drainage tube clamping is a favorable method for the gastric cancer patients with postoperative chylous ascites

In Figure 5A, we describe comprehensive treatment for GC patients with postoperative CA; the therapies included DT clamping, somatostatin, antibiotic, TPN, low-fat diet, and continuous low-pressure drainage. DT clamping was performed for seven patients during the hospitalization period when postoperative CA occurred, and the clamped DT patients had a shorter DT removal time than the nor-



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Figure 2 Delayed drainage tube removal has an important influence on gastric cancer patients with postoperative chylous ascites. $^{b}P < 0.01$. $^{c}P < 0.001$. A: The status of drainage tube (DT) after discharged in gastric cancer (GC) patients with postoperative chylous ascites (CA); B: Postoperative hospitalization have a significantly positive correlation with the time of removal DT in GC patients discharged without DT; C: Time of removal DT in GC patients with postoperative CA who discharged without DT; D: The definition of delayed DT removal in GC patients with postoperative CA; E: Delayed DT removal obviously increase medical resources consumption and economic burden in GC patients with postoperative CA. GC: Gastric cancer; CA: Chylous ascites; DT: Drainage tube; PHD: Postoperative hospitalization duration; THD: Total hospitalization duration; AU: time of antibiotic usage; HC: Hospitalization cost.

mal and delayed removal groups (clamped DT group *vs* normal group, 11.8 d *vs* 13.6 d, $P = 0.047$; clamped DT group *vs* delayed group, 13.6 d *vs* 27.4 d, $P < 0.001$) (Figure 5B and Supplementary Figure 3). Moreover, similar clinical characteristics and treatment strategies were present in the three sub-groups (Supplementary Table 2). Our result further indicated that DT clamping significantly decreased total and postoperative hospitalization time, duration of antibiotic usage, and hospitalization costs in the GC patients with CA (Supplementary Figure 4). In Figure 5C, we dynamically observed the variation in daily drainage volume before DT removal (day 1 to day 7). Compared with the delayed removal group, start from day 3 before remove DT, the normal group and the clamped-DT group had relatively high drainage volumes. Additionally, the results of the drainage variation analysis indicated that 3 consecutive days of drainage volume less than 300 mL may be a suitable remove DT threshold in the GC patients with postoperative CA. Among two patients underwent DT clamping, computed tomography imaging of the abdomen showed that, after about 1 wk of DT clamping, the fluid in the abdominal cavity was reduced (Figure 5D).

Novel conservative therapeutic strategies for gastric cancer patients with postoperative chylous ascites

As the results mentioned above, we subsequently summarized the experiences of the GC patients with postoperative CA treatment in our department (Figure 6). First, the CA patients were divided into two sub-groups according to their postoperative infection status. Second, in the patients with infection, based on traditional treatments, antibiotic therapy was a vital supplement. Third, in the patients without infection, DT clamping was a viable option. Finally, for patients with 3 consecutive days of drainage less than 300 mL, DT removal might be the appropriate management.

DISCUSSION

In this study, we retrospectively analyzed 53 cases of GC with postoperative CA at the FUSCC. Our results indicated that hospitalization duration was closely associated with DT removal time. Furthermore, postoperative infection and antibiotic usage were important independent factors that

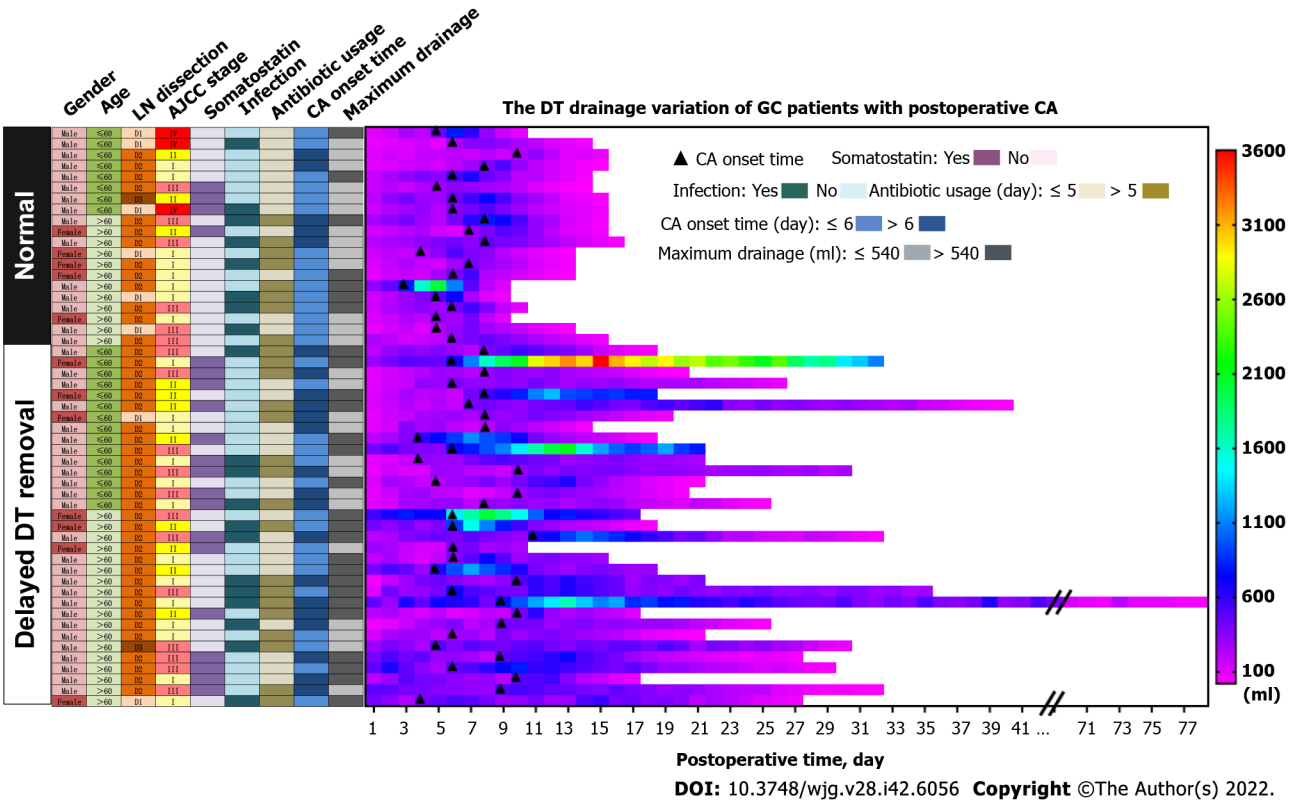


Figure 3 Overview of characteristic differences between the normal and delayed drainage tube removal groups in gastric cancer patients with postoperative chylous ascites. GC: Gastric cancer; DT: Drainage tube; LN: Lymph node; CA: Chylous ascites.

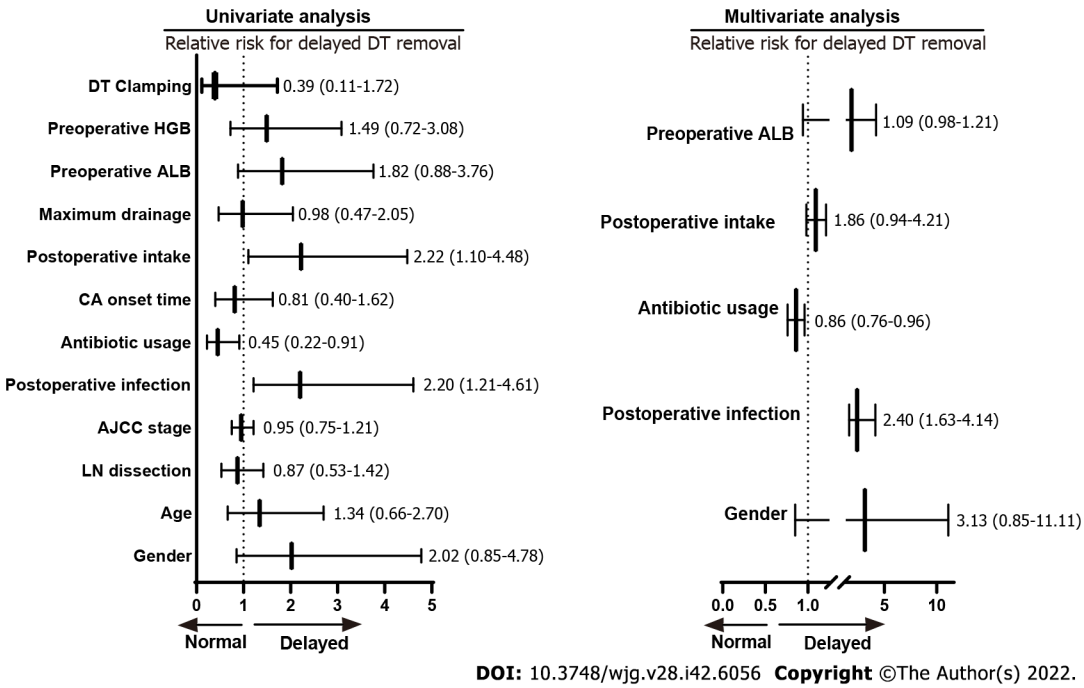
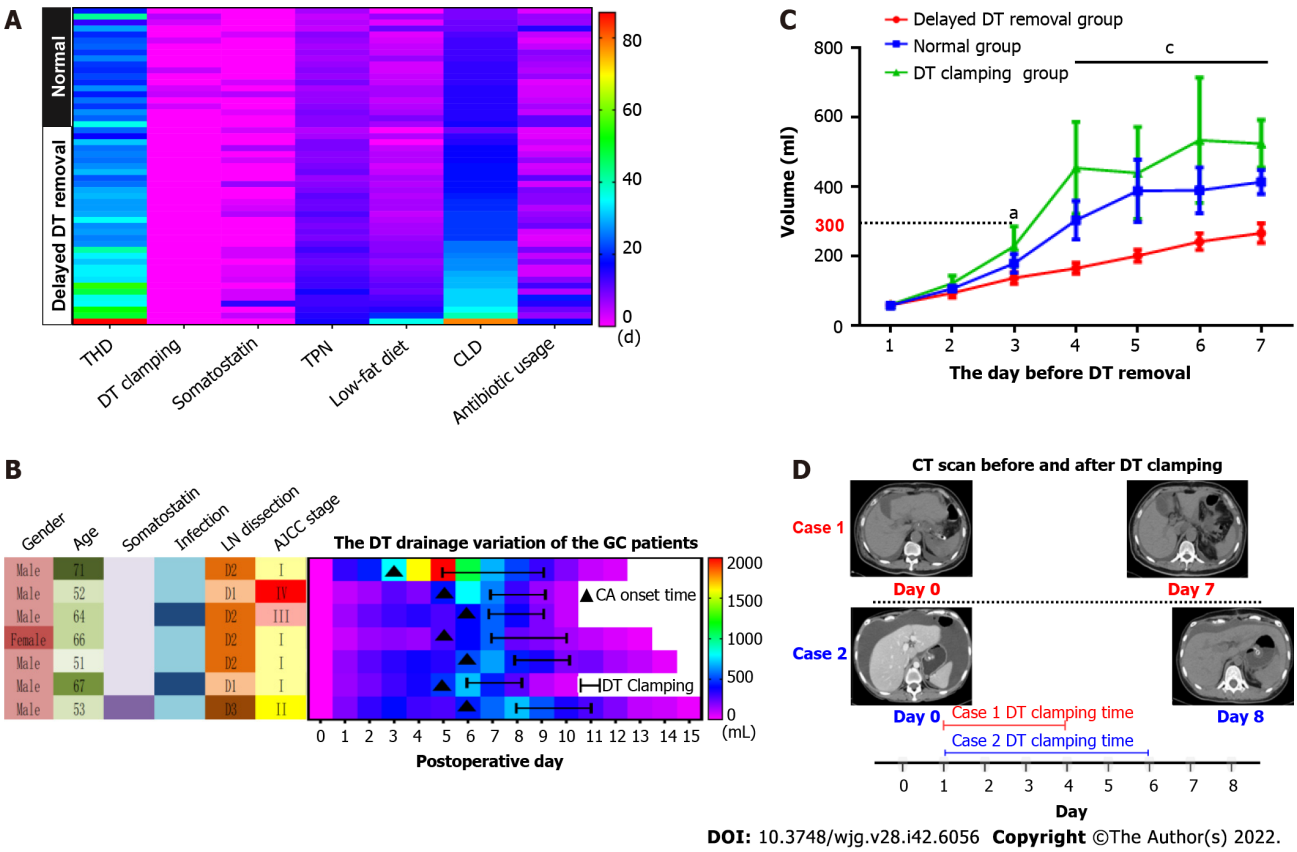


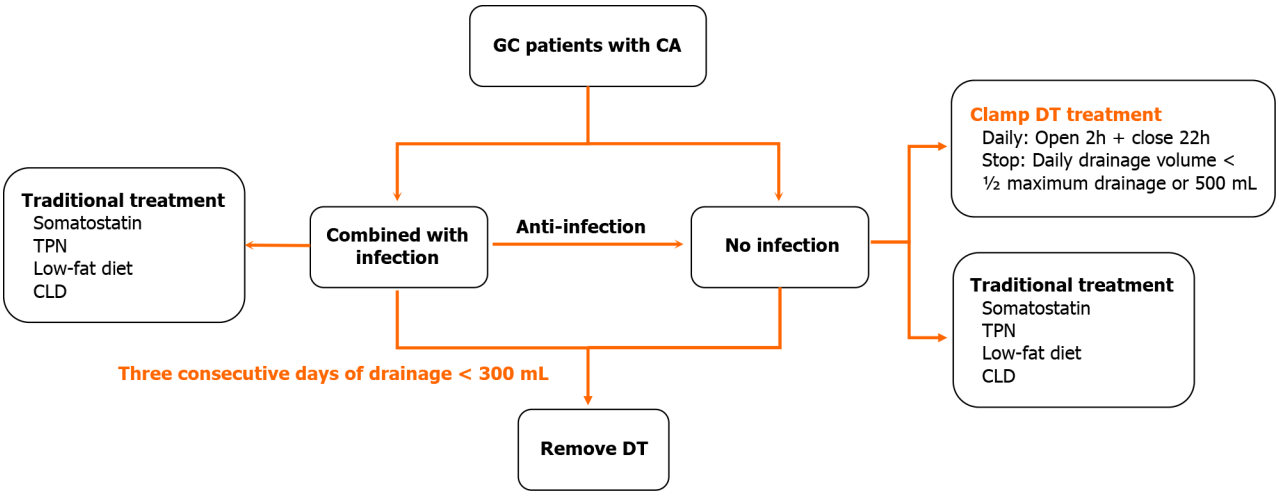
Figure 4 Exploration of multiple factors influence drainage tube removal in gastric cancer patients with postoperative chylous ascites. GC: Gastric cancer; DT: Drainage tube; LN: Lymph node; CA: Chylous ascites.

influenced delayed DT removal in GC patients with postoperative CA. Our study also implied that DT clamping was an appropriate method of postoperative CA treatment for patients without postoperative infection. More importantly, appropriate and lenient indications for DT removal can significantly reduce the duration of hospitalization.



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Figure 5 Drainage tube clamping is a favorable method for the gastric cancer patients with postoperative chylous ascites. A: Overview of overall treatment in the gastric cancer (GC) patients with postoperative chylous ascites; B: The drainage tube (DT) drainage variation of the GC patients underwent the treatment of DT clamping; C: The drainage of GC patients with postoperative chylous ascites in different groups before DT removal; D: Computed tomography scan indicate that the fluid in abdominal cavity was clearly reduced after the DT was clamped. THD: Total hospitalization duration; TPN: Total parenteral nutrition; CLD: Continuous low-pressure drainage; GC: Gastric cancer; DT: Drainage tube; CA: Chylous ascites; LN: Lymph node. $^{a}P < 0.05$. $^{c}P < 0.001$.



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Figure 6 Novel conservative therapeutic strategies for gastric cancer patients with postoperative chylous ascites. TPN: Total parenteral nutrition; CLD: Continuous low-pressure drainage; GC: Gastric cancer; DT: Drainage tube; CA: Chylous ascites.

In most of the GC patients, postoperative CA cannot be discharged at a routine time and have a significant impact on subsequent adjuvant treatment[6,7]. Normally, patients are discharged within 7 d after undergoing GC surgery and most start adjuvant treatment within 30 d at our center. However, in the 53 patients with postoperative CA in our study, the average postoperative hospitalization duration was 21.9 d, and 8 patients' postoperative hospital stays were longer than 30 d. As previously reported, lymphadenectomy was a key influencing factor in GC patients with postoperative CA[5,9]. As shown in

Supplementary Table 3, the clinical characteristics of patients with CA tended to be consistent among those who underwent variety of lymphadenectomy, similar with previous studies that CA was found to be a rare complication even for gastric carcinoma patients undergoing D3 dissection[5,17].

Our results clearly indicated that hospitalization duration is mainly dependent on the time of DT removal in GC patients with CA. A multi-center prospective study recommended the criterion for DT removal be drainage flow between 500 and 1000 mL/d[6]. In fact, we previous performed relatively rigorous standards for DT removal in the patients with CA. Usually, while the volume of drainage less than 100 mL/d, the DT removal will be truly considered. Although all the patients' DTs were removed until the flow volume less than 300 mL/d, and the delayed removal group preferred to perform a significantly high criterion. Therefore, we have a sufficient reason to conclude that, after excluding the influence of postoperative infection, early DT removal is a better choice in GC patients with CA. Moreover, our study found that postoperative infection and antibiotic usage were vital independent factors that influenced delayed DT removal in patients with CA, and clearly clarified anti-infection were an effective supplemental therapy for conservative treatment of postoperative CA. Similarly, Lu *et al*[7] reported the patients with CA had a certain higher level of postoperative white blood cell counts than the other patients in GC.

Previously, the primary treatment for CA was conservative therapy, which mainly involved TPN, continuous low-pressure drainage, somatostatin, and a low-fat diet[18]. Recently, DT clamping has been presented as a potential alternative for patients with CA in other malignancies[19]. In this study, the DTs of 7 patients were clamped until the daily drainage was less than 500 mL/d (or ½ the maximum drainage). After DT clamping, the flow amount was significantly reduced, and the patients were successfully discharged without DT. For the reason of clamping DT facilitates DT removal, previous research has demonstrated absorption and lymphatic drainage increase along with the interstitial hydrostatic pressure[20]. Furthermore, DT clamping could help to evaluate the feasibility of DT removal by conveniently simulating the removal and conversion back to drainage[21]. Several studies have suggested DT clamping as an important alternative, and the detailed suggestion was daily drainage ranging from 1000 to 1500 mL[6,22]. However, a consensus on the threshold of drainage volume for DT clamping has not yet been reached. Therefore, determination of an appropriate criterion for DT removal and DT clamping is urgently needed for GC patients with postoperative CA.

There are certain limitations to our study. First, due to the retrospective study design, it was difficult to individually balance a variety of influencing factors; thus, various biases were unavoidable. Second, despite routine chyle test and TG were measured, the definition of CA is slightly less rigorous. In particular, CA with co-infection cannot fully rule-out the influence of pancreatic and anastomotic leakage, and other infection-related complications. In addition, small-volume CA (*i.e.*, daily drainage volume ranging from 30 to 200 mL) was not considered in this study.

CONCLUSION

In conclusion, postoperative infection and antibiotic usage were vital independent factors that influenced delayed DT removal in GC patients with CA. Appropriate and lenient standards for DT removal can significantly reduce the duration of hospitalization. Furthermore, DT clamping might be a recommend alternative for conservative treatment of postoperative CA.

ARTICLE HIGHLIGHTS

Research background

Chylous ascites (CA) is relatively common postoperative complication in patients undergoing received gastric cancer (GC) surgery that obviously prolongs hospitalization and has a major impacts on subsequent adjuvant treatments.

Research motivation

Drainage tube (DT) clamping has been presented as a potential alternative conservative treatment for GC patients with CA.

Research objectives

This study aimed to explore key factors influencing CA treatment and recommend novel conservative treatment strategies for postoperative CA in GC patients.

Research methods

Data from patients with CA after GC surgery performed at the Fudan University Shanghai Cancer Center between 2006 and 2021 were retrospectively evaluated. Patients were classified into two distinct

groups with respect to DT removal time. We further explored the differences in clinical-pathological features of the different DT removal groups.

Research results

Fifty-three patients underwent surgery for GC and experienced postoperative CA during the study period. Postoperative hospitalization and DT removal time showed a significant positive association (R^2 = 0.979, P < 0.001), while delayed DT removal significantly extended total and postoperative hospitalization times, antibiotic usage duration, and hospitalization cost. In addition, postoperative infection and antibiotic usage were independent factors for delayed DT removal.

Research conclusions

Postoperative infection and antibiotic usage were vital independent factors that influenced delayed DT removal in GC patients with CA. Appropriate and lenient standards for DT removal may significantly reduce the duration of hospitalization.

Research perspectives

DT clamping could be recommended as an alternative for conservative treatment of postoperative CA.

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FOOTNOTES

Author contributions: Kong PF and Xu YH contributed equally to this work; Xu DZ designed the research study; Kong PF, Xu YH, Lai ZH and Sun B performed the research; Kong PF, Xu YH, Ma MZ and Duan YT analyzed the data and wrote the manuscript; all authors have read and approve the final manuscript.

Institutional review board statement: The study was reviewed and approved by Ethics Committee of Fudan University Shanghai Cancer Center Review Board [Approval No. FUSCC-D-2021-164].

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Dataset available from the corresponding author at xudzh@shca.org.cn. Participants gave informed consent for data sharing.

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ORIGINAL ARTICLE

Clinical Trials Study

Computed tomography perfusion in liver and spleen for hepatitis B virus-related portal hypertension: A correlation study with hepatic venous pressure gradient

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Abstract

BACKGROUND

Hepatic venous pressure gradient (HVPG) is the gold standard for diagnosis of portal hypertension (PH). However, its use can be limited because it is an invasive procedure. Therefore, it is necessary to explore a non-invasive method to assess PH.

AIM

To investigate the correlation of computed tomography (CT) perfusion of the liver with HVPG and Child-Pugh score in hepatitis B virus (HBV)-related PH.

METHODS

Twenty-eight patients (4 female, 24 male) with gastroesophageal variceal bleeding induced by HBV-related PH were recruited in our study. All patients received CT perfusion of the liver before transjugular intrahepatic portosystemic stent-shunt (TIPS) therapy. Quantitative parameters of CT perfusion of the liver, including liver blood flow (LBF), liver blood volume (LBV), hepatic artery fraction, splenic blood flow and splenic blood volume were measured. HVPG was recorded during TIPS therapy. Correlation of liver perfusion with Child-Pugh score and HVPG were analyzed, and the receiver operating characteristic curve was analyzed. Based on HVPG (> 12 mmHg *vs* ≤ 12 mmHg), patients were divided into moderate and severe groups, and all parameters were compared.

RESULTS

Based on HVPG, 18 patients were classified into the moderate group and 10 patients were classified into the severe group. The Child-Pugh score, HVPG, LBF and LBV were significantly higher in the moderate group compared to the severe group (all $P < 0.05$). LBF and LBV were negatively associated with HVPG ($r = -0.473$, $P < 0.05$ and $r = -0.503$, $P < 0.01$, respectively), whereas splenic blood flow was positively associated with hepatic artery fraction ($r = 0.434$, $P < 0.05$). LBV was negatively correlated with Child-Pugh score. Child-Pugh score was not related to HVPG. Using a cutoff value of 17.85 mL/min/100 g for LBV, the sensitivity and specificity of HVPG ≥ 12 mmHg for diagnosis were 80% and 89%, respectively.

CONCLUSION

LBV and LBF were negatively correlated with HVPG and Child-Pugh scores. CT perfusion imaging is a potential non-invasive quantitative predictor for PH in HBV-related liver cirrhosis.

Key Words: Hepatic venous pressure gradient; Portal hypertension; Computed tomography perfusion; Hepatitis B; Liver cirrhosis

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Core Tip: Hepatic venous pressure gradient (HVPG) is the gold standard for the diagnosis of portal hypertension (PH), but its use is limited because it is an invasive procedure. Non-invasive assessment of HVPG requires further research. Computed tomography perfusion of the liver may be a useful tool for the evaluation of HVPG. Our results showed that a cutoff of 17.85 mL/min/100 g for liver blood volume yielded an 80% sensitivity and 89% specificity for severe PH. Therefore, computed tomography perfusion of the liver has the potential as a non-invasive quantitative predictor for PH in hepatitis B virus-related liver cirrhosis.

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INTRODUCTION

Gastroesophageal variceal bleeding is a common complication of portal hypertension (PH) in decompensated liver cirrhosis. There is a 60% recurrence rate and 20% mortality rate in the 1st year, and it is the leading cause of liver transplantation and mortality[1-4]. The diagnostic criteria for PH include hepatic venous pressure gradient (HVPG) ≥ 5 mmHg. Notably, when HVPG is higher than 12 mmHg, patients have a significantly increased risk of gastroesophageal bleeding. It was reported that HVPG was positively associated with individual risk of gastroesophageal variceal bleeding, and the incidence of variceal bleeding increased proportionally with an increase in HVPG[1,5-8]. In addition, HVPG can be applied clinically for risk stratification, therapeutic adoption, drug efficacy and adverse events for PH [4,9-12]. However, HVPG is an invasive procedure, which has limited its wide application for the evaluation of therapeutic effects or long-term follow-up. Therefore, studies continue to focus on non-invasive evaluation of HVPG, including anatomy (*e.g.*, liver volume, maximal diameter of spleen), lab results (*e.g.*, platelet level, coagulation function), liver function (*e.g.*, Child-Pugh score, model for end-stage liver disease [commonly known as MELD] score), liver and spleen stiffness (*e.g.*, FibroScan, FibroTouch, magnetic resonance elastography), and even computation simulation modeling. However, none of these methods has demonstrated satisfactory accuracy and reproducibility.

Computed tomography (CT) perfusion of the liver is traditionally utilized to evaluate liver cancer, metastatic tumors, and liver cirrhosis. Decreased blood flow perfusion from the portal vein system and increased blood flow perfusion from the hepatic artery system can be identified with CT perfusion of the liver[13-16]. Furthermore, liver blood perfusion after transjugular intrahepatic portosystemic stent-shunt (TIPS) can be quantitatively assessed with CT perfusion[17]. However, very few reports have focused on the correlation between HVPG and CT perfusion in gastroesophageal bleeding. Talakić *et al* [13] reported that HVPG had no correlation with CT perfusion in end-stage cirrhosis. Therefore, we aimed to explore the relationship between quantitative indices of CT perfusion with HVPG and the Child-Pugh score and to investigate the feasibility of CT perfusion as a non-invasive imaging tool for

HVPG in gastroesophageal variceal bleeding induced by hepatitis B virus (HBV)-related PH.

MATERIALS AND METHODS

Patients

This prospective study was approved by the Institutional Ethics Committee at our hospital, and all written informed consents were obtained from each participant. Patients with recurrent gastroesophageal variceal bleeding resulting from HBV-related PH were randomly recruited from January 1, 2019 to June 30, 2019. All patients previously underwent drug and/or endoscopic therapy and were prepared for the TIPS procedure. The inclusion criteria were as follows: (1) Gastroesophageal bleeding as a consequence of HBV-related PH; (2) CT perfusion and Child-Pugh score available 1 wk before TIPS surgery; and (3) HVPG measured during TIPS and HVPG ≥ 5 mmHg. The exclusion criteria were as follows: (1) Gastroesophageal bleeding caused by any other etiology; (2) liver tumors, including primary and metastatic; (3) any other conditions leading to hemodynamic changes in the liver, such as partial hepatectomy, splenectomy, hepatic tumor surgery, TIPS, *etc*; (4) any factors affecting liver blood perfusion, such as portal vein thrombosis, cavernous transformation, Budd-Chiari syndrome, *etc*; (5) dysfunction in vital organs, such as cardiac, renal or respiratory damage/failure; and (6) any factors that reduced the quality of CT images, such as motion and metal artifacts.

CT perfusion and post-processing

CT perfusion was performed by a Revolution CT scanner (GE Healthcare, Chicago, IL, United States) with 16 cm Z-axis coverage axial scanning mode to cover most parts of the liver. Scanning parameters were set as tube voltage 100 kVp, automatic tube current from 50 mA to 200 mA with noise index as 14, slice thickness of 5 mm, rotation speed of 1.0 sec, helical pitch of 0.992:1.000 and 80% adaptive statistical iterative reconstruction (commonly known as ASIR). Initially, 50 mL nonionic contrast media (Omnipaque 350; GE Healthcare) followed by a 50-mL saline chaser were injected through the antecubital vein at a rate of 5 mL/sec, using a dual-head pump injector (Stellant; Medtron, Saarbrucken, Germany). The scanning was fixed with a 9-sec time delay after injection. Then, CT perfusion was performed. The CT perfusion was compromised of 26 pass acquisitions and 25 interscan gap without table movement, including 10 early acquisitions with an interscan gap of 1 sec, 12 acquisitions with an interscan gap of 2 sec, and 4 acquisitions with an interscan gap of 4 sec. Thus, total scanning time was 80 sec. All patients were instructed to avoid deep and irregular breathing during the procedure. A band compressing the upper abdomen was used to reduce respiratory motion artifacts.

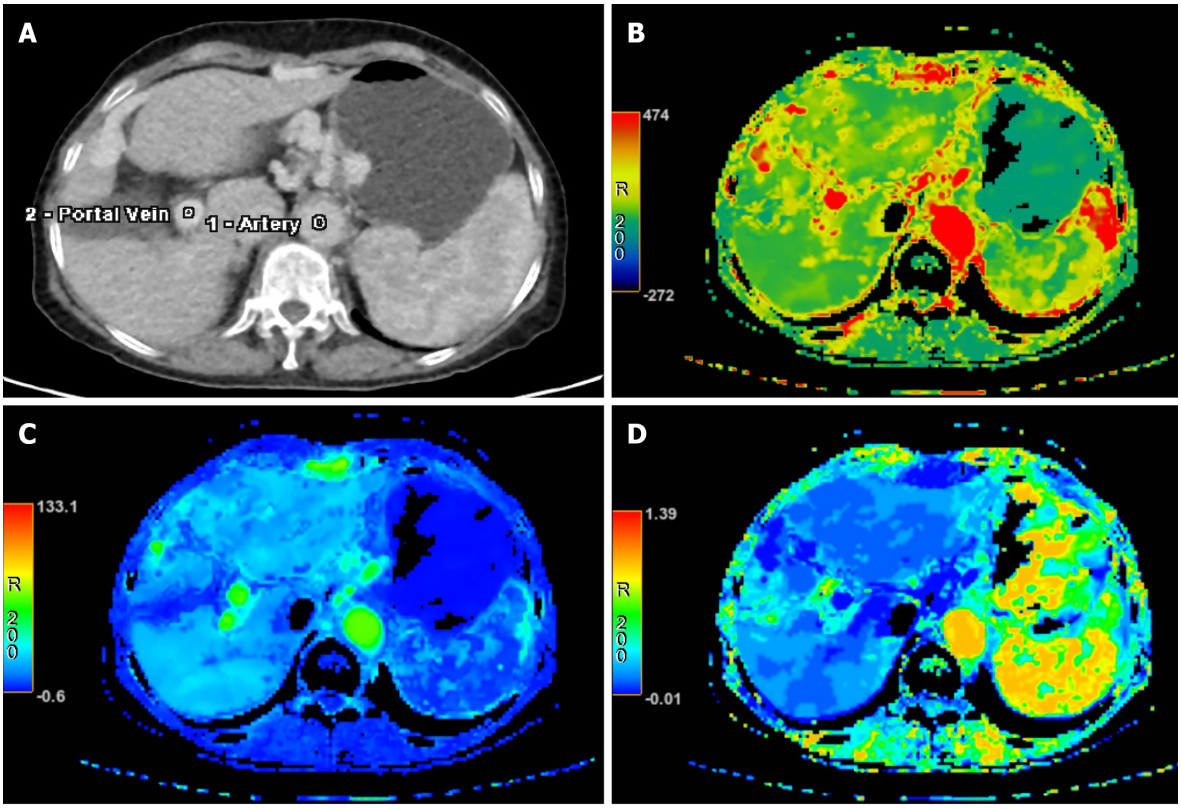
Raw data generated by CT perfusion were reconstructed with a thickness of 2.5 mm. Post-processing was performed separately by two radiologists with 11 years and 7 years respectively of experience in the CT perfusion procedure. First, iterative registration reconstruction was performed to correct respiratory motion between each dynamic acquisition. Second, corrected data were post-processed with a commercial software (CT Perfusion 4D AW 4.7; GE Healthcare). Third, regions of interest were placed in the abdominal aorta and portal vein separately for liver perfusion (Figure 1). The region of interest was placed in the abdominal aorta only for splenic perfusion (Figure 2). Then, the perfusion map would be generated automatically for the liver and spleen (Figures 1 and 2). Finally, three volumes-of-interest would be selected in the left and right liver parenchyma without any hepatic vessels. By contrast, three volumes-of-interest were also selected in the superior, medial and inferior splenic parenchyma. Then, average values of perfusion parameters, including liver blood volume (LBV) (mL/100 mL), liver blood flow (LBF) (mL/100 mL/min), hepatic arterial fraction (HAF) (%), splenic blood volume (SBV) (mL/100 mL/min) and splenic blood flow (SBF) (mL/100 mL/min) were calculated and recorded.

HVPG measurement

HVPG was measured according to established standards[18,19] during the TIPS procedure. After fasting for more than 8 h, all patients underwent local anesthesia. The right internal jugular vein was cannulated using the Seldinger technique, and a 5-French balloon catheter (Edwards Lifesciences LLC, Irvine, CA, United States) was placed into the right hepatic vein, and the wedged and free hepatic venous pressure was measured three times in each patient. Then, HVPG was calculated as the difference between average wedged and free hepatic venous pressure.

Statistical analysis

Statistical analysis was performed with SPSS 24.0 software (IBM Corp., Armonk, NY, United States). All data were described as mean ± SD or range [95% confidence interval (CI)]. Kolmogorov-Smirnov was performed for the normal distribution test. Pearson or Spearman was used to evaluate the relationship among quantitative indices. Kappa was applied to analyze the agreement between observers. Patients were classified into two groups according to the HVPG value [> 12 mmHg (moderate) *vs* ≤ 12 mmHg (severe)]. Quantitative indices, including LBV, HAF, LBF, and SBV, were compared between the two groups. Receiver operating characteristic (ROC) was performed to calculate a cutoff value for differen-



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Figure 1 Computed tomography perfusion of the liver post-processing data. A: Regions of interest were placed in the abdominal aorta and main portal vein as the input blood vessels for calculation of liver computed tomography perfusion; B-D: The parameters of liver computed tomography perfusion were calculated automatically to include hepatic artery fraction (B), liver blood flow (C), and liver blood volume (D).

tiation between moderate and severe PH. A P value of less than 0.05 was considered significant.

RESULTS

General data analysis

Initially, 35 patients had portal vein thrombosis. Then, 13 patients with splenectomy, 3 patients with liver tumors and 2 patients with motion artifacts (leading to unavailable CT perfusion) were excluded. Finally, 28 patients (4 female and 24 male) were included in our study, with an age range of 28-years-old to 68-years-old and an average age of 53.7 years ± 10.4 years. Patient characteristics are summarized in Table 1, including demographics, medical history, Child-Pugh class, and HVPG.

Comparisons of Child-Pugh scores in different types of PH

Ten patients had moderate PH (HVPG < 12 mmHg), and the remaining eighteen patients had severe PH (HVPG ≥ 12 mmHg). The median HVPG was 10 mmHg (interquartile range: 9.0 mmHg; range: 8.0-11.0 mmHg) in the moderate PH group and 21 mmHg (interquartile range: 17.5 mmHg; range: 14.0-31.0 mmHg) in the severe PH group. In the moderate PH group, 6 patients were Child-Pugh A and 4 patients were Child-Pugh B. In the severe PH group, 5 patients were Child-Pugh A, 12 patients were Child-Pugh B, and 1 patient was Child-Pugh C. For the moderate PH group, HVPG and Child-Pugh scores were lower than those in the severe PH group (9.6 mmHg ± 1.3 mmHg *vs* 18.9 mmHg ± 4.4 mmHg, P < 0.001) (Table 2).

Correlation of CT perfusion parameters with HVPG

The two radiologists demonstrated good agreement (Kappa = 0.821, P < 0.01) in the evaluation of the CT perfusion parameters. Quantitative parameters of CT perfusion of the liver are summarized in Table 2. Both LBF and LBV in moderate PH were higher than in severe PH (114.6 ± 36.0 *vs* 87.9 ± 24.8 and 19.7 ± 3.0 *vs* 15.5 ± 2.2, respectively, all P < 0.05). No significant difference was observed between the two groups for the other indices (Table 2).

LBF was negatively associated with HVPG (r = -0.398, P < 0.05). LBV was negatively related to HVPG (r = -0.504, P < 0.01) and Child-Pugh (r = -0.563, P < 0.01). SBF was positively related to HAF (r = 0.498, P

Table 1 Characteristics of the patients

Characteristic	Value
Sex as female/male, *n*	4/24
Age, yr, mean ± SD	53.7 ± 10.4
Height, cm, mean ± SD	169.4 ± 5.8
Weight, kg, mean ± SD	62.9 ± 11.6
Previous episodes of variceal bleeding, mean ± SD	3 ± 2
Treatment history, *n* (%)	
β blockade only	3 (10.7)
Sclera therapy only	4 (14.3)
β blockade and sclerotherapy	21 (75.0)
Child-Pugh stage, *n* (%)	
A	11 (39.3)
B	16 (57.1)
C	1 (3.6)
Ascites, *n* (%)	
None	17 (60.7)
Mild	2 (7.1)
Severe	9 (32.1)
HVPG, mmHg, *n* (%)	
< 12	10 (35.7)
≥ 12	18 (64.3)

HVPG: Hepatic venous pressure gradient; SD: Standard deviation.

< 0.01). No association was observed among HAF, SBV, SBF, Child-Pugh score and HVPG. The ROC of LBV for differentiation between moderate and severe PH resulted in an area under the curve of 0.864 with a standard error of 0.075 (95%CI: 0.72-1.00) (Figure 3). Using a cutoff value of 17.85 mL/min/100 mL for LBV, the sensitivity and specificity for detection of severe PH was 80% and 89%, respectively. ROC of LBF resulted in an area under the curve of 0.797 with a standard error of 0.100 (95%CI: 0.60-1.00) (Figure 3). Using a cutoff value of 111.3 mL/min/100 mL for LBF, the sensitivity and specificity for detection of severe PH was 60% and 94%, respectively.

DISCUSSION

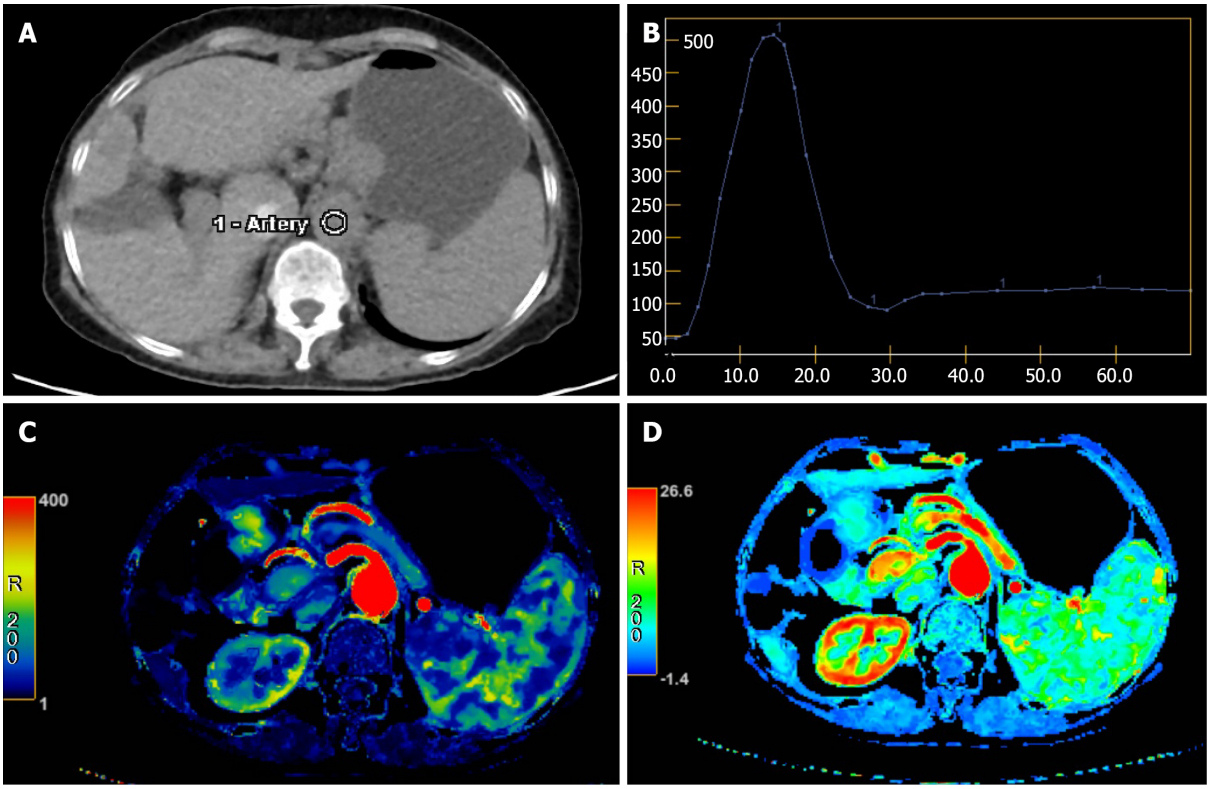
HVPG is the gold standard for diagnosis of liver cirrhosis-induced PH and is an independent risk factor for evaluating the prognosis of decompensated liver cirrhosis[5,19,20]. However, as an invasive measurement requiring a complex operation, wide clinical application of HVPG has been limited. It was reported that quantitative parameters (*e.g.*, LBF, LBV) from CT perfusion of the liver can be used to evaluate the blood supply changes in the liver and spleen with good sensitivity and specificity[13,21,22]. Therefore, our study investigated the correlation of CT perfusion for quantitative assessment of PH in HBV-related PH. Our results suggested that LBV and LBF were negatively correlated with HVPG and Child-Pugh scores, and CT perfusion imaging is a potential non-invasive quantitative predictor for PH in HBV-related liver cirrhosis.

In our study, LBV and LBF were negatively correlated with HVPG. This was explained by a significant decrease in hepatic flow[20-22] after hepatitis B infection when patients were suffering from cirrhosis-induced PH. A decrease in hepatic flow results from hepatocyte damage caused by HBV, deconstruction in normal liver structure, deposition of collagen fibers in the perisinusoidal space and formation of pseudo-lobules and fibroses, which together remarkably increases the resistance of the portal vein blood flow into the liver[1,4]. In this study, LBV and LBF were negatively related to HVPG. It is possible that the decrease of LBV and LBF is the consequence of the increase of HVPG, suggesting significantly reduced blood perfusion in the liver as PH increases. Therefore, CT perfusion is potentially

Table 2 Comparison of the moderate and severe portal hypertension groups

Index	Moderate PH	Severe PH	*P* value
Sex as female/male	2/8	2/16	0.520
Age, yr	54.2 ± 10.9	53.4 ± 10.5	0.848
Height, cm	168.0 ± 6.0	170.1 ± 5.6	0.362
Weight, kg	64.8 ± 12.3	61.8 ± 11.4	0.528
Child-Pugh score	7.1 ± 1.9	7.8 ± 1.8	0.023
HVPG	9.6 ± 1.3	18.9 ± 4.4	0.000
Perfusion CT			
LBF	114.6 ± 36.0	87.9 ± 24.8	0.029
LBV	19.7 ± 3.0	15.5 ± 2.2	0.000
HAF as $\times 10^{-2}$	8.2 ± 2.3	8.7 ± 4.7	0.731
SBF	96.0 ± 30.0	108.7 ± 31.4	0.308
SBV	13.9 ± 2.9	11.9 ± 2.5	0.084

Data are presented as *n* or mean ± SD. CT: Computed tomography; HAF: Hepatic arterial fraction; HVPG: Hepatic venous pressure gradient; LBF: Liver blood flow; LBV: Liver blood volume; PH: Portal hypertension; SBF: Splenic blood flow; SBV: Splenic blood volume.

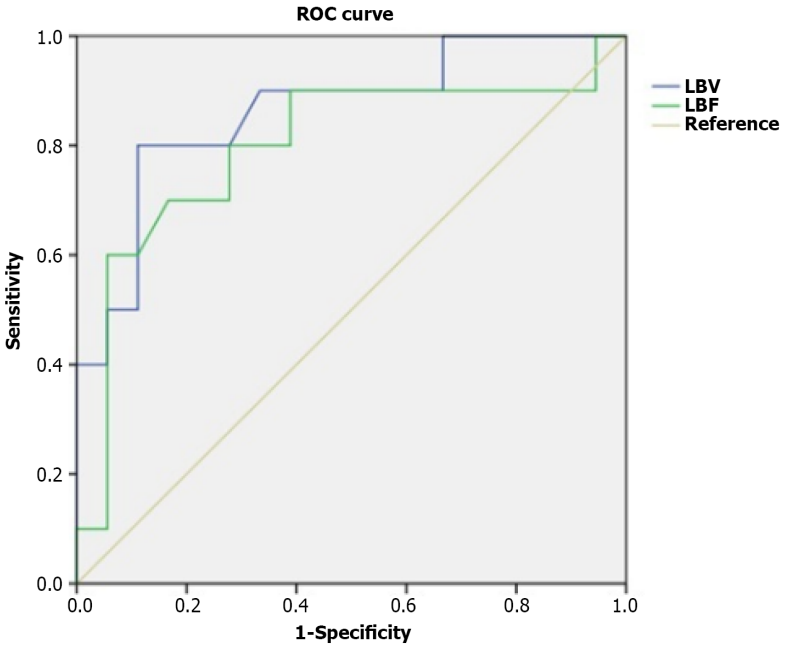


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Figure 2 Computed tomography perfusion of the spleen post-processing data. A: Regions of interest were placed in the abdominal aorta as the input blood vessel; B: The time-density curve was generated automatically for calculation of splenic perfusion; C and D: The parameters of computed tomography perfusion of the spleen were calculated automatically, including splenic blood flow (C) and splenic blood volume (D).

feasible for the non-invasive evaluation of HVPG using LBV and LBF in patients with HBV-related PH.

In this study, liver blood perfusion parameters (*e.g.*, LBV and LBF) in the moderate PH group were significantly higher than those in the severe PH group. For distinguishing moderate PH from severe PH, LBV had a ROC curve with a sensitivity and specificity of 80% and 89%, respectively. LBF had a sensitivity and specificity of 60% and 94%, respectively. Therefore, CT perfusion parameters (LBV and



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Figure 3 Receiver operating characteristic curves to differentiate moderate and severe portal hypertension. For discriminating severe portal hypertension, liver blood volume had an area under the curve of 0.864 with a standard error of 0.075 [95% confidence interval (CI): 0.72-1.00], while liver blood flow had an area under the curve of 0.797 with a standard error of 0.100 (95%CI: 0.60-1.00). LBF: Liver blood flow; LBV: Liver blood volume; ROC: Receiver operating characteristic.

LBF) can be used to distinguish moderate PH and severe PH in PH-induced gastroesophageal variceal bleeding in patients with HBV-related PH.

LBV was negatively correlated with Child-Pugh score, suggesting that liver reserve function decreases with reduced LBV. Moreover, the Child-Pugh score in the moderate PH group was significantly lower than that in the severe PH group. Similarly, liver reserve function was better in the moderate PH group than the severe PH group. This was related to pathophysiological mechanisms underlying hepatitis B cirrhosis and PH. In addition, HVPG in the severe PH group was significantly higher than the moderate PH group. The intrahepatic portal venous system pressure in severe PH may increase, leading to progressively decreased blood flow and gradually weakening the reserve capacity of liver function. However, in this study, the Child-Pugh score was not associated with HVPG, which was consistent with previous studies[3,7,10,23]. The Child-Pugh score is mainly used to evaluate liver reserve function, which can only provide a crude evaluation of PH.

HAF was not related to HVPG, suggesting no correlation between the hepatic artery perfusion ratio and PH in liver cirrhosis. HAF mainly indicates the proportion of hepatic artery blood supply to the total liver blood supply in cirrhosis. When cirrhosis occurs due to damage in the liver sinusoid and liver lobule structure, the blood in the portal vein meets increasing resistance against its return to the liver. When portal vein pressure increases, the blood supply flowing to the liver is reduced. Likewise, compensatory hepatic artery blood perfusion can increase. However, the portal vein blood supply accounts for about three-quarters of the total liver blood supply[24]. The compensatory increase in hepatic artery blood supply could not compensate for a substantial decrease in blood flow in the liver caused by reduced portal vein blood supply. This buffering effect is not enough to maintain the hepatic blood supply[22-24]. In addition, HAF is affected by various factors, such as blood pressure, blood volume and cardiac function. This might explain why HAF was not correlated with HVPG.

The perfusion parameters of the spleen (*e.g.*, SBF, SBV) were not related to HVPG and Child-Pugh classification. This was consistent with a previous study. However, in that cohort, blood flow and blood volume of the liver were not associated with HVPG[13]. This may be related to different samples included in our study, where patients suffering from liver cirrhosis caused by hepatitis B were classified as relatively moderate cases. Among them, according to the Child-Pugh classification, 11 cases were defined as grade A, 16 cases as grade B, and 1 case as grade C. By contrast, patients included in the previous study were primarily suffering from alcoholic cirrhosis with Child-Pugh grade B and C. Furthermore, in the previous study, all patients were suffering from more severe diseases and were planning for liver transplantation as treatment. Moreover, our study excluded factors that may affect portal vein hemodynamics (such as splenic resection, portal vein thrombosis), which may explain the differences between the two studies.

Limitations existed in our study. First, our study only included cases of HBV-related PH, with a remarkable disproportion in patient sex. The majority of patients were Child-Pugh A and Child-Pugh B. A larger sample size is required to identify the clinical application of CT perfusion in patients with different causes of cirrhosis and higher Child-Pugh scores, including alcoholic cirrhosis, drug-induced metabolic liver disease and autoimmune liver disease. Second, our study primarily targeted patients who were suffering from gastric fundus esophageal variceal bleeding as a consequence of PH and excluded other factors like thrombosis, cavernous transformation and splenectomy that could affect liver hemodynamics. Nonetheless, further research is required to determine its application in PH with multiple complications. Finally, our study did not focus on pathology, laboratory and comparative imaging evaluation (such as volume and elasticity of the liver and spleen). Thus, further research is required.

CONCLUSION

Quantitative parameters of CT perfusion imaging, in particular LBV and LBF, were negatively correlated with HVPG and Child-Pugh scores. Therefore, CT perfusion imaging is a potential application for non-invasive quantitative evaluation of HVPG in patients with HBV-related PH.

ARTICLE HIGHLIGHTS

Research background

Hepatic venous pressure gradient (HVPG) is the gold standard for diagnosis of portal hypertension (PH), but the measurement of HVPG is an invasive procedure, which has limited its widespread use. Therefore, we aimed to investigate the feasibility of computed tomography (CT) perfusion as a non-invasive imaging tool for HVPG in PH.

Research motivation

To date, no satisfactory non-invasive method has been proposed as an alternative for HVPG. Determining the feasibility of CT perfusion indices as a non-invasive tool to assess HVPG would be beneficial to patients.

Research objectives

To investigate the correlation of CT perfusion of the liver with HVPG and Child-Pugh score in hepatitis B virus (HBV)-related PH.

Research methods

We prospectively selected 28 HBV-related PH patients in our hospital from January 2019 to June 2019. CT perfusion was performed in all patients, and quantitative parameters of CT perfusion were applied to evaluate HVPG non-invasively. Quantitative indices, including liver blood volume (LBV), liver blood flow (LBF), hepatic artery fraction, splenic blood volume and splenic blood flow, were calculated. The correlation analysis was calculated, and receiver operating characteristic curve analysis was performed.

Research results

Quantitative parameters of CT perfusion imaging, in particular LBV and LBF, were negatively correlated with HVPG and Child-Pugh scores.

Research conclusions

Our findings showed that CT perfusion parameters, LBV and LBF, were negatively correlated with HVPG and Child-Pugh scores. CT perfusion imaging showed potential as a non-invasive quantitative method for the evaluation of HVPG in HBV-related PH.

Research perspectives

Non-invasive assessment of HVPG has been an area of interest for decades, and multi-modality research should be explored in the future, including CT perfusion, anatomical information, lab results, liver and spleen stiffness and computation simulation modeling.

FOOTNOTES

Author contributions: Dong J, Liu FQ, and Wang L designed the report; Zhang Y, Wu YF, Yue ZD, Fan ZH, and

Zhang CY collected the clinical data; Wang L and Zhang Y analyzed and wrote the paper; Dong J and Liu FQ performed quality control; Liu FQ contributed to administrative and financial support; all authors have read and approved the final version of the manuscript.

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EDITORIAL

Kyoto classification of gastritis: Advances and future perspectives in endoscopic diagnosis of gastritis

Osamu Toyoshima, Toshihiro Nishizawa

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Abstract

This editorial provides an update of the recent evidence on the endoscopy-based Kyoto classification of gastritis, clarifying the shortcomings of the Kyoto classification, and providing prospects for future research, with particular focus on the histological subtypes of gastric cancer (GC) and *Helicobacter pylori* (*H. pylori*) infection status. The total Kyoto score is designed to express GC risk on a score ranging from 0 to 8, based on the following five endoscopic findings: Atrophy, intestinal metaplasia (IM), enlarged folds (EF), nodularity, and diffuse redness (DR). The total Kyoto score reflects *H. pylori* status as follows: 0, ≥ 2, and ≥ 4 indicate a normal stomach, *H. pylori*-infected gastritis, and gastritis at risk for GC, respectively. Regular arrangement of collecting venules (RAC) predicts non-infection; EF, nodularity, and DR predict current infection; map-like redness (MLR) predicts past infection; and atrophy and IM predict current or past infection. Atrophy, IM, and EF all increase the incidence of *H. pylori*-infected GC. MLR is a specific risk factor for *H. pylori*-eradicated GC, while RAC results in less GC. Diffuse-type GC can be induced by active inflammation, which presents as EF, nodularity, and atrophy on endoscopy, as well as neutrophil and mononuclear cell infiltration on histology. In contrast, intestinal-type GC develops *via* atrophy and IM, and is consistent between endoscopy and histology. However, this GC risk-scoring design needs to be improved.

Key Words: Kyoto classification; Gastritis; Endoscopy; Gastric cancer; Histology; *Helicobacter pylori*

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Core Tip: Endoscopy-based Kyoto classification of gastritis assesses gastric cancer (GC) risk and *Helicobacter pylori* (*H. pylori*) infection status. Total Kyoto scores of 0, ≥ 2, and ≥ 4 indicate a normal stomach, *H. pylori*-infected gastritis, and gastritis at risk for GC, respectively. Atrophy, intestinal metaplasia (IM), and enlarged folds (EF) increase *H. pylori*-infected GC incidence. Map-like redness is a specific risk factor for *H. pylori*-eradicated GC, while regular arrangement of collecting venules result in less GC risk. Diffuse-type GC is induced by active inflammation, depicting EF, nodularity, and atrophy. Intestinal-type GC develops through atrophy and IM; however, the GC risk-scoring design still needs to be improved.

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INTRODUCTION

The Kyoto classification of gastritis aims to match the endoscopic and histopathological findings of gastritis. It further aims to evaluate gastric cancer (GC) risk and *Helicobacter pylori* (*H. pylori*) infection of gastritis. The Kyoto classification was first advocated by the Japan Gastroenterological Endoscopy Society in 2013 and is widely used in recent clinical practice worldwide[1]. Technological advances in endoscopy have significantly improved the accuracy of identifying premalignant mucosal changes[2]. This editorial provides an update of the recent evidence on the Kyoto classification, clarifying the shortcomings of the Kyoto classification, and providing prospects for future research. This article is divided into the following four chapters: (1) *H. pylori* infection according to the Kyoto classification; (2) The histological consistency of the Kyoto classification; (3) Risk of GC according to the Kyoto classification; and (4) Future prospects in the Kyoto classification.

In the Kyoto classification, the total Kyoto score has been developed as a GC risk score. The total Kyoto score is calculated as the sum of the following 5 endoscopic findings: Atrophy, intestinal metaplasia (IM), enlarged folds (EF), nodularity, and diffuse redness (DR); and ranges from 0 to 8 (Table 1 and Figure 1)[1]. The Kyoto DR score includes the disappearance of the regular arrangement of collecting venules (RAC). Map-like redness (MLR) frequently appears after *H. pylori* eradication, and is generally pathologically consistent with IM[3]. This article describes the total Kyoto score and its five individual findings along with RAC and MLR.

GCs consist of two distinct histological subtypes: Lauren's diffuse and intestinal GC[4]. Diffuse-type GC develops directly from highly active inflammation, whereas intestinal-type GC develops through destruction and replacement of tissues, such as atrophy and IM, and is termed Correa's cascade[5-7]. GCs can also be described according to the different rates of incidence[8,9], lesion characteristics[10-12], and prognoses[13-16] as per the corresponding *H. pylori* infection status. In this editorial, we specifically describe the histological subtypes of GC and *H. pylori* infection status.

H. PYLORI INFECTION IN THE KYOTO CLASSIFICATION

H. pylori non-infection

Evidence of RAC as an indicator of non-infection has been reported in both in Japan[17,18] and several other countries[19-21], including in the west[22-24], as shown in Table 2. Two recent meta-analyses reported that the sensitivity and specificity of RAC for predicting non-infection were 78%-80% and 94%-97%, respectively[25,26]. The high reliability of RAC for non-infectious cases has also been verified.

H. pylori current and past infection

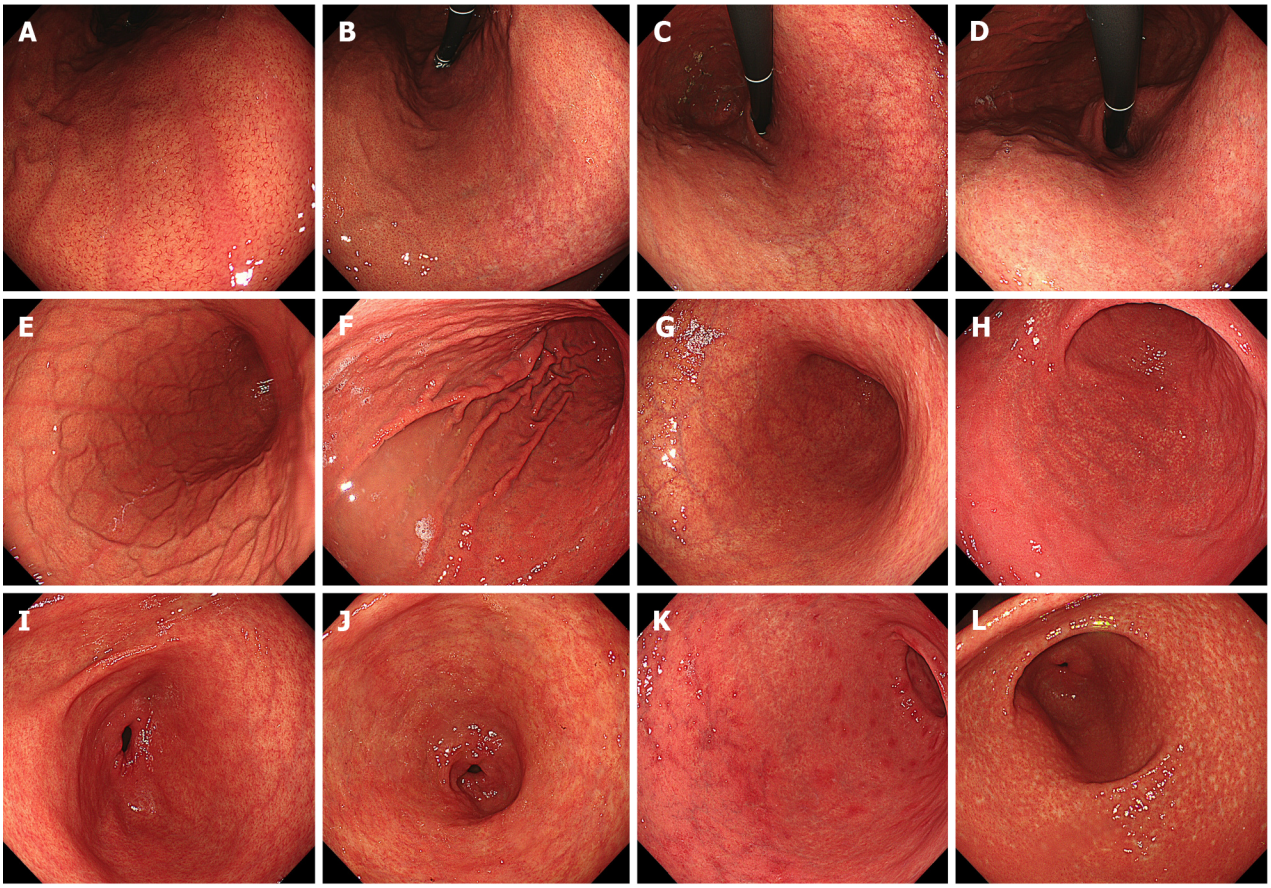
All five Kyoto scores, atrophy (61.1%-85.8% and 58.5%-85.3%)[19,23,27], IM (95.6% and 86.0%)[27], EF (96.6%-99.1% and 85.0%-85.3%)[17,27], nodularity (98.3%-100% and 76.5%-89.1%)[17,20,27,28], and DR (73.6%-97.6% and 65.0%-89.7%)[17,19,20,27], commonly offer high specificity and accuracy for categorizing current infections (Table 2).

Three studies have previously compared patients with non-infectious, current, and past infections, all of which reported that RAC was strongly correlated with non-infection [odds ratios (ORs) = 4.6-55.0]; MLR was a highly specific finding indicative of past infection (ORs = 7.8-12.9), and DR, EF, and nodularity provided high ORs of 10.5-26.4, 6.0-8.6, and 4.0-22.5, respectively, for current infection. Atrophy and IM were associated with both current (ORs = 1.9-21.6 and 4.3) and past infections (ORs = 1.9-22.8 and 4.4), respectively[17,19,25]. A previous study reported an algorithm with an accuracy of 80.0% for defining the presence of RAC as non-infection, DR and mucosal edema as current infection,

Table 1 Kyoto classification score

Endoscopic findings	Kyoto score		
	0	1	2
Atrophy[1]	None, C1	C2, C3	O1-O3
Intestinal metaplasia	None	Antrum	Corpus and antrum
Enlarged folds	Absence	Presence	-
Nodularity	Absence	Presence	-
Diffuse redness	None	Mild with RAC	Severe without RAC

[1]According to the Kimura-Takemoto classification[80].
RAC: Regular arrangement of collecting venules.



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Figure 1 Representative images of the Kyoto classification. A: Normal with regular arrangement of collecting venules; B: Atrophy score 1; C: Atrophy score 2; D: Intestinal metaplasia score 2; E: Normal; F: Enlarged folds score 1; G: Diffuse redness score 1; H: Diffuse redness score 2; I: Normal; J: Intestinal metaplasia score 1; K: Map-like redness; L: Nodularity score 1. A-D: Corpus lesser curvature; E-H: Corpus greater curvature; I-L: Antrum.

and MLR as post-eradication[24]. *H. pylori* eradication decreases the Kyoto EF, nodularity, and DR scores, but does not improve the Kyoto atrophy and IM scores[29]. These results indicate that the presence of RAC predicts non-infection; EF, nodularity, and DR predict current infection; MLR predicts past infection; and atrophy and IM predict current or past infection.

Total Kyoto score

Several studies have previously focused on the association between the total Kyoto score and *H. pylori* infection. The sensitivity and specificity of the total Kyoto score for current infection were good at 78.3%-98.7% and 92.0%-98.4%, respectively (Table 2)[27,30]. The area under the curve (AUC) of the total Kyoto score for predicting current infection was 0.85, with a cutoff value of 2[27]. Current infection rates increased stepwise, with total Kyoto scores of 0-1, 2-3, and ≥ 4 (8.6%, 61.4%, and 85.7%, respectively)

Table 2 Diagnostic performance of Kyoto classification for *Helicobacter pylori* infection

	Ref.	Country	No. of patients	Sensitivity	Specificity	Accuracy
Non-infection						
RAC	Garcés-Durán *et al*[22], 2019	Spain	140	100	49.0	65.0
	Yoshii *et al*[17], 2020	Japan	485	89.1	79.8	85.6
	Zhao *et al*[19], 2020	China	583	62.4	73.7	69.3
	Ebigbo *et al*[23], 2021	Germany	200	80.8	57.4	-
	Fiuza *et al*[20], 2021	Brazil	187	70.7	87.2	74.9
	Glover *et al*[24], 2021	UK	153	78.4	64.3	75.8
	Yuan *et al*[21], 2021	China	165	51.4	96.7	76.4
	Hirai *et al*[18], 2021	Japan	1761	93.2	83.2	90.6
Current infection						
Atrophy	Toyoshima *et al*[27], 2018	Japan	136	82.6	85.8	85.3
	Zhao *et al*[19], 2020	China	583	54.9	61.1	58.5
	Ebigbo *et al*[23], 2021	Germany	200	80.4	69.7	-
Intestinal metaplasia	Toyoshima *et al*[27], 2018	Japan	136	39.1	95.6	86.0
Enlarged folds	Toyoshima *et al*[27], 2018	Japan	136	17.4	99.1	85.3
	Yoshii *et al*[17], 2020	Japan	494	23.1	96.6	85.0
Nodularity	Toyoshima *et al*[27], 2018	Japan	136	8.7	100	84.6
	Yoshii *et al*[17], 2020	Japan	494	6.4	98.3	83.8
	Toyoshima *et al*[28], 2020	Japan	265	33.3	99.6	89.1
	Fiuza *et al*[20], 2021	Brazil	187	10.6	98.6	76.5
Diffuse redness	Toyoshima *et al*[27], 2018	Japan	136	52.2	93.8	86.8
	Yoshii *et al*[17], 2020	Japan	485	60.0	94.7	89.7
	Zhao *et al*[19], 2020	China	583	20.3	97.6	65.0
	Fiuza *et al*[20], 2021	Brazil	187	80.9	73.6	75.4
Total Kyoto score	Toyoshima *et al*[27], 2018[1]	Japan	136	78.3	92.0	89.7
	Sumi *et al*[30], 2022[2]	Japan	561	98.7	98.4	98.6

[1]The total Kyoto score ≥ 2 is defined as current infection.
[2]The total Kyoto score ≥ 1 is defined as current infection.
RAC: Regular arrangement of collecting venules.

[31]. The mean total Kyoto scores differed among patients with current, past, and non-infection (3.4, 1.1, and 0.0, respectively)[32]. A combination of the total Kyoto score and serum *H. pylori* antibody titer allows for the accurate diagnosis of current infection[33]. The total Kyoto score decreases from 3.9 to 2.8 following *H. pylori* eradication[29]. In summary, total Kyoto scores of 0 and ≥ 2 express non-infection and current infection, respectively.

HISTOLOGICAL CONSISTENCY OF KYOTO CLASSIFICATION

The purpose of the Kyoto classification is to match endoscopic and histological findings of gastritis. Regarding atrophy and IM, considerable evidence exists to indicate the consistency between endoscopy and histology. In recent studies, a high Kyoto atrophy score and severe endoscopic IM are associated with histologically advanced stages of operative link for gastritis assessment and operative link for gastric IM assessment, respectively[34,35].

Consistency between the endoscopic findings of the Kyoto scores and histological grading of the updated Sydney system (USS) scores has been examined individually. All five Kyoto scores were associated with histological inflammation, namely the USS score for neutrophil and mononuclear cell

infiltration, which is an indicator of *H. pylori* infection. The Kyoto atrophy and IM scores correlated with both histological atrophy and IM in the corpus[34,36]. Among *H. pylori*-infected patients, the Kyoto EF, nodularity, and DR scores indicated histologically high inflammation in the corpus[36-38]. In summary, the Kyoto atrophy and IM scores were concordant with histological corpus atrophy and IM scores. The Kyoto EF and nodularity scores were associated with the histological corpus inflammation.

GC RISK OF KYOTO CLASSIFICATION

Significant evidence to indicate endoscopic atrophy as a risk factor for GC has been accumulated. The incidence of GC based on atrophy is summarized in Table 3. GC incidences for mild, moderate, and severe atrophy are 0.06%-0.15%, 0.12%-0.34%, 0.31%-1.60%, respectively, indicating the severity of atrophy as a risk factor for GC development, even after *H. pylori* eradication[39-41]. A recent study from Western countries also showed that a Kyoto atrophy score of 2 was associated with GC development with a hazard ratio of 6.4 in patients with baseline IM[42].

The ORs for the histological subtypes of GC based on the Kyoto classification are summarized in Table 4. The Kyoto atrophy score is a predictor of GC with ORs of 2.5-7.4[43-45]. Two recent meta-analyses showed that a Kyoto atrophy score of 2 had high risk ratios (2.8-8.0 for developing GC)[46,47]. In an examination based on histological subtypes, a high Kyoto atrophy score was found to be associated with both diffuse-type and intestinal-type GCs with ORs of 2.3 and 6.2, respectively[44].

A high Kyoto IM score indicates a high risk for GC (OR = 1.6), especially intestinal-type GC (OR = 1.7), but a low risk for diffuse-type GC (OR = 0.2)[44,45,48,49]. In a direct comparison of diffuse-type and intestinal-type GCs, a high Kyoto IM score was associated with intestinal-type GC (ORs = 1.7-2.1)[44,49]. Furthermore, a high Kyoto IM score was associated with multiple GCs[50].

In a study on asymptomatic *H. pylori*-infected patients, the hazard ratio of patients with EF for GC development during the 5 years was high at 43.3[51]. In contrast, EF was associated with a low risk of intestinal-type GC (OR = 0.5)[44]. Furthermore, a direct comparison between diffuse-type and intestinal-type GCs indicated EF as a risk factor for diffuse-type GC (OR = 1.3)[44]. EF is reported to be an indicator of submucosal invasion in patients with GC (OR = 3.4; submucosal invasion *vs* intramucosal depth)[52].

The risk of nodularity is controversial. Previous studies found that nodularity was associated with a high risk for diffuse-type GC (OR = 10.0)[53], notably in young *H. pylori*-infected patients (OR = 64.2)[54]. In contrast, nodularity was described as a low risk factor for GC (OR = 0.5), especially intestinal-type GC (OR = 0.3)[44]. Nodularity decreases with age and the risk of intestinal-type GC increases with age[28]. Therefore, the risk of nodularity in GC should be stratified according to age.

Previously, RAC has been revealed as a predictor of non-GC[45]. Collectively, the Kyoto atrophy, EF, and nodularity scores were associated with diffuse-type GC, whereas the Kyoto atrophy and IM scores were related to intestinal-type GC, as shown in Figure 2.

GC risk after H. pylori eradication

Recently, the risk of GC after *H. pylori* eradication has been intensively investigated. Table 5 shows the risk of GC following *H. pylori* eradication. A Kyoto atrophy score of 2 and MLR are both indicators of GC after eradication, with ORs of 8.1 and 1.8-5.3, respectively[55-57]. Additionally, RAC was inversely associated with eradicated GC (ORs = 0.3-0.4)[56,58]. Studies have further revealed that the hazard ratios of Kyoto atrophy 2 and MLR for GC development were 4.9 and 3.6, respectively[55,59]. Take *et al*[41] previously reported the long-term incidence of GC after eradication based on endoscopic atrophy. The incidence of diffuse-type GC was higher in the second decade of follow-up than in the first decade. This increase was only observed in patients with mild-to-moderate gastric atrophy, indicating that even if atrophy is not severe, the risk of GC can persist long after eradication.

Total Kyoto score

The total Kyoto scores of patients with GC, *H. pylori*-infected GC, and *H. pylori*-eradicated GC were 4.0-4.6, 4.8-5.6, and 4.2, respectively[44,50,58,60]. A high total Kyoto score was associated not only with GC (ORs = 1.5-1.6), but also with both diffuse-type and intestinal-type GCs (ORs = 1.3 and 1.7, respectively, Table 4)[44,61]. Additionally, some investigators showed that the incidence of GC increased stepwise with the total Kyoto scores of 0-1, 2-3, ≥ 4, and that the AUC of the nomogram to predict GC using the total Kyoto score was 0.79[31,61]. Taken together, a total Kyoto score of 4 or more is useful for determining GC risks, including histological subtypes, even after *H. pylori* eradication.

FUTURE PERSPECTIVES IN KYOTO CASSIFICATION

The total Kyoto score was developed to evaluate GC risk, with a score ≥ 4 indicating risk. However, designing a method to simply add each component of the Kyoto score is problematic. First, the GC risks

Table 3 Gastric cancer incidence based on endoscopic atrophy

Ref.	Population	No. of subjects	No. of cancers	Duration, yr	Gastric cancer incidence, %/yr		
					Atrophy (mild)	Atrophy (moderate)	Atrophy (severe)
Shichijo *et al*[39], 2016[1,2]	Post eradication	573	21	6.2 ± 4.8	0.07	0.34	1.60
Kaji *et al*[40], 2019[3]	Screening	12941	63	3.7 ± 0.8	0.10	0.16	0.31
	Post eradication	2571	20	3.7 ± 0.8	0.06	0.12	0.42
Take *et al*[41], 2020[1]	Post eradication	2737	68	7.1 ± 5.4	0.15	0.29	0.67

[1]Mild, moderate, and severe atrophy represent Kimura-Takemoto's C1-2, C3-O1, and O2-3, respectively.
[2]Incidence was divided the incidence per 10 years by 10.
[3]Mild, moderate, and severe atrophy represent Kyoto atrophy scores 0, 1, and 2, respectively.

Table 4 Odds ratio for histological subtype of gastric cancer based on the Kyoto classification

	Ref.	*H. pylori* status	No. of subjects	No. of GC	No. of diffuse-type GC	No. of intestinal-type GC	OR for GC	OR for diffuse-type GC	OR for intestinal-type GC
Atrophy	Sekikawa *et al* [43], 2016	Current, past, and no infection	1823	29	3	26	7.4[1]		
	Toyoshima *et al* [44], 2021	Current infection	499	132	39	93	2.8	2.3	6.2
	Kawamura *et al* [45], 2022	Current, past, and no infection	380	115	19	96	2.5[1]		
Intestinal metaplasia	Shichijo *et al*[48], 2017	Current, past, and no infection	3392	107	22	85		0.2[2]	
	Toyoshima *et al* [44], 2021	Current infection	499	132	39	93	1.6		1.7
Enlarged folds	Nishibayashi *et al* [81], 2003	Current infection	276	135	69	66	5.0		
	Toyoshima *et al* [44], 2021	Current infection	499	132	39	93			0.5
Nodularity	Nishikawa *et al* [53], 2018	Current infection	674	25	9	16		10.0	
	Toyoshima *et al* [44], 2021	Current infection	499	132	39	93	0.5		0.3
RAC	Kawamura *et al* [45], 2022	Current, past, and no infection	380	115	19	96	0.2[3]		
Total Kyoto score	Toyoshima *et al* [44], 2021	Current infection	499	132	39	93	1.6	1.3	1.7
	Lin *et al*[61], 2022	Current, past, and no infection	1848	37	-	-	1.5		

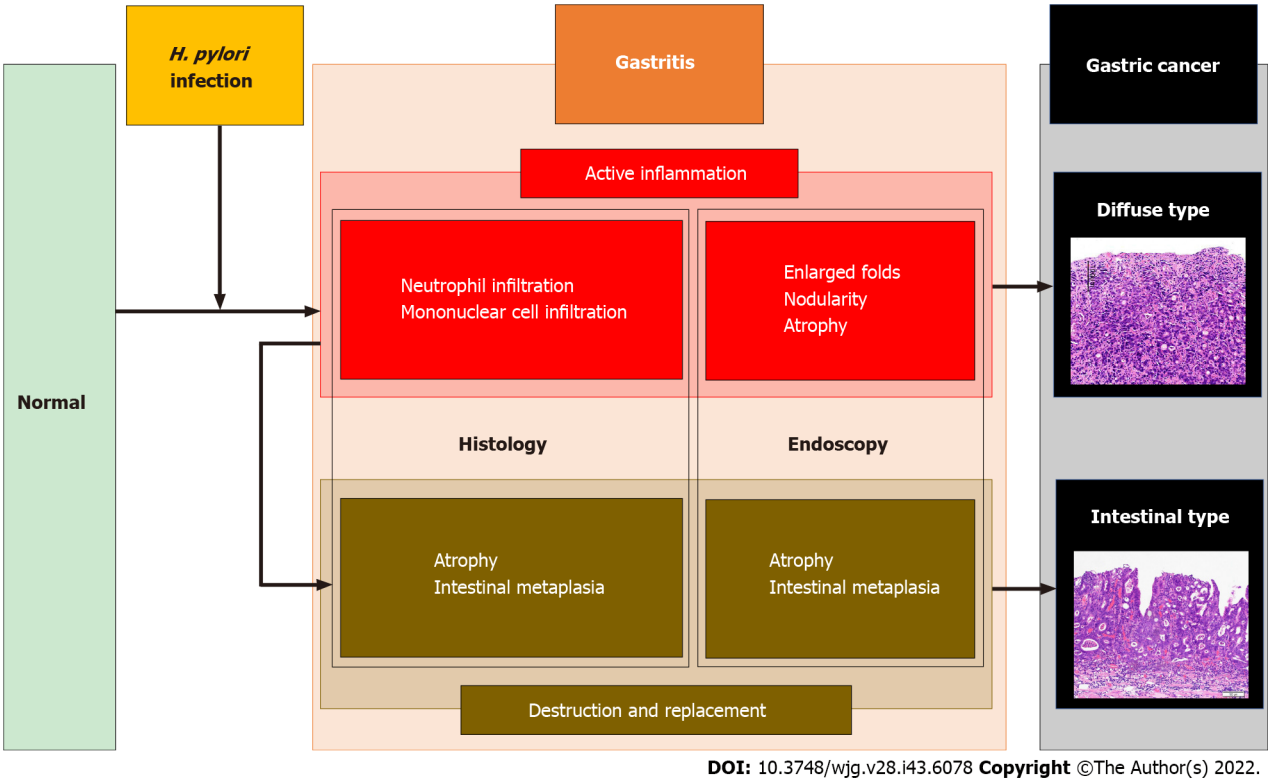
[1]Odds ratio for Kyoto score 2 *vs* Kyoto scores 0 + 1.
[2]Odds ratio for Kyoto scores 1 + 2 *vs* Kyoto score 0.
[3]Odds ratio for regular arrangement of collecting venules presence *vs* absence.
Odds ratios were calculated per 1 rank of Kyoto score. *H. pylori*: *Helicobacter pylori*; GC: Gastric cancer; OR: Odds ratio; RAC: Regular arrangement of collecting venules.

of the diffuse and intestinal types were distinctly different. For example, IM is associated with a high risk of intestinal-type GC but a low risk of diffuse-type GC. Conversely, EF and nodularity are high risk factors for diffuse-type GC, but indicate a low risk of intestinal-type GC (Table 4 and Figure 2). The majority of GC cases are classified as intestinal type, which indicates that the intestinal-type GC risk

Table 5 Odds ratio of gastric cancer after *Helicobacter pylori* eradication based on the Kyoto classification

	Ref.	No. of subjects	No. of GC	Odds ratio
Atrophy	Yan *et al*[57], 2021	1961	132	8.1[1]
Map-like redness	Moribata *et al*[55], 2016	122[2]	22[3]	5.3
	Majima *et al*[56], 2019	194	109	2.1
	Yan *et al*[57], 2021	1961	132	1.8
RAC	Majima *et al*[56], 2019	194	109	0.4
	Ohno *et al*[58], 2020	162	43	0.3

[1]Odds ratio for Kyoto atrophy score 2.
[2]Patients after endoscopic resection of gastric cancer.
[3]Metachronous gastric cancer.
GC: Gastric cancer; RAC: Regular arrangement of collecting venules.



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Figure 2 Pathogenesis of diffuse-type and intestinal-type gastric cancers. *H. pylori*: *Helicobacter pylori*.

may be overestimated, whereas the diffuse-type GC risk may be underestimated. Second, two points were assigned to Kyoto atrophy, IM, and DR scores in the total Kyoto score. The verification of the weighting of the total Kyoto score is a future task. Therefore, this scoring method should be revised in the future. A modified Kyoto score has been suggested as the sum of the following points: 2 points for invisible RAC, and 1 point each for Kyoto atrophy score 2, Kyoto IM score 2, and corpus MLR. Compared with the scores of 0-1, the ORs of the GC morbidity for the modified Kyoto scores of 2-3 and 4-5 were higher, at 8.6 and 28.0, respectively. Although statistical significance was not reached, the AUC of the modified Kyoto score had a higher predictive ability than that of the original total Kyoto score (0.75 *vs* 0.71, respectively)[45]. Furthermore, a scoring system specific to histological GC subtypes is needed. Third, MLR has been shown to predict GC after *H. pylori* eradication. Since IM manifests as MLR after *H. pylori* eradication[3], MLR may be more suitable than IM to assess the risk of eradicated GC.

In Western countries, RAC and endoscopic IM have been extensively studied; however, other endoscopic findings, such as atrophy, EF, nodularity, DR, and MLR, have been less extensively explored, and further studies in more varied populations are required. This article does not mention a

variety of important endoscopic findings, including spotty redness as a predictor of *H. pylori* infection [62]; xanthoma[57,63,64], foveolar hyperplastic polyp[65], refluxed bile[66], and a lack of fundic gland polyp[64] to predict GC; and depressive erosion and fundic gland polyp as indicators of functional dyspepsia[32,67]. Further research is required to confirm these findings. RAC provides high kappa values of intra-observer and inter-observer agreements of 0.88-0.91 and 0.74-0.79, respectively[21,68]; however, agreement between the other endoscopic findings needs to be clarified.

Autoimmune gastritis (AIG) is gaining attention as an important factor owing to the decrease in *H. pylori* infection[69]. Both severe endoscopic atrophy and a Kyoto IM score of 2 have been reported as AIG features[70,71]. Further steps should be taken to elucidate the differential diagnosis between AIG and *H. pylori*-associated gastritis using the Kyoto classification.

Recently, image-enhanced endoscopy (IEE) has been widely used in clinical practice. Two meta-analyses previously reported the utility of narrow-band imaging (NBI) for the diagnosis of IM[72,73]. Additionally, an improved diagnostic accuracy on using NBI, blue laser imaging, and linked color imaging have been reported[74-77]. In the future, research on endoscopic assessment using IEE, including texture and color enhancement imaging[78,79] will be required.

CONCLUSION

In conclusion, the total Kyoto score and individual Kyoto score, including atrophy, IM, EF, nodularity, and DR, can predict GC risk and *H. pylori* infection. Total Kyoto scores of 0, ≥ 2, and ≥ 4 indicate a normal stomach, *H. pylori*-infected gastritis, and gastritis at risk for GC, respectively; RAC predicts non-infection; EF, nodularity, and DR predict current infection; MLR predicts past infection; and atrophy and IM predict current or past infection. Atrophy, IM, and EF all increase in *H. pylori*-infected GC, MLR is a specific risk factor for *H. pylori*-eradicated GC, while RAC indicates a lesser GC risk. Diffuse-type GC can be induced by active inflammation, which presents as EF, nodularity, and atrophy on endoscopy, and neutrophil and mononuclear cell infiltration on histological examination. In contrast, intestinal-type GC develops *via* atrophy and IM, and is consistent on endoscopy and histology. However, the GC risk-scoring design still needs to be improved.

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OPINION REVIEW

Current status of minimally invasive liver surgery for cancers

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Abstract

Hepatocellular carcinoma (HCC) patients have chronic liver disease with functional deterioration and multicentric oncogenicity. Liver surgeries for the patients should be planned on both oncological effects and sparing liver function. In colorectal patients with post-chemotherapy liver injury and multiple bilateral tumors, handling multiple tumors in a fragile/easy-to-bleed liver is an important issue. Liver surgery for biliary tract cancers is often performed as a resection of large-volume functioning liver with extensive lymphadenectomy and bile duct resection/reconstruction. Minimally invasive liver surgery (MILS) for HCC is applied with the advantages of laparoscopic for cases of cirrhosis or repeat resections. Small anatomical resections using the Glissonian, indocyanine green-guided, and hepatic vein-guided approaches are under discussion. In many cases of colorectal liver metastases, MILS is applied combined with chemotherapy owing to its advantage of better hemostasis. Two-stage hepatectomy and indocyanine green-guided tumor identification for multiple bilateral tumors are under discussion. In the case of biliary tract cancers, MILS with extensive lymphadenectomy and bile duct resection/reconstruction are developing. A robot-assisted procedure for dissection of major vessels and handling fragile livers may have advantages, and well-simulated robot-assisted procedure may decrease the difficulty for biliary tract cancers.

Key Words: Minimally invasive liver surgery; Laparoscopic liver resection; Robot-assisted liver resection; Hepatocellular carcinoma; Colorectal liver metastases; Biliary tract carcinoma

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Core Tip: Minimally invasive liver surgery (MILS) for hepatocellular carcinoma is applied with the advantages of laparoscopic "caudal approach" for cases of cirrhosis or repeat resections. Small anatomical resections using newly developing approaches are under discussion. In many cases of colorectal liver metastases, MILS is applied combined with chemotherapy, owing to its advantage of better hemostasis. Two-stage hepatectomy and indocyanine green-guided tumor identification for multiple bilateral tumors are under discussion. In the case of biliary tract cancers, MILS with extensive lymphadenectomy and bile duct resection/reconstruction are developing. A robot-assisted procedure may have advantages.

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INTRODUCTION

Liver surgery for cancer is mainly performed in patients with hepatocellular carcinoma (HCC), liver metastasis of colorectal carcinoma (CRCLM), or biliary tract carcinomas (BTC)[1-4]. It is usually performed as curative-intent liver resection (LR) without other comparable alternatives, except for ablation therapy for small HCC and liver transplantation for patients with severe cirrhosis and non-advanced HCC. However, LR for each disease has its own specificity based on disease characteristics and background liver condition. This editorial describes the characteristics of LR for each disease. Thereafter, the advantages, disadvantages, and current status of the minimally invasive approach for each disease, and its potential are discussed.

LR FOR HCC

Patients with HCC mostly have a history of chronic liver disease (CLD), which causes functional deterioration and multicentric oncogenicity in the injured liver[1]. Therefore, depending on the tumor and pre-existing liver conditions, LR, ablation therapy, trans-arterial chemoembolization, liver transplantation, or recently emerged systemic chemo-immune therapy are chosen. The rates of LR application to primary HCC cases and the 5 year-survival rate thereafter are approximately 30% and 50%, respectively[1]. However, after a successful first treatment, many patients eventually enter a long-term treatment course with repeated treatments for recurrent/multicentric metachronous HCC raised from an oncogenic CLD background. One of the above-listed treatment options is selected based on the tumor and liver conditions of the patients at the time of each treatment. On LR indication, an evaluation of the liver function and, accordingly, estimation of resectable functional liver volume should be performed preoperatively[5], since HCC patients with CLD have a potential risk factor for postoperative morbidity and liver failure. Furthermore, potential repeated treatments over a long period of the treatment course should be considered in the treatment strategy of patients with HCC/CLD.

However, dissemination of cancer cells within the same portal territory as HCC tumors is well known, and anatomical resection of HCC is recommended[6]. Hemi-hepatectomy, sectionectomy, and sometimes segmentectomy, are widely accepted anatomical resections. However, since clear margins of segments one, five, six, and seven have sometimes been difficult to define, the Tokyo 2020 terminology of liver anatomy and resections was recently issued for segment or smaller anatomical resections[7]. These small anatomical resections and their combinations are under discussion for their oncological advantages. Liver surgeries for HCC patients should be planned to be appropriate in terms of both oncological effects on the currently existing HCC and sparing function of the liver with CLD, not only to minimize postoperative morbidity but also from the long-term perspective of potential repeated treatments.

LR FOR CRCLM

LR is the only curative-intent treatment for patients with CRCLM, with a 5-year overall survival rate of approximately 40%-50%[3]. However, due to the advancement of chemotherapy over the last few decades, combination strategies with chemotherapy, including adjuvant, neoadjuvant, and conversion strategies, for expanding indications and improving outcomes have become increasingly common[3]. Based on the current situation, patients with multiple tumors in the bilateral lobes often undergo LR. Several procedures are advocated to ensure that extended LR for multiple tumors is feasible and safer,

such as residual liver hypertrophy with percutaneous transhepatic portal embolization (PTPE)[8,9], two-stage hepatectomy[10], or associating liver partition or portal vein embolization for staged hepatectomy (ALPPS)[11]. In contrast, parenchymal-sparing LR is recommended for tumors occurring in small numbers, since tumor cell spreading *via* the portal vein system is rare, contrary to HCC[12]. Repeat LR for resectable recurrences improves long-term outcomes[13], and parenchymal-sparing LR reportedly improves salvageability and survival at recurrence within the liver[14]. The background liver condition is usually not fibrotic without CLD but is often associated with post-chemotherapy liver injury, such as steatosis and congestion of microcirculation with sinusoidal obstructive syndrome[15,16]. Liver steatosis can lead to liver fragility during surgery and postoperative elevation of transaminase levels. Congestion of microcirculation with sinusoidal obstruction can cause increased blood loss during surgery and, sometimes, postoperative morbidity, including ascites, similar to portal hypertension in patients with cirrhosis[17].

LIVER SURGERY FOR BTC

Liver surgery (LS) for BTC is performed in intrahepatic cholangiocarcinoma (ICC; peripheral and central types), perihilar cholangiocarcinoma, and gall bladder carcinoma[4]. Small peripheral ICC of the mass-forming type, often with CLD backgrounds and rarely with lymph node metastases, can be treated with an HCC-like approach, though it usually lacks an HCC-like tumor capsule with more invasive features than HCC[18]. Others have more aggressive features like spreading along the Glissonian pedicle with perineural, lymphatic, and venous invasions, as well as direct liver parenchymal invasion and lymph node metastases[4,18]. Since the invasion often involves hilar and intrahepatic Glissonian pedicles, LRs for those diseases sacrifice large volumes of functioning non-cancerous liver parenchyma to remove the cancer cells deeply infiltrated from the hilum into the peripheral Glissonian pedicle. PTPE is sometimes applied in such cases to enlarge the residual liver volume preoperatively[8]. Extensive lymphadenectomy and bile duct (sometimes including vasculature: Portal vein and hepatic artery) resection plus reconstruction are required. The patients mostly have a normal liver; however, sometimes damage from biliary obstruction may be present. In cases with jaundice, preoperative biliary drainage is needed[19].

MINIMALLY INVASIVE LIVER SURGERY

Laparoscopic liver resection (LLR) emerged in the early 1990s, and its indications have expanded thereafter[20,21]. The cancers previously mentioned are all within the indication of LLR. Less intraoperative blood loss, shorter hospital stays, and less morbidity in some conditions with comparable long-term outcomes have been generally reported with LLR for HCC and CRCLM[22,23]. LLRs for BTC, especially for those requiring extensive lymphadenectomy and bile duct (and vasculature) resection plus reconstruction, are now in the developmental stage with some early reports[24]. Although laparoscopic techniques for liver parenchymal transection, mobilization, and hemostasis have been established, those for extensive lymphadenectomy and bile duct (and vasculature) resection plus reconstruction are performed only in specialized centers. Robot-assisted LLR is also in its developmental stage and is performed mainly in specialized centers[25]. Moreover, devices that can be used in robotic procedures are still limited. However, some of the robotic procedures, such as bile duct/vasculature resection plus reconstruction, are expected to have advantages due to increased dexterity from stable endo-wrist instruments and stable high-definition three-dimensional visualization.

Minimally invasive LR for HCC

HCC with pre-existing CLD is thought to be the cancer for which the specific approach of minimally invasive LR (MILR) is most beneficial. In 2013, we presented the novel concept of the "caudal approach to LLR"[26]. Researchers followed[27,28] and defined the concept as a major conceptual changing of LLR in the statement of the 2nd international consensus conference[29]. LR is a procedure for handling the liver while it is protected inside the subphrenic "rib cage". In open LR, the cage is opened with a large subcostal incision and the liver is mobilized. In LLR, the laparoscope and instruments get directly into the space from the caudal direction with minimal damage on the cage, and minimal mobilization and compression on the liver[30]. This leads to minimal damage to the adherent structures as well as the liver. Since HCC patients mostly have underlying CLD, they have a higher risk of post-LR morbidity. LR patients are exposed to three types of surgical stress: (1) General and whole-body surgical stress; (2) Decreased liver function from the decrease of functioning liver parenchyma after LR; and (3) Surgery-induced damage on the environment structures and residual liver (such as disruption of collateral vessels in CLD patients by laparotomy/dissection of peritoneal attachments and parenchymal damage by compression). The third type of surgical stress can be reduced by laparoscopic-specific caudal approach[26-28] in LLR for HCC/CLD patients and that decreases short-term morbidity[30]. We

evaluated the short-term outcomes of liver surface small LLR for patients with severe CLD and our findings showed comparable short-term outcomes to those from patients of mild-to-moderate liver dysfunction[31]. Direct access to the surface tumor and minimal dissection of attachments made this surgery possible. This setting of LR can be achieved only with a laparoscopic approach. An international retrospective study using propensity score matching analysis of Child-Pugh B patients who underwent LR has shown that LLR is beneficial for these patients[32]. Moreover, LLR is thought to be advantageous also in less post-LR liver functional deterioration for those patients by smaller damage caused in surgical manipulation[30]. It can be beneficial for long-term and repeated treatment courses for the patients.

The treatment of intrahepatic recurrence is also important. Modifications of the anatomy and the formation of adhesions make repeating LR more difficult. Laparoscopic surgeries make following procedures easier from reduced formation of adhesions[33]. Furthermore, LLR allows for better visibility and manipulation even in a small surgical field between adhesions[30]. It can lead to unnecessity of total adhesiolysis in repeat procedures. Our international retrospective study for repeat LR compared laparoscopic and open approaches[34] and showed that laparoscopic repeat LR is feasible and has the short-term advantages of less bleeding and morbidity for selected patients. The overall survival curves were clearly separated, with a better tendency in LLR, though the disease-free survival curves were overlapped. The post-LR overall survival of HCC/CLD patients is determined not only by the treatment results of the resected lesion but by those of metachronous lesions and liver insufficiency during the long-term course[35,36]. During the long course of repeated treatments, patients with HCC/CLD should have adequate residual liver function after each intervention, which enables them to get future repeat treatments. We hypothesized that better overall survival after laparoscopic procedure may be from less liver functional deterioration[30]. Accompanied with less adhesion, it can make the repeat treatments easy to access and decrease deceased patients by liver insufficiency. LLR with the specific caudal approach, performed as a unique strong local treatment, is beneficial for patients with HCC/CLD.

However, LLR also has certain disadvantages. Disorientation can be easily happened due to lack of fine perceptible sensation and overview of the whole surgical field, and difficulty in performing precise intraoperative ultrasonography. Simulation and navigation from pre- and intra-operative imaging studies have been used to overcome these disadvantages. Well-simulated small anatomical resections in LLR can secure the tumor location in the resected area and provide adequate surgical margins, and lead to less postoperative bile leakage and less residual ischemic/congestive parenchyma, which possibly leads to recurrence[37]. For HCC for which anatomical resection is recommended, there are several reports of LLR that can lead to new developments, such as landmark (hepatic veins, *etc.*)-guided small anatomical resection[38], indocyanine green (ICG)-guided anatomical resection and tumor identification [39], and LLR with a Glissonian approach to more peripheral smaller branches from the hilum (cone unit resection)[40]. Robot-assisted LLR is an important emerging tool under discussion[25,41]. It could be advantageous, for example, in cases exposing a wide range of Glissonian pedicles and major hepatic veins.

MILR for CRCLM

As mentioned before, patients with post-chemotherapy liver injury and those with multiple tumors in bilateral lobes increasingly undergo MILR with recent advancements in neoadjuvant/conversion chemotherapy[15,16]. Handling multiple tumors in a fragile/easy-to-bleed liver is an important issue.

In MILR for CRCLM, disorientation can be easily happened due to lack of fine perceptible sensation and overview of the whole surgical field, and difficulty in performing precise intraoperative ultrasonography, as mentioned in MILR for HCC. Simulation and navigation from pre- and intraoperative imaging studies to assure tumors in the resected area with enough surgical margin are important. Landmark (such as hepatic veins)-oriented small anatomical resection[38], ICG-guided anatomical resection and tumor identification[39], and LLR with a Glissonian approach to more peripheral smaller branches from the hilum[40,42] are also applied to CRCLM. However, because the liver does not have CLD and the need for anatomical resection is low, LR for CRCLM with a large number of tumors is often planned as a combination of large anatomical resection plus partial resections or multiple partial resections. For the combination of large anatomical resection plus partial resections, the application of two-stage hepatectomy or ALPPS in MILR is now advocated[43,44]. For multiple partial resections, ICG-guided tumor identification is reported to be effective in addition to conventional intraoperative navigation[45,46].

In terms of handling fragile and easy-to-bleed injured liver after chemotherapy, bleeding can be controlled more easily in LLR than in open procedures by optimal visualization and pneumoperitoneum accompanied by Pringle's maneuver. However, handling a fragile liver can sometimes be difficult due to congenital motion restrictions in LLR, especially in complicated resections. Robot-assisted LLR may give us a chance to overcome this difficulty with its advantages, such as increased dexterity from stable endo-wrist instruments.

Theoretically, early recovery after MILR can be advantageous when it enables the earlier introduction of adjuvant chemotherapy. Although there are no solid data on the combination of MILR and chemotherapy, the combination of these treatments is a matter of further investigation.

Minimally invasive liver surgery for BTC

BTC is an emerging indication of minimally invasive liver surgery (MILS) under discussion[47]. Surgery for BTC consists of three different procedures: LR, lymphadenectomy, and resection plus reconstruction of the bile duct (and vasculature). The laparoscopic procedure for LR has already been established during its application in HCC and CRCLM. Lymphadenectomy around the hepatic hilum and hepatoduodenal ligaments is often performed in laparoscopic pancreatic surgery[48,49]. Laparoscopic resection plus reconstruction of the bile duct (and vasculature) for malignant diseases should be added to MILS for BTC, although there are reports of bile duct resection plus reconstruction for benign diseases[50].

Surgeries for small peripheral mass-forming ICC rarely with lymph node metastases and early-stage gall bladder carcinoma without invasion to the hepatic hilum or hepato-duodenal ligament (candidates for radical cholecystectomy) requires only peripheral LR and mild lymphadenectomy. They are the first good candidates for the MILS application. On the other hand, advanced gallbladder carcinoma, central (invasive) ICC, and perihilar carcinoma with pedicle invasions and a high potential for lymph node metastases should be handled with a complicated combination of LR, extended lymphadenectomy, and resection plus reconstruction of the bile duct (and vasculature). There are only a few reports of MILS applications in those conditions[24,51]. Among these, minimally invasive surgery for perihilar carcinoma is the most difficult. Bile duct dissection, division, and reconstruction should be performed at the more peripheral part of the small-sized bile duct to obtain a cancer-free margin. Under laparoscopic conditions, disorientation is easy to occur due to the lack of good overview/tactile sensation and motion restriction is also a congenital problem. Setting an adequate resection line without cancer invasion and performing reconstruction of the small orifice of the residual bile duct is more difficult than the open procedure. From this perspective, a well-simulated and planned robot-assisted procedure may be a potential tool to overcome this situation.

CONCLUSION

MILS for HCC with CLD background and recommendation for anatomical resection has been established and applied to most cases with advantages in cirrhotic patients and repeat LR by laparoscopic specific "caudal approach". Small anatomical resections using Glissonian approach, ICG-guided approach, landmark (*i.e.*, hepatic vein)-guided approach are under discussion and developments. Robot-assisted procedure for long range dissection of major vessels may have advantages.

MILS for CRCLM with combination treatments to chemotherapy has been established and applied to many cases with the advantage in better hemostasis by pneumoperitoneum and optimal visualization. LLRs with two-stage hepatectomy, ALPPS, ICG-guided tumor identification are under discussion and developments. Robot-assisted procedure for handling the fragile liver after chemotherapy may have advantages.

MILS for BTC with spreading invasion along Glissonian pedicle, lymph node metastases and direct invasion to liver parenchyma is developing, especially for extensive lymphadenectomy and bile duct (vasculatures) resection /reconstruction, and possible in experienced center. Well simulated and planned robot-assisted procedure may decrease the difficulty in setting tumor free resection line and reconstruction of small bile duct orifice (Table 1).

Table 1 Summary of current status of minimally invasive liver surgery for cancers

Disease	Backgrounds of disease and liver for surgery	Status of minimally invasive liver resection
HCC	Chronically injured background of liver[1,2]: (1) Deteriorated liver function–postoperative morbidity; and (2) Multicentric carcinogenesis–repeated treatments	Established/applied with some merit to most cases[21,29]: Advantages in LR of LC patients[30,31] and repeat LR[30,34] by laparoscopic specific "caudal approach"[26-28]
	Anatomical resection[6]	Under discussion and development: (1) Small anatomical resection using Glissonian approach[40,42], ICG-guided approach[39], hepatic vein-guided approach[38]; and (2) Robot for long-range dissection of major vessels?
CRCLM	Combination treatments with chemotherapy[3]: (1) Multiple bilateral tumors–TSH[10], ALPPS[11]; and (2) Injured liver–fragility from steatosis[15], easy bleeding form congestion[16,17]	Established/applied to many cases[20,23]: Advantages of better hemostasis[20,29] by pneumoperitoneum/optimal visualization
	Parenchymal sparing resection[12,14]	Under discussion and development: (1) LLR with TSH[43], ALPPS[44]–possible in experienced centers; (2) ICG-guided tumor identification [39,45]; and (3) Robot for handling the fragile liver?
BTC	Spreading invasion along Glissonian pedicle[4], LN metastases and direct invasion to liver parenchyma: (1) Extended LR of normal	Developing and possible in specialized center[24]: (1) Lymphadenectomy and bile duct (vasculature) resection/reconstruction in

	liver–PTPE[8]; (2) Lymphadenectomy; and (3) Bile duct (vasculature) resection/reconstruction-Needs of setting tumor free resection line and reconstruction of small bile duct orifice	specialized center[24,51]; and (2) Difficulties in setting tumor free resection line and reconstruction of small bile duct orifice: Well simulated robot-assisted procedure?

HCC: Hepatocellular carcinoma; CRCLM: Liver metastasis of colorectal carcinoma; BTC: Biliary tract carcinomas; LR: Liver resection; LLR: Laparoscopic liver resection; LC: Liver cirrhosis; ICG: Indocyanine green; TSH: Two-stage hepatectomy; ALPPS: Associating liver partition or portal vein embolization for staged hepatectomy; PTPE: Percutaneous transhepatic portal embolization; LN: Lymph node.

FOOTNOTES

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MINIREVIEWS

Management of non-alcoholic fatty liver disease patients with sleep apnea syndrome

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is strongly associated with sleep apnea syndrome (SAS). Many NAFLD patients have SAS, and obstructive sleep apnea hypopnea syndrome is also considered to be an independent risk factor for NAFLD, as it contributes to the progression of NAFLD *via* oxidative stress, lipid peroxidation, inflammation, and insulin resistance. This review aims to provide some recommendations for the management of NAFLD patients with SAS, including diet, exercise, weight loss, and continuous positive airway pressure. This review also highlights the importance of effective strategies in NAFLD prevention and treatment.

Key Words: Nonalcoholic fatty liver disease; Sleep apnea syndrome; Obesity; Obstructive sleep apnea hypopnea syndrome; Continuous positive airway pressure; Management

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Core Tip: Nonalcoholic fatty liver disease (NAFLD) is strongly associated with sleep apnea syndrome (SAS). This minireview presents the relationship between NAFLD and SAS; addresses the role of obesity, insulin resistance, and oxidative stress, and emphasizes the management of NAFLD with SAS, which mainly includes lifestyle interventions and continuous positive airway pressure therapy. This review also highlights the importance of effective strategies in NAFLD prevention and treatment.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is defined as hepatic steatosis greater than 5% and excludes causes such as alcohol, viral infection and hereditary factors[1]. Due to numerous studies that have characterized the association of NAFLD with metabolic syndromes, such as obesity and type 2 diabetes (T2DM), an international consensus in 2020 proposed renaming NAFLD as metabolic-associated fatty liver disease (MAFLD)[2]. NAFLD can progress into nonalcoholic steatohepatitis (NASH) with or without fibrosis. Currently, there are two main hypotheses about the pathogenesis of NASH. One is the "two-hit" hypothesis proposed by James and Day[3] in 1998: The first strike is induced by insulin resistance and excessive accumulation of hepatic lipids, while oxidative stress and inflammation are considered to compose the second strike. As research in this field continues to advance, the "parallel multihits" hypothesis is thought to more accurately explain the complex mechanisms of NAFLD progression, which involves genetic and epigenetic factors, insulin resistance, endoplasmic reticulum stress, oxidative stress, and gut dysbiosis[3-5].

NAFLD patients are prone to sleep apnea syndrome (SAS), a common respiratory disease. It is estimated that nearly 1 billion adults aged 30-69 years worldwide have SAS. A real-world study of uncomplicated NAFLD patients in South Korea showed that during a median follow-up of 5.3 years, 1351 patients (0.4%, total 334334) were diagnosed with SAS; among patients with a fatty liver index > 31.0, SAS occurred in 0.8% of patients[6]. Based on the differences in their pathogenesis, SAS is broadly categorized into three types: Obstructive sleep apnea hypopnea syndrome (OSAHS), central SAS and mixed SAS. OSAHS is the most common type of SAS, and obesity is a strong risk factor for OSAHS. Approximately 40% of individuals with obesity are reported to have OSAHS, and 70% of OSAHS patients have obesity[7]. The etiology of OSAHS is complex, with the main causes including anatomical narrowing of the upper airway and local soft tissue collapse. Obesity exacerbates upper respiratory obstruction due to compression of the pharyngeal cavity and airway by neck fat[8,9]. It has been reported that central obesity and neck circumference thickening are significantly and positively associated with the apnea hypoventilation index (AHI), and the AHI is also positively associated with insulin resistance[10]. In addition, the incidence of metabolic syndrome is elevated in patients with OSAHS compared to non-OSAHS; in particular, components of metabolic syndrome, such as T2DM, obesity, and dyslipidemia, are more strongly associated with OSAHS[11]. A German observational cohort study also found a higher incidence of hepatic steatosis in patients with moderate-to-severe OSAHS, and the assessment of a snoring index may help to identify the risks of associated liver disease in OSAHS patients[12]. More importantly, a previous meta-analysis found that OSAHS is associated with an increased risk of advanced fibrosis in NAFLD patients, independent of age, sex and body mass index (BMI)[13]. Another Italian observational cohort study suggested that evaluating the mean oxyhemoglobin percentage in obese patients with OSAHS is of great clinical value in identifying the risks of NAFLD progression[14]. However, because comorbidities coexist and are independently associated with systemic inflammation, it is very difficult to clarify the effects of OSAHS on the development and progression of NAFLD[15,16]. The exact mechanisms of NAFLD and SAS are still largely unknown, but some biological processes are considered to be integrated in NAFLD patients with SAS.

Hypoxia and oxidative stress

Lipid accumulation and subsequent oxidative stress in the liver are considered the initial causes of NAFLD progression and the development of NASH. The accumulation of free fatty acids in hepatocytes due to insulin resistance, increased *de novo* lipogenesis or excessive lipid uptake from dietary sources can disrupt the mitochondrial microstructure, leading to impaired fatty acid β-oxidation and the production of reactive oxygen species (ROS)[17,18]. Overwhelmed ROS can trigger oxidative stress, which is a stressful state involving an imbalance between oxidative and antioxidant actions, usually due to excess ROS interfering with endogenous antioxidant defense system[5]. Oxidative stress can exacerbate lipid accumulation in hepatocytes, disrupt the structure and function of mitochondria; activate Kupffer cells; and promote the release of inflammatory factors such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-8, thereby exacerbating the inflammatory response of hepatocytes[19].

OSAHS is characterized by chronic intermittent hypoxia (CIH) that occurs at night, and prolonged CIH can lead to tissue hypoxia and promote oxidative stress, lipid peroxidation, and systemic inflammation. The disturbed lipid metabolism and excessive oxidative stress in the liver during NAFLD progression may further affect OSAHS. Accordingly, the combination of OSAHS aggravates hepatic impairment, promotes lipid metabolism disorder and increases insulin resistance in patients with NAFLD[20-22]. This was also demonstrated by the positive correlation between the severity of CIH in OSAHS patients and the severity of liver fibrosis measured by liver elastography[23]. Evidence from clinical investigations suggests an increased incidence of NAFLD in patients with OSAHS even in the absence of obesity or metabolic syndrome, while the severity of NAFLD is parallelly associated with the severity of OSAHS in NAFLD patients with OSAHS[24]. More importantly, even in children with NAFLD, nocturnal hypoxia-induced oxidative stress promotes disease progression[25]. In addition, patients with OSAHS are at risk of hypoxemia and even hypercapnia due to the collapse of the pharynx

during sleep, resulting in upper airway obstruction and airflow restriction, as well as respiratory distress and even interruption of breathing, leading to a decrease in oxygen saturation.

CIH, insulin resistance and disorders of lipid metabolism caused by OSAHS may exacerbate NAFLD in patients with obesity and lead to an increased risk of NASH and more serious diseases[26,27]. It has been reported that polymorphisms of proinflammatory cytokine genes including the highly sensitive C-reactive protein, IL-6 and leptin receptor genes are associated with increased risk of OSA and NAFLD in Asian Indians[28]. Moreover, Asian Indian subjects carrying the Gly972Arg polymorphism of insulin receptor substrate 1 are predisposed to developing OSA and NAFLD[29]. However, as mentioned previously, we do not yet fully understand the association between OSAHS and NAFLD. Current evidence suggests that OSAHS-related CIH triggers excessive lipolysis, manifesting as an increase in plasma free fatty acids[30,31]. Excessive free fatty acids lead to ectopic fat accumulation, insulin resistance, vascular dysfunction and dyslipidemia[32,33], which may be one of the mechanisms by which OSAHS promotes the progression of NAFLD[34]. Furthermore, CIH has also been hypothesized to promote oxidative stress through increased ROS production and angiogenesis, increased sympathetic activation with elevated blood pressure, and systemic and vascular inflammation with endothelial dysfunction[35].

Diagnosis

The diagnosis of NAFLD is primarily an exclusionary diagnosis, which is different from the diagnostic criteria for MAFLD. Secondary causes of hepatic steatosis and causes of liver complications due to other diseases must be excluded to make the diagnosis of NAFLD, including alcohol and drug use, hepatic viral infections and autoimmune liver diseases, Wilson's disease and lipodystrophy[1,36,37]. Currently, the gold standard for NAFLD diagnosis (*e.g.*, assessment of steatosis, steatohepatitis and the stage of liver fibrosis) remains liver biopsy and histological staining; however, it is inappropriate to routinely perform liver biopsy; because it is invasive and may pose a risk of complications, sampling errors and expert interpretation inconsistencies. Therefore, many noninvasive assessment and clinical scoring systems are used to diagnose and assess the degree of steatosis and fibrosis in patients with NAFLD. For example, abdominal ultrasonography, as a noninvasive and convenient method, and controlled attenuation parameter measurement based on transient elastography for ultrasonography attenuation are commonly used to detect the extent of liver steatosis[38]. Magnetic resonance imaging (MRI) is also applied in the noninvasive diagnosis and grading of NAFLD, and is considered to be efficient in quantifying liver fat, stiffness, and visceral adipose tissue[39]. The further developed MRI-proton density fat fraction and proton magnetic resonance spectroscopy are reported to be more concise technologies for NAFLD diagnosis[40]. Furthermore, hepatologists from the American Gastroenterology Association and Chronic Liver Disease Foundation summarized several commonly used noninvasive scores[41]. Among them, the fibrosis-4 index is the most widely studied and preferred simple noninvasive algorithm and it is used to evaluate the degree of liver fibrosis based on age and levels of platelet, aspartate aminotransferase (AST) and alanine aminotransferase (ALT)[42].

Treatment

The recommended treatment for NAFLD focuses on lifestyle interventions, including dieting, exercise, weight loss, and promotion of energy expenditure. In 2020, the Asia Pacific Association for the Study of the Liver proposed clinical practice guidelines for NAFLD. This guideline points out that conservative management of lifestyle changes remains the best choice for the treatment of NAFLD[37]. Notably, when NAFLD patients are combined with SAS, supplemental treatment with continuous positive airway pressure (CPAP) is the most appropriate approach. CPAP therapy has been reported to help improve sleep apnea and hypoventilation, as well as stabilize or delay the progression of NAFLD[24]. Inflammation, a hypoxic environment and oxidative stress are key pathogenic mechanisms in patients with NAFLD and OSAHS. Therefore, antioxidants such as vitamin E may be effective, and anti-obesity medications have also shown considerable potential in the treatment of NAFLD and OSAHS[43,44]. The glucagon-like peptide-1 receptor agonist, liraglutide and semaglutide may improve AHI and protect against NASH and obesity, especially in relation to T2DM[45,46]. Notably, liraglutide at a dose of 3 mg is well tolerated and is effective at improving NAFLD and OSAHS[47,48].

Diet

A reasonable dietary structure and appropriate dietary intake are beneficial for obesity, especially for NAFLD patients with OSAHS. Severely obese patients with NAFLD should strictly limit calorice intake, and if necessary, scientific recipes should be prescribed by nutritional experts to help alter the metabolic pattern. A Mediterranean diet is recommended for patients with NAFLD and NASH. Based on high amounts of monounsaturated fatty acids derived from olive oil, fruits, grains, whole grains and low-fat dairy products, the Mediterranean dietary pattern can reduce the incidence of metabolic syndrome, obesity, T2DM and cardiovascular disease, as well as certain cancers[49-51]. Remarkably, the Mediterranean diet may potentially counteract the inflammation and oxidative stress that occur in OSAHS and improve upper-airway neuromuscular control and muscle force-generating capacity[52]. In an interdisciplinary weight loss and lifestyle intervention trial, participants who adhered to the Mediterranean diet

demonstrated greater weight loss as well as more significant improvement in OSAHS[53].

In addition, the diversity of foods should be increased for patients with NAFLD, and the foods need to be enriched for antioxidants and high-quality proteins, which can contribute to NAFLD and OSAHS improvement. However, NAFLD patients should reduce the consumption of specific foods, including red meat and overprocessed foods[54]. Moderate coffee consumption is allowed for NAFLD patients, but the intake of alcohol, fructose and sugar-containing drinks should be avoided because alcohol or sugar consumption can increase lipid synthesis, visceral fat accumulation and insulin resistance, and increase the risk of liver fibrosis in patients with NAFLD.

Exercise

Exercise is beneficial for NAFLD and SAS. Previous studies have shown that sedentary and reduced physical activity is associated with the progression of NAFLD and the development of moderate to severe OSAHS[55,56]. There is growing evidence showing that aerobic exercise training is beneficial for patients with SAS[57]. Active physical activity may reduce body weight, improve blood circulation, contribute to sleep quality and address excessive daytime sleepiness. Therefore, regular exercise facilitates weight loss and improves NAFLD and associated complications[58,59]. The recommended minimum level of exercise is 5 d per week (> 30 min each day) of moderate intensity aerobic exercise or 3 d per week (> 20 min each day) of vigorous exercise.

Metabolic bariatric surgery

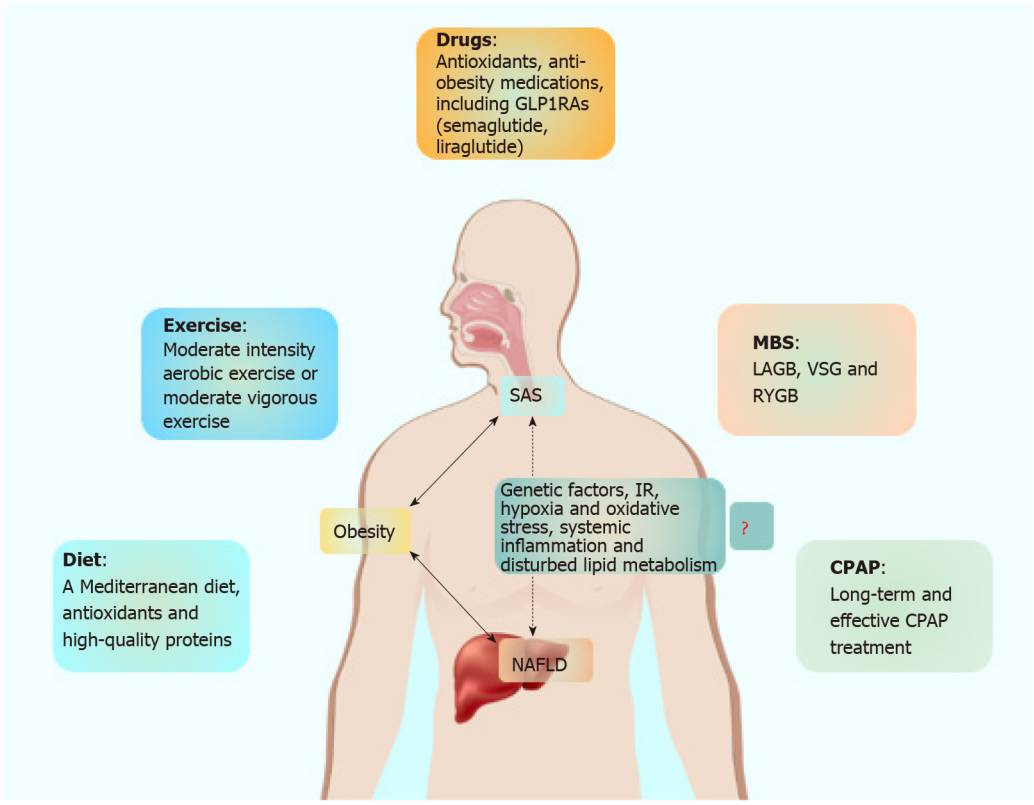
For NAFLD patients with severe obesity, weight loss is very difficult due to their large weight base and may require metabolic bariatric surgery (MBS) intervention. Over the past few decades, MBS has evolved from the simple gastric volume-limiting procedure laparoscopic adjustable gastric banding to surgical options with hormonal effects, including vertical sleeve gastrectomy (VSG) and Roux-en-Y gastric bypass (RYGB), both of which induce appetite changes by modulating intestinal and central hormones, thereby reducing food consumption and increasing satiety[60]. Usually, MBS is the most effective treatment for obesity and often results in dramatic improvements in glycemic control, insulin resistance and NAFLD[61,62].

SAS is also one of the comorbidities with the greatest response rate to MBS. The 6th IFSO Global Registry Report shows that MBS leads to a reduction of SAS ranging from 58%-65%, depending on the type of operation (LSG, RYGB, OAGB). Evidence from clinical studies has confirmed the efficacy of MBS for patients with obesity and OSAHS[63,64]. A retrospective study comparing the effect of VSG and RYGB on weight loss and their comorbidity remission also showed that both MBSs are effective in improving OSAHS symptoms[65]. Most surprisingly, in a study observing the effect of VSG in patients with a BMI ≥ 50 kg/m^2, complete remission was observed in all 13 patients with comorbid OSAHS[66]. MBS can effectively improve sleep apnea and nocturnal hypoxia, as well as hepatic steatosis and fibrosis [67,68]. More importantly, the severity of OSAHS determines the risk of NAFLD before MBS, and after surgery, it also determines the improvement of NAFLD[67].

CPAP

CPAP is the first-line recommendation for the treatment of OSAHS. For NAFLD patients with OSAHS, early intervention is even more important. Additionally, for obesity and OSAHS patients scheduled for MBS, guidelines recommend preoperative CPAP for at least 4 wk[69]. CPAP acts in multiple pathways to prevent airway collapse, reduce pharyngeal edema and upper airway resistance, increase the action of upper airway opening muscles through vagal reflexes, and restore chemoreceptor sensitivity and respiratory center drive. CPAP treatment has been reported to significantly reduce sleep apnea and hypoventilation in patients with OSAHS, improve quality of life; reduce daytime sleepiness, and decrease the occurrence of hypertension, diabetes, cardiovascular and cerebrovascular complications [70]. This may be because CPAP treatment reduces markers of oxidative stress and thus improves metabolic syndrome[71]. In addition, a randomized controlled trial showed that patients with OSAHS had increased markers of liver injury and atherogenic risk[72], whereas CPAP treatment helped stabilize or delay the progression of NAFLD and demonstrated improvements in metabolic and cardiovascular function[24]. A meta-analysis of 192 obesity patients combined with OSAHS showed a significant reduction in serum AST and ALT levels after CPAP treatment for 3 mo[73]. Another meta-analysis of six randomized controlled trials involving 699 subjects also showed that CPAP treatment reduces total cholesterol and triacylglycerol levels[74]. More importantly, CPAP treatment of patients with OSAHS significantly reduces serum inflammatory markers, including CRP and TNF-α[75]. More interestingly, in another randomized controlled trial, Sundaram *et al*[76] found that CPAP treatment reverses the parameters of liver injury, reduces oxidative stress in children with NAFLD can also be used to predict NAFLD progression in obese children with OSAHS.

Although these reports point to a beneficial effect of CPAP on NAFLD-associated parameters[73,74, 77,78], short-term (usually 4-12 wk) CPAP treatment may not be as effective[72,79,80]. The most significant result of CPAP treatment is a case report published in 2018 that reported the reversal of NAFLD and the normalization of liver enzymes and the associated lipidome in a patient with both NAFLD and severe OSAHS after 6 years of treatment[81]. In addition, long-term follow-up data based



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Figure 1 Management of nonalcoholic fatty liver disease with sleep apnea syndrome. Healthy lifestyle management remains the current first-line recommendation for nonalcoholic fatty liver disease (NAFLD) with sleep apnea syndrome (SAS), including diet, exercise, and weight loss. For patients with obesity and type 2 diabetes, drug intervention should also include anti-obesity medications and insulin-sensitizing drugs. Metabolic bariatric surgery can be considered when lifestyle changes and medical interventions are not effective. In addition, continuous positive airway pressure therapy may improve both intermittent hypoxia and liver injury in patients with NAFLD and SAS. MBS: Metabolic bariatric surgery; CPAP: Continuous positive airway pressure; IR: Insulin resistance; LAGB: Laparoscopic adjustable gastric banding; VSG: Vertical sleeve gastrectomy; RYGB: Roux-en-Y gastric bypass; SAS: Sleep apnea syndrome; NAFLD: Nonalcoholic fatty liver disease.

on a Taiwanese population also showed a lower cumulative incidence of NAFLD and cirrhosis in CPAP-treated patients than in nontreated patients[82].

Therefore, the beneficial effects of short-term CPAP therapy on OSAHS and NAFLD may be elusive, and although long-term and effective CPAP treatment can show significant improvements in both OSAHS and NAFLD, most patients are not amenable to long-term oral and nasal mask therapy. In addition, CPAP therapy is not yet commonly used due to the high cost of the device.

CONCLUSION

Obesity is a high-risk factor for NAFLD and SAS. Most patients living with obesity and metabolic syndrome are prone to SAS, and the severity of disease in patients with SAS is significantly associated with NAFLD progression. It is important to note that, even in nonobese patients, there is a relationship between NAFLD progression and SAS severity. SAS is usually one of the complications of NAFLD. However, at present, we can only confirm that lipid disorders, hypoxia, oxidative stress, and inflammation are involved, and a deeper understanding of the pathogenesis remains unclear. More in-depth studies are needed to elucidate the causal relationship between them. More importantly, an understanding of the pathogenesis will help us to develop more individualized treatment plans.

Collectively, healthy lifestyle management remains the most important strategy for the treatment of NAFLD, including diet, exercise and weight loss. Furthermore, the use of antioxidants such as vitamin E and the insulin-sensitizing drug pioglitazone is also necessary. It needs to be emphasized again that timely CPAP intervention is also important for NAFLD patients with SAS. We have summarized our recommendations for NAFLD combined with SAS management in Figure 1, and the improvements in SAS and NAFLD after intervention with lifestyle measures, medications and MBS are summarized in Table 1.

Table 1 Effects of current strategies on nonalcoholic fatty liver disease with sleep apnea syndrome

Category		Effects	Ref.
Lifestyle measures	Mediterranean diet	Inhibition of inflammation and oxidative stress that occur in OSAHS and improvement of upper-airway neuromuscular control and muscle force-generating capacity	[51]
	Dietary behavior change, moderate-intensity aerobic exercise, sleep hygiene, and tobacco and alcohol avoidance	Increases in adherence to the Mediterranean diet	[52]
		Reduced AHI and oxygen desaturation index	
		Increased sleep quality	
		Decease of body weight, fat mass, visceral adipose tissue, and neck, chest, and waist circumferences	
	Aerobic exercise training	Reduced body weight improved blood circulation, better sleep quality and less daytime sleepiness	[56-58]
Medications	Phentermine plus extended-release topiramate	Significant improvements in overnight oxygen saturation and reduction in blood pressure	[42]
	Liraglutide and semaglutide	Histological resolution of NASH and improved metabolic control	[43-48]
		Decreased AHI, body weight, SBP and HbA1c	
MBS	VSG or other MBS	Reduced AHI	[62-64]
	VSG	100% remission rate in patients with OSAHS who also underwent hiatal hernia repair	[65]
	MBS	Improved sleep apnea and nocturnal hypoxia, as well as liver steatosis and fibrosis	[66]

OSAHS: Obstructive sleep apnea hypopnea syndrome; AHI: Apnea hypoventilation index; NASH: Nonalcoholic steatohepatitis; SBP: Systolic blood pressure; HbA1c: Glycated hemoglobin; MBS: Metabolic bariatric surgery; VSG: Vertical sleeve gastrectomy.

FOOTNOTES

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Abstract

BACKGROUND

Intestinal micro-ecological imbalances impair the intestinal barrier and induce intestinal inflammation, for example, ulcerative colitis (UC). According to the latest research, abnormalities in intestinal microbiota structure and their metabolites play a dominant role in UC progression; in addition, they could affect the mucus barrier based on different factors. Although numerous studies have confirmed the important role of intestinal microbiota in UC pathogenesis, the intricate connection between microbiota and metabolites and mucus barrier in UC occurrence remains unclear, and correlation analyses of differential microbiota and their metabolites under UC are relatively scarce.

AIM

To reveal the differential intestinal microbiota and metabolites in UC pathogenesis and explore more sensitive biomarker compositions.

METHODS

We used the antibiotic combination method to establish intestinal pseudo-aseptic mice; afterward, dextran sulfate sodium (DSS) was applied to establish an acute experimental colitis mice model. Colitis severity, assessed based on disease activity index, colorectal length, colorectal wet weight, and histological lesions, and mucus-related staining (mucopolysaccharide alcian blue and immunofluorescence of mucin), was compared between the pseudo-aseptic and bacterial colitis mice. Finally, differential intestinal microbiota, metabolites, and their association and correlations, were analyzed by 16s rDNA sequencing in combination with non-targeted metabolomics, through gas chromatography-mass spectrometry.

RESULTS

Compared with the pseudo-aseptic mice, intestinal bacteria positive mice were more severely ill and their intestinal mucus loss was more pronounced in DSS-induced colitis ($P < 0.05$), suggesting that different microbiota and metabolites could cause the different degrees of colitis. Subsequently, we observed that in addition to *Klebsiella*, and *Bacteroides*, which were widely associated with colitis, *Candidatus Stoquefichus*, *Anaerobiospirillum*, *Muribaculum*, and *Negativibacillus* may be involved in protection against colitis. Furthermore, differential metabolites of the microbiota were mainly enriched in the synthesis-related pathways of key structural sequences of mucin. In combination with the mucin-related staining and immunofluorescence results, the findings indicate that the differential microbiota and their metabolites potentially regulate the composition and function of mucus under colitis.

CONCLUSION

Microbiota and their metabolites are major factors regulating the composition and function of mucus, in turn influencing the function and structure of intestinal mucus barrier under colitis. The different microbiota and metabolites identified in the present study could be novel biomarkers for colitis.

Key Words: Ulcerative colitis; Gut microbiota; Metabolites; Dextran sulfate sodium; Mucin

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Core Tip: We observed that the differences in microbiota and their metabolites can cause different degrees of colitis. The differential metabolites of the microbiota in colitis are mainly enriched in pathways related to the key structural sequence synthesis of mucin, and the different levels of the metabolites lead to differential expression of mucin and mucus. Therefore, the differential metabolites of colitis could regulate the composition and function of mucus. In addition, the differential intestinal microbiota and their metabolites in mice with colitis were largely associated with amino acid and energy metabolism.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic nonspecific inflammatory disease of the colorectum and has an unknown cause. UC is more likely to occur in young and middle-aged people. Its main clinical symptoms include diarrhea, mucopurulent bloody stools, and a variety of extraintestinal manifestations, such as peripheral arthritis, erythema nodosum, anterior uveitis, and others that seriously affect the quality of life of patients. The World Health Organization lists UC as one of the most difficult modern diseases and as one of the most significant research topics in the field of digestion because it is difficult to cure, easily recurs, has a high risk of cancer, and often requires lifelong medication.

UC pathogenesis is complex and considered to be mediated by genetic susceptibility, microbial dysregulation, and environmental factors[1]. The intestinal barrier is a complex and prominent defense system that not only prevents the transfer of various toxins through the intestines into tissues and organs but also prevents the invasion of endogenous microorganisms, which is particularly prominent in maintaining homeostasis in the intestine and even the body[2]. The intestinal barrier consists of a mechanical, chemical, biological, and immune barrier. The role of biological barriers in UC is receiving

increasing attention. The latest research shows that abnormalities of the intestinal microbiota and its metabolites play a more important role in the progression of the course of UC[3]. In addition to being involved in constituting a biological barrier, the intestinal microbiota and metabolites also have the functions of biological antagonism, defense against infection, participation in the maturation of the immune system, regulation of intestinal mucus, regulation of intestinal epithelial metabolism, and nutrition[3].

Studies have confirmed that the number of goblet cells in the intestine of aseptic reared mice decreases, their morphology becomes smaller, the mucus layer becomes thinner, and the permeability increases compared with conventionally reared mice[4]. Jakobsson *et al*[5] found that in C57BL/6J mice with two different intestinal microbiotas, although the thickness of the colonic mucus was similar, there was a significant difference in the permeability of the mucus barrier. Several other studies have found that intestinal microbiota and metabolites can affect mucus through the following effects: (1) Regulation of mucus synthesis: the microbiota's structures, such as lipopolysaccharides, flagella proteins, and inflammatory factors induced by induction, can directly regulate the mucus 2 protein, Mucin2 (MUC2) to produce mucus; probiotics can also provide the relevant nutrients required for MUC2 synthesis to upregulate its transcription; bacterial products and the inflammatory factor secretion caused by them also induce the formation of sulfotransferase, sulfate MUC2, and enhance the anti-inflammatory capacity of the intestine[6,7]; (2) Mucus secretion regulation: active substances, such as lipopolysaccharides and secondary bile salts, stimulate goblet cells to secrete MUC2 into the intestinal cavity to flush bacteria and resist invasion, but excessive MUC2 secretion causes goblet cell depletion and reduces the anti-infection ability of the intestinal mucosa[8,9]. It has also been suggested that inflammasomes can maintain the intestinal environment and microbiota homeostasis by promoting the production of interleukin-18 in sensing intestinal bacterial products or cell damage[10]; and (3) Mucus degradation regulation: primarily the slow degradation of probiotics represented by *Akkermansia muciniphila*, which is used to maintain the dynamic balance of the mucus layer and the rapid degradation of *Escherichia coli*, a representative of pathogenic bacteria that destroys the integrity of the mucus layer.

Therefore, the intestinal microbiota and its metabolites can directly constitute a biological barrier and affect the mucus barrier to play a multi-dimensional regulatory role. It can be seen that the intestinal microbiota and metabolites have a central position in the pathogenesis of UC. At present, although many studies have confirmed the important role of intestinal microbiota in the pathogenesis of UC, the intricate connection between microbiota and metabolites in UC occurrence remains unclear, and the correlation analysis of UC differential microbiota and its metabolites is relatively scarce, suggesting the need to carry out further studies.

Based on the intestinal microbiota and metabolism, this study uses a combination of antibiotics to establish pseudo-aseptic mice. We followed the internationally accepted dextran sulfate sodium method to establish a UC model. By comparing the severity of the disease, mucus-related protein expression, and 16s rDNA sequencing of pseudo-aseptic and bacterial UC mice, we evaluated the practical significance of intestinal microbiota in UC pathogenesis in multiple dimensions. Further, through microbiota sequencing combined with non-targeted metabolomics association analysis, we tried to reveal the differential intestinal microbiota and microbiota metabolites in UC pathogenesis, exploring more sensitive biomarker compositions and providing new ideas for diagnosing UC.

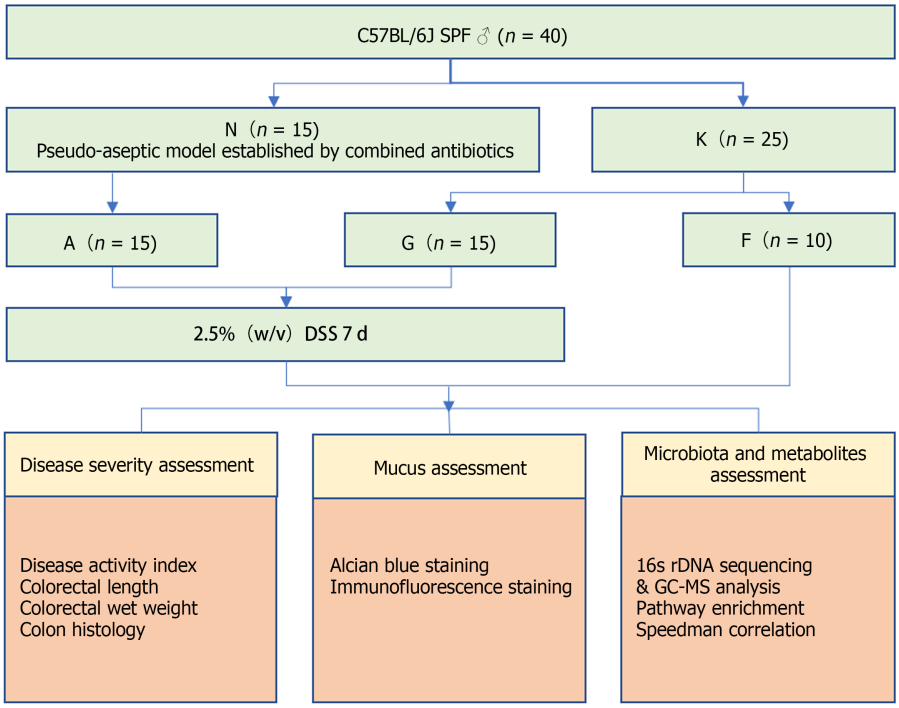
MATERIALS AND METHODS

Experimental animals

The animal protocol was designed to minimize pain or discomfort to the animals. Forty male C57BL/6J mice, specific pathogen free (SPF) grade, weighing 20 ± 2 g were purchased from SPF Biotechnology Co., Ltd. (Beijing, China, certificate No. SCXK-2019-0010). They were routinely raised in the SPF-level animal room of Beijing University of Chinese Medicine, with a 12-h light/night cycle, temperature of 22 ± 2 °C, and humidity of 50%-60% and were given access to unlimited food and drinking water. The experimental process complied with the ethical requirements formulated by the Laboratory Animal Ethics Subcommittee of the Academic Committee of Beijing University of Chinese Medicine (No. BUCM-2020-01162). The study design was shown in Figure 1.

Establishment of pseudo-aseptic models

Amphotericin B (catalog No. 1397-89-3), vancomycin hydrochloride (catalog No. 1404-93-9), neomycin sulfate (No. 1405-10-3), metronidazole (No. 443-48-1), and ampicillin sodium (No. 69-52-3) were all purchased from China Shanghai Macklin Biochemical Co., Ltd. After 1 wk of adaptive rearing of all mice, 40 mice were randomly divided into the pseudo-aseptic group (N group, n = 15) and the non-intervention group (K group, n = 25) using the random number table method. A schematic diagram of the model to replicate the pseudo-aseptic model of N group mice is shown in Figure 2A. Gastric lavage (0.1 mL/10 g) was first given with amphotericin B (1 mg/kg body weight) for 3 d, and from day 4, 1 mg/mL of ampicillin was added to drinking water. Meanwhile, mice were given a mixture of antibiotics



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Figure 1 Study design. N: Pseudo-aseptic group; K: Blank group; A: Microbiota$^-$ colitis group; F: Blank group; G: Microbiota$^+$ colitis group; DSS: Dextran sulfate sodium; GC-MS: Gas chromatography-mass spectrometry.

that included vancomycin 50 mg/kg body weight, neomycin 100 mg/kg body weight, metronidazole 100 mg/kg body weight, and amphotericin B 1 mg/kg body weight every 12 h for a total of 21 d (*i.e.*, 4-24 d)[11].

Establishment of a colitis model

Pseudo-aseptic mice were labeled as pseudo-aseptic colitis model groups (group A, n = 15), and non-intervention mice were randomly divided into the following two groups using the random number table method: Blank group (group F, n = 10) and bacterial colitis model group (group G, n = 15). In addition to the blank group, the remaining 30 mice were free to drink 2.5% (w/v) dextran sulfate sodium (DSS) solution (molecular weight: 36000-50000 Da; MP Biomedicals, Santa Ana, CA, United States) to establish an acute experimental colitis model.

Analysis of the disease activity index

The disease activity index (DAI) score includes the following three indicators: weight loss rate, stool consistency, and degree of fecal occult blood. The DAI score = (weight loss rate score + stool consistency score + fecal occult blood score)/3[12], the specific scoring criteria are shown in Table 1.

Measurement of colorectal length

The abdominal cavity was exposed, and the pelvis was cut from the anus with scissors. The fascia and other structures from the rectum were cut with forceps and ophthalmic scissors upwards. An ileocecal mass, a large bulge, was discovered, cutting from the lower end of the ileocecal to the rectum, and the colorectal length was measured.

Measurement of colorectal wet weight

The removed colorectal wet weight was measured before dipping in the phosphate buffer solution, and the intestinal tube was cut along the longitudinal axis before weighing. The feces in it were cleaned up, and the wet weight of the intestinal tube was recorded.

Colonic histological lesions score

After the specimen was fixed with 4% paraformaldehyde for 24 h, 4 μm sections were prepared after paraffin embedding. The histological lesions of the colon were observed under light microscopy after hematoxylin-eosin (HE) staining. The standard histological lesions scoring was used[13]. Table 2 provides the scores, with a total score of 0-16. One slice was made per mouse, and the average score of seven items per specimen was calculated.

Table 1 Disease activity index assessment standards

Score	Weight loss rate	Stool consistency	Fecal occult blood
0	< 1%	Normal	Negative
1	1%-5%		
2	5%-10%	Soft stool (mushy)	Positive
3	10%-15%		
4	> 15%	Watery stools (mostly near the anus)	Visible bloody stools or hematochezia

Weight loss rate = (weight the day before the material is taken - weight 2 d before the material is taken)/the weight of the day before the material is taken × 100%.

Table 2 Scoring criteria for histological lesions in the colon

Content	Score				
	0	1	2	3	4
Goblet cells	No loss	Mild loss	Moderate loss	Severe loss	
Mucosal thickness	No change	Mild thickening	Moderate thickening	Severe thickening	
Inflammatory cells	No infiltration	Mild increase	Moderate increase	Severe increase	
Submucosal inflammatory cell infiltration	No infiltration		Mild increase	Moderate increase	Severe increase
Degree of disruption of mucosal structures	No damage			Mild destruction	Moderate destruction
Percentage of epithelial cell ulcer area	No ulcers	0%-25%	25%-50%	50%-75%	75%-100%
Number of crypt abscesses	No	1–3	4–6	7–9	> 10

Colon tissue mucopolysaccharide alcian blue staining

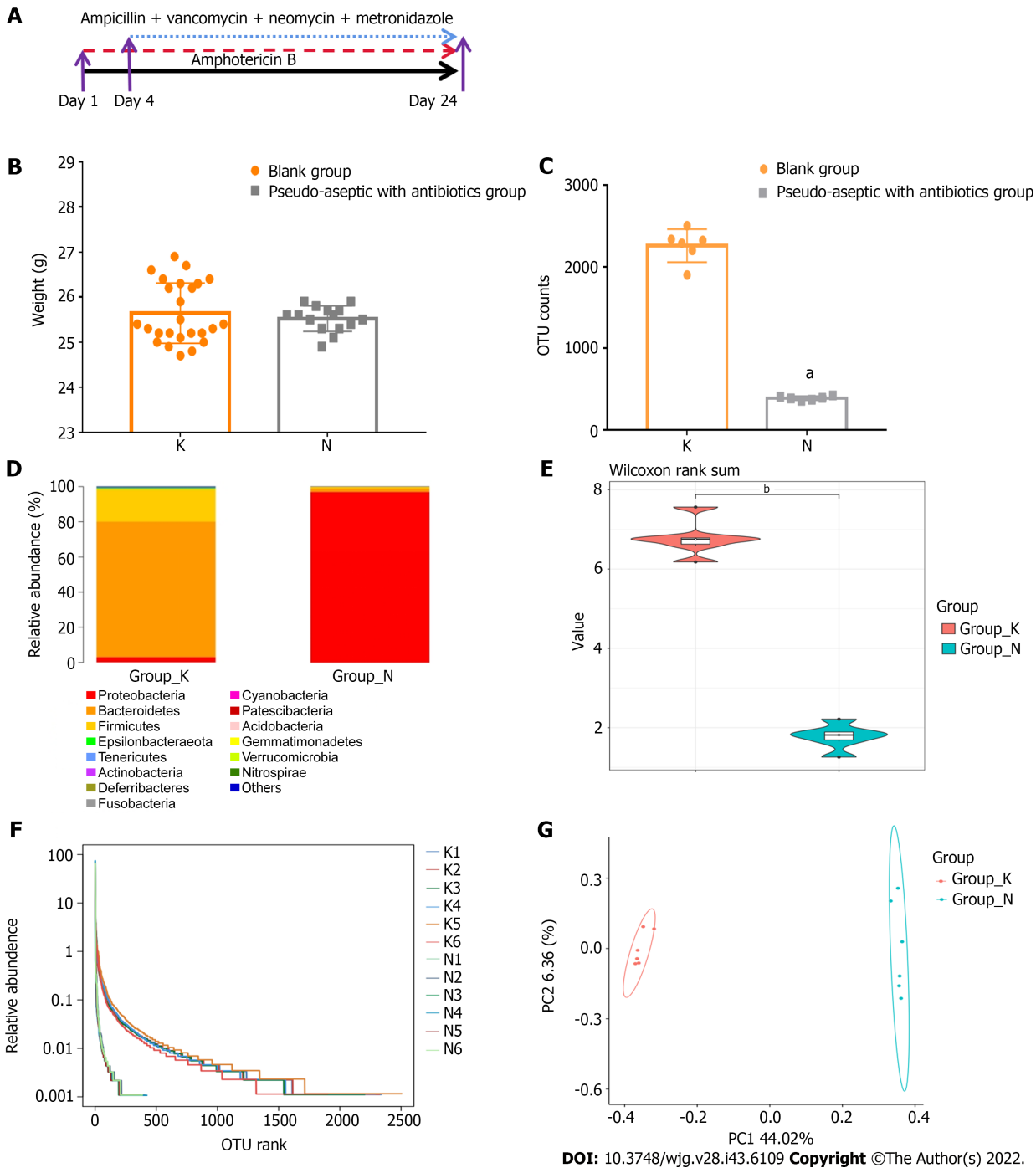
The paraffin sections were stained with alcian blue dye, and the HE staining step was continued. This was followed by washing, counterstaining, dehydrating, and clearing post-sealing. A panoramic scanner was used to scan the specimen, and the Caseviewer software (Hungarian 3D HISTECH, Pannoramic 250/MIDI, version 2) took pictures. Each sample was photographed after setting the white balance of the high magnification field of view (× 400), and Image J software (National Institutes of Health, United States) was used to analyze the images. The area of positive staining was selected for statistical analysis.

Colonic MUC2 immunofluorescence staining

Paraffin sections were de-waxed and then repaired by antigens. Primary antibodies (MUC2, 1:200, GB11344, Wuhan Servicebio Biotechnology Co., Ltd., Wuhan China), secondary incubated antibodies, and 4′,6-diamidino-2-phenylindole-stained nuclei were added after fluorescence quenching and serum blocking. Each sample was photographed and collected after setting a high magnification field of view (× 200) white balance; the image was analyzed using the Image J software mentioned above. The percentage of the positively stained area (area%) was selected for statistical analysis.

16s rDNA sequencing of the intestinal microbiota

The genomic DNA of the sample was extracted using a DNA extraction kit, followed by agarose gel electrophoresis. NanoDrop2000 was used to detect the concentration of the DNA. Takara's Tks Gflex DNA Polymerase was used for polymerase chain reaction (PCR), ensuring amplification efficiency and accuracy using genomic DNA as a template and specific primers with barcodes. Identification of the microbiota diversity corresponding V3-V4 regions was done using primers 343F and 798R. This study sample was assayed for mouse fecal microbiota, and the V3-V4 zone forward primer was set as 343F-5′-TACGGRAGGCAGCAG-3′, and the reverse primers used were: 798R-5′- AGGGTATCTAATCCT-3′. The PCR products were detected by electrophoresis, purified with magnetic beads after detection, purified as a two-round PCR template, amplified by two-round PCR, and then used for electrophoresis detection, purification with magnetic beads after detection, and Qubit quantification of the PCR products after purification. The PCR products were mixed in equal amounts according to PCR product concentrations and sequenced on a PCR machine (580BR10905; Bio-Rad Laboratories, Hercules, CA, United States). Using Vsearch (version 2.4.2) software, the high-quality sequence valid tags obtained by quality control were operational taxonomic unit (OTU) classified according to 97% similarity. The



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Figure 2 The combination of five antibiotics could effectively build an intestinal pseudo-aseptic mouse model. A: Antibiotics applied; B: Body weight; C: Operational taxonomic unit counts; D: Microbiota structure; E: Shannon index of alpha-diversity; F: Rank abundance of alpha-diversity; G: Beta-diversity. N: Pseudo-aseptic with antibiotics group; K: Blank group. Compared with the blank group, $^{a}P < 0.01$, $^{b}P < 0.05$.

sequence with the largest abundance in each OTU was selected as the representative sequence of the OTU. The Ribosomal Database Project classifier naive Bayesian classification algorithm was used to compare the representative sequence with the database to obtain the OTU annotation information.

Aseptic conditions were used to collect mouse feces. The feces were retained on clean filter paper, immediately picked up with disinfected forceps, placed in cryopreservation tubes, frozen in liquid nitrogen, and stored in a -80 °C ultra-low temperature freezer for subsequent use in the sequencing of fecal microbiota. The biological information for differential microbiota compared on a website (https://cloud.oebiotech.cn/task/) included the following: OTU abundance, community distribution statistics of microbiota, alpha diversity analysis, beta diversity analysis, and microbial multivariate statistical analysis [differential species heat map and 16S-based Kyoto Encyclopedia of Genes and Genomes (KEGG) function prediction].

Gas chromatography-mass spectrometry analysis and identification of intestinal microbiota metabolites

Gas chromatography-mass spectrometry (GC-MS) was used to detect and identify metabolites of the fecal microbiota. Processes included: sample pretreatment, metabolite extraction, metabolite derivatization, GC-MS detection, data pretreatment, and statistical analysis. The chromatographic conditions of this study were: DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J&W Scientific, Folsom, CA, United States), high purity helium carrier gas (purity not less than 99.999%), flow rate 1.0 mL/min, and a temperature of 260 °C at the inlet. The injection volume was 1 μL, the injection was not shunted, and the solvent was delayed by 5 min. Program heating was as follows: The initial temperature of the column oven was 60 °C, maintained for 0.5 min, heated to 125 °C at a rate of 8 °C/min, heated up to 210 °C at a rate of 5 °C/min, heated up to 270 °C at a rate of 10 °C/min, and heated up to 305 °C at a rate of 20 °C/min for 5 min. Mass spectrometry conditions: Electron bombardment ion source, ion source temperature of 230 °C, four-stage rod temperature of 150 °C, and electron energy of 70 eV. The scanning method was full scan mode, and the quality of scanning ranged from m/z 50-500. Metabolite data analysis included multivariate statistical analysis, univariate statistical analysis, differential metabolite screening, correlation analysis, and metabolic pathway enrichment analysis.

Statistical analysis

SPSS 22.0 software (SPSS Inc., Chicago, IBM, United States) was used to analyze all data. Measurements that conform to the normal distribution were expressed as mean ± SD, with *t*-test analysis for comparisons between two groups and one-way ANOVA for multi-group comparisons. Fischer's least significant difference was used for homogeneous variances and Tamhane's T2 for unequal variances. The measurement data of the abnormal distribution were expressed as median and quartile spacing (P25, P75), with the Mann–Whitney nonparametric test used for comparison between two groups and the Kruskal-Wallis nonparametric test used for multi-group comparison. The intergroup comparison of the counting data was expressed using median and interquartile spacing (P25, P75), and the Kruskal-Wallis nonparametric test was used for multi-group comparisons of the counting data. The statistical methods of the present study were reviewed by Prof. Zhao-Lan Liu from the Center for Evidence-based Chinese Medicine, Beijing University of Chinese Medicine.

RESULTS

Combination of five antibiotics could effectively establish an intestinal pseudo-aseptic mouse model

After 24 d of combined antibiotic application, there was no significant difference in body weight between pseudo-aseptic mice and mice in the non-intervention group (25.52 *vs* 25.64, $P > 0.05$, Figure 2B). The number of OTUs in the intestinal microbiota of non-intervention mice was significantly higher than that of pseudo-aseptic mice (2259.50 *vs* 389.83, $P < 0.01$, Figure 2C), indicating that the abundance of intestinal microbiota in pseudo-aseptic mice was significantly reduced. Subsequently, a columnar accumulation of species relative abundance at the phylum taxonomic level was plotted, showing the top 15 species in abundance, and it was found that the intestines of non-intervention mice were dominated by the *Bacteroidetes* (77.1%) and *Firmicutes* phyla (18.8%). In contrast, the intestinal communities of pseudo-aseptic mice were dominated by the *Proteobacteria* phylum (96.9%), with a distinctly single community structure (Figure 2D). A further selection of the Shannon index for the violin plot showed that the species richness and microbiota community diversity of the intestinal microbiota of the non-intervention mice were significantly greater than those of the pseudo-aseptic group (6.75 *vs* 1.78, $P < 0.01$, Figure 2E). The Rank Abundance analysis found that the span of the horizontal axis of the non-intervention mice curve was significantly larger than that of the pseudo-aseptic mice, indicating that the composition of the non-intervention mouse microbiota was relatively rich, and the span of the longitudinal axis of the non-intervention mice curve was smaller than that of the pseudo-aseptic mice, indicating that the species composition of the non-intervention mice microbiota was more uniform (Figure 2F). Further beta diversity analysis found that the two groups of microbiota differed substantially between groups and could be significantly separated (Figure 2G). Based on the above results, it was confirmed that the combined antibiotic method adopted by this study could effectively remove the intestinal microbiota of mice and establish an intestinal pseudo-aseptic mouse model, and its microbiota abundance, community structure, and alpha and beta diversity are significantly reduced.

Mice with bacteria were more severely ill in DSS-induced colitis

On day 2 of taking the DSS solution, stool changes and fecal occult blood test were positive, and the mice gradually lost weight from day 3 onwards. The symptoms gradually worsened with the prolongation of the DSS drinking time. During the entire molding period, four mice died in the pseudo-aseptic group and five mice died in the bacterial group. Performing DAI scoring on the last day of molding revealed an increase in both groups of colitis mice and greater severity of illness in the bacterial

mice (Figure 3A). After dissecting the intestinal tube, the length of the colorectum and the wet weight of the intestinal tube were measured, and it was found that the intestines of the two groups of colitis mice were significantly shortened and that the wet weight was significantly increased, especially in the bacterial mice. (Figures 3B-D). Subsequently, 10 mice in each group were selected for histological lesion evaluation of the intestinal mucosa, and the results showed that the four layers of colonic mucosa structure in the blank group were clear, the goblet cells were abundant, there was no obvious inflammatory cell infiltration, and the mucosal thickness was normal. In contrast, the mucosal structure of the colitis mice was destroyed; the four-layer structure disappeared, the inflammatory cells infiltrated, the submucosal layer was partially affected, and no obvious crypt abscess was seen (Figure 3E). The histological performance of pseudo-aseptic mice was between blank and bacterial mice, and the histological lesions were light. The score was significantly lower than that of bacterial mice (Figure 3F).

Intestinal mucus loss was more pronounced in DSS-induced colitis mice with bacteria

HE staining revealed that goblet cells in the intestinal mucosa of colitis mice were reduced. Then, through alcian blue staining, it was found that the colon mucosa of blank mice was rich in mucopolysaccharides and goblet cells. The mucopolysaccharides and goblet cells in the colons of mice with colitis were significantly reduced, the mucus layer was interrupted, and the goblet cells were significantly reduced and vacuolated (Figures 4A and B), and the characteristics of mucus of pseudo-aseptic mice came in between those of blank and bacterial mice. Immunofluorescence staining of MUC2, the most important structural and functional mucus protein, was significantly decreased ($P < 0.01$) in the intestines of the bacterial colitis model compared with those of the blank group. Expression of MUC2 in the pseudo-aseptic colitis mice came in between that of the blank and bacterial mice (Figures 4C and D).

The above two studies suggest that different intestinal microbiota have different effects on mucin and mucus, and the degree of colitis that eventually forms is different. The method of free drinking DSS solution can successfully establish a colitis model, but the symptoms of colitis, disease activity, intestinal mucosal histological structure, and mucus distribution in pseudo-aseptic mice are less than those in intestinal bacterial mice. The appearance of this difference may be related to the diversity of the intestinal microbiota or the difference in microbiota metabolites between pseudo-aseptic and bacterial colitis mice. The study then used 16s rDNA sequencing combined with non-targeted metabolomics technology to reveal this characteristic difference, further explore the potential differential intestinal microbiota and metabolites of colitis mice, and screen out the characteristic differential microbiota and metabolites in colitis.

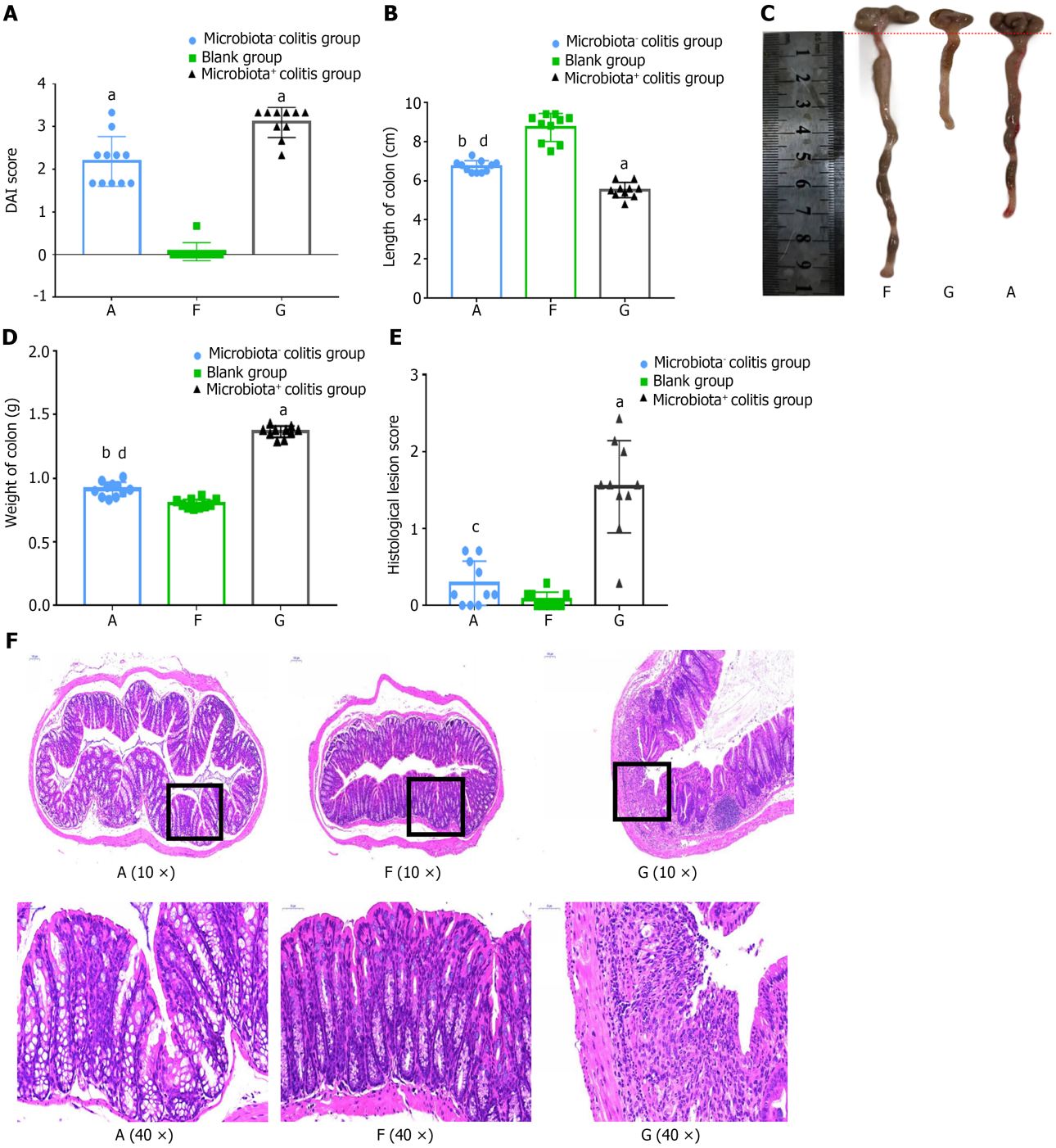
Analysis of different characteristic microbiota in DSS-induced colitis mice

Six mice were randomly selected from each group by the random number table method for 16s rDNA microbiota sequencing, and by comparing the number of OTUs, it was found that the OTU number of the intestinal microbiota of blank group mice was significantly higher than that of mice with bacteria ($P < 0.01$, Figure 5A). Subsequently, the diversity index dilution curve with goods coverage index and specaccum species accumulation curves were constructed, and the results showed that the two curves tended to be stable at the end, indicating that the sequencing data of each sample were sufficient and that sampling was sufficient (Figures 5B and C). The observed species index was used to plot the box plot and found that the alpha diversity of the intestinal microbiota of mice with bacterial colitis was significantly lower than that of the blank group (Figure 5D). Beta diversity analysis of the composition of the intestinal microbiota of the two groups of mice was further studied, and the results showed that the two groups were separated farther apart, which proved that there was a significant difference (Figure 5E).

Subsequently, Wilcoxon rank sum test was used for clustering at the genus taxon level of the microbiota, and Wilcoxon was performed on the different species of intestinal microbiota, and 50 different species with P (FDR) < 0.05 and names were selected for heat map cluster analysis (Figure 5F). Compared with that in the blank group, it was found that the following bacterial genera in the intestinal microbiota of mice with bacterial colitis were significantly increased ($P < 0.05$): *Anaerobiospirillums*, *Rikenellaceae RC9* gut group, *Prevotellaceae Ga6A1* group, *Escherichia-Shigella*, *Klebsiella*, *Anaerotruncus*, *Negativibacillus*, *Candidatus Stoquefichus*, *Blautia*, *Verrucomicrobium UBA1819*, *Helicobacter*, and *Lachnoclostridium*. The above bacterial genera were significantly elevated in mice with colitis and can be considered harmful bacteria that cause the occurrence or aggravation of colitis.

The bacterial genera that significantly decreased ($P < 0.05$) in the intestinal microbiota of mice with bacterial colitis were: *Bacteroides*, *Prevotellaceae UCG-001*, *Prevotellaceae NK3B31*, *Acetatifactor*, *Muribaculum*, *Lactobacillus*, *Eubacterium coprostanoligenes*, *Candidatus Saccharimonas*, and *Eubacterium brachy*. The above bacterial genera were significantly reduced in colitis mice, and we think that they may be able to prevent or mitigate the occurrence and development of colitis.

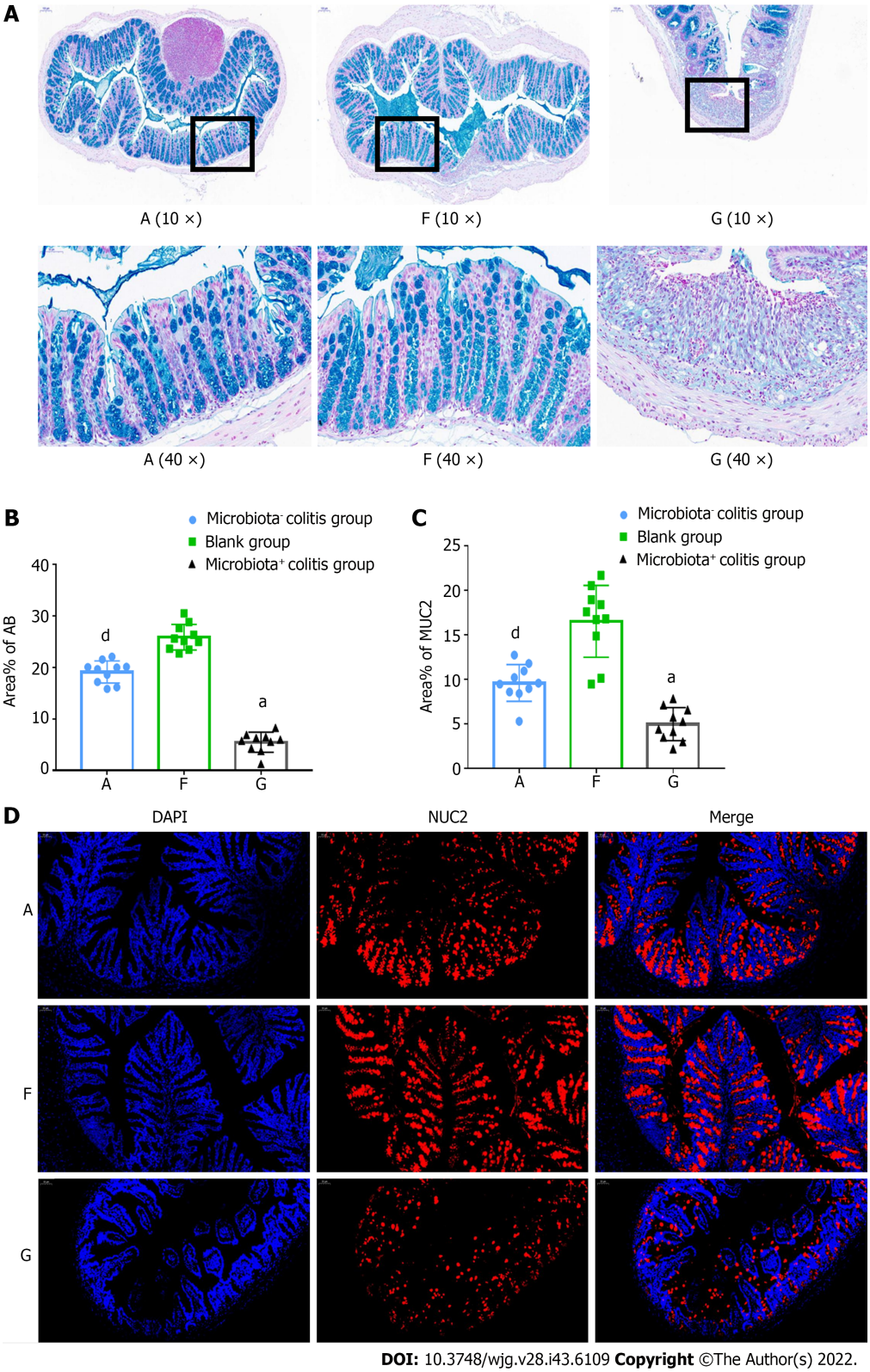
Phylogenetic Investigation of Communities by Reconstruction of Unobserved States analysis was then used to predict the composition of differential microbial gene functions of known phylum level classifications, and the predicted KEGG results were displayed using cluster heat maps (Figure 5G). The study found that in the classification of the phylum level there were 28 KEGG signaling pathways with obvious enrichment of different microbiota in mice with colitis. Combined with the characteristics of the colitis disease itself and P (FDR) values, we deduced that the pathways involved were, from large to



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Figure 3 Mice with bacteria were more severely ill in dextran sulfate sodium-induced colitis. A: Disease activity index score; B: Colon length; C: Colon photos; D: Colon weight; E: Histological lesions score; F: Hematoxylin-eosin staining. A: Microbiota⁻ colitis group, F: Blank group, G: Microbiota⁺ colitis group. DAI: Disease activity index. Compared with the blank group, $^{a}P < 0.01$, $^{b}P < 0.05$. Compared with microbiota⁺ colitis group, $^{c}P < 0.01$, $^{d}P < 0.05$.

small according to the difference in significance: biodegradation and metabolism of symbionts, cell growth and death, biosynthesis and metabolism of glycans, energy metabolism, immune system diseases, digestive system, nucleotide metabolism, metabolic diseases, metabolism of terpenoids and polyketone compounds, amino acid metabolism, protein folding, classification and degradation, cancer, metabolism of cofactors and vitamins, immune system, metabolism of other amino acids, transport and catabolism, lipid metabolism, cellular processes and signaling, signaling molecules and interactions, and enzyme families. In summary, it was observed that the functional prediction of differential intestinal microbiota was mainly concentrated in the pathways related to the metabolism of various substances. Thus, we carried out further research on microbiota metabolism.

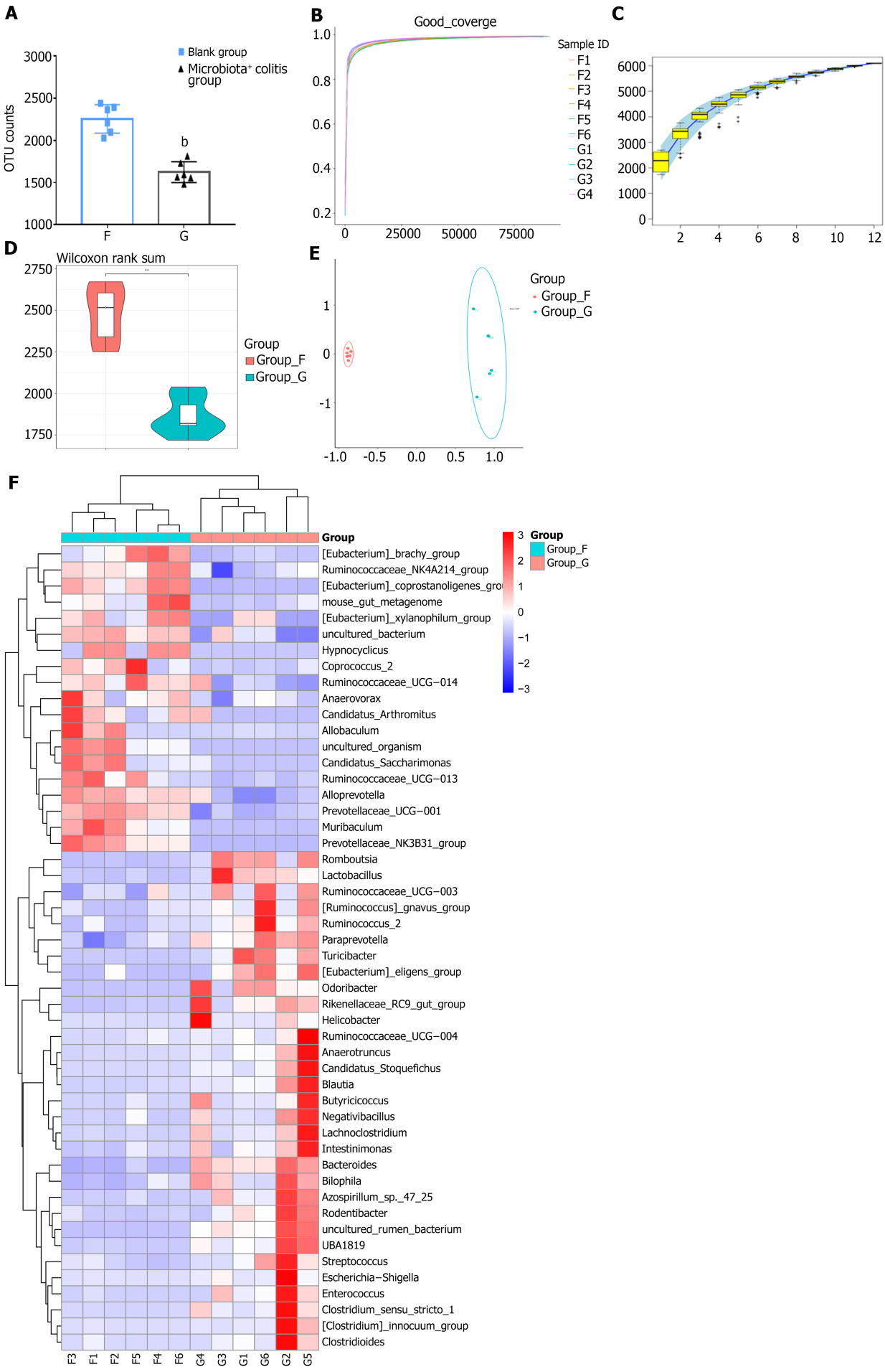


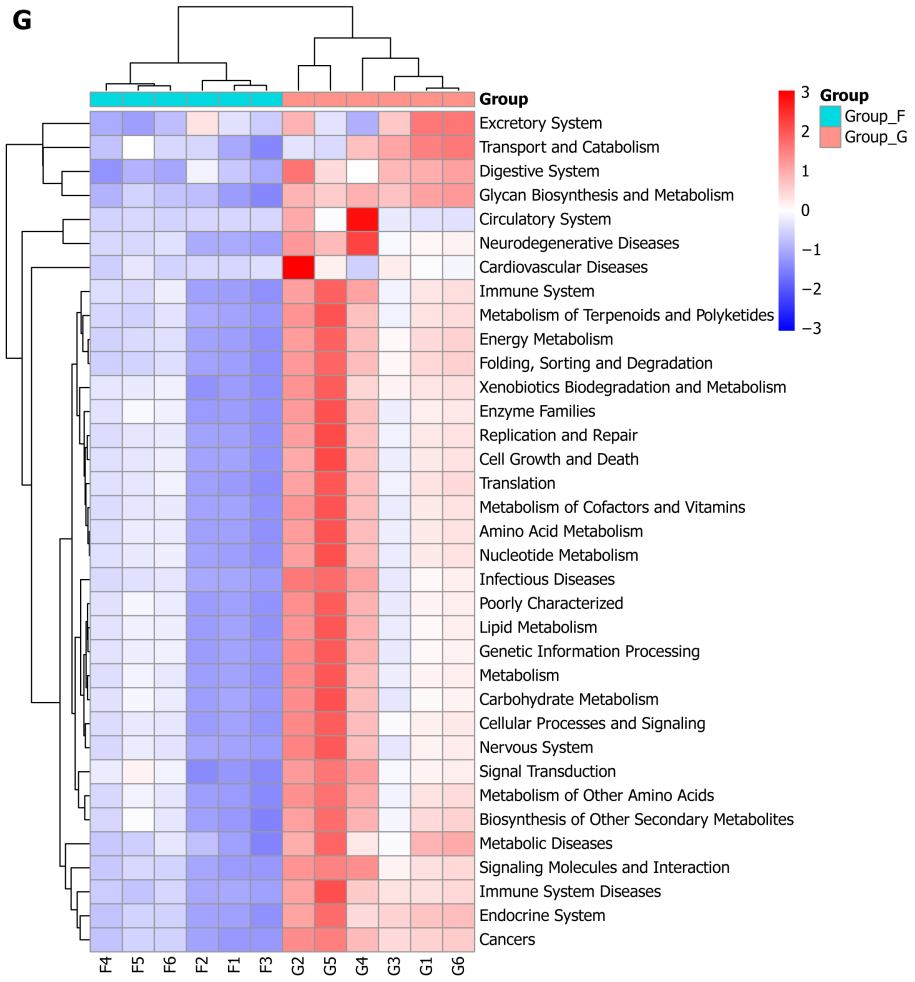
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Figure 4 Intestinal mucus loss was more pronounced in dextran sulfate sodium-induced colitis mice with bacteria. A: Alcian blue staining; B: Percentage of positively stained areas; C: Percentage of MUC2-positive stained areas; D: Mucin2 (MUC2) immunofluorescence staining. A: Microbiota⁻ colitis group, F: Blank group, G: Microbiota⁺ colitis group. MUC2: Mucin2. Compared with the blank group, $^{a}P < 0.01$. Compared with the microbiota⁺ colitis group, $^{d}P < 0.05$.

Analysis of different microbiota metabolites in DSS-induced colitis mice

Orthogonal partial least-squares discrimination analysis was used to distinguish the overall differences in metabolic profiles between groups and look for differential metabolites between groups. It can be found that the microbiota metabolites of the blank group and the colitis mice have good polymerization, the intra-group difference is small, and the separation between the groups is obvious, indicating that there are obvious differences between the two groups of metabolites (Figure 6A). The *P* values and fold


A
Blank group
Microbiota+ colitis group
OTU counts
3000
2500
2000
1500
1000
b
F
G
B
Good_coverge
1.0
0.8
0.6
0.4
0.2
0
25000
50000
75000
Sample ID
F1
F2
F3
F4
F5
F6
G1
G2
G3
G4
C
6000
5000
4000
3000
2000
1000
0
2
4
6
8
10
12
D
Wilcoxon rank sum
2750
2500
2250
2000
1750
Group
Group_F
Group_G
E
Group
Group_F
Group_G
1
0
-1
-1.0
-0.5
0.0
0.5
1.0
F
Group
Group_F
Group_G
3
2
1
0
−1
−2
−3
[Eubacterium]_brachy_group
Ruminococcaceae_NK4A214_group
[Eubacterium]_coprostanoligenes_gro
mouse_gut_metagenome
[Eubacterium]_xylanophilum_group
uncultured_bacterium
Hypnocyclicus
Coprococcus_2
Ruminococcaceae_UCG−014
Anaerovorax
Candidatus_Arthromitus
Allobaculum
uncultured_organism
Candidatus_Saccharimonas
Ruminococcaceae_UCG−013
Alloprevotella
Prevotellaceae_UCG−001
Muribaculum
Prevotellaceae_NK3B31_group
Romboutsia
Lactobacillus
Ruminococcaceae_UCG−003
[Ruminococcus]_gnavus_group
Ruminococcus_2
Paraprevotella
Turicibacter
[Eubacterium]_eligens_group
Odoribacter
Rikenellaceae_RC9_gut_group
Helicobacter
Ruminococcaceae_UCG−004
Anaerotruncus
Candidatus_Stoquefichus
Blautia
Butyricicoccus
Negativibacillus
Lachnoclostridium
Intestinimonas
Bacteroides
Bilophila
Azospirillum_sp._47_25
Rodentibacter
uncultured_rumen_bacterium
UBA1819
Streptococcus
Escherichia−Shigella
Enterococcus
Clostridium_sensu_stricto_1
[Clostridium]_innocuum_group
Clostridioides
F3
F1
F2
F5
F4
F6
G4
G3
G1
G6
G2
G5



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Figure 5 Analysis of different characteristic microbiota in dextran sulfate sodium-induced colitis mice. A: Operational taxonomic unit counts; B: Goods coverage index dilution curve; C: Specaccum species accumulation curve; D: Observed species violin graph of alpha-diversity; E: Beta-diversity; F: Heat map cluster analysis of 50 different species at the genus level; G: Heat map cluster analysis of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States analysis with Kyoto Encyclopedia of Genes and Genomes pathways at the phylum level. F: Blank group; G: Microbiota^{+} colitis group. Compared with the blank group, $^{b}P < 0.01$.

change values of the two groups performed on the *t*-test were visualized using the volcano plot (Figure 6B), and the differential metabolites between the two groups were further screened (the standard was set to the variable weight value > 1, and the *P* value of the *t*-test < 0.05). Subsequently, all significantly different metabolites were hierarchically clustered, and a clustered heat map was plotted (Figure 6C). After analysis, it was found that there were 7 kinds of metabolites with significant downregulation in the intestinal microbiota of mice with bacterial colitis, and according to the significant differences, the order was: Galacturonic acid, maltose, lactitol, D-ribose, pyrophosphate, lactulose, and N-acetyl-5-hydroxytryptamine; there were 50 kinds of metabolites significantly upregulated in the intestinal microbiota of mice with bacterial colitis, the top 10 metabolites by the significant difference in size were: D-tagletose, o-phosphateserine, 5-methoxytryptamine, spermine, ribonic acid, creatinine, pinitol, sarcosine, 3-hydroxybutyric acid, and phenol (Table 3). Subsequently, through correlation data matrix analysis (Figure 6E), the degree of correlation between significantly different metabolites was quantified, and it was found that the significantly upregulated differential metabolites in colitis were most closely related to pyrophosphate, lactulose, galacturonic acid, maltose, and lactitol, and the significantly upregulated metabolites were closely related to o-phosphate, sonol, L-tryptophan, butylenediamine, 5-methoxytryptamine, spermine, p-hydroxyphenylpropionic acid, and l-lactic acid.

Based on the KEGG database, the study used the KEGG ID of differential metabolites to enrich the metabolic pathways of differential metabolites, and the *P* (FDR) < 0.05 in the metabolic pathway was used as the significance of the enrichment of the metabolic pathway, and the significant enrichment signaling pathway of the top 20 was selected for bubble mapping (Figure 6D). The analysis found that the differential metabolites of colitis mice were mainly enriched in: biosynthesis of aminoacyl tRNA; arginine and proline metabolism; ABC transporter; biosynthesis of valine, leucine, and isoleucine; cAMP

Table 3 Differential metabolites of gut microbiota in dextran sulfate sodium-colitis mice

VIP	*P* value	log2 (FC)	Metabolites	Expression
1.760	0.011	2.207	Digalacturonic acid	↓
2.128	0.017	3.470	D-maltose	↓
1.747	0.017	1.927	Lactitol	↓
1.898	0.000	-2.342	D-tagatose	↑
2.323	0.000	-3.519	O-phosphoserine	↑
1.111	0.000	-0.845	5-methoxytryptamine	↑
1.659	0.000	-1.867	Spermine	↑
2.493	0.000	-4.048	Ribonic acid	↑
1.726	0.001	-1.952	Creatinine	↑
2.983	0.001	-5.714	Pinitol	↑
1.716	0.001	-2.032	Sarcosine	↑
1.327	0.002	-1.259	3-hydroxybutyric acid	↑
1.593	0.002	-1.715	Phenol	↑
2.771	0.002	-5.062	Ascorbic acid	↑
2.181	0.003	-3.167	Montanic acid	↑
1.985	0.003	-2.701	L-tryptophan	↑
1.857	0.003	-2.522	2,3-dihydroxypyridine	↑
3.490	0.003	-5.993	Putrescine	↑
1.090	0.004	-0.853	Shikimic acid	↑
1.303	0.004	-1.191	Malonic acid	↑
1.539	0.004	-1.639	L-lactic acid	↑
1.010	0.004	-0.779	L-threonine	↑
1.549	0.005	-1.665	Xanthosine	↑
1.277	0.007	-1.301	hydroxy-3-(4'-hydroxy-3'4-methoxyphenyl)propionic acid	↑
1.297	0.007	-1.238	Leucine	↑
1.113	0.008	-0.947	7-hydroxynicotinic acid	↑
1.766	0.008	-2.298	3,6-anhydro-d-galactose	↑
2.103	0.009	-3.424	3-deoxyhexitol	↑
1.162	0.011	-1.079	Thymidine	↑
1.259	0.013	-1.171	4-aminobutyric acid	↑
1.118	0.015	-1.076	Aminomalonate	↑
2.490	0.016	-4.348	Maltitol	↑
2.607	0.016	-4.806	Butane-2,3-diol	↑
1.388	0.018	-1.622	(4-hydroxyphenyl)-4-propionic acid	↑
1.138	0.018	-0.998	Threose	↑
1.283	0.020	-1.266	2-hydroxybutanoic acid	↑
1.148	0.021	-1.130	3-hydroxypalmitic acid	↑
1.081	0.022	-0.983	Epicatechin	↑
1.219	0.022	-1.150	L-valine	↑
1.415	0.023	-1.560	Erythronic acid	↑
2.556	0.026	-2.451	Ornithine	↑

1.935	0.028	-3.003	Diacetone alcohol	↑
1.000	0.029	-0.917	Beta-glutamic acid	↑
1.442	0.029	-1.651	Sinapinic acid	↑
1.874	0.031	-2.768	Atropine	↑
1.211	0.031	-1.903	L-asparagine	↑
1.103	0.035	-0.988	4',5-dihydroxy-7-glucosyloxyflavanone	↑
1.334	0.037	-1.861	L-glutamine	↑
2.530	0.039	-4.230	Adenosine	↑
1.070	0.039	-0.948	Pyruvic acid	↑
1.051	0.041	-0.985	Cerotinic acid	↑
1.026	0.043	-0.942	L-tyrosine	↑
1.646	0.043	-2.361	Urocanic acid	↑

Up-arrows represent upregulated metabolites, and down-arrows represent downregulated metabolites in microbiota$^+$ colitis mice.

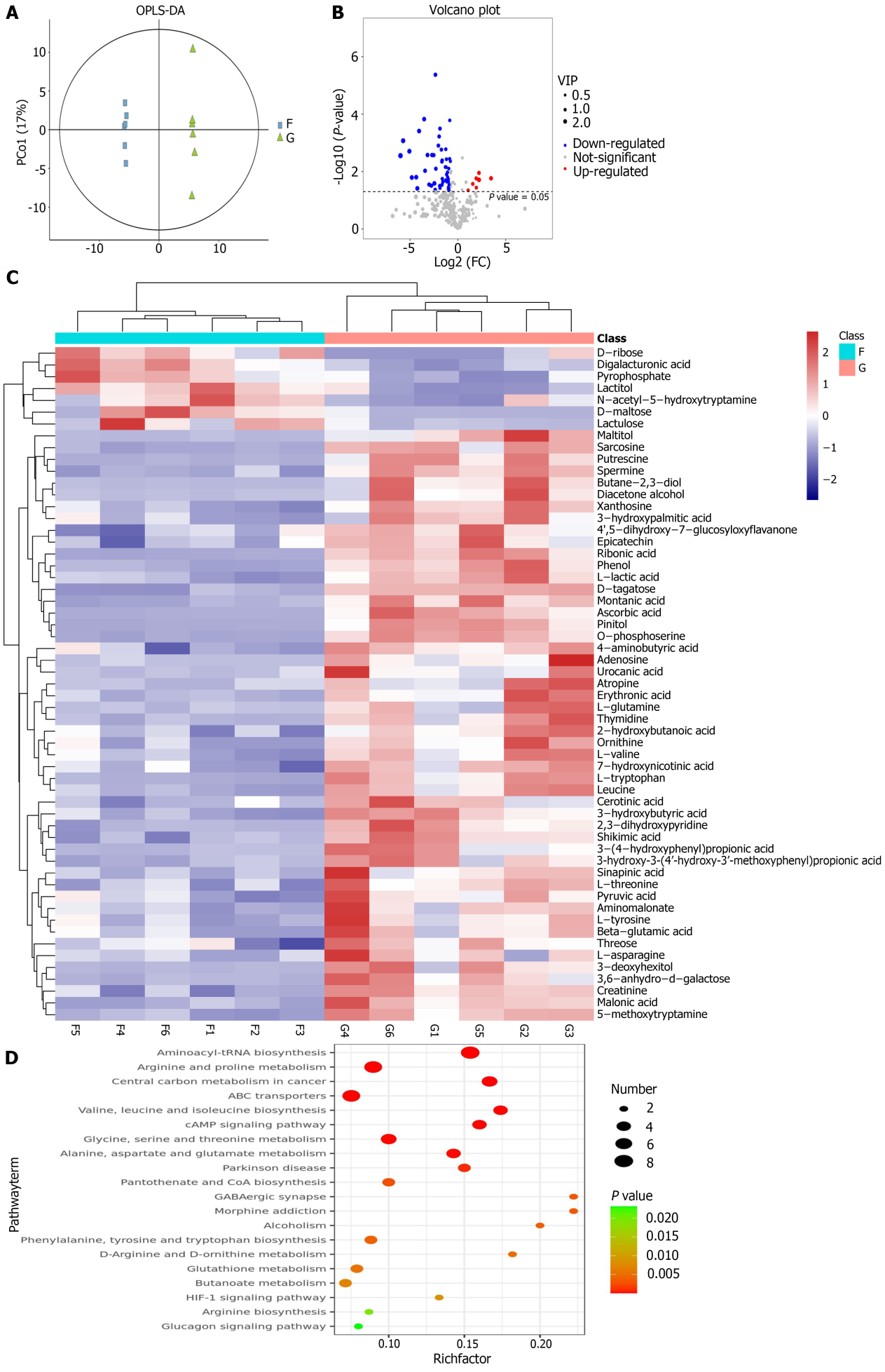
signaling pathway; metabolism of glycine, serine, and threonine; metabolism of alanine, aspartic acid, and glutamic acid; pantothenic acid and CoA biosynthesis; GABA synapses; biosynthesis of phenylalanine, tyrosine, and tryptophan; D-arginine and D-ornithine metabolism, glutathione metabolism, butyrate metabolism, and hypoxia-inducible factor-1 signaling pathways. In general, the differential metabolites of the intestinal microbiota of colitis mice are mainly concentrated in amino acid and energy metabolism.

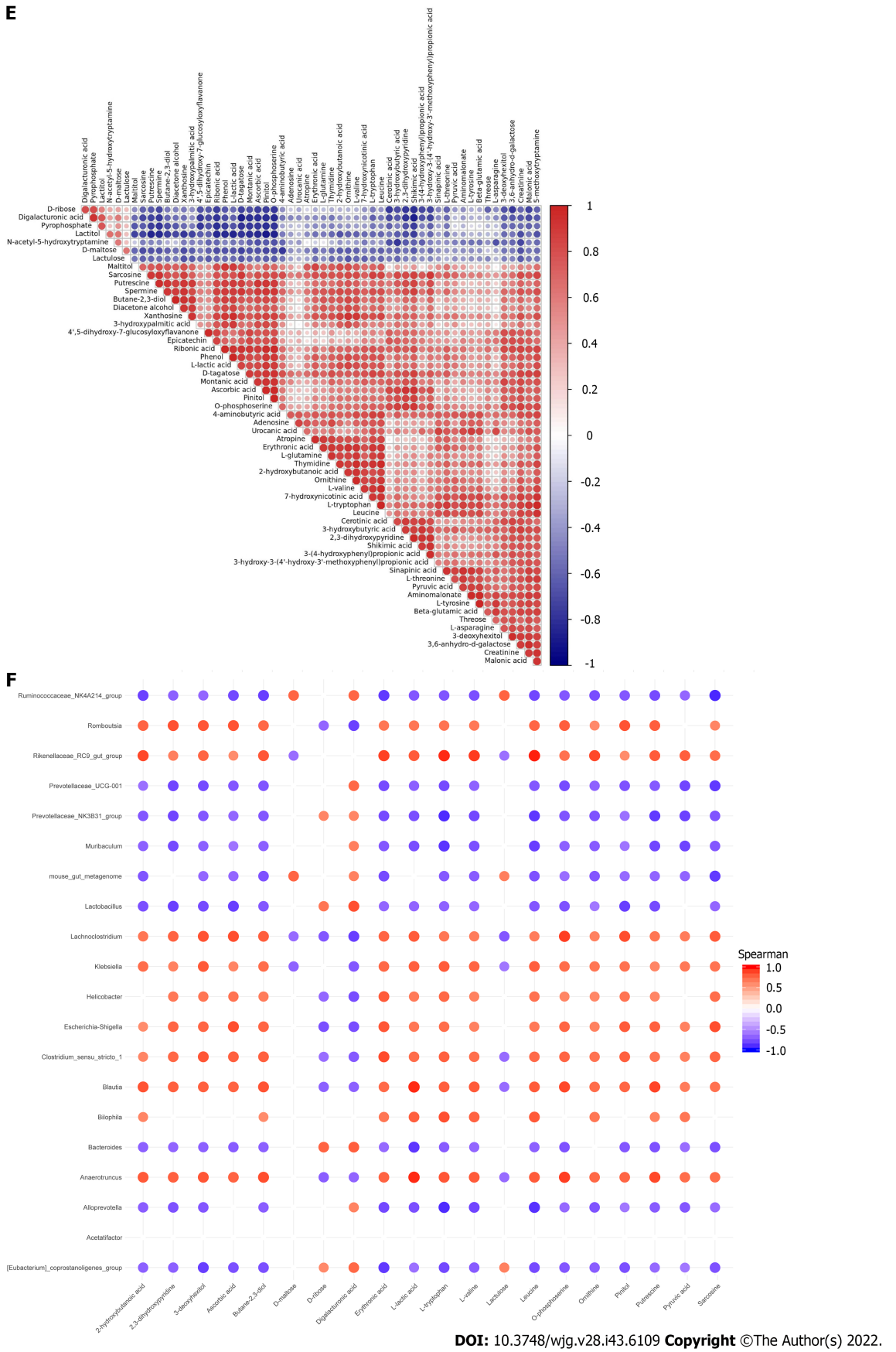
Finally, the correlation analysis of differential metabolites and differential microbiota species of the two groups of intestinal microbiota was carried out using Speedman correlation analysis, and the correlation matrix of the differential metabolites of top 20 and the microbiota at the genus taxonomic level was plotted (Figure 6F). The results showed that 3-deoxyhexanol, ascorbic acid, 2,3-butanediol, trihydroxybutyric acid, L-tryptophan, levo valine, leucine, o-phosphoserine, pineol, butylamine, and sarcosine were not only positively correlated with the genera significantly elevated in colitis ($P < 0.05$) but also significantly negatively correlated with the significantly reduced genera ($P < 0.05$), from which it can be speculated that the above substances are differential metabolites that can cause or aggravate colitis, and are positively correlated with their occurrence and development. Galacturonic acid was positively correlated with the significantly reduced genera in colitis ($P < 0.05$), while lactulose was negatively correlated with the significantly elevated genera ($P < 0.05$). Therefore, it can be considered that galacturonic acid and lactulose may play a beneficial role in the occurrence and development of colitis.

DISCUSSION

This study found that bacterial colitis caused more mucosal damage and inflammation than bacteria pseudo-aseptic colitis. The destruction of intestinal mucus was more pronounced, speculating that the intestinal microbiota contributed to colitis. The differences in the microbiota could determine the severity of colitis. However, in the past, the academic community has discussed the mechanism of the intestinal microbiota in UC, mostly based on research on the characteristics of the microbiota itself, such as flagellar protein, lipopolysaccharide, *etc*., often ignoring the co-metabolism of the host and the microbiota or the self-metabolism of the microbiota. Its metabolites can be used by the host intestinal epithelial cells again, thereby participating in and maintaining the intestinal microbiome balance of the host[14]. Modern research has gradually concluded that the intestinal microbiota in UC can affect the intestinal microenvironment through different characteristics of the species itself and metabolites produced by metabolism, including affecting the function of epithelial structures, changing the state of mucus synthesis, secretion, and degradation, and promoting or inhibiting immune responses and inflammatory responses[15,16].

We found that certain microbiota and metabolites were significantly elevated in colitis mice after comparing them to blank mice, suggesting that the microbiota and metabolites were involved in colitis or aggravated it. Significantly reduced microbiota and metabolites in colitis are deemed to have a protective effect on colitis. Further, the correlation analysis between the differential microbiota and metabolites and the analysis of KEGG pathway enrichment found that the intestinal differential microbiota and its metabolites in mice with colitis were mainly concentrated in amino acid and energy metabolism.


A
OPLS-DA
PCo1 (17%)
F
G
B
Volcano plot
-Log10 (P-value)
Log2 (FC)
P value = 0.05
VIP
0.5
1.0
2.0
Down-regulated
Not-significant
Up-regulated
C
Class
F
G
D-ribose
Digalacturonic acid
Pyrophosphate
Lactitol
N-acetyl-5-hydroxytryptamine
D-maltose
Lactulose
Maltitol
Sarcosine
Putrescine
Spermine
Butane-2,3-diol
Diacetone alcohol
Xanthosine
3-hydroxypalmitic acid
4',5-dihydroxy-7-glucosyloxyflavanone
Epicatechin
Ribonic acid
Phenol
L-lactic acid
D-tagatose
Montanic acid
Ascorbic acid
Pinitol
O-phosphoserine
4-aminobutyric acid
Adenosine
Urocanic acid
Atropine
Erythronic acid
L-glutamine
Thymidine
2-hydroxybutanoic acid
Ornithine
L-valine
7-hydroxynicotinic acid
L-tryptophan
Leucine
Cerotinic acid
3-hydroxybutyric acid
2,3-dihydroxypyridine
Shikimic acid
3-(4-hydroxyphenyl)propionic acid
3-hydroxy-3-(4'-hydroxy-3'-methoxyphenyl)propionic acid
Sinapinic acid
L-threonine
Pyruvic acid
Aminomalonate
L-tyrosine
Beta-glutamic acid
Threose
L-asparagine
3-deoxyhexitol
3,6-anhydro-d-galactose
Creatinine
Malonic acid
5-methoxytryptamine
F5
F4
F6
F1
F2
F3
G4
G6
G1
G5
G2
G3
D
Aminoacyl-tRNA biosynthesis
Arginine and proline metabolism
Central carbon metabolism in cancer
ABC transporters
Valine, leucine and isoleucine biosynthesis
cAMP signaling pathway
Glycine, serine and threonine metabolism
Alanine, aspartate and glutamate metabolism
Parkinson disease
Pantothenate and CoA biosynthesis
GABAergic synapse
Morphine addiction
Alcoholism
Phenylalanine, tyrosine and tryptophan biosynthesis
D-Arginine and D-ornithine metabolism
Glutathione metabolism
Butanoate metabolism
HIF-1 signaling pathway
Arginine biosynthesis
Glucagon signaling pathway
Pathwayterm
Richfactor
Number
P value



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Figure 6 Analysis of different microbiota metabolites in dextran sulfate sodium-induced colitis mice. A: Orthogonal partial least-squares discrimination analysis; B: Metabolite volcano plot; C: Clustered heat map of significantly expressed metabolites; D: Bubble mapping of the top 20 significant Kyoto Encyclopedia of Genes and Genomes pathways of metabolites; E: Correlation matrix analysis with significant metabolites; F: Correlation matrix of the top 20 differential metabolites and the microbiota at the genus levels. F: Blank group; G: Microbiota+ colitis group.

There are 10 to 100 trillion microorganisms in the human gastrointestinal tract, and in the past few decades, the impact of the gut microbiota on human health has received widespread interest from science and the general public. The gut microbiome comprises bacteria, viruses, fungi, and archaea that live in different states in the human gastrointestinal tract. More and more studies have confirmed that the intestinal microbiota is closely related to inflammatory bowel disease, metabolic disease, liver disease, hypertension, and other diseases[17,18]. As probiotics and other means to restore and rebuild the normal intestinal microbiota have shown good therapeutic effects for a variety of diseases, the intestinal microbiota is considered to be a new target for the occurrence or treatment of many diseases [19].

However, because the intestinal microbiota not only has a large number but also has a complex distribution and structural composition, the driving mechanism of the intestinal microbiota in many diseases is still unclear, which hinders the construction of intestinal microbiota models for specific diseases and the search for disease characteristic intestinal microbiota. Therefore, the development of aseptic mice is the most effective tool for conducting intestinal microbiota research, and aseptic mice can provide scientific methods for solving such problems[20].

Currently, two main methods have emerged in academia to study the effects of the microbiota on mouse physiology and disease: aseptic mice and antibiotic treatment regimens (pseudo-aseptic mice). Aseptic mice do not contain any bacteria, viruses, eukaryotic microorganisms, or other saprophytic or parasitic related life forms[21], but the high labor and cost of generating and maintaining them have resulted in many research teams not being able to use this model. In addition, this chronic lack of stimulation from foreign antigens can leave the development of its organs in an idealized aseptic state, leading to significant limitations in the structure and function of organs in the body, especially the immune system[22].

Scientists have created another method using multiple antibiotics for gut sterilization to solve this problem. Broad-spectrum antibiotic therapy usually eliminates most of the intestinal microbiota of mice. It can be easily applied to any genotype or condition in mice. Still, because it is impossible to eliminate the intestinal microbiota, it is generally referred to as pseudo-aseptic mice relative to aseptic mice[23]. Different antibiotics selectively deplete different members of the microbiota, with metronidazole and clindamycin targeting anaerobic bacteria; vancomycin being effective only against gram-positive bacteria; polymyxin B specifically targeting gram-negative bacteria[24]; amphotericin B having strong antimicrobial activity against candida spores, *etc*.; neomycin being effective against both gram-positive and gram-negative bacteria; and ampicillin being more effective against gram-positive bacteria, *Viridans streptococci* and *Enterococcus*[25]. Pseudo-aseptic mice can therefore be established by extensively depleting the intestinal microbiota by using different kinds of antibiotic mixtures. In this study, the establishment of intestinal pseudo-aseptic mice was built by combining species antibiotics, and the 16s rDNA sequencing of the microbiota was confirmed.

The pathogenesis of ulcerative colitis is complex, and most of the current views are that the interaction of multiple factors causes it. The academic community summarizes the pathogenesis of UC as follows: in genetically susceptible people, such as those suffering from depression and anxiety-related conditions, the intestinal microbiota involved in the intestinal barrier is destroyed, and the immune system function is disrupted, resulting in an excessive immune-inflammatory response[26]. The pathogenesis of UC involves multiple mechanisms, and the colitis model established by different methods is suitable for different research purposes, among which DSS is the most widely used chemical drug for the preparation of colitis models. In the DSS model, sulfated polysaccharides act as a direct chemical toxin of the colon epithelium by reducing the number of mucins and destroying the intestinal epithelial mucus layer[27], causing intestinal epithelial cells damage and structural destruction of the tightly connected complex, while breaking the distribution of the intrinsic intestinal microbiota and affecting the bacterial community structure and diversity[28]. The permeability of the intestinal barrier is increased, and harmful macromolecular antigens, bacteria, toxins, and other substances successfully pass through the intestinal barrier, causing abnormal responses to inherent and adaptive immunity and cascading amplification of inflammation and eventually forming colitis.

A growing number of epidemiological clues point to the onset of UC being closely related to dysbiosis of the gut microbiota. Colonic contents provide bacteria with a rich nutritional environment and become the main settlement of the intestinal microbiota, which provides energy and nutrients to the host through metabolites[29]. Under physiological circumstances, the intestinal microbiota mainly has the following functions: first, it forms a biological barrier with the intestinal mucosal epithelium, blocks exogenous antigenic substances from entering the intestinal mucosa, and produces different metabolic and secondary metabolites through its metabolism to provide energy for the intestinal epithelium; secondly, it participates in the differentiation of T lymphocytes, and jointly regulates mucosal immunity with lymphatic centers and immune cells to regulate immune function; thirdly, it participates in the metabolism and synthesis of a variety of amino acids, proteins, and other substances and participates in the composition of the mucosal barrier[30].

Studies have shown that bacteria that promote mucus degradation, such as *Ruminococcus* and *Entamoeba histolytica*, have been found in larger numbers in patients with UC compared to healthy individuals. *Listeria* that inhibits mucus synthesis has increased significantly, in contrast, *Lactobacillus* that promotes MUC2 secretion has decreased significantly in monocytogenes[31]. *Escherichia coli*

infection can disrupt the balance of intestinal flora and disrupt the intestinal microenvironment[32]. By colonizing the intestinal epithelial cells and secreting enterotoxins, *E. coli* damages the intestinal mucus layer, reduces the protein expression of tight junctions and adhesive junctions, impairs the intestinal mucosal barrier structure, increases intestinal permeability, and induces inflammatory responses and the production of cytokines, such as tumour necrosis factor-α, in addition to increasing MUC2 degradation, which is directly involved in UC pathogenesis[33]. In the stool specimens of patients with UC, the *Proteobacteria* phylum increased significantly, and the phyla *Firmicutes* and *Bacteroidetes* decreased significantly[34], accompanied by different degrees of expression changes in various amino acids, such as decreased valine and glutamate expression; increased tryptophan and isoleucine content; increased pyruvate metabolism; decreased citric acid expression; and decreased short-chain and medium-chain fatty acids in energy metabolism[35]. Other studies have shown that the protective microbiota of *Clostridium*, *Prevoella*, *Bifidobacterium*, and *Lactobacillus acidophilus* were significantly reduced in UC[36], while the invasive *Ekmanella* and *E. coli* were significantly increased. The metabolites of short-chain fatty acids, tryptophan, and bile acids[37] that provide nutrients and energy and protect the epithelium were also significantly reduced[38]. Besides *Klebsiella*, *Escherichia-Shigella*, *Bacteroides*, *Lactobacillus*, which are well-known microbiota in colitis, we also found *Candidatus Stoquefichus*, *Anaerobiospirillum*, *Muribaculum*, *Rikenellaceae RC9* gut group, *Candidatus Saccharimonas*, *Prevotellaceae Ga6A1* group, and *Negativibacillus*. We believe these are emerging microbiota that induces or protects against colitis.

Some studies have reported a certain relationship between other emerging flora and metabolites and colitis. For example, *Faecalibacterium* can influence goblet cell differentiation, mucin synthesis, and glycosylation in colonic epithelium, thereby regulating the intestinal mucus barrier[39]. *Lachnospiraceae* can degrade mucins and are major consumers of mucins. *Lachnospiraceae* over-deplete MUC2, reduce the thickness of the mucus layer and cause severe inflammation in the colon in DSS-induced UC mice[40]. Allobaculum mucolyticum secretes a high amount of mucin O-glycan targeting carbohydrate active enzymes, which enables it to efficiently degrade intestinal mucins, thereby degrading the host's protective mucus layer and disrupting the mucus barrier[41]. Blautia's excessive concentrations in the body may lead to the elevation of secondary bile acids, such as lithocholic acid and deoxycholic acid, which in turn induce UC[42]. A study observed significant differences in the contents of *Brautia* in the fecal and mucosal microbial communities of inflammatory bowel disease (IBD) patients and healthy people[43]. It has also been demonstrated intestinal inflammation in IBD patients is associated with increased *Faecalibaculum* relative abundance[44].

In addition, substantial changes have been evident in colitis, including the marked elevation in 3-deoxyhexanol and o-phosphoserine levels and the reduction in galacturonic acid and other microbiota-derived metabolites. Such findings have not been widely reported with regard to the occurrence and development of colitis. By reviewing the literature, we found that MUCs, as a large and complex class of glycosylated proteins, are characterized by important "mucin domains" composed of a protein core. This core contains proline, threonine, and serine, all three of which are known as rich-PTS sequences. Interestingly, through enrichment analysis, we found that these differential metabolites in this study are primarily enriched in the mucin domain of the rich-PTS sequence synthesis pathways. Levy *et al*[45] studies have shown that taurine and arginine can further promote the synthesis and expression of MUC2 by modulating the NLRP6 signaling pathway, thereby protecting the intestinal epithelium and mucus barrier. Therefore, we believe that these differential microbiota metabolites in colitis are likely to regulate the composition and function of mucus.

This study found differential intestinal microbiota and their metabolites in colitis. Preliminary studies, such as enrichment analysis, have initially found that these differential microbiota and metabolites are likely to be directly involved and affect the mucus barrier of the intestine. However, this study has shortcomings; namely, the lack of verification links for transplanting differentially expressed intestinal microbiota or metabolites into colitis mice; if an experimental design of microbiota or metabolite transplantation is added, we believe that the scientific nature of this study can be further improved, which is a research question we will evaluate further.

CONCLUSION

Candidatus Stoquefichus, Anaerobiospirillum, Rikenellaceae RC9 gut group, *Prevotellaceae Ga6A1* group and *Negativibacillus* are potentially emerging flora that induce or aggravate colitis, and *Candidatus Saccharimonas* and *Muribaculum* are potentially emerging flora that prevent or alleviate colitis. 3-Deoxyhexitol and o-phosphoserine may cause or aggravate colitis, whereas galacturonic acid may play beneficial roles in colitis alleviation and recovery. The differential metabolites of the flora are mainly enriched in the synthesis-related pathways of the rich-PTS sequence of the mucin MUC2 domain. They can influence the composition and function of mucus by regulating mucin expression, and finally act on the mucus barrier to induce aggravation or reduce colitis prevention.

ARTICLE HIGHLIGHTS

Research background

The role of gut microbiota in ulcerative colitis (UC) cannot be ignored; however, most of current research is only based on the microbiota itself, ignoring microbiota metabolism. Microorganisms can reduce many biologically active substances, such as short-chain fatty acids, which have strong immunomodulatory effects. Modern studies have reported that the destruction of the integrity of the mucus barrier is an early pathological change in UC. Different gut microbiota and their metabolites can influence the intestinal mucus barrier through different pathways, including altering epithelial structure, affecting mucin synthesis, secretion and degradation, and modulating immune responses.

Research motivation

At present, although many studies have confirmed the important role of gut microbiota in UC, the intricate relationship between microbiota and metabolites in UC has not been fully clarified. Association analysis of differential flora and their metabolites are required. It is worthwhile to conduct this study based on the intestinal mucus barrier to further reveal the potential differential biomarkers of UC.

Research objectives

The aim of the present study was to reveal the differential gut microbiota and metabolites that affect mucus in UC pathogenesis. The regulatory effect provides new evidence and new ideas for UC diagnosis.

Research methods

In the present study, based on the intestinal mucus barrier, taking the intestinal flora and metabolism as the breakthrough point, a combination of antibiotics was used to establish pseudo-aseptic mice, and the widely used dextran sodium sulfate was used to establish colitis mice. Disease severity, mucus-associated protein expression, bacterial 16s rDNA sequences, and non-targeted metabolomes of bacterial and bacterial colitis mice were examined. The tract microbiota and metabolites play potentially important roles in UC pathogenesis by affecting the mucus barrier.

Research results

This study found that: (1) The antibiotics combination can effectively remove the intestinal flora of mice, and reduce bacterial abundance, and α and β diversity, alter community structure, and successfully establish pseudo-aseptic mice; (2) Comparing the bacteria-bearing mice with the pseudo-aseptic mice, the bacteria-bearing mice had more severe colitis based on disease activity index, more severe intestinal mucosal damage, and more obvious intestinal mucus loss; (3) In the intestinal flora of colitis mice, *Candidatus Stoquefichus*, *Anaerobiospirillum*, *Rikenellaceae RC9* gut group, *Prevotellaceae Ga6A1* group, and *Negativibacillus* were significantly increased, while *Candidatus Saccharimonas* and *Muribaculum* were significantly decreased. The significantly up-regulated metabolites in the intestinal flora of colitis mice included 3-deoxyhexitol and ortho-phosphoserine, and the significantly down-regulated metabolites were galacturonic acid, *etc.*; and (4) Further enrichment analysis found that the above differential metabolites were mainly associated with amino acid and energy metabolism. Spearman correlation analysis found that 3-deoxyhexitol, o-phosphoserine, *etc.* were positively correlated with the occurrence and development of colitis, and galacturaldehyde was positively correlated with the occurrence and development of colitis. There was a significant positive correlation between galacturonic acid and lactulose and the reduction and colitis recovery.

Research conclusions

Candidatus Stoquefichus, Anaerobiospirillum, Rikenellaceae RC9 gut group, *Prevotellaceae Ga6A1* group, and *Negativibacillus* are potentially emerging flora that induce or aggravate colitis, and *Candidatus Saccharimonas* and *Muribaculum* are potentially emerging flora that prevent or alleviate colitis. 3-Deoxyhexitol and o-phosphoserine may cause or aggravate colitis, while galacturonic acid may play a beneficial role in colitis alleviation and recovery. The differential metabolites of the flora are mainly enriched in the synthesis-related pathways of the rich-PTS sequence of the mucin MUC2 domain. They can affect the composition and function of mucus by regulating the expression of mucin, and finally act on the mucus barrier to induce aggravation or reduce colitis prevention.

Research perspectives

This study identified some less-reported differential gut microbiota and their metabolites in colitis, which could affect UC progression by modulating mucin synthesis, altering mucus status and the mucus barrier. In future studies, we will carry out in-depth transplantation experiments of fecal bacteria and metabolites to further verify the experimental conclusions of this study.

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FOOTNOTES

Author contributions: Wang JL and Han X have contributed equally to this work, and they are co-first authors; Shi L and Li JX conceived and designed the study; Wang JL and Han X performed major experimental work; Wang JL and Shi R acquired and analyzed the results and edited the manuscript; Liu LL, Wang K, Liao YT, Jiang H, Zhang Y, Hu JC, and Zhang LM performed the experiments and statistical analyses; Shi L and Li JX revised the manuscript; All authors read and approved the final version of the manuscript.

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ORIGINAL ARTICLE

Basic Study

Salvia miltiorrhiza extract may exert an anti-obesity effect in rats with high-fat diet-induced obesity by modulating gut microbiome and lipid metabolism

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Abstract

BACKGROUND

Studies have shown that a high-fat diet (HFD) can alter gut microbiota (GM) homeostasis and participate in lipid metabolism disorders associated with obesity. Therefore, regulating the construction of GM with the balance of lipid metabolism has become essential for treating obesity. *Salvia miltiorrhiza* extract (Sal), a common traditional Chinese medicine, has been proven effective against atherosclerosis, hyperlipidemia, obesity, and other dyslipidemia-related diseases.

AIM

To investigate the anti-obesity effects of Sal in rats with HFD-induced obesity, and explore the underlying mechanism by focusing on GM and lipid metabolism.

METHODS

Obesity was induced in rats with an HFD for 7 wk, and Sal (0.675 g/1.35 g/2.70 g/kg/d) was administered to treat obese rats for 8 wk. The therapeutic effect was evaluated by body weight, body fat index, waistline, and serum lipid level. Lipid factors (cAMP, PKA, and HSL) in liver and fat homogenates were analyzed by ELISA. The effect of Sal on GM and lipid metabolism was assessed by 16S rRNA-based microbiota analysis and untargeted lipidomic analysis (LC-MS/MS),

respectively.

RESULTS

Sal treatment markedly reduced weight, body fat index, serum triglycerides (TG), total cholesterol (TC), low-density lipoprotein, glucose, free fatty acid, hepatic lipid accumulation, and adipocyte vacuolation, and increased serum high-density lipoprotein (HDL-C) in rats with HFD-induced obesity. These effects were associated with increased concentrations of lipid factors such as cAMP, PKA, and HSL in the liver and adipose tissues, enhanced gut integrity, and improved lipid metabolism. GM analysis revealed that Sal could reverse HFD-induced dysbacteriosis by promoting the abundance of *Actinobacteriota* and *Proteobacteria*, and decreasing the growth of *Firmicutes* and *Desulfobacterita*. Furthermore, LC-MS/MS analysis indicated that Sal decreased TGs (TG18:2/18:2/20:4, TG16:0/18:2/22:6), DGs (DG14:0/22:6, DG22:6/22:6), CL (18:2/18:1/18:1/20:0), and increased ceramides (Cers; Cer d16:0/21:0, Cer d16:1/24:1), (O-acyl)-ω-hydroxy fatty acids (OAHFAs; OAHFA18:0/14:0) in the feces of rats. Spearman's correlation analysis further indicated that TGs, DGs, and CL were negatively related to the abundance of *Facklamia* and *Dubosiella,* and positively correlated with *Blautia* and *Quinella*, while OAHFAs and Cers were the opposite.

CONCLUSION

Sal has an anti-obesity effect by regulating the GM and lipid metabolism.

Key Words: *Salvia miltiorrhiza* extract; Obesity; Gut microbiota; Lipid metabolism; High fat diet

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Core Tip: Obesity is a major public health issue today and an obesity-related change in gut microbiota composition and its metabolite profile has been demonstrated. As a commonly used traditional Chinese medicine, *Salvia miltiorrhiza* extract (Sal) has many pharmacological effects, including anticoagulant, anti-inflammation, antioxidation, anti-fibrosis, anti-tumor, and organ protection. Although it has not been documented, Sal has a regulatory effect on obesity, which may be related to the gut microbiota. In the present study, we found that Sal plays a role in weight loss, lowering serum lipid levels, regulating the gut microbiota, and improving intestinal fecal metabolites in obese rats.

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INTRODUCTION

Obesity, a condition whose incidence increases yearly, can lead to several chronic metabolic syndromes such as diabetes, hyperlipidemia, and atherosclerosis. According to 2016 epidemiological statistics, more than 1.9 billion adults worldwide suffer from obesity, and its prevalence reaches 70% in the United States[1]. According to the WHO, adults with a body mass index (BMI) > 25 are considered overweight, while adults with a BMI > 30 are considered obese[2].

Gut microbiota (GM) is among the key regulators of metabolism[3]. Dysregulation of the GM is closely connected with obesity and its complications[4]. Under physiological circumstances, there is a symbiotic relationship between the GM and the host, which keeps the intestinal dynamic equilibrium of the body and metabolism[5]. On the other hand, the GM-host imbalance[6] has been associated with the occurrence of many diseases, such as obesity, enteritis, and colitis. Recent data indicated that there are significant differences in the structural composition of the GM of obese patients and that of non-obese people. For example, fewer *Bacteroides* (beneficial bacteria which metabolize oligosaccharides and polysaccharides, providing nutrition to the host) and more *Pachyderma* were found in the intestinal tract of obese patients[7]. Consistent findings suggest that obesity is associated with decreased abundance in some taxa, such as *Bifidobacterium*, *Christensenella*, and *Ackermannia,* which are considered beneficial microbes. Moreover, as a key regulator of host metabolism, the GM can influence lipid metabolism and blood and tissue lipid levels in humans and rats[8]. The GM has a major role in the fermentation of carbohydrates, fermenting carbohydrates and producing short-chain fatty acids (SCFAs), such as acetic

acid, propionic acid, and butyric acid, to prevent and treat obesity and its complications. As a result, mounting evidence suggests treating obesity or obesity-related disease by improving the structure of the GM balance to regulate metabolism, particularly lipid metabolism.

Currently, bioactive substances of natural drugs are becoming increasingly popular as a new safe and effective approach to prevent and treat obesity. *Salvia miltiorrhiza* extract (Sal) is a traditional Chinese medicine that includes water-soluble components such as salyanolic acid, tanshinaldehyde, and comfrey acid that can inhibit early adipogenesis[9] and alleviate lipid metabolism disorders[10]. Moreover, its fat-soluble components, such as dihydrodanhinone I, tanshinone IIA and IIB, and cryptotanshinone can reduce glycerol release[11], promote adipocyte differentiation, and reduce the contents of triglycerides (TG) and cholesterol[12] to treat fatty liver or coronary atherosclerosis[13]. The main pharmacological effects of Sal include restraining the activation of IκB-α and NF-κB, inhibiting the oxidation of low-density lipoprotein (LDL-C) from regulating lipid metabolism processes and antioxidant effects, and improving the body's sensitivity to insulin by activating the AMPK pathway [14]. Sal is also used to treat atherosclerosis[15], hyperlipidemia, obesity, and other dyslipidemia-related diseases. Furthermore, Wang *et al*[16] found that the effect of Sal in regulating hepatic steatosis may be related to the intestinal flora. Hence, this study further investigated the anti-obesity effects of Sal in rats with high-fat diet (HFD)-induced obesity and explored the underlying mechanism by focusing on the GM and lipid metabolism.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (aged 7-8 wk, weighing 160 g ± 20 g) were provided by the Hunan Silaike Jingda Experimental Animal Co. Ltd. (Changsha, China; Animal Certificate Number: SCXK (Xiang) 2019-0004) and housed in specific pathogen-free conditions (23.0 °C ± 2.0 °C ambient temperature, 50%-60% relative humidity, and 12/12 h light/dark cycle) in Laboratory Animal Science and Technology Center of Jiangxi University of Traditional Chinese Medicine (Animal use license SYXK 2021-0007). The rats had free access to food and water throughout the experiment. The protocol (Permit Number: JZLLSC2021-236) was approved by the Jiangxi University of Chinese Medicine Animal Care and Use Committee and performed according to the guidelines prescribed by the committee. Experimental manipulation was performed after 7 d of acclimatization.

Drug

Salvia miltiorrhiza (batch number: 200701) was supplied by Baishixin Chinese Herbal Pieces Co., Ltd. (Millizhou, China). Preparation of *Salvia miltiorrhiza* extract was prepared as follows: The radix *Salvia miltiorrhiza* bunge was crushed into powder, dried at 58 °C, placed in 5000 mL round bottom flask, mixed with 75% ethanol (material-liquid ratio 1:10), soaked for 18 h, and placed in a water bath temperature 90 °C. A reflux extraction device was used to heat the reflux for 3 h, after which the filtrate was collected with four layers of gauze and left for 18 h after hot filtration.

The prepared Sal was freeze-dried with a vacuum freeze-dryer (Scientz-100F) and then smashed with zirconia beads using a mixer mill (MM 400, Retsch) at 30 Hz for 1.5 min. Then, 100 mg was dissolved with 1.2 mL of 70% methanol solution and vortexed for 30 s every 30 min 6 times, and the samples were placed in a refrigerator at 4 °C overnight. After centrifugation at 15984 *g* for 10 min, the extracts were filtered and then analyzed by ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS). Analytical conditions and mass spectrometry data were based on Wang *et al*[17] and Chen *et al*[18], respectively. Linear ion trap and triple quadrupole scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP) with the AB4500 QTRAP UPLC/MS/MS System operating in positive and negative ion mode (Figure 1A and B). For each period, a particular set of MRM transitions was observed by the metabolites eluted during this period (Figure 1C and D). Table 1 shows some of the metabolites identified in this study along with their metabolite numbers, integral values, and names.

Experimental design

According to previous studies[19-23], an HFD with purified ingredients and a total caloric value of 475 Kcal/100 g, with lard as the main source of fat (D12451, Research Diets Inc.) was used to induce obesity. The experiment scheme for the modeling of HFD-induced obesity in rats and drug administration is shown in Figure 2. At the initial phase, all the rats were divided into either a control group (given normal diet; n = 8) or an HFD group (given HFD; n = 40) for 7 wk and housed at an ambient temperature of 22.0 °C ± 1.0 °C. Then, the HFD groups were randomly subdivided into five groups: Control (HFD + normal saline), Sal_L (HFD + 0.675 g/kg/d Sal), Sal_M (HFD + 1.35 g/kg/d Sal), Sal_H (HFD + 2.70 g/kg/d Sal), and orlistat (HFD + 32.4 mg/kg/d). All the treatments lasted for 8 wk. The body weight was measured every 3 d and intake of food was measured per cage daily. After deducting the residual food from the initially supplied, the food intake (g/rat/wk) was determined. Rats were randomly selected from multiple cages, and cages were changed every 2 wk to control for potential cage

Table 1 Some metabolites detected in *Salvia miltiorrhiza* extract

Index	Compounds	Class I
pme2292	Putrescine	Alkaloids
pmf0096	Oxalic acid	Organic acids
Zmyn000268	2,3-Dihydroxypropanal	Others
pme2601	3-Hydroxypropanoic acid	Organic acids
MWS1787	2-Picoline; 2-Methylpyridine	Alkaloids
pma6298	3-Hydroxypyridine	Alkaloids
MWSmce460	2-Piperidone	Alkaloids
MWS1990	4-Pentenoic acid	Organic acids
MWSmce461	L-Azetidine-2-carboxylic acid	Alkaloids
MWStz073	5-Hydroxy-2-pyrrolidinone	Alkaloids

effects and sex/age differences.

Macroscopic observation

On the final day of the trial, all rats were euthanized with pentobarbital sodium (40 mg/kg intraperitoneally) and weighed, and the liver and fat were quickly removed and weighed after blood was drawn from the abdominal aorta. The body fat index (BFI) was calculated as follows: BFI = Total weight of fat/Body weight of rat × 100%.

Histological analysis

Fresh liver and adipose tissues were fixed with 4% polyformaldehyde (PFA) at 4 °C, embedded in paraffin, and then cut into 4-μm thick slices. Samples were then stained with hematoxylin-eosin (H&E) and observed under a light microscope to examine the histopathology according to the published criteria by Yerian *et al*[24] and Liew *et al*[25].

Determination of serum biochemical parameters

The serum was centrifuged for 15 min at 999 *g* at room temperature. Serum TG, total cholesterol (TC), high-density lipoprotein (HDL-C), LDL-C, glucose (GLU), and free fatty acids (FFAs) were measured with a Beckmann COULTERAU480 automatic biochemical analyzer.

Enzyme-linked immunosorbent assay (ELISA)

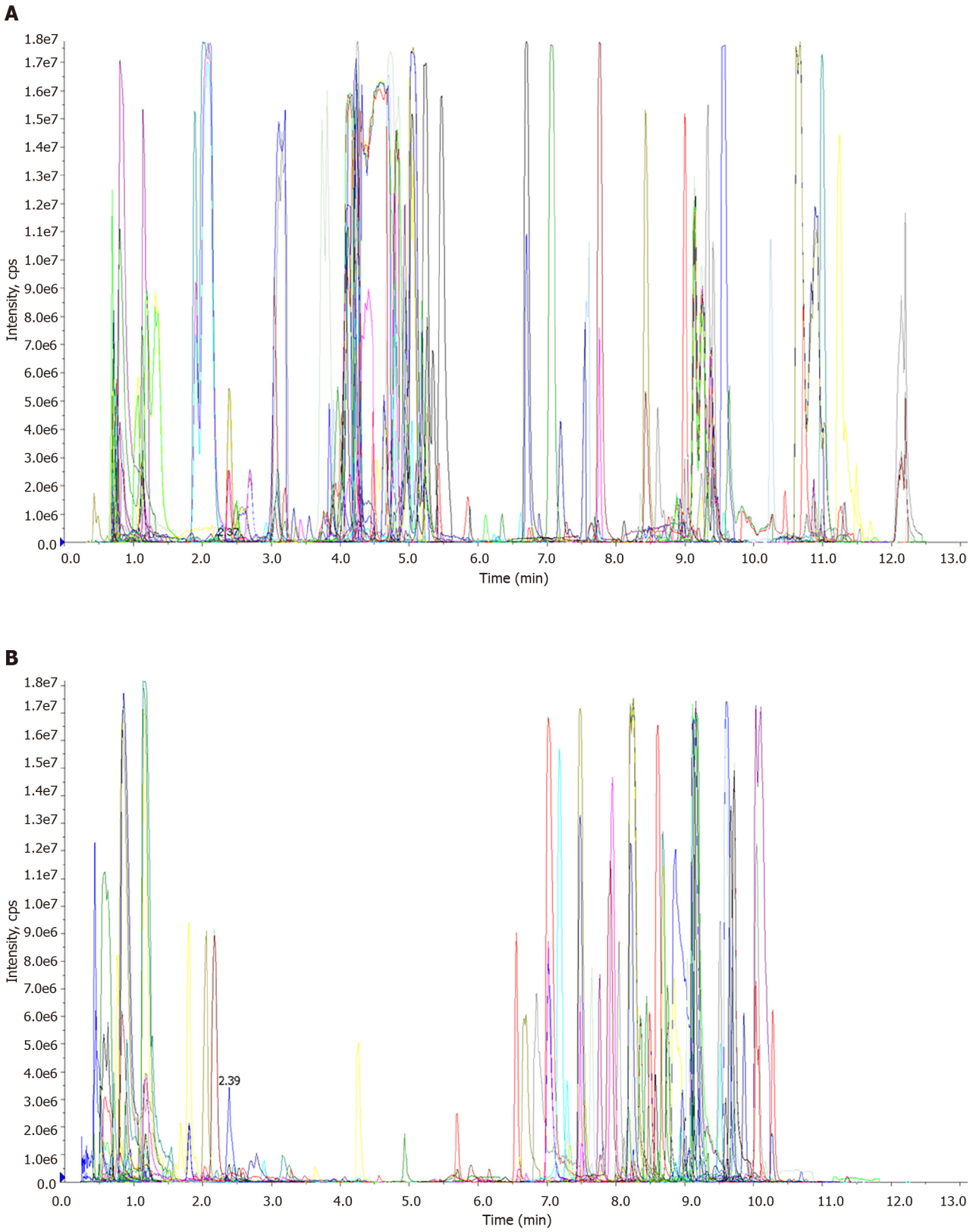
To detect the contents of cAMP in liver tissue, and hormone-sensitive lipase (HSL) and PKA in adipose tissue, parts of the liver and adipose tissue were homogenized under slow rotation (4 °C, 30 min) in 300 μL of RIPA buffer. The supernatant was obtained by centrifugation at 18759 *g* for 30 min. The concentrations of cAMP, HSL, and PKA were determined with commercial ELISA kits (Thermo Fisher Scientific, Waltham city, MA, United States), and the absorbance was measured at 450 nm with a microplate reader (Thermo, Varioskan, MA, United States).

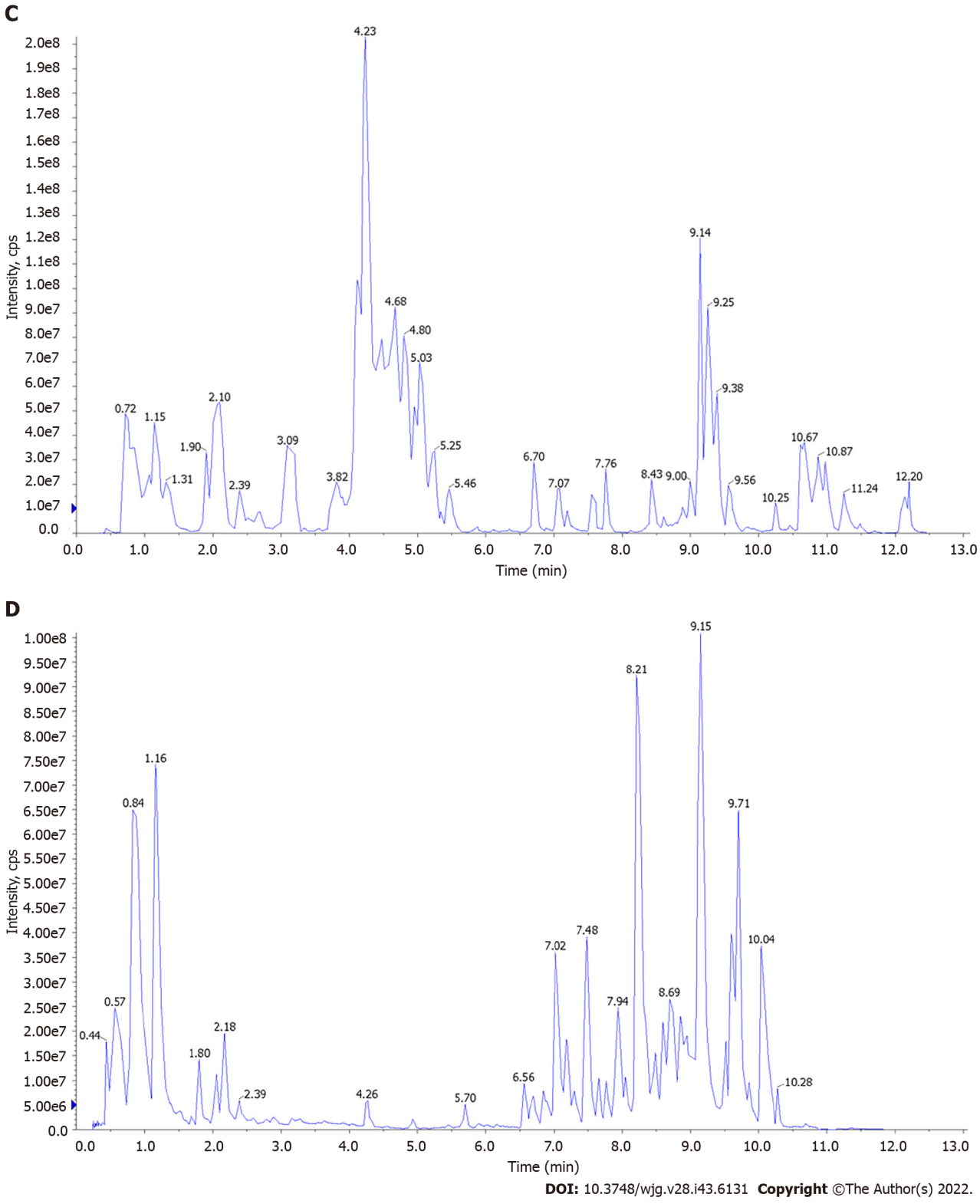
Microbial diversity analysis

Intestinal contents of all rats were collected in cryopreservation tubes and preserved at -80 °C. The microbial diversity analysis was conducted using I-sanger (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China; www.i-sanger.com). Microbial community genomic DNA was extracted from intestinal contents using the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, United States). The DNA extract was tested on a 1% agarose gel, and the concentration and purity of the DNA were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, United States). PCR reactions were run in triplicate.

PCR products were extracted from 2% agarose gel, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions, and quantified using a Quantus™ Fluorometer (Promega, United States). Purified amplicons were equimolar and end-to-end sequenced on the MiSeq PE300 platform/NovaSeq PE250 (United States) under the standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were uploaded to the NCBI Sequence Read Archive (SRA).

Multiplexing of raw 16S rRNA gene sequencing reads was performed, followed by quality filtering achieved with Fast version 0.20.0 and merging with FLASH version 1.2.7. UPARSE version 7.1 was used for clustering operational taxonomic units (OTUs) with a 97 percent similarity cutoff. The RDP classifier version 2.2 was used for classification analysis of the 16S rRNA database with a confidence threshold of


A
Intensity, cps
Time (min)
2.37
B
Intensity, cps
Time (min)
2.39



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Figure 1 UPLC-MS/MS analysis of *Salvia miltiorrhiza* extract. A: Total ions Current-N of QC_MS; B: Total ions Current-P of QC_MS; C: MRM detection of multimodal maps-N; D: MRM detection of multimodal maps-P.



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Figure 2 *In vivo* experiment scheme. Sal: *Salvia miltiorrhiza*.

70%. Calculation of the Shannon index and Sobs diversity was performed, and principal coordinates analysis (PCoA) was used to assess alpha diversity using Mothur (version v.1.30.1). The Kruskal-Wallis H test and the Wilcoxon rank-sum test were used to identify taxa that significantly differed (biomarkers) between groups ($P < 0.05$). I-sanger was used to perform community bar plot and heatmap analysis, correlation analysis, and co-occurrence network analysis.

LC-MS/MS analysis

A total of 50 mg of rat feces were mixed with 80 μL of methanol and 400 μL of methyl-tert-butyl ether for lipid extraction. The mixtures were vortexed for 30 s, followed by sonication for 30 min, and then precipitated at -20 °C for 30 min. The organic phase was separated by centrifugation at 18759 *g* for 15 min at 4 °C. Then, 350 μL of lipid extracts contained in the upper phase were transferred to EP vials and dried in a vacuum concentrator. Consequently, the lipid extract was re-dissolved in 100 μL of isopropanol:acetonitrile (1:1, v/v) solution, followed by 2 min vortexing and 5 min ultrasonication on an ice water bath. Finally, 80 μL of supernatant was transferred carefully to sample vials for LC-MS/MS analysis.

Mass spectral data were collected using a Thermo Q-Exactive Mass Spectrometer equipped with an electrospray ionization[26] source that can operate in either positive or negative ion mode. The raw data from the LC-MS analyses were imported into Lipid Search (Thermo, CA, United States) for peak detection, alignment, and identification. MS/MS fragments were used to identify the lipids. At least 80% of the lipidomic features detected in any set of samples were retained. After filtering, minimum lipid values were performed for specific samples with lipid levels below the lower limit of quantification, and each lipid profile was summed and pooled. After pooling procedures and imputation, log-transformed data were statistically analyzed to determine significant differences in metabolite levels between comparable groups.

Statistical analysis

Most of the data were statistically analyzed and figures produced using GraphPad Prism 7.0 software (San Diego, CA, United States). One-way ANOVA was performed on multiple groups, followed by Duncan's test to analyze statistical differences. For the data on the GM, we used the online platform of the Majorbio Cloud. The Wilcoxon rank-sum test was used to analyze alpha diversity. Weighted UniFrac distances were used to generate the PCoA plots. Kruskal-Wallis rank sum tests were used to analyze species differences between groups. All results are represented as the mean ± SE. $P < 0.05$ was considered statistically significant.

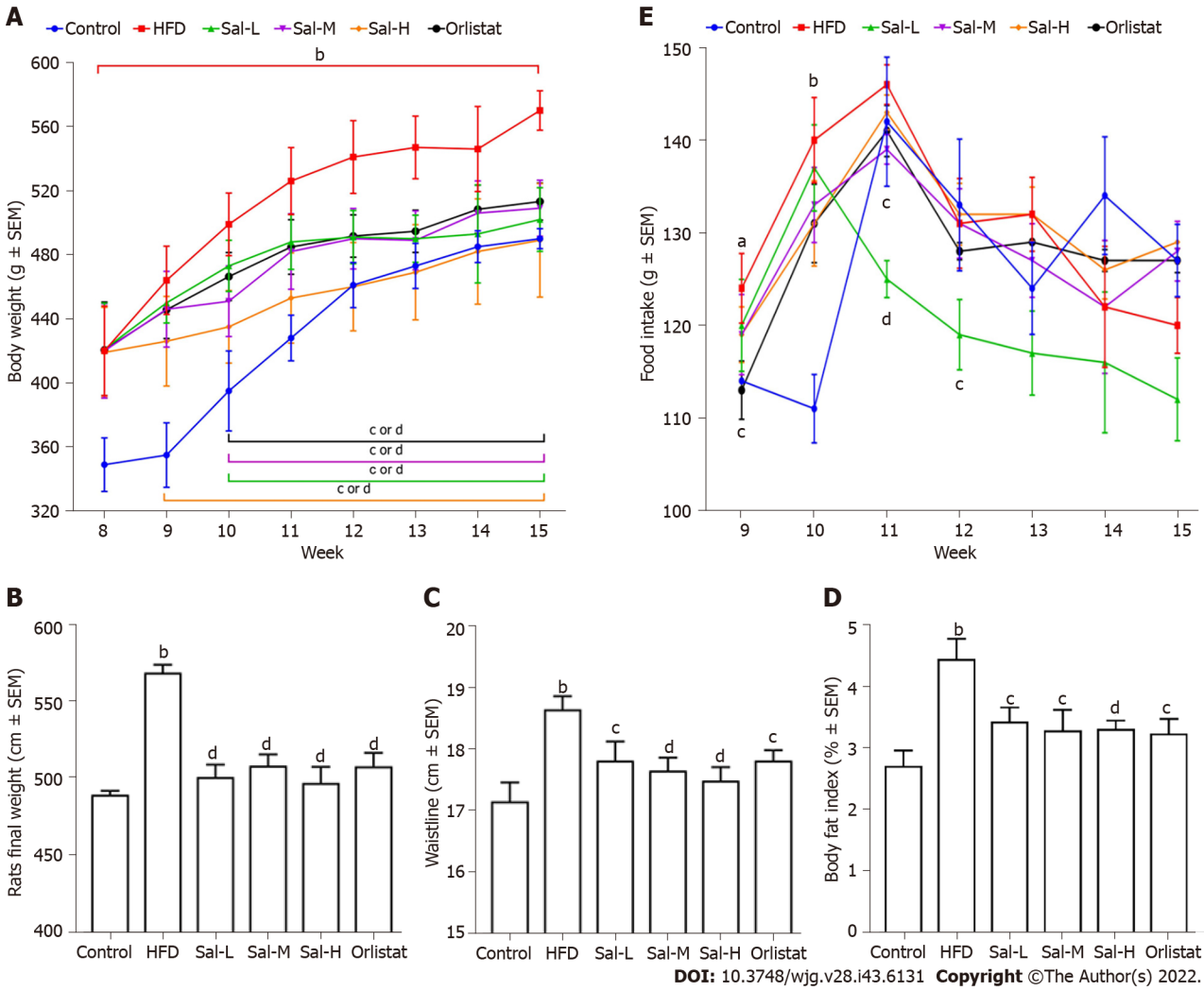
RESULTS

Effects of Sal on body weight gain and fat accumulation in obese rats

After 8 wk of feeding, the body weight of HFD-induced obese rats was significantly higher ($P < 0.05$) compared to the control group ($n = 8$) (Figure 3A). However, the body weight decreased significantly in the Sal_H group ($n = 8$) from the 9th wk, and in Sal_L ($n = 8$) and Sal_M ($n = 8$) groups from the 10th wk compared with the HFD group ($P < 0.05$ for all). On the last day, the final body weight (Figure 3B), waistline (Figure 3C), and body fat index (Figure 3D) in the HFD group were observably increased compared with those of the control group, while Sal supplementation significantly reduced these indices without dose dependence ($P < 0.05$ for all). No significant difference was found among these groups in food intake (Figure 3E) ($P > 0.05$). The above results suggest that Sal can reduce the indices of HFD-induced obesity without affecting appetite intake.

Effects of Sal on serum lipid profiles, liver tissue, and adipose histopathology in HFD group rats

A long-time HFD intake often results in abnormal lipid metabolism[27]. Serum lipid content can reflect the lipid metabolism in the body, among which TG, TC, LDL-C, and HDL-C are the most critical



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Figure 3 Therapeutic evaluation of *Salvia miltiorrhiza* in ameliorating high-fat diet-induced obesity in rats. A: Body weight change; B: Final weight; C: Waistline; D: Body fat index; E: Food intake. Values are expressed as the mean ± SE and analyzed by one-way ANOVA followed by Duncan's multiple comparison test, $n = 8$. HFD: High-fat diet. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* HFD group.

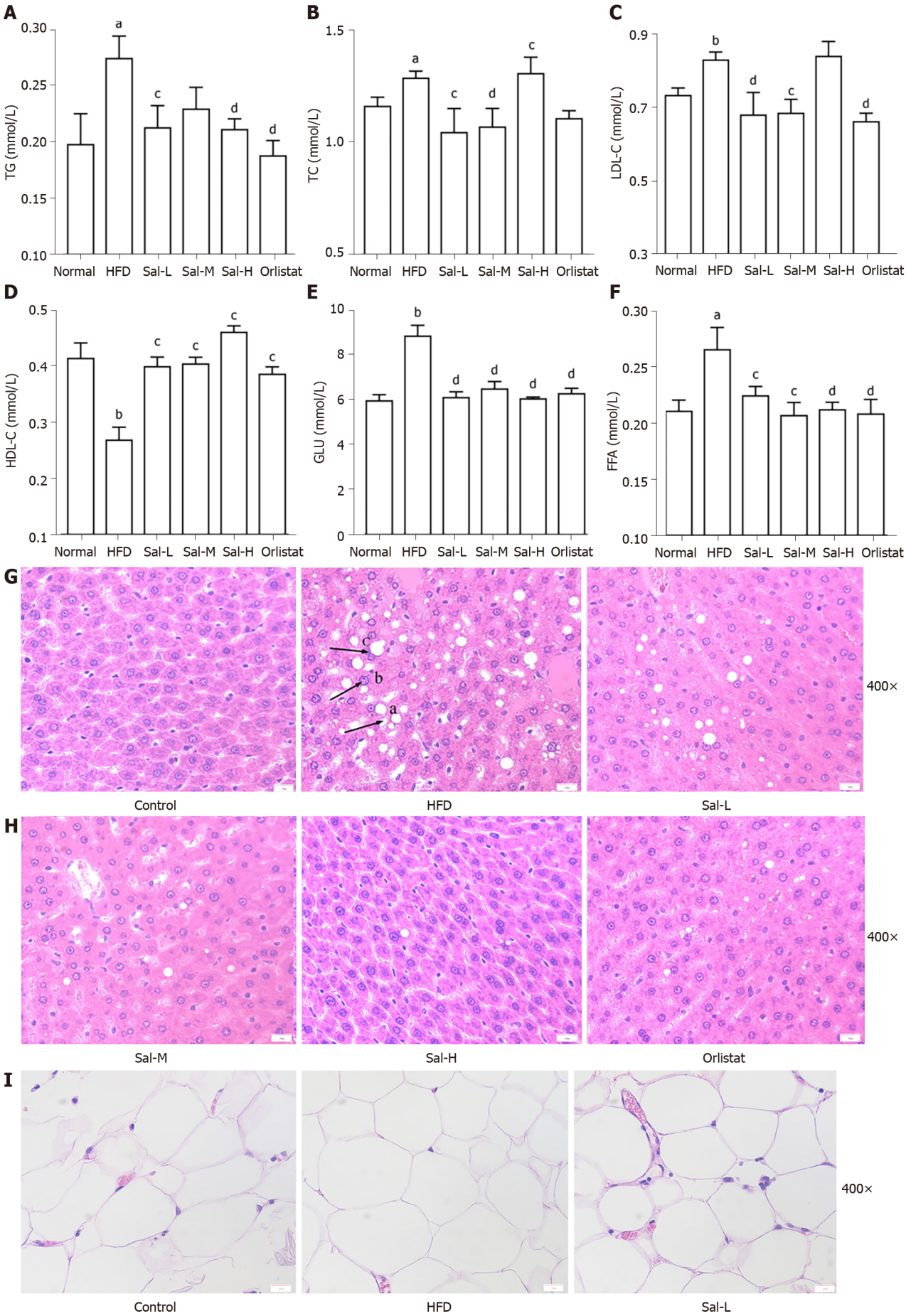
indicators to measure lipid metabolism. In our study, HFD feeding led to a significant increase in serum TG (Figure 4A), TC (Figure 4B), LDL-C (Figure 4C), GLU (Figure 4E), and FFAs (Figure 4F) levels and a significant decrease in the HDL-C (Figure 4D) level ($P < 0.05$ for all), while Sal_L, Sal_M, and orlistat significantly reduced TG and LDL-C levels without a dose-dependent relationship compared with the HFD group ($P < 0.05$ for all). However, Sal_H had no significant effect on TC or HDL-C ($P > 0.05$).

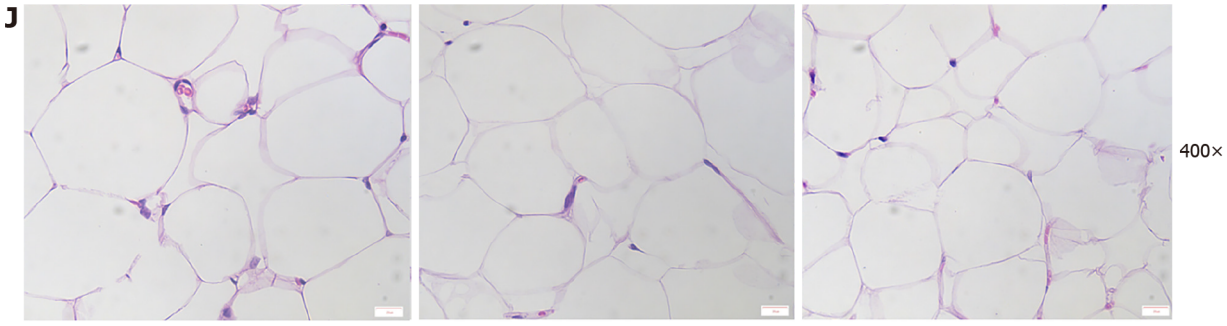
To assess the pathological injury in liver and fat tissues, pathological samples were prepared and stained using H&E. In the liver tissue in the control group, the hepatic lobule structure was ordered and tight (Figure 4G and H). By contrast, the sample in the HFD group exhibited pitting necrosis with balloon-like changes in hepatocytes, which revealed obvious accumulation of lipid droplets in the liver of HFD-induced obese rats. However, Sal and orlistat treatment alleviated these pathological changes. These data further suggest that the HFD intervention for 7 wk can lead to obesity, while Sal administration can effectively reduce dyslipidemia and hepatic lipid accumulation caused by HFD.

H&E-stained fat tissue was observed under a microscope, which showed that the outline of fat cells and volume became larger and the arrangement was loose in the HFD group (Figure 4I and J) compared with the control group. After treatment with Sal and orlistat, both the outline and volume of fat cells were reduced. The results showed that Sal alleviates hepatic steatosis and adipocyte hyperplasia in HFD-induced obese rats.

Sal increases the activities of cAMP, PKA, and HSL in HFD rats

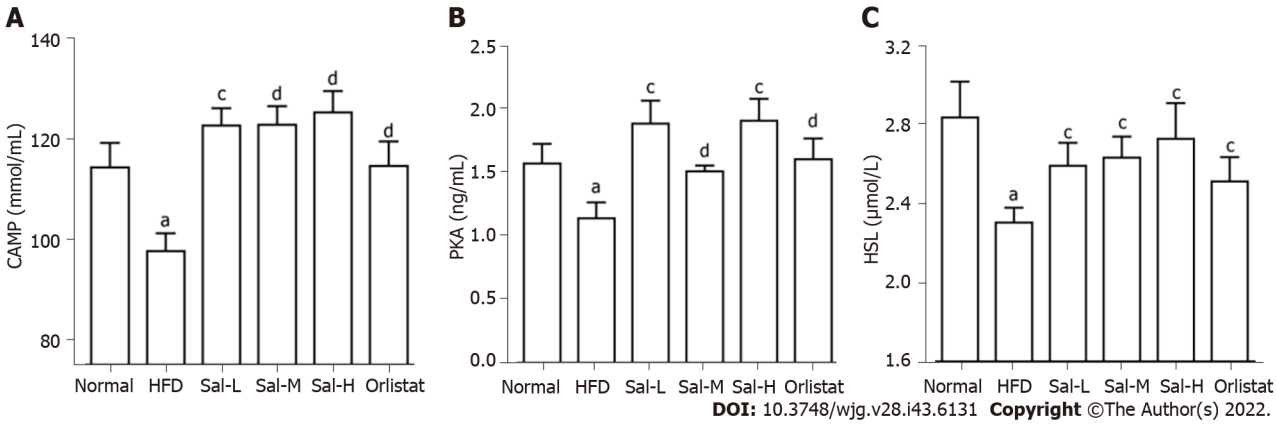
The PKA and HSL signaling system is involved in promoting lipolysis and has a central role in regulating metabolism in all organ systems affected by obesity. In the present study, the levels of cAMP (Figure 5A) in liver tissue, and HSL (Figure 5B) and PKA (Figure 5C) in adipose tissue of the HFD group were significantly lower than those of the control group ($P < 0.05$ for all); contrarily, these levels were significantly increased in HFD-induced obese rats treated with Sal and orlistat ($P < 0.05$ *vs* HFD group


A
TG (mmol/L)
0.30
0.25
0.20
0.15
0.10
a
c
d
d
Normal HFD Sal-L Sal-M Sal-H Orlistat
B
TC (mmol/L)
1.5
1.0
0.5
a
c
d
c
Normal HFD Sal-L Sal-M Sal-H Orlistat
C
LDL-C (mmol/L)
0.9
0.7
0.5
0.3
b
d
c
d
Normal HFD Sal-L Sal-M Sal-H Orlistat
D
HDL-C (mmol/L)
0.5
0.4
0.3
0.2
0.1
b
c
c
c
Normal HFD Sal-L Sal-M Sal-H Orlistat
E
GLU (mmol/L)
10
8
6
4
2
0
b
d
d
d
d
Normal HFD Sal-L Sal-M Sal-H Orlistat
F
FFA (mmol/L)
0.30
0.25
0.20
0.15
0.10
a
c
c
d
d
Normal HFD Sal-L Sal-M Sal-H Orlistat
G
c
b
a
400×
Control
HFD
Sal-L
H
400×
Sal-M
Sal-H
Orlistat
I
400×
Control
HFD
Sal-L



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Figure 4 Effects of *Salvia miltiorrhiza* on serum lipid profiles, glucose, free fatty acid levels, and histopathological changes of the liver and adipose tissue in HFD-fed rats. A-D: Serum triglyceride, total cholesterol, low-density lipoprotein, and high-density lipoprotein levels; E: Serum glucose level; F: Serum free fatty acid level; G and H: Representative images of hematoxylin-and eosin-stained (H&E) sections of liver tissue (400 ×) in the six groups (a: Lipid droplet accumulation; b: Punctate necrosis of hepatocytes with inflammatory cell infiltration; c: Balloon-like changes) (Scale bars: 20 μm); I and J: Representative images of H&E sections of adipose tissue (400 ×) in the six groups (Scale bars: 20 μm). Significance between groups was calculated using one-way ANOVA followed by Duncan's multiple comparison test. Data are shown as the mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* HFD group. Error bars represent standard error.



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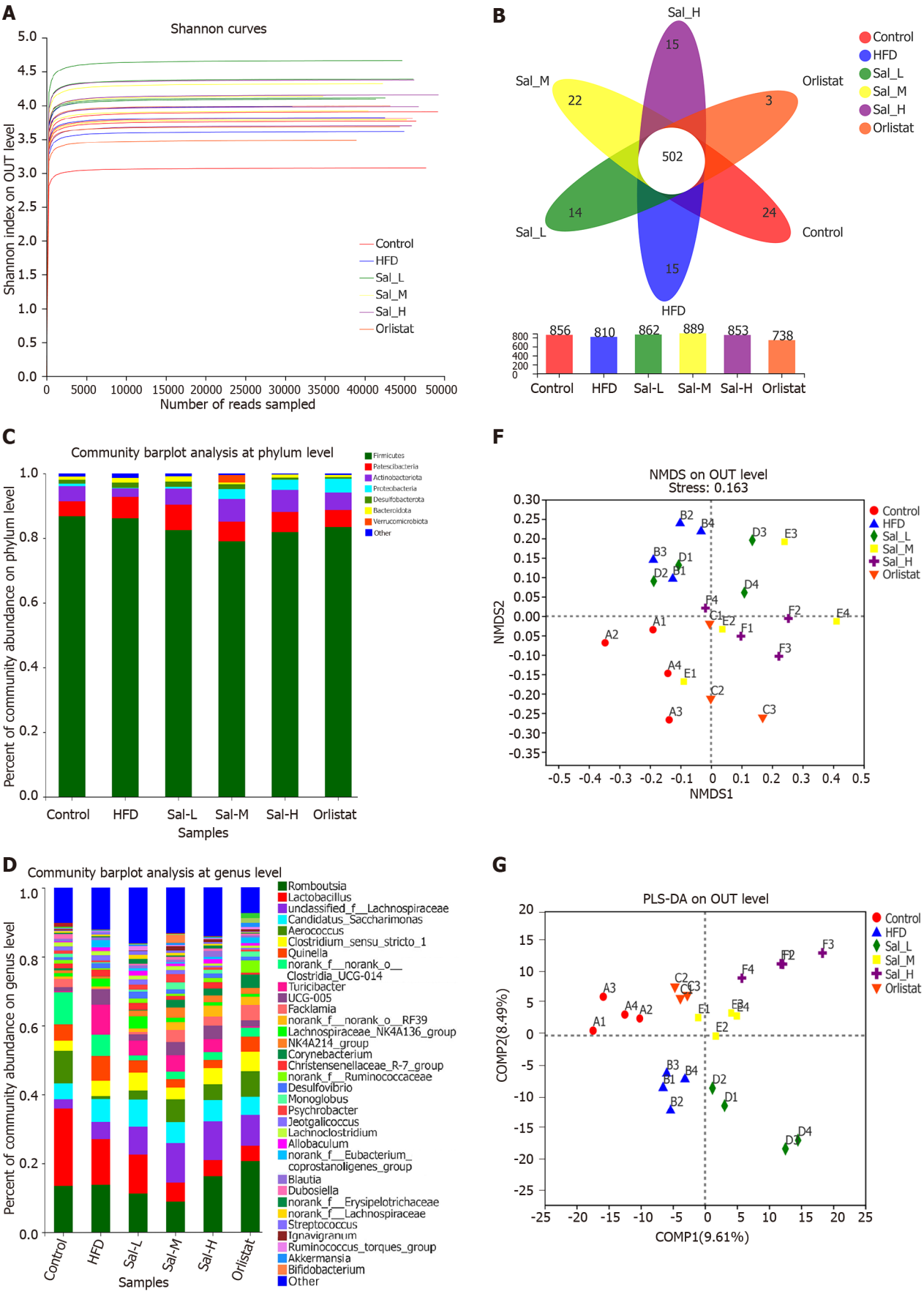
Figure 5 Levels of cAMP in liver tissue and HSL and PKA in adipose tissue of high-fat diet-fed rats. A: cAMP; B: PKA; C: HSL. Data are presented as the mean ± SE ($n = 8$). HFD: High-fat diet. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* HFD group.

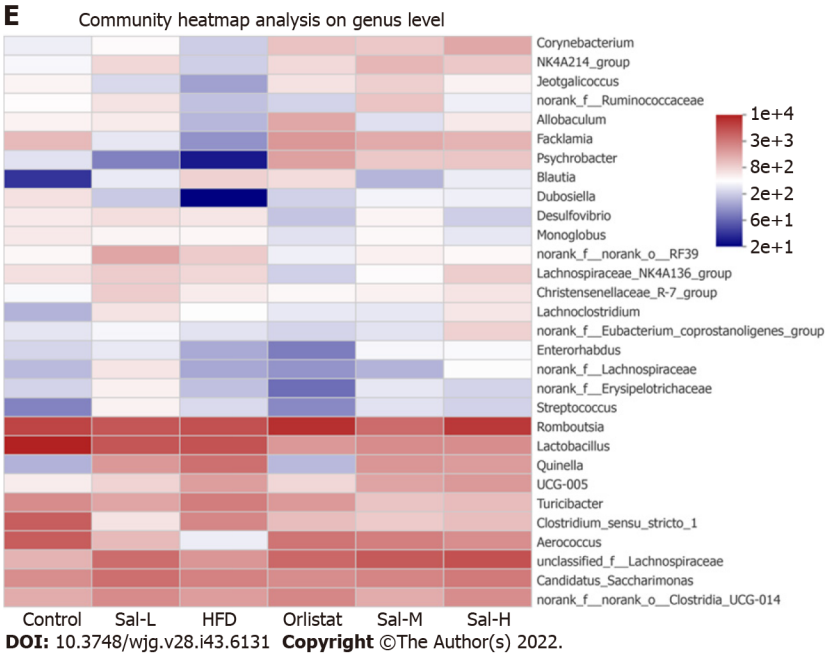
for all). The results indicate that Sal regulates lipid metabolism by enhancing lipolysis in obese rats induced with an HFD.

Sal improves the composition of gut microbiome in HFD rats

Many studies have shown that GM dysbiosis has an essential role in the pathogenetic process of human obesity and animal obesity induced by an HFD. Fecal samples from various groups were analyzed by 16S rRNA sequencing to investigate the regulatory effect of Sal on the GM composition in HFD-induced obese rats. Twenty-four samples yielded a total of 1224808 sequencing reads. The Shannon index curve (Figure 6A), reflecting the alpha diversity of the intestinal flora, shows an adequate amount of sample sequencing data; the Venn diagram (Figure 6B) shows the overlap among the six groups at the OTU level. A total of 856, 810, 862, 889, 853, and 738 OTUs were identified in the control, HFD, Sal_L, Sal_M, Sal_H and orlistat groups, respectively. Statistical analysis of bioinformatics for OTUs at 97% similar levels found that 502 OTUs overlapped among groups. Compared with the control group, the number of OTUs in the GM was reduced in the HFD group, while Sal reversed the change. We think the main reason for the invalid effect of orlistat on OTUs of GM is that there was an anomalous sample in the orlistat group.

To determine which bacteria were improved through Sal and thus intervened in the disease progression of obesity, we analyzed the composition of GM in different groups. The community barplot analysis at the phylum level (Figure 6C) showed that the relative abundances of *Proteobacteria* and *Actinobacteriota* decreased in the HFD group, and that of *Desulfobacterota* increased compared with those in the control, Sal, and orlistat groups. Compared with the control group at the genus level (Figure 6D), the relative abundances of *Aerococcus*, *Dubosiella*, *Psychrobacter*, and *norank_f_Lachnospiraceae* were significantly decreased, and those of *Quinella* and *Turicibacter* were increased in the HFD group. The


A
Shannon curves
Shannon index on OUT level
Number of reads sampled
Control
HFD
Sal_L
Sal_M
Sal_H
Orlistat
B
Sal_H
Sal_M
Orlistat
Sal_L
Control
HFD
15
22
3
502
14
24
15
856
810
862
889
853
738
Control
HFD
Sal-L
Sal-M
Sal-H
Orlistat
C
Community barplot analysis at phylum level
Percent of community abundance on phylum level
Samples
Firmicutes
Patescibacteria
Actinobacteriota
Proteobacteria
Desulfobacterota
Bacteroidota
Verrucomicrobiota
Other
F
NMDS on OUT level
Stress: 0.163
NMDS1
NMDS2
D
Community barplot analysis at genus level
Percent of community abundance on genus level
Samples
Romboutsia
Lactobacillus
unclassified_f__Lachnospiraceae
Candidatus_Saccharimonas
Aerococcus
Clostridium_sensu_stricto_1
Quinella
norank_f__norank_o__Clostridia_UCG-014
Turicibacter
UCG-005
Facklamia
norank_f__norank_o__RF39
Lachnospiraceae_NK4A136_group
NK4A214_group
Corynebacterium
Christensenellaceae_R-7_group
norank_f__Ruminococcaceae
Desulfovibrio
Monoglobus
Psychrobacter
Jeotgalicoccus
Lachnoclostridium
Allobaculum
norank_f__Eubacterium_coprostanoligenes_group
Blautia
Dubosiella
norank_f__Erysipelotrichaceae
norank_f__Lachnospiraceae
Streptococcus
Ignavigranum
Ruminococcus_torques_group
Akkermansia
Bifidobacterium
Other
G
PLS-DA on OUT level
COMP2(8.49%)
COMP1(9.61%)



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Figure 6 Beneficial effects of *Salvia miltiorrhiza* are associated with improved gut microbiome composition in high-fat diet-fed rats. A: α-diversity analysis: Shannon curves at the operational taxonomic unit (OTU) level; B: Venn diagram; C: Community bar plot analysis at the phylum level; D: Community bar plot analysis at the genus level; E: Community heatmap analysis of 30 species at the genus level; F: Non-metric multidimensional scaling (NMDS) at the OTU level; G: Partial least squares discriminant analysis (PLS-DA) score at the OTU level.

community heatmap analysis at the genus level (Figure 6E) showed that the relative abundance of *Blautia* was increased in the HFD group than in the control, Sal, and orlistat groups, while those of *Facklamia*, *Jeotgalicoccus*, *NK4A214_group*, and *Corynebacterium* were decreased.

Furthermore, β-diversity analysis, including non-metric multidimensional scaling (NMDS) and partial least squares discrimination analysis (PLS-DA), was used to assess the diversity variance among these six groups. NMDS (stress = 0.15) (Figure 6F) revealed that the GM composition of the HFD group was completely separated from that of the control group. Although the aggregation of the Sal_L group was similar to that of the HFD group, the two groups were significantly separated, and the aggregation of the control group was significantly enhanced as the dose of Sal increased.

PLS-DA (Figure 6G) was further used to analyze the similarity and differences among the grouped samples. It demonstrated that the species distribution of the HFD group was separated from that of each dose group of Sal and the control and orlistat groups, while the distance between Sal_M and the control group was shorter than that between the HFD and control groups. These findings suggest that Sal effectively improves GM composition in obese rats.

To further discover the regulatory effect of Sal on specific GM in obese rats, we analyzed species differences among the control, HFD, Sal_L, Sal_M, Sal_H, and orlistat groups. The genus-level Kruskal-Wallis H test bar plot (Figure 7A) showed that Sal and orlistat treatment markedly decreased the relative abundances of *Quinella* and *Blautia* in obese rats and significantly increased the abundances of *Facklamia*, *Corynebacterium*, *Psychrobacte*, and *norank_f_Ruminococcaceae*. In addition, the Wilcoxon rank-sum test at the genus level showed that compared with the control, Sal_L, Sal_M, Sal_H, and orlistat groups, the relative abundances of these species in the HFD group were significantly decreased, including *Facklamia*, *Jeotgalicoccus*, *Aerococcus*, and *Dubosiella*, while those of *Lactobacillus*, *Turicibacter*, and *Quinella* were increased significantly (Figure 7B-F). These findings suggest that Sal is important in treating obesity by regulating the GM composition in obese rats.

Correlation analysis of gut microbiota

To further clarify the distribution between groups and species, we analyzed the correlation of species abundance information among different samples through network analysis to obtain the coexistence relationship of species in environmental factors. The association and model prediction by network analysis at the genus level (Figure 7G) showed that the top species were most closely related to these six groups according to the degree of weighting (Table 2): *Romboutsia, Aerococcus, Turicibacter, Quinella, Facklamia*, *Corynebacterium* and *Desulfobacterota*. The evolution analysis by the phylogenetic tree at the genus level (Figure 7H) indicated that the top species of the closest consanguinity relation were *Romboutsia*, *Lactobacillus*, *unclassified_Lachnospiraceae*, *Aerococcus*, *Turicibacter*, and *Quinella*, which is consistent with the conclusion of community bar plot analysis, indicating that Sal exerts an anti-obesity role by regulating the above bacterial flora structure.

Table 2 Species nodes

Node name	Degree	Weighted degree
g_Romboutsia	6	36385.41667
g_Aerococcus	6	13881.41667
g_Turicibacter	6	10477.08333
g_Quinella	6	269.16667
g_Facklamia	6	6077.25000
g_Corynebacterium	6	4418.00000
g_Desulfovibrio	6	2716.58333

Node name represent species nodes. Degree is the degree of the node. Weighted degree, which means the degree weight of the node, is the number of species sequences corresponding to the node.

Finally, the functional prediction analysis (Figure 7I) revealed that these bacteria were primarily concentrated in energy production and conversion, amino acid transport and metabolism, carbohydrate transport and metabolism, biosynthesis, transport, and catabolism of secondary metabolites, lipid transport, and lipid oxidation, according to COG and KEGG orthology information and abundance. These findings suggest that the mechanism of Sal in obesity treatment involves energy and lipid metabolism.

Sal regulates lipid metabolism in obese rats

Lipid metabolism disorders are closely related to obesity and metabolic syndrome[28]. Here, non-targeted lipidomic studies of intestinal contents in rats were conducted to explore the effects of Sal on lipid metabolism. The number of differential metabolites between the control and HFD groups and between the HFD and the Sal groups is visualized in Figure 8A. In a statistically significant analysis, different metabolites were identified where $P < 0.05$ and VIP > 1 were used to identify the different metabolites. In order to understand the metabolic differences between the control, Sal, and HFD groups, the significant lipid data were analyzed using a heatmap of metabolite cluster analysis, which showed the variation of each lipid in each group directly and illustrated the relative increase (red) or decrease (blue) tendency in the HFD group compared with the control and Sal groups (Figure 8B). Interesting, the expression of the control group and the HFD group showed opposite trends; the Sal_M group was the closest to the control group trend in each Sal group, while the low and high dose groups of Sal showed the opposite trend when compared to the control group, which could be explained by the dose-response curve. Therefore, the Sal_M group was selected to make further comparisons of differential metabolites. Relative contents of identified lipids and fold changes in metabolites in the control and Sal_M groups compared with those in the HFD group were calculated to further investigate the magnitude of change in the significant lipids (Table 3). Increased TGs (TG18:2/18:2/20:4, TG16:0/18:2/22:6, TG16:0/14:0/22:6), DGs (DG14:0/22:6, DG22:6/22:6), and CL (18:2/18:1/18:1/20:0) were observed in the HFD rats. In contrast, the lipids including ceramides (Cers; Cer d18:0/20:4, Cer d16:0/21:0, Cer d16:1/24:1), (O-acyl)-ω-hydroxy fatty acids (OAHFAs; OAHFA18:0/14:0), and Hex1Cers (Hex1Cer d18:0/16:0 + O, Hex1Cer d18:1/18:2 + 2O, Hex1Cer t18:0/16:0 + O, Hex1Cer t18:1/18:1 + 2O) showed a decreasing tendency in HFD-induced obese rats. These results identified the up-regulated and down-regulated lipids in the obesity rat induced by an HFD.

In order to further obtain the variation of the expression trend of the different metabolites among the control, HFD, and Sal_M groups, we performed VIP value analysis (Figure 8C and D). TG (16:0/14:0/22:6), TG (16:0/18:2/22:6), TG (18:2/18:2/20:4), Cer (d16:0/21:0), and DG (22:6/22:6) were the most significant metabolites among these groups. These metabolites are closely related to the development of obesity and might have an important role in the metabolism dysfunction in HFD-induced obesity.

In pathway analysis, matched metabolic pathways were displayed based on the *P* value and KEGG pathway enrichment analysis (Figure 8E). A total of 7 pathways were found with $P < 0.05$, five of which were closely associated with obesity, including regulation of lipolysis in adipocytes, insulin resistance, glycerolipid metabolism, fat digestion and absorption, and cholesterol metabolism. In general, these results demonstrate that Sal may exert an anti-obese effect by regulating lipid metabolism in obese rats.

The above results show that Sal can effectively regulate the blood lipid level and GM composition of obese rats. The distance-based redundancy analysis (db-RDA analysis) and Spearman correlation heatmap were used further to investigate the correlation between lipid metabolism and the GM. Three sets of environmental factors were selected to establish the correlation between lipid metabolism and GM, including the physiological and biochemical indexes (FFAs, weight, BFI, and GLU), the key factors of lipid metabolism (cAMP, PKA, and HSL), and the lipid molecules screened by lipidomics. According

Table 3 Differential metabolites in feces after Sal treatment

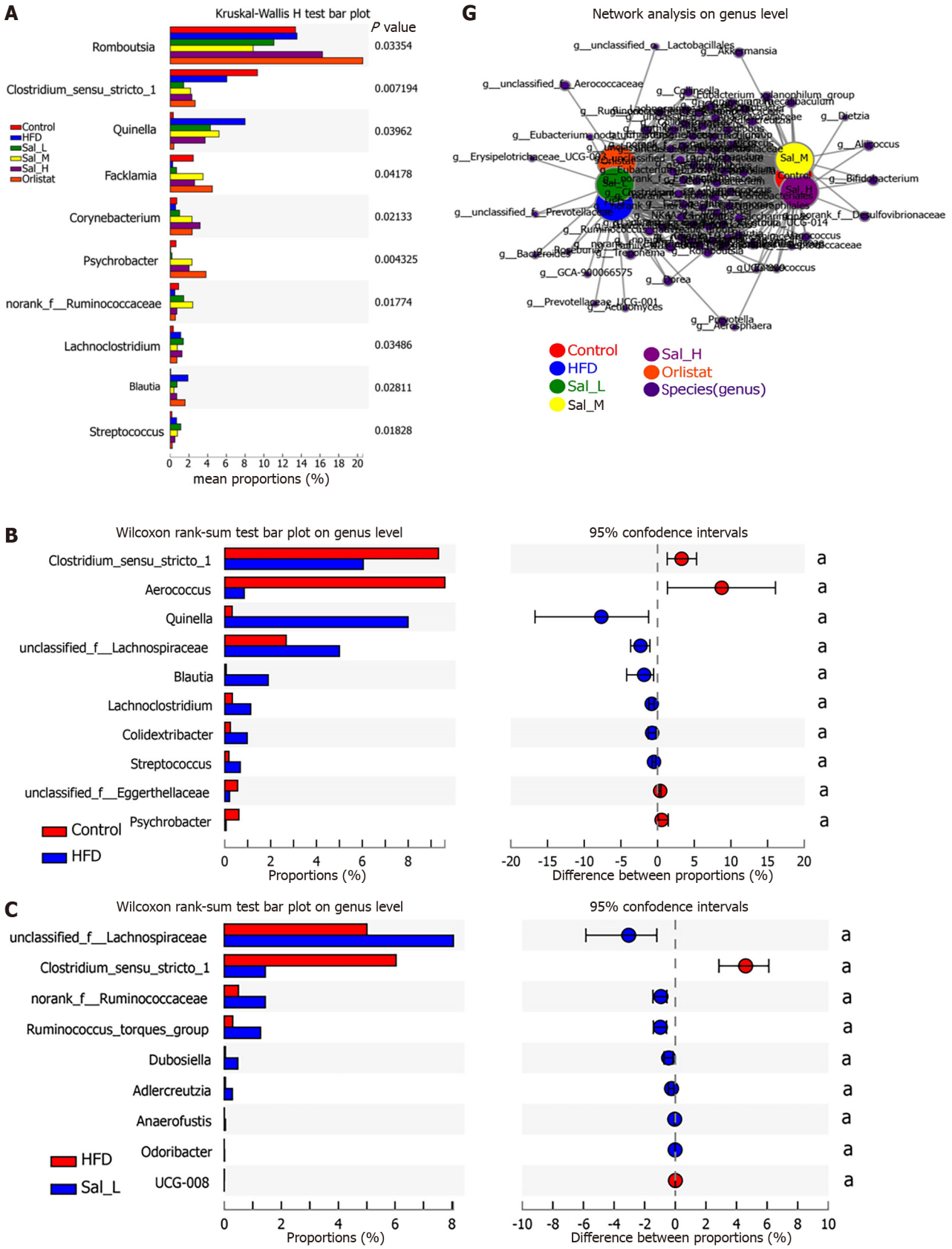
NO	Metabolite	Rt (min)	m/z	Formula	VIP		FC		Trend	
					H *vs* C	S *vs* H	H *vs* C	S *vs* H	H *vs* C	S *vs* H
1	Cer (d18:0/20:4)	6.72	588.54	$C_{38}H_{70}O_3N_1$	1.34	1.67	1.07	0.90	↓[a]	↑[c]
2	DG (14:0/22:6)	6.62	630.51	$C_{39}H_{68}O_5N_1$	1.51	1.91	0.91	1.14	↑[a]	↓[d]
3	DG (22:6/22:6)	6.56	730.54	$C_{47}H_{72}O_5N_1$	1.54	2.01	0.91	1.15	↑[a]	↓[c]
4	Hex1Cer (d18:0/16:0 + O)	6.01	718.58	$C_{40}H_{80}O_9N_1$	1.07	1.01	1.03	1.01	↓[a]	↑[c]
5	Hex1Cer (d18:1/18:2 + 2O)	5.76	756.56	$C_{42}H_{78}O_{10}N_1$	1.13	1.12	1.04	1.12	↓[a]	↑[c]
6	Hex1Cer (t18:0/16:0 + O)	5.79	734.58	$C_{40}H_{80}O_{10}N_1$	1.26	1.20	1.05	1.20	↓[a]	↑[c]
7	Hex1Cer (t18:1/18:1 + 2O)	5.80	756.56	$C_{47}H_{72}O_5N_1$	1.13	1.14	1.04	1.14	↓[a]	↑[c]
8	MGDG (16:1/17:2)	5.70	756.56	$C_{47}H_{72}O_5N_1$	1.13	1.14	1.04	0.96	↓[a]	↑[c]
9	TG (6:0/9:0/18:2)	4.39	615.46	$C_{36}H_{64}O_6Na_1$	1.23	1.36	0.95	1.06	↑[a]	↓[c]
10	TG (4:0/14:1/18:3)	6.29	631.49	$C_{39}H_{67}O_6$	1.34	1.52	0.93	1.08	↑[a]	↓[c]
11	TG (12:1e/6:0/18:4)	5.82	615.50	$C_{39}H_{67}O_5$	1.82	1.86	0.90	1.12	↑[b]	↓[c]
12	TG (16:0/14:0/22:6)	12.21	868.74	$C_{55}H_{98}O_6N_1$	1.87	2.27	0.87	1.18	↑[a]	↓[d]
13	TG (16:0/18:2/20:5)	12.82	877.73	$C_{57}H_{97}O_6$	1.46	1.68	0.94	1.08	↑[a]	↓[c]
14	TG (15:0/18:2/22:6)	11.72	895.74	$C_{58}H_{96}O_6Li_1$	1.45	1.92	0.92	1.13	↑[a]	↓[d]
15	TG (18:2/18:2/20:4)	11.79	920.77	$C_{59}H_{102}O_6N_1$	1.83	2.22	0.87	1.19	↑[a]	↓[c]
16	TG (16:0/18:2/22:6)	12.36	925.73	$C_{59}H_{98}O_6Na_1$	2.24	3.01	0.80	1.36	↑[a]	↓[e]
17	TG (18:2/17:1/22:6)	11.60	921.75	$C_{60}H_{98}O_6Li_1$	1.49	1.78	0.91	1.12	↑[a]	↓[c]
18	CL (18:2/18:1/18:1/20:0)	5.57	741.53	$C_{83}H_{152}O_{17}P_2$	1.50	1.31	0.95	1.05	↑[a]	↓[c]
19	Cer (d16:0/21:0)	8.47	626.57	$C_{38}H_{76}O_5N_1$	2.14	1.89	1.12	0.88	↓[a]	↑[c]
20	Cer (d16:1/24:1)	9.02	664.59	$C_{41}H_{78}O_5N_1$	1.87	1.66	1.11	0.89	↓[a]	↑[c]
21	OAHFA (18:0/14:0)	5.70	756.56	$C_{47}H_{72}O_5N_1$	1.32	1.18	0.95	0.94	↓[a]	↑[c]

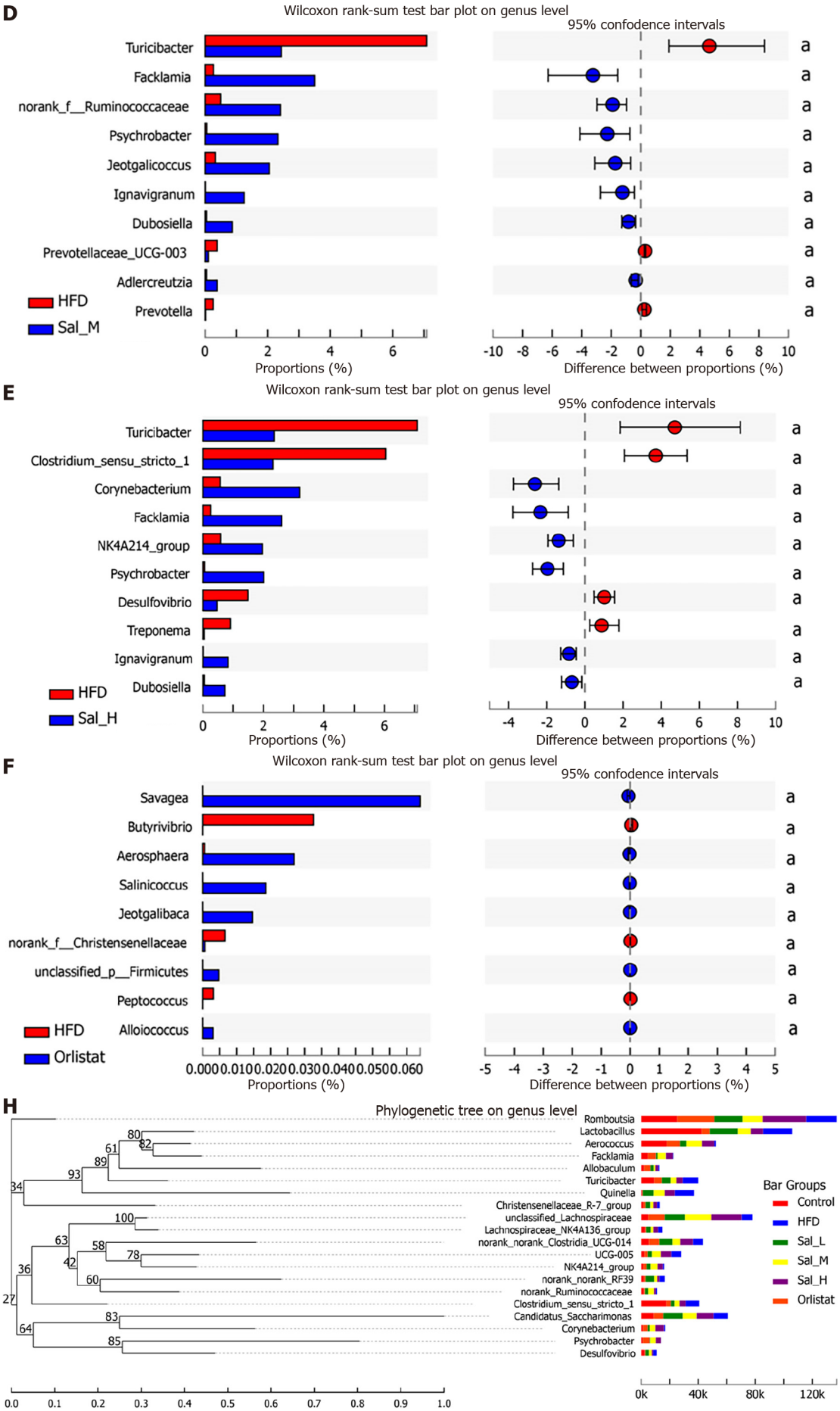
[a]$P < 0.05$.
[b]$P < 0.01$ *vs* control group.
[c]$P < 0.05$.
[d]$P < 0.01$.
[e]$P < 0.01$ *vs* high-fat diet (HFD) group.
Control, HFD, and Sal_M ($n = 8$ per group) groups. ↑: Metabolite content increased; ↓: Metabolite content decreased; C: Control group; H: High-fat diet group; S: Sal_M group.

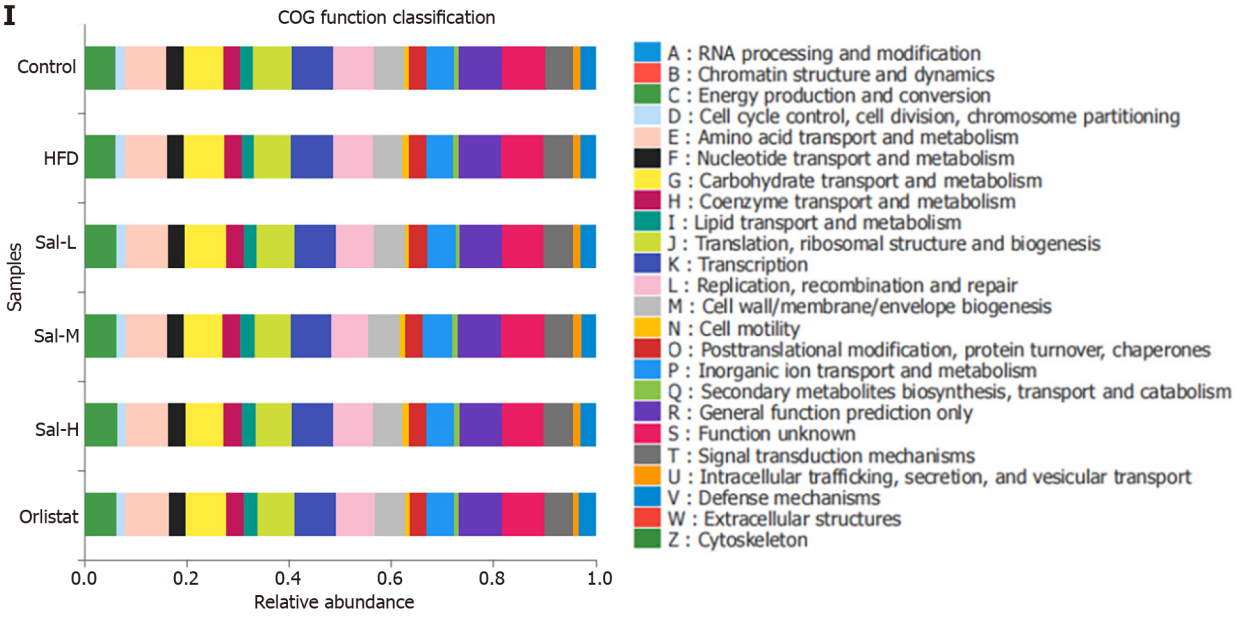
to the db-RDA analysis, the levels of FFAs, weight, BFI, and GLU of obese rats in the HFD group were opposite to the GM abundance in the control, Sal_L, Sal_M, and Sal_H groups at the genus level (Figure 8G). Analysis of the Spearman-related heatmap (Figure 8F) at the genus level found that TGs, DGs, and Cers were positively correlated with *Quinella*, *Desulfovibrio*, *Blautia*, and *Turicibacter*, and negatively correlated with *Aerococcus*, *Dubosiella*, *Facklamia*, and *Jeotgalicoccus*; cAMP, PKA, and HSL were positively correlated with *Corynebacterium*, *Aerococcus*, *Dubosiella*, and *Facklamia* and negatively correlated with *Desulfovibrio* and *Blautia* (Figure 8H), which agreed with our previous findings about the structure of the microbiome. The above analysis hints that Sal participates in the regulation of the microbial community structure and environmental characteristics, as well as the regulation of lipid metabolism, which helps to promote lipolysis and prevent adipogenesis.

DISCUSSION

In the present study, the weight losing effect of Sal on HFD-induced obese rats is evident, which was proven by the decrease in weight and BFI without changing food intake compared to the HFD groups with increased weight, obesity indexes, and lipid profiles. Moreover, we observed that Sal significantly down-regulated the levels of TG, TC, LDL-C, GLU, and FFAs in the serum of obese rats. Additionally, H&E analysis revealed that the liver tissue from HFD-induced obese rats treated with Sal displayed


A
Kruskal-Wallis H test bar plot
P value
Romboutsia
Clostridium_sensu_stricto_1
Quinella
Facklamia
Corynebacterium
Psychrobacter
norank_f__Ruminococcaceae
Lachnoclostridium
Blautia
Streptococcus
0.03354
0.007194
0.03962
0.04178
0.02133
0.004325
0.01774
0.03486
0.02811
0.01828
Control
HFD
Sal_L
Sal_M
Sal_H
Orlistat
0 2 4 6 8 10 12 14 16 18 20
mean proportions (%)
G
Network analysis on genus level
Control
HFD
Sal_L
Sal_M
Sal_H
Orlistat
Species(genus)
B
Wilcoxon rank-sum test bar plot on genus level
Clostridium_sensu_stricto_1
Aerococcus
Quinella
unclassified_f__Lachnospiraceae
Blautia
Lachnoclostridium
Colidextribacter
Streptococcus
unclassified_f__Eggerthellaceae
Psychrobacter
Control
HFD
0 2 4 6 8
Proportions (%)
95% confodence intervals
a
-20 -15 -10 -5 0 5 10 15 20
Difference between proportions (%)
C
Wilcoxon rank-sum test bar plot on genus level
unclassified_f__Lachnospiraceae
Clostridium_sensu_stricto_1
norank_f__Ruminococcaceae
Ruminococcus_torques_group
Dubosiella
Adlercreutzia
Anaerofustis
Odoribacter
UCG-008
HFD
Sal_L
0 2 4 6 8
Proportions (%)
95% confodence intervals
a
-10 -8 -6 -4 -2 0 2 4 6 8 10
Difference between proportions (%)


D
Wilcoxon rank-sum test bar plot on genus level
95% confodence intervals
Turicibacter
Facklamia
norank_f__Ruminococcaceae
Psychrobacter
Jeotgalicoccus
Ignavigranum
Dubosiella
Prevotellaceae_UCG-003
Adlercreutzia
Prevotella
HFD
Sal_M
Proportions (%)
Difference between proportions (%)
E
Wilcoxon rank-sum test bar plot on genus level
95% confodence intervals
Turicibacter
Clostridium_sensu_stricto_1
Corynebacterium
Facklamia
NK4A214_group
Psychrobacter
Desulfovibrio
Treponema
Ignavigranum
Dubosiella
HFD
Sal_H
Proportions (%)
Difference between proportions (%)
F
Wilcoxon rank-sum test bar plot on genus level
95% confodence intervals
Savagea
Butyrivibrio
Aerosphaera
Salinicoccus
Jeotgalibaca
norank_f__Christensenellaceae
unclassified_p__Firmicutes
Peptococcus
Alloiococcus
HFD
Orlistat
Proportions (%)
Difference between proportions (%)
H
Phylogenetic tree on genus level
Romboutsia
Lactobacillus
Aerococcus
Facklamia
Allobaculum
Turicibacter
Quinella
Christensenellaceae_R-7_group
unclassified_Lachnospiraceae
Lachnospiraceae_NK4A136_group
norank_norank_Clostridia_UCG-014
UCG-005
NK4A214_group
norank_norank_RF39
norank_Ruminococcaceae
Clostridium_sensu_stricto_1
Candidatus_Saccharimonas
Corynebacterium
Psychrobacter
Desulfovibrio
Bar Groups
Control
HFD
Sal_L
Sal_M
Sal_H
Orlistat



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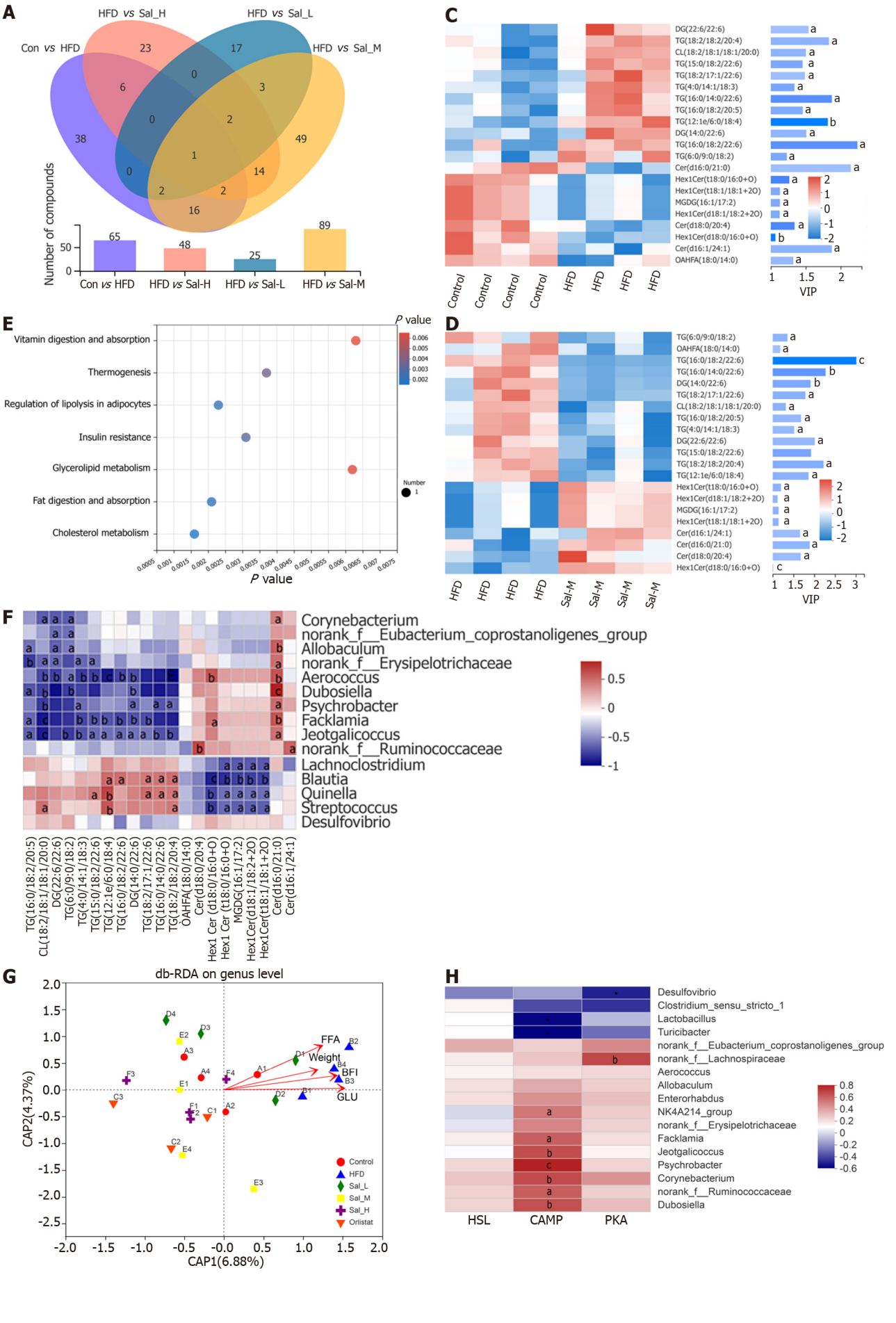
Figure 7 Effect of *Salvia miltiorrhiza* on intestinal microbiota composition in six groups of rats. A: Differential analysis among these six groups at the genus level; B-F: Differential analysis between control and high-fat diet (HFD) groups, HFD and Sal_L groups, HFD and Sal_M groups, HFD and Sal_H groups, and HFD and orlistat groups at the genus level ($^aP < 0.05$, $^bP < 0.01$); G: Network analysis at the genus level; H: Phylogenetic tree at the genus level; I: COG functional classification.

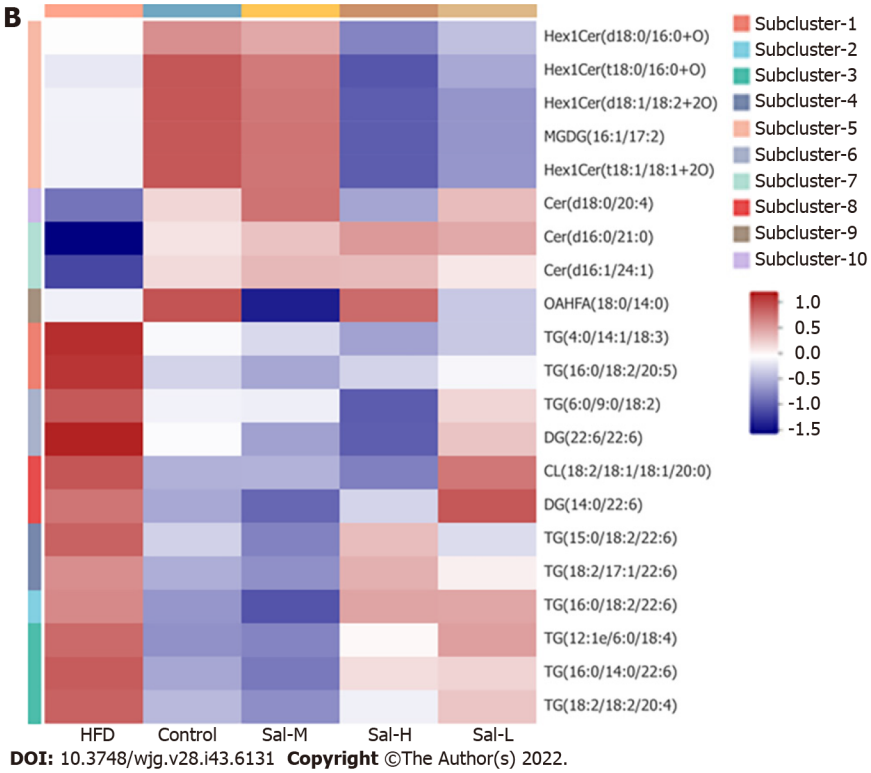
reduced mean adipocyte size, increased smaller adipocytes, and alleviated hepatic steatosis. Sal can improve dyslipidemia and reduce obesity by preventing the buildup of excess lipid compositions in the liver that can cause fatty liver and dyslipidemia in obese people. Similarly, after Sal intervention, smaller adipocytes were observed in adipose tissue. These results suggest that Sal has promising anti-obesity effects associated with reducing HFD-induced body weight independent of food intake.

Sal has been proven to have anti-inflammation[29] and antioxidation effects, which is widely used to treat cardiovascular disease[30], diabetes[31], fatty liver disease[32], and other dyslipidemia diseases [33]. Jung *et al*[9] reported that tanshinone, an ingredient in the Sal, can inhibit HFD-induced obesity by preventing early adipogenesis and improving lipid metabolism. Importantly, we found that Sal exerts anti-HFD-induced obesity effects by improving blood lipid levels, and regulating GM and metabolites, thus further suggesting the potential of Sal for clinical treatment of obesity.

As an environmental factor, the GM interaction with the host[34] has an essential role in the occurrence and development of obesity[35]. Previous studies reported lower GM diversity in obese mice than in the control group, and obese mice showed a decrease in *Bacteroides* and an increase in *Firmicutes* [36,37]. Armougom *et al*[38] found more *Methanobacter*, *Bacteroidetes*, and *Lactobacillus* in anorexic patients. GM imbalance is one of the pathogeneses of obesity. In this study, we found that obese rats had a different gut flora composition than lean rats, with higher abundances of *Desulfobacterota*, *Quinella*, and *Turicibacter* and lower abundances of *Jeotgalicoccus*, *NK4A214*, *Aerococcus*, *Dubosiella*, *Psychrobactor*, *norank_f_Lachnospiraceae*, and *norank_f_Erysipelotrichaceae* at the genus level and *Proteobacteria* and *Actinomycetes* at the phylum level. Studies have suggested that *Firmicutes*, including *Lachnoaceae*, *Erysipelotrichaceae*, *Oscillospiraceae*, and *Ruminococcaceae*, can promote energy absorption in obese and diabetic rats[39]. In this study, we found that Sal decreased the abundance of *Firmicutes*, including *norank_f_Lachnospiraceae* and *Dubosiella*, in HFD-induced obese rats. Similar results were observed in HFD-induced obese rats treated with polyphenols and procyanidins[40,41]. *Bacteroidetes*, a beneficial bacterium, have been reported to be negatively associated with obesity and hyperlipidemia. Previous studies have suggested that the proportion of *Bacteroidetes* and *Proteobacteria* is slightly higher than that of the control population[27]. Interestingly, when HFD was used to successfully induce obesity in rats, we found an increased abundance of *Bacteroidetes* at the phylum level. Our results are consistent with Schwiertz's evidence of a significant increase in *Bacteroidetes* in overweight and obese subjects[42]. We believe that this is because factors, such as heredity, diet, and environment, may have similar or even opposite effects on the flora. Lipid factors involved in metabolism may also interfere with the structure of GM through various mechanisms. Studies have shown that FXR agonists regulate lipid metabolism and GM by increasing the presence of *Ackermann* and decreasing the presence of *Desulfuricans* in obese mice[43], which is consistent with our findings.

One of the consequences of dyslipidemia is inefficient lipid metabolism, which in turn accelerates the development of obesity and dyslipidemia[44,45]. Lipid metabolism disorders provide the "first hit" in the progression of metabolic diseases such as obesity[46]. Hence, this study applied lipidomics to


A
HFD vs Sal_H
HFD vs Sal_L
Con vs HFD
HFD vs Sal_M
Number of compounds
Con vs HFD
HFD vs Sal-H
HFD vs Sal-L
HFD vs Sal-M
C
VIP
D
VIP
E
P value
Vitamin digestion and absorption
Thermogenesis
Regulation of lipolysis in adipocytes
Insulin resistance
Glycerolipid metabolism
Fat digestion and absorption
Cholesterol metabolism
Number
F
Corynebacterium
norank_f__Eubacterium_coprostanoligenes_group
Allobaculum
norank_f__Erysipelotrichaceae
Aerococcus
Dubosiella
Psychrobacter
Facklamia
Jeotgalicoccus
norank_f__Ruminococcaceae
Lachnoclostridium
Blautia
Quinella
Streptococcus
Desulfovibrio
G
db-RDA on genus level
CAP1(6.88%)
CAP2(4.37%)
FFA
Weight
BFI
GLU
Control
HFD
Sal_L
Sal_M
Sal_H
Orlistat
H
Desulfovibrio
Clostridium_sensu_stricto_1
Lactobacillus
Turicibacter
norank_f__Eubacterium_coprostanoligenes_group
norank_f__Lachnospiraceae
Aerococcus
Allobaculum
Enterorhabdus
NK4A214_group
norank_f__Erysipelotrichaceae
Facklamia
Jeotgalicoccus
Psychrobacter
Corynebacterium
norank_f__Ruminococcaceae
Dubosiella
HSL
CAMP
PKA



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Figure 8 Lipidomics analysis and associations of gut microbial species with environmental factors. A: Numbers of differential metabolites between the control and high-fat diet (HFD) groups, HFD and Sal_L groups, HFD and Sal_M groups, and HFD and Sal_H groups (Venn diagram); B: Hierarchical clustering of metabolites in Control, HFD, Sal_L, Sal_M, and Sal_H groups; C: Heatmap of the VIP expression profile of the metabolites between control and HFD groups; D: Heatmap of the VIP expression profile of the metabolites between HFD and Sal_M groups; E: KEGG pathway enrichment; F: Spearman's correlation between metabolites and gut microbiota; G: Correlation between free fatty acids, weight, body fat index, glucose, and microbial flora structure displayed by distance-based redundancy analysis (db-RDA analysis); H: Spearman's correlation between cAMP, PKA, HSL, and gut microbiota. $^{a}P < 0.05$, $^{b}P < 0.01$, or $^{c}P < 0.001$.

analyze the composition of lipid metabolites in obesity and observed the alteration of lipid metabolites by increasing the treatment of Sal to analyze the molecular mechanism of the anti-obesity and cholesterol-lowering effects of Sal, thus providing new insights into the treatment of obesity. A range of significant changes occurred in the lipidome with progressive obesity. Most notably, the contents of DGs, TGs, and CLs were increased in the HFD group compared to the control group, which was similar to previous studies[47]. Contrary, Sal decreased the levels of these lipid species in the lipidome profile, suggesting that Sal could improve the imbalance between HFD-induced lipid synthesis and catabolism. The concentration of FFAs in serum is related to lipid metabolism and glucose metabolism, and diseases such as diabetes and obesity can increase the concentration of FFAs. When energy metabolism is abnormal, FFAs will accelerate the accumulation of TG in hepatocytes or convert to lipid intermediates such as DGs, CLs, and Cer, depleting cellular functions and, in turn, leading to obesity and other metabolic disorder diseases[48]. Interestingly, we found that the supplementation of Sal effectively decreases the hepatic accumulation of TGs, DGs, and CLs. Evidence suggests that Cer is involved in obesity-induced metabolic disorders by various mechanisms, including inflammation, apoptosis, and autophagy. However, in our study, Cer was not entirely increased in the HFD-induced obese rats. We wondered whether Cer might interfere with obesity by participating in other pathways or whether the balance of sphingolipid (SP) metabolism, rather than Cer accumulation, is correlated with the development of obesity[49]. SP species are not only increased in obese patients but are also associated with hepatic oxidative stress, suggesting that these lipids may participate in the progression of obesity [50]. In general, TG accumulation is a sign of obesity and is positively correlated with hexosylceramide (HexCer); yet, in our experiment, Hex1Cer, which acts as a SP, was reduced in the HFD-induced obese rats and negatively correlated with TG. Part of that has to do with the small number of animals analyzed, and the role of Hex1Cer in obesity is not quite clear yet. Our results were consistent with Eisinger's study[51]. Some researchers also believe that pharmacological blockage of glucosylceramide prevents obesity and liver steatosis[3,52,53]. In addition, we found that a few fatty acids, such as OAHFA, a novel lipid in structure with functions such as stimulating insulin secretion and improving glycolipid transport *in vivo*[54,55], were increased in HFD-induced obese rats treated with Sal. These lipids are structurally novel lipids that are provided. It is worth mentioning that some beneficial lipids are important to the development of obesity, such as phosphatidylcholines[56], which can regulate lipid and cholesterol metabolism[57]. Although it did not appear in our study, it is also one of the focuses of our future research.

Pathway enrichment analysis can assess the biochemical pathways involved in significantly different metabolites and their functional classification. Significant pathways enriched by KEGG, including lipolysis in adipocytes, insulin resistance, glycerolipid metabolism, fat digestion and absorption, and cholesterol metabolism, have been shown to be closely associated with the development of obesity, among which metabolism of cholesterol had the greatest significance, with a *P*-value of 0.0016. Moreover, the dysfunction of glycerolipid metabolism may disturb the energy metabolism of hepatocytes and adipocytes. Furthermore, obese patients are often accompanied by hormonal dysregulation, which aggravates lipid metabolic disorders. For example, as one of the anti-lipolysis hormones, insulin has an important role in reducing lipolysis by reducing cAMP concentration in adipocytes. The increase of cAMP catalyzes the hydrolysis of triacylglycerol to fatty acids and glycerol and then completes the lipolysis process, and PKA with HSL is involved[2]. Similarly, increases in FFAs can also cause insulin-mediated decreases in glucose oxidation and utilization, increase insulin resistance, and cause hyperinsulinemia and obesity. In our study, the levels of cAMP, PKA, and HSL were decreased in the HFD group compared to the control group, while GLU and FFA were increased. Spearman-related heatmap analysis further showed that cAMP, PKA, and HSL had a negative correlation with the structure of the GM in the HFD group, while FFAs, weight, BFI, and GLU were positively correlated with GM.

It is widely believed that the regulatory effect of intestinal microbiota on lipid metabolism is closely related to bile acids and short-chain fatty acids (SCFAs). According to Zhang *et al*[58], increasing the SCFA-producing genera *Blautia* and *Allobaculum* could enhance intestinal integrity and reduce the body weight in obese mice[59], thus rivaling obesity. *Allobaculum* is a member of *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae*, a family that affects host metabolism[60] and protects body weight gain from HFD. *Allobaculum* can produce butyric acid and propionic acid[61,62]. In our research, analysis of the Spearman-related heatmap showed that lipids including TGs, DGs, and CL that increased in the HFD group were inversely associated with *Allobaculum* and positively related to *Blautia,* which suggests that Sal may improve *Allobaculum* but not *Blautia* to produce SCFAs and then regulate the GM and lipid metabolism. Acetate, propionate, and butyrate are examples of SCFAs produced by bacteria, and research showed that SCFA-induced activation of the PPAR pathway could modulate lipid metabolism by increasing energy consumption[63], reducing body weight, and decreasing liver TG accumulation [64]. It should be noted that the genus *Blautia,* as a member of the *Lachnospiraceae* family, could produce SCFAs, which can regulate inflammation and metabolism[65]. Consequently, the observation in our research that Sal promoted a decrease in *Lachnospiraceae* and *Allobaculum* indicated that Sal likely confers beneficial effects *via* modulating the GM composition and host lipid metabolism with SCFAs, which is consistent with previous reports[66,67]. However, whether *Lachnospiraceae* has a key role in the regulation of obesity and lipids still needs to be confirmed by accurate tests.

Desulfovibrio has been reported to be positively correlated with metabolism[68,69] and may increase after an HFD[70,71]. Similar to our findings, *Desulfovibrio* was reported to be decreased by the intervention of Sal. We also found that the improvement of gut microecology resulting in the increase of SCFAs may be responsible for the cholesterol-lowering effects of obesity. There is a reciprocal relationship between bile acids and cholesterol metabolism, which is closely related to intestinal flora. Bile acids are synthesized from cholesterol in the liver and have a role in maintaining cholesterol homeostasis and promoting lipid absorption[72]. Intestinal flora regulates lipid metabolism in the host by affecting bile acid composition. GM could influence bile acid metabolism by performing structural modifications, including oxidation, deconjugation, or hydroxylation[73]. It has been confirmed that bile acids and bile acid signaling pathways are involved in the control of plasma lipid and lipoprotein levels and that hepatic bile acid synthesis has an important role in the regulation of plasma TG levels in obese individuals[74], which is consistent with the present study that the cholesterol metabolism was most concentrated in KEGG pathway with the elevated cholesterol and TG in the serum and the significant elevation of TGs in lipidomics, while Sal may regulate the relationship between bile acids and cholesterol metabolism by reducing cholesterol accumulation and regulating lipid metabolism.

The above experimental results suggest that HFD-induced obesity causes dyslipidemia and dysregulation of GM and metabolites, accompanied by weight gain, hepatic steatosis, and abdominal fat accumulation. Importantly, we found that Sal effectively improved blood lipids and reshaped the balance of GM and lipid metabolism in obese rats (Figure 9), reversing weight gain and fat accumulation. Correlation analysis further demonstrated that Sal exerted anti-obesity effects through lipid metabolites of intestinal flora, which laid a good foundation for the subsequent study. In the following study, the key components of Sal's anti-obesity effects will be further investigated by pharmacokinetic and high-performance liquid chromatography techniques. The key flora and metabolites of Sal's anti-obesity effects will be further demonstrated by intestinal flora transplantation and lipid metabolite supplementation, and the key targets of Sal's anti-obesity effects will be further explored by molecular docking or protein interaction and gene enrichment and editing techniques, which will become a promising way to find new targets for obesity.

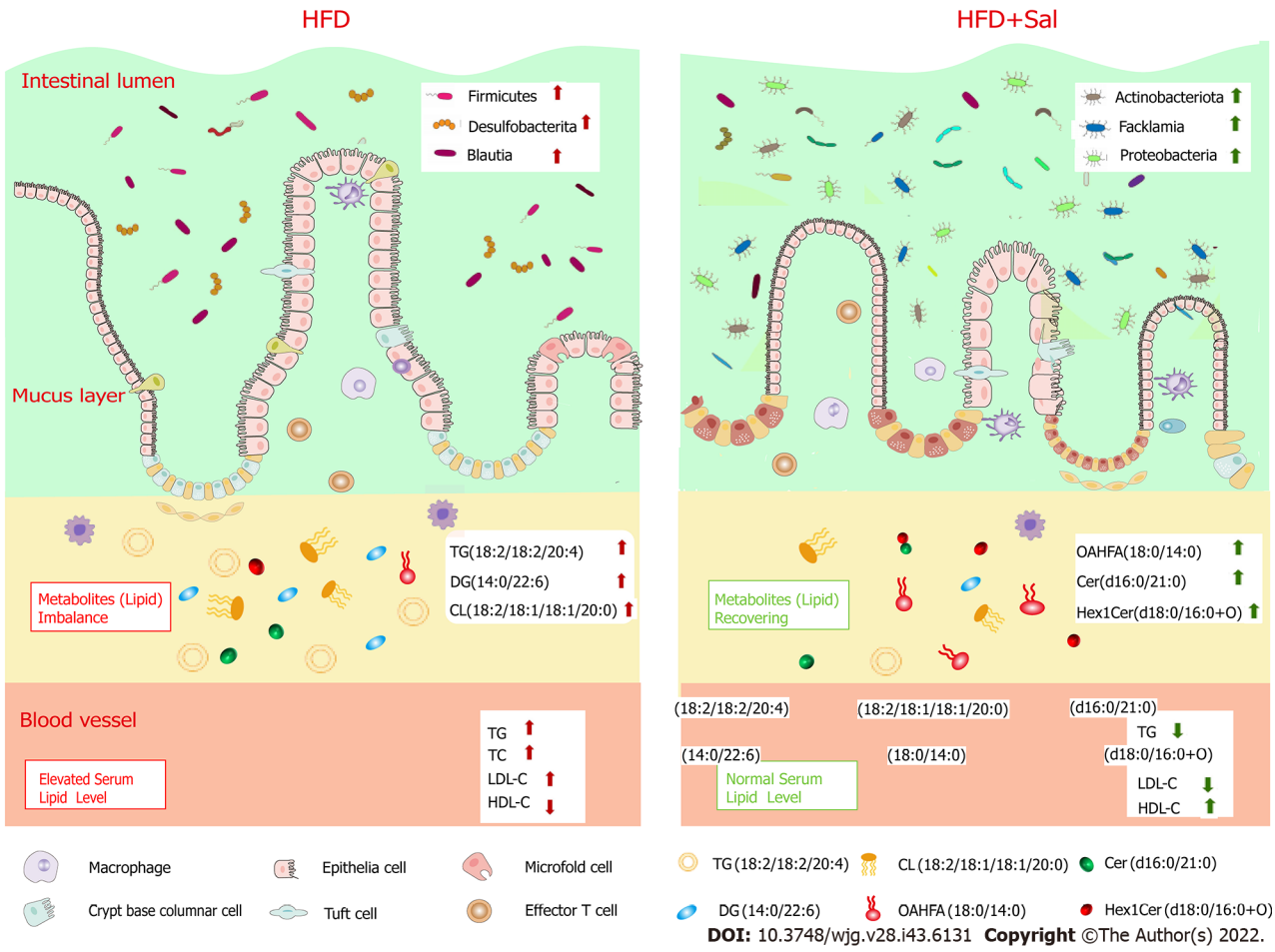


Figure 9 Graphical illustration of mechanism of action of *Salvia miltiorrhiza* to alleviate obesity. *Salvia miltiorrhiza* ameliorated obesity by reshaping the balance of gut microbiota, modulating the lipid metabolites, and improving blood lipids. Sal: *Salvia miltiorrhiza* extract; HFD: High-fat diet; TG: Triglycerides; TC: Total cholesterol; HDL-C: High-density lipoprotein; LDL-C: Low-density lipoprotein; GLU: Glucose; FFA: Free fatty acids.

CONCLUSION

Our results showed that Sal reduces body weight, body fat index, serum lipid level, hepatic lipid accumulation, and adipocyte vacuolation in HFD-induced obese rats, which may be associated with enhanced gut integrity and improved lipid metabolism. 16s RNA sequence analysis revealed that Sal could reverse HFD-induced dysbacteriosis while LC-MS/MS analysis indicated that Sal could improve the lipid composition of HFD rats, which provides research basis and evidence to study the mechanism of Sal in the treatment of obesity.

ARTICLE HIGHLIGHTS

Research background

Obesity is a world health problem. A growing number of studies have suggested that gut microbiota is an important regulator of host metabolism, and the dysregulation or imbalance of gut microbiota (GM) is closely related to obesity and its complications. Mounting evidence suggests that improving the structure of the GM balance to regulate metabolism, particularly lipid metabolism, is a viable strategy for treating obesity or obesity-related disease.

Research motivation

Salvia miltiorrhiza extract (Sal) has shown good efficacy in experimental obese rats induced by a high-fat diet (HFD). Also, disturbances in gut microbiota have been observed in various diseases, including metabolic disease. However, few studies have explored the role of Sal on gut microbiota and lipid metabolism when treating obesity.

Research objectives

To investigate whether Sal can alleviate obesity induced by an HFD by regulating gut microbiome and lipid metabolism.

Research methods

Rats were given an HFD (with purified ingredients and a total caloric value of 475 Kcal/100 g, with lard as the main source of fat) for 7 wk, while Sal (0.675 g/1.35 g/2.70 g/kg/d) was administered for 8 wk. Serum lipid test, liver and fat tissue histopathologic examination, ELISA, 16S RNA sequencing, and LC-MS/MS analysis were used to evaluate the efficacy of Sal on obesity.

Research results

Sal effectively improved blood lipids and reshaped the balance of gut microbiota and lipid metabolism in obese rats, reversing weight gain and fat accumulation caused by HFD. Correlation analysis further demonstrated that Sal exerted anti-obesity effects through lipid metabolites of intestinal flora, which laid a good foundation for the subsequent study.

Research conclusions

Sal may exert an anti-obesity effect in HFD-induced obese rats by modulating the gut microbiome and lipid metabolism.

Research perspectives

This study addressed an important topic of the development of obesity, *i.e.*, the role of gut microbiota and lipid metabolism in the development of obesity, using an extract from a Chinese herb that has been found to have anti-obesity effects in various diseases.

FOOTNOTES

Author contributions: Ai ZL and Zhang X contributed equally to this work; Zhong YB and Ge W performed the experiments; Liu DY, Wang HY, and Zuo ZY contributed reagents/materials/analytical tools; Liu DY and Ge W analyzed the data; Ai ZL and Liu DY wrote the paper; Zuo ZY and Wang HY conceived and designed the experiments.

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ORIGINAL ARTICLE

Retrospective Study

Upper gastrointestinal endoscopic findings in celiac disease at diagnosis: A multicenter international retrospective study

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Abstract

BACKGROUND

Gastroduodenal endoscopy and biopsy following positive specific serology is considered the gold standard to diagnose celiac disease (CeD) in adults. Whether upper endoscopy helps detect comorbid conditions is unknown.

AIM

To investigate the prevalence of non-celiac endoscopic findings in patients in whom endoscopy was performed to confirm CeD diagnosis.

METHODS

This is an observational, descriptive, multicenter, retrospective study that reports endoscopic findings obtained in adult patients enrolled in local registries from four tertiary centers. We collected data reported on first endoscopy, indicated for investigation of CeD. Diagnosis of CeD was performed by histology (≥ Marsh 2

type mucosal damage) and specific serology. Two European and one North American center included biopsy-confirmed CeD following positive serology. A fourth center (South America) included symptomatic patients undergoing endoscopy, irrespective of CeD serology. The latter cohort included a non-CeD control group.

RESULTS

A total of 1328 patients (80% female; 35 years median age) were enrolled, of whom 95.6% had positive specific serology. In 135 patients, endoscopy revealed 163 abnormalities unrelated to CeD (prevalence: 10.1%). Erosive reflux esophagitis (6.4%), gastric erosions (2.0%), and suspicion of esophageal metaplasia (1.2%) were the most common findings. Biopsy-confirmed Barrett's esophagus was infrequent (0.2%). No endoscopic cancer was detected. Older patients (≥ 51 years of age) had a higher prevalence of endoscopic findings than those ≤ 50 ($P < 0.01$). Within the South American cohort, CeD was associated with a lower rate (8.2%) of comorbid endoscopic findings compared with controls (29.1%; $P < 0.001$). In the adjusted multivariate analysis of this cohort, having CeD was associated with a 72% reduction in the risk of any endoscopic abnormality ($P < 0.0001$), and having alarm symptoms was associated with a 37% reduction in the risk of finding at least one endoscopic lesion ($P < 0.02$).

CONCLUSION

In this large multicenter study, young adults with positive CeD serology had few comorbid endoscopic findings. Although patients over 51 years had a high prevalence of non-CeD gastroduodenal mucosal damage, no malignancy or premalignant lesions were found.

Key Words: Celiac disease; Upper gastrointestinal endoscopy; Concomitant endoscopic lesions; Malignancies; Multicenter study

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Core Tip: We offer novel data on the prevalence of non-celiac endoscopic findings at the time of endoscopy performed to confirm celiac disease (CeD) diagnosis. Based on the very high performance of specific serology tests, the diagnosis of CeD without duodenal biopsy has been proposed in recent years. However, some guidelines do not recommend avoiding endoscopy because relevant comorbid diagnosis can be missed. Our results found that comorbid upper gastrointestinal endoscopic pathology is uncommon in patients with positive CeD serology at the time of diagnostic endoscopy suggesting that a non-biopsy strategy is unlikely to clinically miss significant concomitant endoscopic findings unrelated to CeD.

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INTRODUCTION

Celiac disease (CeD) is one of the most common life-long chronic diseases affecting people with a genetic predisposition conferred by HLA-DQ2 or DQ8[1]. Current recommendations for diagnosing CeD in adult patients involve a combination of specific serology and a duodenal biopsy demonstrating some degree of intestinal atrophy[2,3]. When CeD is clinically suspected, upper gastroduodenal endoscopy with duodenal biopsy confirms diagnosis[4]. Based on the very high specificity and predictive values of specific serology tests[5], the diagnosis of CeD without duodenal biopsy has been proposed in recent years[6-8]. Indeed, European pediatric societies recommend a non-biopsy approach under specific and strict criteria[9,10]. However, other pediatric societies (*e.g.*, the North American Pediatric Gastroenterology Society) do not recommend this, in part because relevant comorbid diagnosis could be missed[11]. This is of particular concern in patients with alarm symptoms such as weight loss, anemia, or abdominal pain[2,12,13]. However, relatively few studies have explored this in-depth, particularly in adult patients undergoing endoscopy to confirm CeD diagnosis[14-16].

Thus, we conducted a multicenter study involving four cohorts of patients diagnosed in three countries to investigate the prevalence of coincidental upper gastrointestinal endoscopic findings in CeD patients at the time of diagnosis. We also compared upper gastrointestinal mucosal injury

diagnoses across centers and age groups. Finally, we studied the pathological findings in patients with a confirmed diagnosis of CeD *vs* those in whom the disease was ruled out.

MATERIALS AND METHODS

Design

We conducted a descriptive multicenter retrospective study on endoscopic findings from adult patients who met standard clinical, serological, and histological criteria for CeD. Patients from four different CeD-specialized centers were included. Two European cohorts (Universities of Naples/Salerno and Padua; Italy) and a North American cohort (McMaster University, Hamilton; Canada) recruited consecutive patients enrolled in local registers. CeD was diagnosed by positive serology and confirmed by biopsy. The Naples/Salerno cohort included consecutive patients seen between 1987 and 2021, the Padua cohort between 2017 and 2021, and the Hamilton cohort between 2018 and 2020. A fourth (Small Bowel Section, Dr. C. Bonorino Udaondo Gastroenterology Hospital, Buenos Aires; Argentina) included patients referred for endoscopy and duodenal biopsy due to the presence of symptoms and/or signs compatible with CeD but, irrespective of serology, all of them part of prior research and study[7,15]. Thus, the fourth cohort included CeD and non-CeD participants (controls). Figure 1 and Table 1 summarize the demographic characteristics of the cohorts. The Ethics and Research Board of the Dr. C. Bonorino Udaondo Gastroenterology Hospital approved the study because of the prospective design and intervention in the Buenos Aires cohort. Ethics approval was obtained from Hamilton Integrated Research Ethics Board (HiREB# 14460/5415). In Italy, Ethical Committee review was not required for retrospective studies while patient data remained anonymously coded.

Endoscopic procedures

In all CeD centers, experienced gastroenterologists performed upper gastrointestinal endoscopies and obtained duodenal biopsies *per* shared standard of care protocols. Endoscopic reports were generated using a standard format, and the data were entered into a common database. Duodenal biopsies were sent to each institution's experienced pathologist. A standard number of biopsies were taken when any endoscopic abnormality was detected (*e.g.*, endoscopic evidence of esophageal metaplasia). Endoscopic abnormalities were defined as follows[17]: (1) Erosive esophagitis: Esophageal mucosal damage characterized by one or more mucosal breaks that do not extend across the top of mucosal folds and confluent lesions or ulcers of any size; (2) Suspected esophageal metaplasia: Endoscopically suspected columnar mucosa without histological confirmation of specialized intestinal metaplasia; (3) Barrett's esophagus confirmed by biopsy: Metaplastic columnar epithelium replacing the stratified squamous epithelium in biopsies from suspected metaplasia or presence of intestinal metaplasia; (4) Gastric and duodenal erosions: Presence of erythema and erosions in stomach or duodenum; (5) Esophageal, gastric, or duodenal ulcers extending into the *muscularispropria*; and (6) Esophageal, gastric, or duodenal cancer: Suspected endoscopic lesions were confirmed by specialized pathology.

CeD diagnosis

CeD was diagnosed based on duodenal histology (Marsh's classification)[1,18]. Inclusion criteria were Marsh 2 enteropathy or higher and positive CeD-specific serology [presence of either anti-TTG immunoglobulin (Ig)A, Anti-EmA IgA, anti-DGP IgA/IgG]. When serology was negative, CeD was diagnosed based on histology and clinical response to the gluten free diet (GFD)[18]. If patients had known exposure to gluten before the endoscopy, intestinal biopsies were taken. As previously stated, the Buenos Aires cohort was part of a research study in which the diagnosis was made first on histological grounds and then confirmed by serology. The standard specific CeD test for all centers was IgA transglutaminase 2[5]. Patients with normal biopsy or minimal inflammation (Marsh 0 or 1) were excluded from the study, regardless of serology or GFD response. In the Hamilton cohort, diagnosis of seronegative CeD patients was based on histology and a clinical and histological response to the GFD.

Data analysis

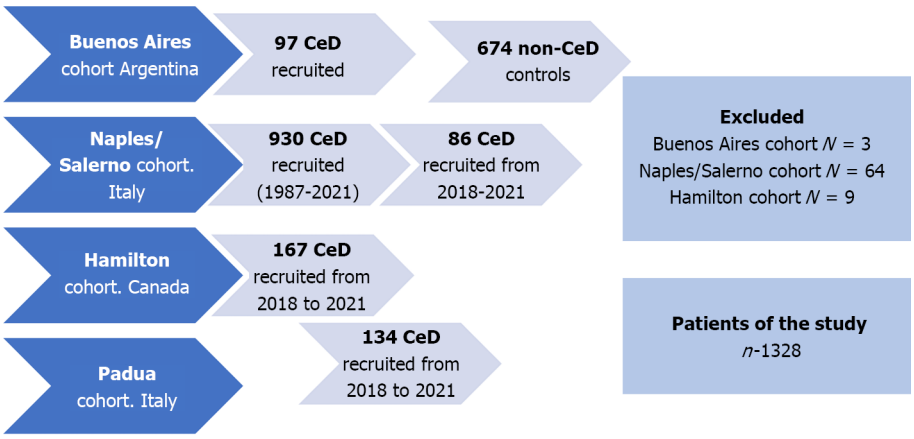
Statistical analysis was carried out using STATA (STATA version 14.0 Corp, College Station, TX, United States). Categorical variables were reported as frequencies and percentages, while continuous variables were reported as mean ± SD and/or median and 25%-75% interquartile ranges, according to their distribution. Comparisons of categorical variables between groups were made using the χ^2 test or Fisher's exact test. P values < 0.05 were considered statistically significant. For comparisons of continuous variables the analysis of variance test was used. Logistic regression was used to assess the risk of endoscopic lesions. The model included the report of significant lesions in endoscopy and/or histology reports as a dependent variable and factors such as age, sex, personal history, and signs/symptoms as independent variables.

Given the different recruitment times between centers, a subgroup analysis was performed to compare results in the Naples/Salerno cohort, focusing on cases diagnosed between 2018 and 2021 *vs*

Table 1 Demography, celiac disease serology, and endoscopic findings by cohorts and the overall population

Demographic data and upper GI endoscopic findings	Naples/Salerno cohort	Buenos Aires cohort	Hamilton cohort	Padua cohort	Overall CeD population
Total population	930 (70.0)	97 (7.3)	167 (12.6)	134 (10.1)	1328
Age in yr	34 (26-42)	35 (27-44)	39 (27-54)	35 (23-46)	35 (26-43)
Female sex	754 (81.1)	88 (90.7)	122 (73.0)	100 (74.6)	1064 (80.1)
Patients with positive serology	899 (96.7)	97 (100)	147 (88.0)	130 (97.0)	1269 (95.6)
Patients with at least one significant endoscopic abnormality	89 (9.6)	8 (8.2)	34 (20.4)	4 (3.0)	135 (10.2)
Reflux esophagitis with erosions	78 (8.4)	1 (1.0)	6 (3.6)	0	85 (6.4)
Esophageal peptic ulcers	0	0	1 (0.6)	0	1 (0.2)
Esophageal malignancy	0	0	0	0	0
Suspicion of esophageal metaplasia	3 (0.3)	0	13 (7.8)	0	16 (1.2)
Biopsy confirmed Barrett's esophagus	2 (0.2)	0	1(0.6)	0	3 (0.2)
Gastric erosions	0	7 (7.2)	16 (9.6)	4 (3.0)	27 (2.0)
Gastric ulcers	1 (0.1)	0	0	0	1 (0.1)
Gastric cancer	0	0	0	0	0
Duodenal erosions	0	0	15 (9.0)	0	15 (1.1)
Duodenal ulcers	8 (0.9)	1 (1.0)	6 (3.6)	0	15 (1.1)
Duodenal cancer	0	0	0	0	0

Data are presented as *n* (%) or median and 25% interquartile range. CeD: Celiac disease; GI: Gastrointestinal; IQR: Interquartile range.



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Figure 1 Flow chart of participants. CeD: Celiac disease.

previous endoscopies, estimating that such analysis could detect differences by using more actualized endoscopic protocols that were temporally concordant with those reported from patients collected in the Padua and Ontario cohorts.

RESULTS

Overall, 1404 patients were diagnosed with CeD and 1328 of them were included in the study (Figure 1). Patients with slightly positive serology but Marsh 0 or 1 (n = 76) were not diagnosed as CeD.

Demographic and clinical characteristics

The number of participants recruited varied between centers (Tables 1 and 2). The Naples/Salerno cohort contained most (70.0%) of the patients, while the Buenos Aires cohort had the fewest patients (7.3%). The European and North American centers differed in the length of time the celiac centers had been operational. The South American center included patients and controls over a specific time previously enrolled in a different study. There was a female predominance in all groups. There was no difference in the age at which diagnostic endoscopy was performed. There were no differences in baseline demographics across centers. The percentage of patients testing positive for celiac specific antibodies ranged from 88% (Hamilton) to 100% (Buenos Aires).

Endoscopic findings in CeD patients and age-related damage

Endoscopy revealed 163 distinct abnormalities in 135 patients with CeD (10.1%) (Table 1). The most common finding was erosive reflux esophagitis (6.4%), with the highest prevalence in the Naples/Salerno cohort (8.4%) and the lowest in the Buenos Aires (1%) and Padua (0%) cohorts. Peptic esophageal ulcers were only found in 1 patient within the total cohort. Although Barrett's esophagus was suspected in 1.2% of the patients, it was biopsy confirmed in 0.2% of cases (18.7% of those suspected and subsequently biopsied). The Hamilton cohort had a higher suspicion of metaplasia (n = 13), but Barrett's esophagus was confirmed in 1 patient (Table 1). Gastric ulcers were found in 1 patient (0.1%) within the Naples/Salerno cohort, while gastric erosions were found in 2.0% of the total population, with a higher prevalence in the Buenos Aires (7.2%) and the Hamilton (9.6%) cohorts. In the latter, 9.1% of patients with duodenal erosion were documented. Overall, 1.1% of duodenal ulcers were discovered, with a higher frequency encountered in the Hamilton cohort (3.6%). No cancers were reported at any level of the upper gastrointestinal (GI) tract of CeD patients.

Patients under the age of 50 had a lower risk of having at least one abnormality compared with patients over the age of 51 (P < 0.01). This indicated a 96.6% increase in lesions found in older patients (8.9% *vs* 17.5%), which was primarily driven by erosive esophagitis and gastric erosions (Table 2). We performed a subgroup analysis of the Naples/Salerno cohort, including patients diagnosed between 2018 and 2021. Compared with the overall Naples/Salerno cohort, patients diagnosed recently (n = 86) had a higher percentage of at least one significant endoscopic abnormality (29.2% *vs* 9.6%, respectively), owing to a higher proportion of cases with erosive reflux esophagitis (20.0% *vs* 8.4%) and duodenal ulcers (8.2% *vs* 0.9%, respectively). These endoscopic features were more common in the Naples/Salerno cohort (after 2018) than in the other cohorts (Padua and Hamilton) (P < 0.01). Compared with the Padua cohort, the Salerno cohort had a higher proportion of patients with at least one endoscopic abnormality (29.1% *vs* 3.0%; P < 0.01) (Supplementary Table 1).

Endoscopic findings in celiac patients and non-celiac controls from the Buenos Aires cohort

We compared CeD patients (n = 97) *vs* non-CeD controls (n = 674) (Table 3) using the Buenos Aires cohort. The median age at endoscopy in non-CeD controls was 11 years higher than in patients with CeD, and the percent of females was lower (P < 0.01 for both). Compared with patients with CeD, a higher proportion of controls were under the age of 50 (P < 0.001) (Table 3). CeD specific serology was positive in 1.3% of non-CeD controls. IgA transglutaminase positive levels in controls were less than three times the upper limit of normal. Endoscopic findings were more frequent in controls than in CeD patients (P < 0.001). In all age groups, gastric erosions were most common. Two control subjects, both older than 51, had a stomach adenocarcinoma and another a duodenal cancer at diagnostic endoscopy. In contrast, no cancers were discovered in CeD patients. Metaplasia was found in 1.0% of controls, with Barrett's esophagus being confirmed after biopsy in two of these cases. Controls over the age of 51 had 12.9% more frequent mucosal damage compared with younger subjects (overall prevalence 31.4% *vs* 27.8%, respectively).

The crude multivariate analysis based on CeD patients and non-CeD controls found that a CeD diagnosis and presence of alarm symptoms(weight loss, anemia, bleeding, dysphagia, epigastric pain, or history of malignancy) reduced the risk of having at least one lesion by 78.0% and 49.0% (P < 0.0001 for both), respectively. According to the adjusted multivariate analysis, having CeD was associated with a 72% reduction in the risk of any endoscopic lesion (P < 0.0001), and having alarm symptoms was associated with a 37% reduction in the risk of having at least one endoscopic lesion (P < 0.02; Table 4).

DISCUSSION

The study's main finding was that upper endoscopy performed concurrently with duodenal biopsies for CeD diagnosis revealed no concomitant damage in 92.0% of cases. Only 1.6% of CeD patients had relevant findings with the potential to progress to severe disease, comprised by esophageal and gastric ulcers and Barrett's esophagus. While 8.9% of patients demonstrated upper GI injury, only 1.3% potentially had dangerous lesions. The low yield of relevant concomitant findings in this study does not support the usefulness of upper endoscopy beyond the need of obtaining biopsies for the diagnosis of CeD.

Table 2 Demography, celiac disease serology, and endoscopic findings of the overall population and by the age of diagnosis

Demographic data and upper GI endoscopic findings	Overall CeD population	≤ 50 yr	51-60 yr	≥ 61 yr
Patients	1328	1140 (85.8)	114 (8.6)	74 (5.6)
Age in yr	35 (26-43)	33 (25-39)	55 (53-56)	67 (63-71)
Female sex	1064 (80.1)	931 (81.7)	82 (71.9)	51 (41.0)
Patients with positive serology	1269 (95.6)	1092 (95.8)	109 (95.6)	68 (91.9)
Patients with at least one significant endoscopic abnormalities	135 (10.1)	102 (8.9)[1]	20 (17.5)	13 (17.6)
Reflux esophagitis with erosions	85 (6.4)	69 (6.0)	12 (10.5)	4 (5.4)
Esophageal ulcers	1 (0.2)	1 (0.1)	0	0
Esophageal cancer	0	0	0	0
Suspicion of metaplasia	16 (1.2)	10 (0.9)	3 (2.6)	2 (2.7)
Biopsy confirmed Barrett's esophagus	3 (0.2)	2 (0.2)	0	1 (1.3)
Gastric erosions	27 (2.0)	20 (1.7)	3 (2.6)	4 (5.4)
Gastric ulcers	1 (0.1)	1 (0.1)	0	0
Gastric cancer	0	0	0	0
Duodenal erosions	15 (1.1)	8 (0.7)	3 (2.6)	4 (5.4)
Duodenal ulcers	15 (1.1)	10 (0.9)	4 (3.5)	1 (1.3)
Duodenal cancer	0	0	0	0

[1]≤ 50-years-old *vs* other age categorizations: *vs* patients > 51-years-old: $P < 0.01$. Data are presented as *n* (%) or median and 25% interquartile range.
CeD: Celiac disease; GI: Gastrointestinal.

The possibility of detecting important or relevant esophageal, gastric, or duodenal pathology during diagnostic endoscopy has been put forward as an added benefit to the confirmation of CeD. Previous findings in CeD patients include reflux esophagitis, esophageal eosinophilia or eosinophilic esophagitis (mostly in children), Barrett's esophagus, *Helicobacter pylori* (*H. pylori*) infection and autoimmune gastritis[14-16]. These were, however, reported in small populations and single center studies. Our study, which included cohorts from the European Union, North America, and South America, gathered the largest sample of patients reported to date. The sample size collectively obtained allowed for subgroup and age category comparisons. The majority of CeD patients were young and female, as expected. The Buenos Aires cohort was prospectively designed to diagnose symptomatic patients suspected of having CeD, which allowed for comparisons between CeD patients and controls biopsy (Marsh's 0 or 1 histology categorization).

Our findings in a large multicenter population confirm recent reports that adult patients with alarm symptoms have a very low prevalence of major endoscopic and histological findings in the upper GI tract other than CeD features at presentation and was comparable to that of patients without alarm symptoms[14,16]. The definition of what constitutes an alarming symptom for CeD at the time of diagnosis appears to be central to this analysis. Weight loss, iron deficiency anemia, pain, or malabsorption symptoms were prevalent among symptomatic patients, which constitutes the vast majority of currently diagnosed CeD patients since a case finding strategy is recommended[19]. However, our findings were limited to the upper GI tract, and the lower GI tract was not explored.

Erosive reflux esophagitis was the most common endoscopic finding at the time of diagnosis (6.4%). Notably, undiagnosed patients with classical or subclinical CeD frequently seek treatment for gastroesophageal reflux symptoms prior to diagnosis, which has been shown to be more common in subjects in whom CeD is ruled out or in those treated with the GFD[20]. We previously reported that up to 30% of newly diagnosed CeD patients perceive moderate to severe reflux symptoms, which does not respond to anti-reflux therapy prior to CeD diagnosis[21,22]. Most of these "non-responsive" patients to anti-reflux therapy will rapidly improve after starting the GFD. Surprisingly, between 2018 and 2021, the Naples/Salerno cohort revealed higher prevalence of overall endoscopic lesions, and specifically of erosive reflux esophagitis, compared with diagnoses made before that time. This could be attributed to the characteristics of the CeD population over time or to differences in the reporting of endoscopic and histology findings.

The possibility of missing severe lesions or potentially dangerous diseases in CeD patients if a diagnostic endoscopy is not performed has been a source of concern in CeD guidelines[2,4]. With respect to Barrett's esophagus or esophageal metaplasia, an Italian study published in 2005 showed

Table 3 Demography and endoscopic findings in celiac disease patients and non-celiac disease controls of the Buenos Aires cohort

Demographic data and upper GI endoscopic findings	CeD population	Non-CeD population	≤ 50 yr non-CeD	51-60 yr non-CeD	≥ 61 yr non-CeD
Patients	97 (12.6)	674 (87.4)	435 (64.5)	135 (20.0)	104 (15.4)
Age in yr	35 (27-44)	45 (33-55)[a]	37 (29-44)	55 (53-58)	68 (63-72)
Female sex	88 (90.7)	472 (70.0)[a]	312 (71.7)	92 (68.1)	68 (65.4)
Patients with positive serology	97 (100)	9 (1.3)[a]	6 (1.3)	3 (2.2)	0
Patients with at least one significant endoscopic abnormalities	8 (8.2)	196 (29.1)[b]	121 (27.8)	48 (35.6)	27 (26.0)
Reflux esophagitis with erosions	1 (1.0)	21 (3.1)	11 (2.5)	2 (1.5)	5 (4.8)
Esophageal ulcers	0	0	0	0	0
Esophageal cancer	0	0	0	0	0
Suspicion of metaplasia	0	7 (1.0)	5 (1.1)	2 (1.5)	0
Biopsy confirmed Barrett's esophagus	0	2 (0.3)	2 (0.5)	0	0
Gastric erosions	7 (7.22)	165 (24.5)[a]	103 (23.7)	43 (31.8)	19 (18.3)
Gastric ulcers	0 (0)	11 (1.6)	4(0.9)	2 (1.5)	5 (4.8)
Gastric cancer	0	2 (0.3)	0	1 (0.7)	1 (1.0)
Duodenal erosions	0	10 (1.5)	6 (1.4)	2 (1.5)	2 (1.9)
Duodenal ulcers	1 (1.0)	5 (0.7)	2 (0.5)	2 (1.5)	1 (1.0)
Duodenal cancer	0	1 (0.1)	1 (0.2)	0	0

[a]$P < 0.01$.
[b]$P < 0.001$.
Data are presented as *n* (%) or median and 25% interquartile range. Controls were grouped according to age at endoscopy. Comparisons between celiac disease patients *vs* non-celiac disease controls.
CeD: Celiac disease; GI: Gastrointestinal; IQR: Interquartile range.

Table 4 Crude and adjusted multivariate analysis for the Buenos Aires cohort

Independent variable	At least one endoscopic lesion[1] (Buenos Aires cohort)			
	OR (95%CI)	*P* value	Adjusted[2] OR (95%CI)	*P* value
Male sex	1.19 (0.83-1.69)	0.34	1.03 (0.72-1.48)	0.860
Age	1.01 (1.00-1.02)	0.14	1.00 (0.99-1.01)	0.550
Celiac disease	0.22 (0.10-0.46)	< 0.0001	0.28 (0.13-0.60)	0.001
Alarm symptoms[3]	0.51 (0.35-0.74)	< 0.0001	0.63 (0.43-0.93)	0.020

[1]At least one endoscopic lesion: Erosive esophagitis, esophageal ulcer, esophageal cancer, Barrett's esophagus, gastric erosion, gastric ulcer, gastric cancer, duodenal erosion, duodenal ulcer, duodenal cancer.
[2]Weight loss, anemia, bleeding, dysphagia, epigastric pain, neoplasia history.
[3]Weight loss, iron deficient anemia, malabsorption, chronic diarrhea.
CI: Confidence interval; M: Male; OR: Odds ratio.

metaplasia in 26.6% of CeD patients compared with 10.9% of the control population[23]. This was not confirmed in studies from the United States[24] and South America[15,21] nor by the present study. Reasons for this discrepancy could be related to differences in populations and in the definition of Barrett's esophagus, which required confirmation by biopsy in our study.

In the present study, we did not find mucosal eosinophilic infiltration. A pediatric prospective longitudinal study based on systematic esophageal biopsies found that diagnoses of eosinophilic esophagitis and/or eosinophilia were not clinically relevant, suggesting esophageal biopsy is not necessary in the absence of clinical suspicion[25]. A 2015 cross-sectional population study in the United States based on a national pathology database involving over 88000 CeD patients with both esophageal and duodenal biopsies, reported a slight increase in comorbid eosinophilic esophagitis and CeD[24]. However, no link

between reflux esophagitis or Barrett's esophagus and CeD has been reported. Finally, autoimmune atrophic gastritis was previously modestly associated with CeD[26]. Our study, as well as other population-based studies and systematic reviews, did not confirm the association[27,28].

An earlier prospective study[15] collected consecutive patients and non-CeD controls in a high-risk population for having CeD, and gastric and duodenal biopsies were performed systematically at the time of the diagnostic endoscopy CeD and biopsy. Gastric biopsies from untreated CeD patients also revealed a significantly higher intraepithelial lymphocyte count in the antrum and corpus when compared with controls[15,29,30]. According to an Irish study, 10% of CeD patients have lymphocytic gastritis, which is twice the rate of non-CeD controls[12,14]. These findings are attributed to *H. pylori* infection, autoimmune atrophic gastritis[15,26], or a pan-mucosal gluten-related inflammation[14,15,29,30].

Our study showed only 1 CeD patient had a gastric peptic ulcer. Previous studies found 18.1% of CeD children with gastric ulcers, with a higher prevalence in *H. pylori* negative patients and those with no history of nonsteroidal anti-inflammatory drug use[31,32]. The rate of *H. pylori* infection across centers was not consistently reported here, and this could explain the difference in results. Previous research, however, has shown that high rates of biopsy-confirmed *H. pylori* infection are not associated with an increased risk of malignancy in the long term[27,33]. However, several studies have also shown that when endoscopic appearance is normal, histological evaluation (both in the stomach and the esophagus) is not cost-effective, especially when performed in experienced academic centers[34-36].

There was no diagnosis of gastric adenocarcinoma in CeD. Despite the small number of cases studied, this is consistent with previous findings that the prevalence of other cancers (breast, colon, pulmonary, and gynecological cancers) in CeD appears to be lower than in the general population[27,28]. Small bowel carcinoma is extremely rare in the general population, and CeD patients are three times more likely to develop it[1,28]. However, malignancies in the duodenum are still uncommon at the time of CeD diagnosis, which implies diagnostic CeD endoscopy should not be recommended as surveillance for upper GI cancer[28]. Overall, the current findings, as well as those from previous studies, suggest that a biopsy-avoiding approach in adult patients who meet recommended and strict serological criteria for CeD is possible[12,37-40].

Study strengths included the multicenter design, the large number of patients diagnosed at specialized centers for CeD in whom confirmatory biopsy diagnosis was obtained, as well as the use of standard endoscopic protocols. Despite the small numbers in sub-analyses, the study also provided novel data related to the association of endoscopic findings according to age and time. Study limitations included the observational design, the retrospective collection of endoscopic reports (with potential missing data), the differences in time of enrollment across the four centers, the lack of systematic collection of biopsies from the esophagus and stomach, and the limited number of non-CeD controls. Although the current study suggests that missing potentially serious events is unlikely, this should be confirmed in a larger population.

CONCLUSION

In conclusion, this multicenter, retrospective study found that comorbid upper GI endoscopic pathology is uncommon in patients with positive CeD serology at the time of diagnostic endoscopy. The risk of severe or premalignant lesions is extremely low, and no malignancies were found in patients who displayed potential warning signs. Our findings suggest that a non-biopsy strategy for diagnosing CeD in adults is unlikely to miss clinically significant concomitant endoscopic findings unrelated to CeD. The results of this study should encourage future population-based or prospective studies in this area.

ARTICLE HIGHLIGHTS

Research background

Celiac disease (CeD) is currently diagnosed in adult patients using a combination of specific serology tests and a duodenal biopsy obtained through an upper endoscopy. Upper endoscopy is also considered necessary for CeD diagnosis because non-CeD comorbidities can be missed.

Research motivation

The prevalence of upper gastrointestinal comorbidities at the time of CeD diagnosis has received little attention.

Research objectives

To investigate the prevalence of coincidental upper gastrointestinal endoscopic findings at the time of diagnostic endoscopy in four cohorts of patients diagnosed in three different countries.

Research methods

We conducted a descriptive multicenter retrospective study reporting endoscopic findings from adult patients who met standard criteria for diagnosing CeD.

Research results

Of 1328 adult patients enrolled, 95.6% had positive specific serology. In 135 patients, endoscopy revealed 163 abnormalities unrelated to CeD (10.1%). Erosive reflux esophagitis (6.4%), gastric erosions (2.0%), and suspicion of esophageal metaplasia (1.2%) were the most common findings. Biopsy-confirmed Barrett's esophagus was infrequent (0.2%). No other neoplastic or malignancies lesions were detected. Patients with alarm symptoms or signs had a lower rate of concomitant findings.

Research conclusions

Adults with positive CeD serology had few comorbid endoscopic findings when CeD was diagnosed.

Research perspectives

These findings raise the possibility that adult patients who meet recommended and strict serological criteria for CeD could be diagnosed without undergoing endoscopy and biopsy.

FOOTNOTES

Author contributions: Stefanolo JP, Ciacci C, Zingone F, Gizzi C, Marsilio I, Espinet ML, Pinto-Sánchez MI, and Niveloni SI contributed with data acquisition; Stefanolo JP performed the statistical analysis; Stefanolo JP, Pinto-Sánchez MI, Ciacci C, Zingone F, and Bai JC contributed to study design; Verdú EF, Smecuol EG, Moreno ML contributed to critical analysis; Bai JC, Verdú EF, Ciacci C, Pinto-Sánchez MI and Zingone F contributed to writing and critical review of the manuscript; All authors read and approved the final manuscript.

Institutional review board statement: The Ethics and Research Board of the Dr. C. Bonorino Udaondo Gastroenterology Hospital approved the study because of the prospective design and intervention in the Buenos Aires cohort. Ethics approval was obtained from the Hamilton Integrated Research Ethics Board (HiREB# 14460/5415). In Italy, Ethical Committee review was not required for retrospective studies while patient data remained anonymously coded.

Informed consent statement: Informed consent was not required by the Ethics and Research Committee of the Hospital de Gastroenterología Dr. C. Bonorino Udaondo (Buenos Aires, Argentina) given the retrospective nature of the study and because this study was categorized as minimal risk by the Committee.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at jbai@intramed.net. Consent was not obtained, but the presented data are anonymized and risk of identification is low.

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Abstract

BACKGROUND

Journal Impact Factor™ (JIF) is often used to evaluate the relative reputation and quality of academic journals in their respective fields, and can greatly influence the quality and scope of subsequent manuscript submissions. Therefore, many if not all academic journals are interested in increasing their JIF, to improve their academic impact.

AIM

To determine the importance of the integrity of the editorial and publication process in improving the academic influence of academic journals and the JIF of academic journals.

METHODS

In this paper, we describe our statistical analysis of bibliometric factors - including the 2021 JIFs released in the *Journal Citation Report*™ 2022, discipline rankings, received and published articles in 2019-2021, and webpage visits and downloads - for seven journals published by Baishideng Publishing Group (Baishideng) and indexed in Science Citation Index Expanded™; ultimately, we introduce and discuss the editing and publishing processes of Baishideng's journals in their entirety, as they form the basis for our objective of safeguarding and bolstering integrity in academic publication.

RESULTS

For the seven journals assessed, their 2021 JIFs were basically unchanged from 2020, with the current metric ranging from 5.374 for *World Journal of Gastroenterology* (*WJG*) to 1.534 for *World Journal of Clinical Cases* (*WJCC*). Further assessments of the journals' bibliometrics from 2019 to 2020, showed that *World Journal of Stem Cells* has the highest self-citation rate (1.43%) and *World Journal of*

Gastrointestinal Surgery has the lowest (0.21%). Additionally, the total 3012 articles published during this period were cited by more than 20000 articles in approximately 8000 academic journals. Of note, the 1102 articles published in *WJG* were cited by articles in 3059 journals, among which 171 journals have a JIF of > 10, including internationally renowned academic journals such as *CA-A Cancer Journal for Clinicians* (2021 JIF 286.130, record count: 1), *Lancet* (2021 JIF 202.731, record count: 4), *Nature Reviews Immunology* (2021 JIF 108.555, record count: 2), *Nature Reviews Gastroenterology & Hepatology* (2021 JIF 73.082, record count: 9), *Lancet Gastroenterology & Hepatology* (2021 JIF 45.042, record count: 8), *Gastroenterology* (2021 JIF 33.883, record count: 19), and *Gut* (2021 JIF 31.793, record count: 21). This suggests that Baishideng's journals have been widely recognized for their academic quality. In the *Reference Citation Analysis* (*RCA*) database, all seven Baishideng-published journals obtained a 2022 *Journal Article Influence Index* (*JAII*). For example, *WJG* has a 2022 *JAII* of 22.048, ranking 18th out of 102 journals in the field of gastroenterology & hepatology in the *RCA*, with 469909 total citations (6/102) and 21313 total articles (5/102). The numbers of manuscripts received and published in 2021 were both higher than those in 2019-2020. For example, *WJCC* received a total of 3650 manuscripts in 2021, which is 91.1% higher than those in 2019-2020 (average: 1910 papers/year). In 2021, *WJCC* published 1296 articles, representing an increase of 105.1% compared to those in 2019-2020 (average: 632 articles/year). The numbers of webpage visits and downloads received by the seven journals have increased year by year. For example, the number of total visits received by *WJG* in 2019-2021 was 1974052 in 2019, 2317835 in 2020 (increased by 17.4% compared with that in 2019), and 2652555 in 2021 (increased by 4.4% compared with that in 2020). The visitors were from more than 220 countries and regions worldwide, such as the United States, China, and the United Kingdom. Open access (OA) plays a vital role in improving the quality, efficiency, transparency, and integrity of academic journal publishing. From 2019 to 2021, a total of 5543 OA articles were published in the seven journals, of which 2083 (37.6%) were invited and published free-of-charge. During the same period, 1683 articles were published in *WJG*, and the authors were from more than 70 countries and regions. For the total 5543 articles published in the seven journals from 2019 to 2021, 3903 article quality tracking reports were received after the online publication of these articles. The quality of the articles was further evaluated through the Baishideng's article quality and author evaluation tracking system, with 4655 articles (84.0%) having received author evaluation and feedback, which contributes to tracking metrics for authors' satisfaction with the collective publication processes. From March 25, 2021 to June 28, 2022, the seven journals received a total of 424 reader evaluations and 229 letters from readers; this subsequent reader engagement demonstrates that the popularity of the published articles and the volume of their readership audience were improved through the reader evaluation system.

CONCLUSION

Ultimately, the findings from our bibliometric assessments indicate that establishing, promoting and actively practicing processes that safeguard and bolster the integrity of the editing and publication process also help to improve the academic influence of academic journals, which itself is the cornerstone for improving JIF.

Key Words: Journal Impact Factor; Academic journal; Editing process; Publishing process; Open access; Peer review; Language polishing; Academic influence

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Core Tip: This study describes a statistical analysis of bibliometric factors including the 2021 Journal Impact Factor™ (JIF) released in the *Journal Citation Report*™ 2022, discipline rankings, received and published articles in 2019-2021, and webpage visits and downloads for seven journals published by Baishideng Publishing Group (Baishideng). This study also introduces and discusses the editing and publishing processes of Baishideng's journals in their entirety, as they form the basis for our objective of safeguarding and bolstering integrity in academic publication. The findings from these bibliometric assessments indicate that establishing, promoting, and actively practicing processes that safeguard and bolster the integrity of the editing and publication process also help to improve the academic influence of academic journals, which itself is the cornerstone for improving JIF.

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INTRODUCTION

In June 2022, Clarivate Analytics officially released its *Journal Citation Report*™ (*JCR*) 2022, and more than 12000 academic journals received their new Journal Impact Factor™ (JIF). The concept of JIF as a metric of a journal's impact in a given field was first proposed by Eugene Garfield in 1955, and was calculated as follows for any given year: Number of that year's citations divided by the source items published in that journal during the previous 2 years[1]. Since then, JIF has been frequently used as a quantitative representation of the relative reputation and quality of academic journals in their respective fields[2]. Although JIF may be misused, it has an undeniable impact on the quality and scope of manuscript submissions[3-5]. Many authors use JIF when selecting an academic journal to which they will submit their manuscript, and the higher the JIF of the academic journal, the better its scientific reputation[6]. As such, managers of academic journals have focused their efforts on developing processes that will increase the respective JIFs and thereby improve the academic influence of each journal.

There are many factors affecting the JIF of an academic journal, such as whether the journal is open access (OA) and whether the journal is specialized[7]; however, few studies have focused on the impact of the integrity of the editing and publishing process on the improvement of the JIF of academic journals. In this study, we examined the importance of the integrity of the editorial and publication process in improving the JIF of academic journals by introducing and discussing the complete editing and publishing process of journals published by Baishideng Publishing Group (Baishideng), and statistically analyzing their 2021 JIFs released in the *JCR* 2022, published articles in recent years, and webpage visits and downloads for Baishideng's seven journals included in Science Citation Index Expanded (SCIE), a Web of Science™ Core Collection index.

MATERIALS AND METHODS

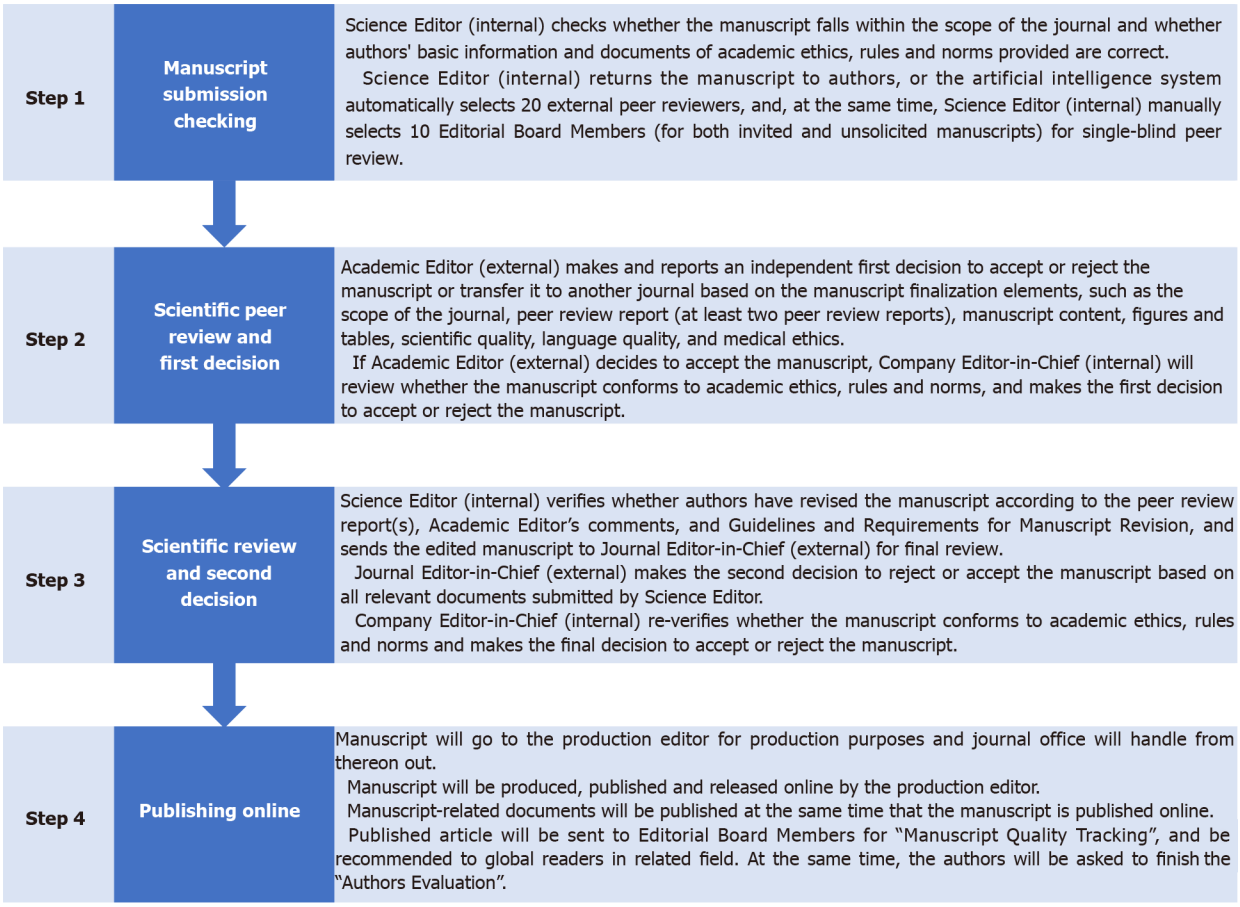
Baishideng's journals undergo a broad and thorough editing and publishing process

The seven SCIE-indexed journals published by Baishideng are all single-blind peer reviewed and OA. All manuscripts received by Baishideng's journals, both invited and freely submitted, are subjected to external peer review. The external peer review assesses the manuscripts for scientific quality, linguistic quality, and adherence to ethical standards and norms.

Baishideng established a broad and thorough academic journal editing and publishing process by focusing on the basic functions of academic journals, including: (1) Registration, to ensure the priority of authors' academic thoughts; (2) Peer review, to ensure the quality of the paper and obtain peer recognition; (3) Communication, to promote the dissemination of scientific findings; and (4) Document storage, to archive publications for future reference and textual research. Through the F6Publishing system, independently developed by Baishideng, each manuscript progresses stepwise through the complete editing and publishing process, including submission review, peer review, first decision, second decision, final acceptance, online publication[8], post-publication tracking of the quality of published articles, authors' evaluation of the publishing process, and readers' evaluation of the article (Figure 1).

Gathered data

We selected the seven SCIE-indexed journals from among the 47 total OA journals published by Baishideng, and performed systematic quantitative and statistical analyses based upon their 2021 JIFs, discipline rankings, and received and published articles in 2019-2021, including the main source countries/regions and the number of published articles. Furthermore, data were obtained from Web of Science, and the journals/articles citing the articles published in the respective seven Baishideng journals from 2019 to 2020 were statistically analyzed. In addition, we statistically analyzed the number of visits and downloads received by the seven journals' respective webpages, the number of non-conforming submissions identified in the submission preview, the number of peer reviewers and editorial board members who participated in the manuscript peer review, and the number of manuscripts that needed language polishing at the second decision, as well as the number of evaluations received *via* the Baishideng article quality tracking system, author evaluation system, and reader evaluation system from 2019 to 2021.



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Figure 1 The stepwise editing and publishing process of Baishideng journals. Each submitted article is subject to this process, with those of demonstrated quality in peer review progressing through the entire process to publication and post-publication qualitative and quantitative tracking.

RESULTS

Overview of 2021 JIFs of Baishideng's seven SCIE-indexed journals

In the *JCR* 2022, all seven of Baishideng's SCIE-indexed journals received their latest JIF. Compared with the JIFs of the seven journals received in 2020, the 2021 JIFs of three journals increased, and those of the remaining four slightly decreased, yielding an overall similarity between the 2 years (Table 1).

The 2021 JIF, number of received and published articles, citations, and number of webpage visits and downloads for World Journal of Gastroenterology in 2019-2021

The 2021 JIF, number of received and published articles, and citations for *World Journal of Gastroenterology* in 2019-2021: According to the *JCR* 2022, the 2021 JIF of *World Journal of Gastroenterology* (*WJG*) is 5.374, and the 5-year JIF is 5.715, ranking 31st among 93 journals in the field of gastroenterology and hepatology, located in quartile (Q) 2 (Figure 2). The 2021 JIF of *WJG* decreased by 0.368 from its 2020 JIF (5.742).

According to the *Reference Citation Analysis (RCA)* database, independently developed by Baishideng, *WJG* has a 2022 *Journal Article Influence Index* [2022 *JAII*, calculated as (Total Citations/Total Articles)] of 22.048, ranking 18th out of 102 journals in the field of gastroenterology & hepatology in the *RCA*, with 469909 total citations (6/102) and 21313 total articles (5/102).

From 2019 to 2020, *WJG* received a total of 3986 articles (average: 1993 articles/year), among which 1247 (31.3%) were invited and 2739 (68.7%) were freely submitted; the acceptance rate was 27.6%. During that same period, *WJG* published 1102 articles (average: 551 articles/year), among which 479 (43.5%) were invited and 623 (56.5%) were freely submitted. As of July 26, 2022, the articles published in *WJG* received a total of 12458 citations (without self-citations: 12341) by 11413 articles (without self-citations: 11314), yielding a self-citation rate of 0.94%; there were a total of 6387 citations in 2021. After excluding self-citations, the 11314 articles that cited the *WJG*-published articles were from 3059 journals (data from Web of Science, Table 2); among these journals, 171 (5.6%) had a JIF of > 10 (data from Web of Science), accounting for 25.9% of the 660 total journals that had received a JIF of > 10 in the *JCR* 2022. Moreover, the journals citing the *WJG*-published articles include internationally renowned academic

Table 1 Comparison of the 2020 and 2021 Journal Impact Factors™ of the seven Baishideng journals indexed in Science Citation Index Expanded

Journal title	2021 JIF	2020 JIF	5-yr JIF	Category: Journal rank/total journals (quartile)
World Journal of Gastroenterology	5.374	5.742	5.715	Gastroenterology and Hepatology: 31/93 (Q2)
World Journal of Stem Cells	5.247	5.326	4.964	Cell and Tissue Engineering: 12/29 (Q2); Cell Biology: 86/194 (Q2)
World Journal of Diabetes	4.560	3.763	5.370	Endocrinology and Metabolism: 62/146 (Q2)
World Journal of Psychiatry	3.500	4.571	7.380	Psychiatry: 89/155 (Q3)
World Journal of Gastrointestinal Oncology	3.404	3.393	3.250	Oncology: 163/245 (Q3); Gastroenterology and Hepatology: 59/93 (Q3)
World Journal of Gastrointestinal Surgery	2.505	2.582	3.099	Surgery: 104/211 (Q2); Gastroenterology and Hepatology: 81/93 (Q4)
World Journal of Clinical Cases	1.534	1.337	1.599	Medicine, General and Internal: 135/172 (Q4)

JIF: Journal Impact Factor™; Q: Quartile.



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Figure 2 The 2021 Journal Impact Factor and category rank of *World Journal of Gastroenterology*. *JCR*: *Journal Citation Report*™; SCIE: Science Citation Index Expanded™.

journals such as *CA-A Cancer Journal for Clinicians* (2021 JIF 286.130, record count: 1), *Lancet* (2021 JIF 202.731, record count: 4), and *Nature Reviews Immunology* (2021 JIF 108.555, record count: 2), and renowned academic journals in the field of gastroenterology & hepatology such as *Nature Reviews Gastroenterology & Hepatology* (2021 JIF 73.082, record count: 9), *Lancet Gastroenterology & Hepatology* (2021 JIF 45.042, record count: 8), *Gastroenterology* (2021 JIF 33.883, record count: 19), and *Gut* (2021 JIF 31.793, record count: 21). From 2019 to 2020, the authors of the 1102 articles published in *WJG* were from 71 countries/regions, represented by 472 articles (42.8%) from China, 165 (15.0%) from the United States, 93 (8.4%) from Italy, 82 (7.4%) from Japan, 51 (4.6%) from South Korea, and 239 (21.7%) from other countries/regions (data from Web of Science, Figure 3). Of note, the article entitled "Gastrointestinal and hepatic manifestations of COVID-19: A comprehensive review", which was contributed by Cha *et al* [9] from Cleveland Clinic in the United States in 2020, is the article with the highest number of citations among the articles published in *WJG*. This article mainly reviews the gastrointestinal and hepatic manifestations of coronavirus disease 2019 (COVID-19). As of June 28, 2022, this article has been cited 77 times.

In 2021, *WJG* received a total of 2109 manuscripts, of which 1116 (52.9%) were invited and 993 (47.1%) were freely submitted; the acceptance rate was 27.5%, which was 5.8% higher than that in 2019-2020 (average: 1993/year). A total of 581 articles were published in *WJG* in 2021, including 360 (62.0%) invited and 221 (38.0%) freely submitted. As of June 28, 2022, the number of total citations was 1039. The number of articles published in *WJG* in 2021 was 5.4% higher than that in 2019-2020 (551 articles/year on average). The authors were from 56 countries/regions, represented by 160 articles (27.5%) from China, 59 (10.2%) from Italy, 53 (9.1%) from the United States, 38 (6.5%) from Japan, 20 (3.4%) from South Korea, 20 (3.4%) from Brazil, and 231 (39.7%) from other countries/regions (Figure 4).

Number of webpage visits and downloads received by *WJG* in 2019-2021: From 2019 to 2021, the *WJG* webpage received a total of 1974052 visits in 2019, 2317835 in 2020 (an increase of 17.4% compared with that in 2019), and 2652555 in 2021 (an increase of 14.4% compared with that in 2020), with visits from

Table 2 Rank and record count of journals that published articles that cited the 1102 articles published in *World Journal of Gastroenterology* in 2019-2020, *n* (%)

Rank	Publication title	Record count
1	*Cancers*	241 (2.13)
2	*International Journal of Molecular Sciences*	241 (2.13)
3	*Frontiers in Oncology*	193 (1.706)
4	*Journal of Clinical Medicine*	138 (1.22)
5	*Nutrients*	119 (1.052)
6	*Scientific Reports*	111 (0.981)
7	*Frontiers in Pharmacology*	100 (0.884)
8	*Frontiers in Immunology*	92 (0.813)
9	*Frontiers in Medicine*	89 (0.787)
10	*World Journal of Clinical Cases*	77 (0.681)
11	*Cells*	76 (0.672)
12	*Digestive Diseases and Sciences*	73 (0.645)
13	*Medicine*	71 (0.628)
14	*Medicine Hagerstown*	70 (0.619)
15	*Cureus*	67 (0.592)
16	*Surgical Endoscopy*	67 (0.592)
17	*Surgical Endoscopy and Other Interventional Techniques*	67 (0.592)
18	*Diagnostics*	64 (0.566)
19	*Diagnostics Basel Switzerland*	64 (0.566)
20	*BMC Gastroenterology*	63 (0.557)
21	*Biomed Research International*	55 (0.486)
22	*Evidence Based Complementary and Alternative Medicine*	55 (0.486)
23	*Evidence Based Complementary and Alternative Medicine ECAM*	55 (0.486)
24	*PLOS One*	53 (0.468)
25	Other journals, *n* = 3025	9013 (79.662)

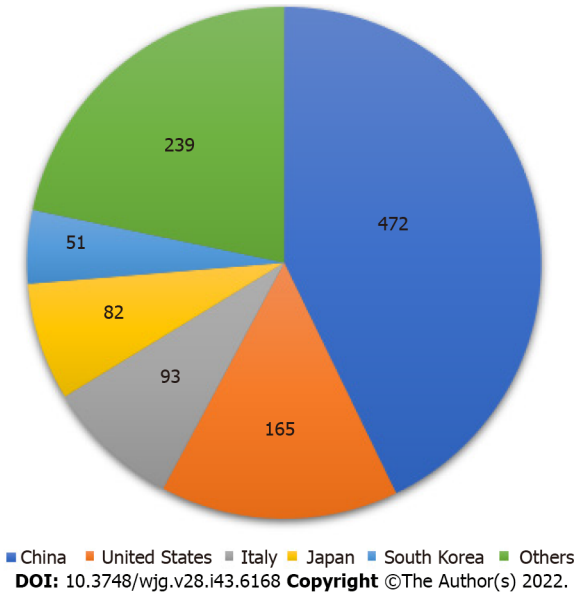
more than 220 countries and regions worldwide (Figure 5). The number of downloads was 1117432 in 2019, 2459893 in 2020 (an increase of 120.1% compared with that in 2019), and 3754483 in 2021 (an increase of 52.6% compared with that in 2020), with downloads from more than 200 countries and regions worldwide.

The 2021 JIF, number of received and published articles, citations, and number of webpage visits and downloads for World Journal of Stem Cells in 2019-2021

The 2021 JIF, number of received and published articles, and citations for *World Journal of Stem Cells* in 2019-2021: According to the *JCR* 2022, the 2021 JIF of *World Journal of Stem Cells* (*WJSC*) is 5.247, and the 5-year JIF is 4.964, ranking 12th among 29 journals in the field of cell & tissue engineering, located in Q2 (Figure 6), and ranking 86th among 194 journals in the field of cell biology. The 2021 JIF of *WJSC* decreased by 0.079 from its 2020 JIF (5.326).

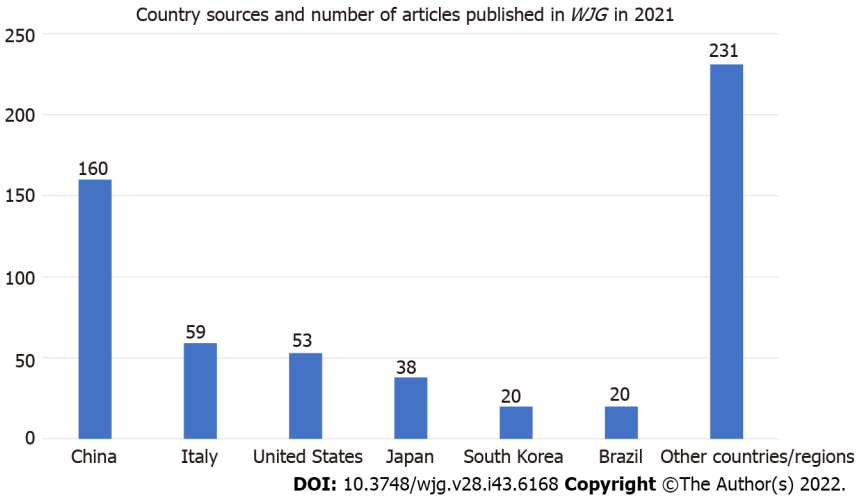
According to the *RCA* database, *WJSC* has a 2022 *JAII* of 17.729, ranking 13th out of 32 journals in the field of cell & tissue engineering in the *RCA*, with 12056 total citations (18/32) and 680 total articles (20/32).

From 2019 to 2020, *WJSC* received a total of 396 articles (average: 198 articles/year), among which 282 (71.2%) were invited and 114 (28.8%) were freely submitted; the acceptance rate was 46.5%. During the same period, *WJSC* published 184 articles (average: 92 articles/year), among which 155 (84.2%) were invited and 29 (15.8%) were freely submitted. As of June 28, 2022, the articles published in *WJSC* received a total of 1956 citations (without self-citations: 1928) by 1813 articles (without self-citations: 1794), yielding a self-citation rate of 1.43%; there were a total of 1000 citations in 2021. After excluding



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Figure 3 Country sources and number of articles published in *World Journal of Gastroenterology* in 2019-2020.



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Figure 4 Country sources and number of articles published in *World Journal of Gastroenterology* in 2021. *WJG*: *World Journal of Gastroenterology*.

self-citations, the 1794 articles that cited the *WJSC*-published articles were from 858 journals (data from Web of Science, Table 3); among these journals, 61 (7.1%) had a JIF of > 10 (data from Web of Science), accounting for 9.2% of the 660 total journals that had received a JIF of > 10 in the *JCR* 2022. Moreover, the journals citing the *WJSC*-published articles include renowned academic journals in the field of cell biology such as *Nature Reviews Molecular Cell Biology* (2021 JIF 113.915, record count: 1) and *Trends in Cell Biology* (2021 JIF 21.167, record count: 1). From 2019 to 2020, the authors of the 184 articles published in *WJSC* were from 41 countries/regions, represented by 78 articles (42.4%) from China, 22 (12.0%) from the United States, 11 (6.0%) from Brazil, 51 (6.0%) from South Korea, 10 (5.4%) from Italy, and 52 (28.3%) from other countries/regions (data from Web of Science, Figure 7). Of note, the article entitled "Mesenchymal stem cells from different sources and their derived exosomes: A pre-clinical perspective", which was contributed by Associate Professor Álvarez-Viejo *et al*[10] from Hospital Universitario Central de Asturias, Spain in 2020, is the article with the highest number of citations among the articles published in *WJSC*. This article mainly reviews the research progress of different sources of mesenchymal stem cells and their derived apoplasts from a preclinical perspective. As of June 28, 2022, this article has been cited 29 times.

In 2021, *WJSC* received a total of 215 manuscripts, of which 167 (77.7%) were invited and 48 (22.3%) were freely submitted; the acceptance rate was 50.7%, which was 8.6% higher than that in 2019-2020 (average: 198 articles/year). A total of 109 articles were published in *WJSC* in 2021, including 96 (88.1%) invited and 13 (11.9%) freely submitted. As of June 28, 2022, the number of total citations was 164. The

Table 3 Rank and record count of journals that published articles that cited the 184 articles published in *World Journal of Stem Cells* in 2019-2020, *n* (%)

Rank	Publication title	Record count
1	*International Journal of Molecular Sciences*	139 (7.748)
2	*Cells*	76 (4.236)
3	*Frontiers in Cell and Developmental Biology*	70 (3.902)
4	*Stem Cell Research Therapy*	69 (3.846)
5	*Stem Cells International*	44 (2.453)
6	*Cancers*	37 (2.062)
7	*Stem Cell Reviews and Reports*	24 (1.338)
8	*Frontiers in Immunology*	23 (1.282)
9	*Frontiers in Bioengineering and Biotechnology*	22 (1.226)
10	*Biomedicines*	18 (1.003)
11	*Scientific Reports*	18 (1.003)
12	*Pharmaceutics*	17 (0.948)
13	*Frontiers in Pharmacology*	16 (0.892)
14	*Current Stem Cell Research Therapy*	14 (0.780)
15	*Frontiers in Oncology*	12 (0.669)
16	*Biology*	10 (0.557)
17	*Biology Basel*	10 (0.557)
18	*Biomolecules*	10 (0.557)
19	*Molecules*	10 (0.557)
20	*Molecules Basel Switzerland*	10 (0.557)
21	*Applied Sciences Basel*	8 (0.446)
22	*Journal of Clinical Medicine*	8 (0.446)
23	*Molecular Biology Reports*	8 (0.446)
24	*Neural Regeneration Research*	8 (0.446)
25	Other journals, *n* = 834	1113 (62.040)

number of articles published in *WJSC* in 2021 was 18.5% higher than that in 2019-2020 (92 articles/year on average). The authors were from 28 countries/regions, represented by 26 articles (23.9%) from China, 12 (11.0%) from Italy, 8 (7.3%) from the United States, 6 (5.5%) from Brazil, 5 (4.6%) from India, and 52 (47.7%) from other countries/regions (Figure 8).

Number of webpage visits and downloads received by *WJSC* in 2019-2021: From 2019 to 2021, the *WJSC* webpage received a total of 99638 visits in 2019, 160901 in 2020 (an increase of 61.5% compared with that in 2019), and 192193 in 2021 (an increase of 19.4% compared with that in 2020), with visits from more than 180 countries and regions worldwide (Figure 9). The number of downloads was 51642 in 2019, 111236 in 2020 (an increase of 115.3% compared with that in 2019), and 180164 in 2021 (an increase of 62.0% compared with that in 2020), with downloads from more than 120 countries and regions worldwide.

The 2021 JIF, number of received and published articles, citations, and number of webpage visits and downloads for World Journal of Diabetes in 2019-2021

The 2021 JIF, number of received and published articles, and citations for *World Journal of Diabetes* in 2019-2021: According to the *JCR* 2022, the 2021 JIF of *World Journal of Diabetes* (*WJD*) is 4.560, and the 5-year JIF is 5.370, ranking 62nd among 146 journals in the field of endocrinology & metabolism, located in Q2 (Figure 10). The 2021 JIF of *WJD* increased by 0.797 from its 2020 JIF (3.763).

According to the *RCA* database, *WJD* has a 2022 *JAII* of 24.495, ranking 48th out of 149 journals in the field of endocrinology & metabolism in the *RCA*, with 20331 total citations (92/149) and 830 total articles (124/149).

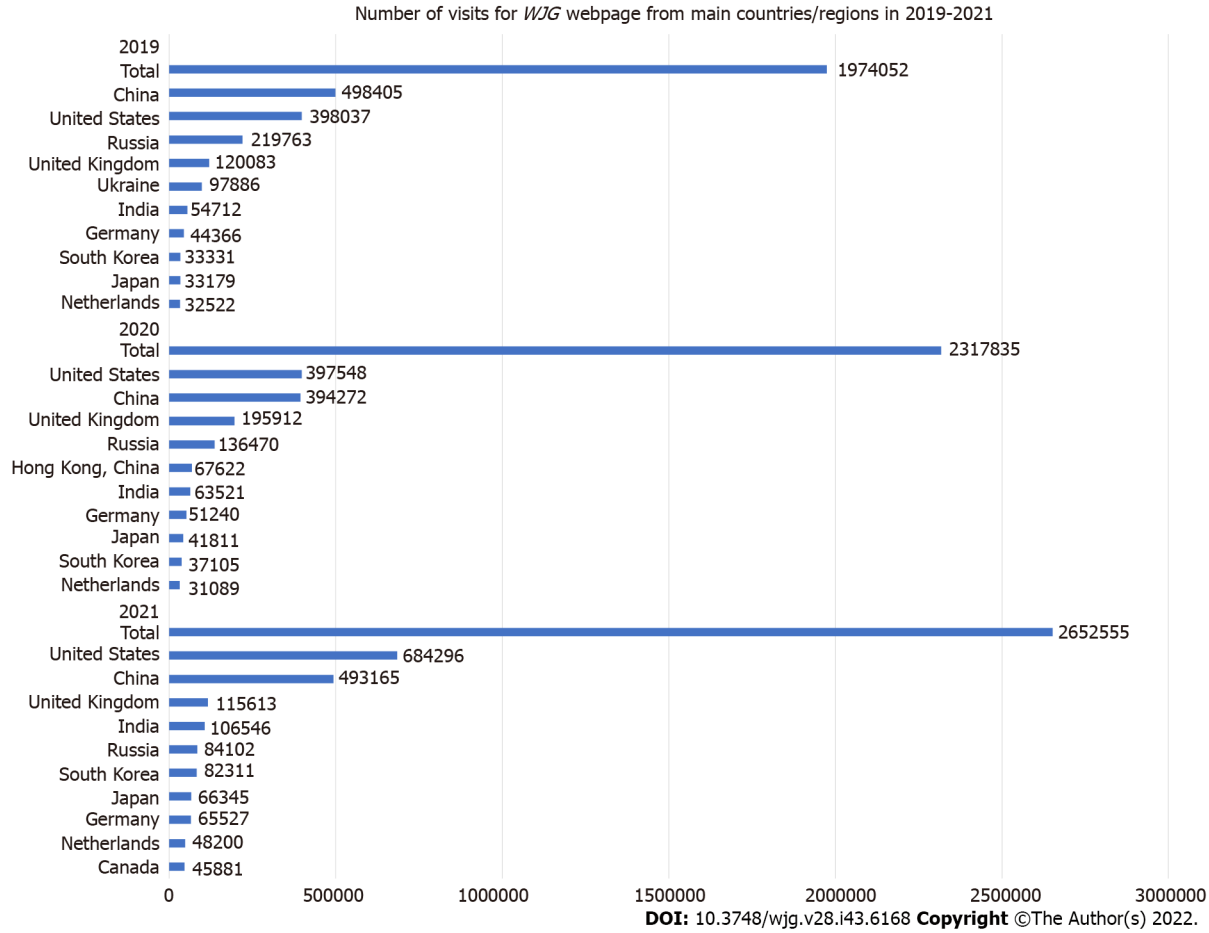


Figure 5 Number of visits to the *World Journal of Gastroenterology* webpage from main countries/regions in 2019-2021. *WJG*: *World Journal of Gastroenterology*.

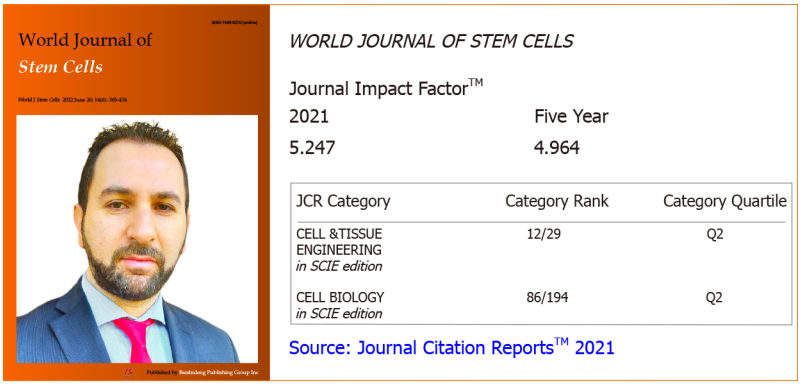


Figure 6 The 2021 Journal Impact Factor and category rank of *World Journal of Stem Cells*. *JCR*: *Journal Citation Report*™; SCIE: Science Citation Index Expanded™.

From 2019 to 2020, *WJD* received a total of 294 articles (average: 147 articles/year), among which 150 (51.0%) were invited and 144 (49.0%) were freely submitted; the acceptance rate was 37.4%. During the same period, *WJD* published 110 articles (average: 55 articles/year), among which 68 (61.8%) were invited and 42 (38.2%) were freely submitted. As of June 28, 2022, the articles published in *WJD* received a total of 964 citations (without self-citations: 959) by 931 articles (without self-citations: 927), yielding a self-citation rate of 0.52%; there were a total of 482 citations in 2021. After excluding self-citations, the 927 articles that cited the *WJD*-published articles were from 669 journals (data from Web of Science, Table 4); among these journals, 30 (4.5%) had a JIF of > 10 (data from Web of Science), accounting for 4.5% of the 660 total journals that had received a JIF of > 10 in the *JCR* 2022. Moreover, the journals citing the *WJD*-published articles include renowned academic journals in the field of endocrinology &

Table 4 Rank and record count of journals that published articles that cited the 110 articles published in *World Journal of Diabetes* in 2019-2020, *n* (%)

Rank	Publication title	Record count
1	*International Journal of Molecular Sciences*	35 (3.776)
2	*Frontiers in Endocrinology*	22 (2.373)
3	*Nutrients*	17 (1.834)
4	*International Journal of Environmental Research and Public Health*	15 (1.618)
5	*Journal of Clinical Medicine*	15 (1.618)
6	*Diabetes Metabolic Syndrome and Obesity Targets and Therapy*	14 (1.510)
7	*Diabetes Metabolic Syndrome*	13 (1.402)
8	*Diabetes Metabolic Syndrome Clinical Research Reviews*	13 (1.402)
9	*Cureus*	9 (0.971)
10	*Diabetes Therapy*	8 (0.863)
11	*Diabetes Therapy Research Treatment and Education of Diabetes and Related Disorders*	8 (0.863)
12	*Antioxidants*	7 (0.755)
13	*Antioxidants Basel Switzerland*	7 (0.755)
14	*BMJ Open Diabetes Research Care*	7 (0.755)
15	*Diabetes Research and Clinical Practice*	7 (0.755)
16	*Medicine*	7 (0.755)
17	*Medicine Hagerstown*	7 (0.755)
18	*Metabolites*	7 (0.755)
19	*PLOS One*	7 (0.755)
20	*Scientific Reports*	7 (0.755)
21	*Biomedicines*	6 (0.647)
22	*Cardiovascular Diabetology*	6 (0.647)
23	*Diabetes Obesity Metabolism*	6 (0.647)
24	*Frontiers in Pharmacology*	6 (0.647)
25	Other journals, *n* = 645	671 (72.384)

metabolism such as *Diabetes Care* (2021 JIF 17.152, record count: 3), *Obesity Reviews* (2021 JIF 10.867, record count: 2), and *Diabetologia* (2021 JIF 10.460, record count: 3).

From 2019 to 2020, the authors of the 110 articles published in *WJD* were from 42 countries/regions, represented by 23 articles (20.9%) from the United States, 18 (16.4%) from China, 8 (7.3%) from India, 6 (5.5%) from Greece, 5 (4.5%) from Turkey, and 50 (45.5%) from other countries/regions (data from Web of Science, Figure 11). Of note, the article entitled "Evaluation of oxidative stress levels in obesity and diabetes by the free oxygen radical test and free oxygen radical defense assays and correlations with anthropometric and laboratory parameters", which was contributed by Dr. Găman *et al*[11] from "Carol Davila" University of Medicine and Pharmacy, Romania in 2020, is the article with the highest number of citations among the articles published in *WJD*. This article focuses on the assessment of oxidative stress in obese and diabetic subjects and the possible correlation between oxidative stress and anthropometric and biochemical parameters. As of June 28, 2022, this article has been cited 39 times.

In 2021, *WJD* received a total of 300 manuscripts, of which 211 (70.3%) were invited and 89 (29.7%) were freely submitted; the acceptance rate was 49.0%, which was 104.1% higher than that in 2019-2020 (average: 147 articles/year). A total of 147 articles were published in *WJD* in 2021, including 111 (75.5%) invited and 36 (24.5%) freely submitted. As of June 28, 2022, the number of total citations was 286. The number of articles published in *WJD* in 2021 was 167.2% higher than that in 2019-2020 (55 articles/year on average). The authors were from 38 countries/regions, represented by 42 articles (28.6%) from China, 12 (8.2%) from the United States, 9 (6.1%) from India, 7 (4.8%) from Italy, 5 (3.4%) from Croatia, 5 (3.4%) from Turkey, and 67 (45.6%) from other countries/regions (Figure 12).

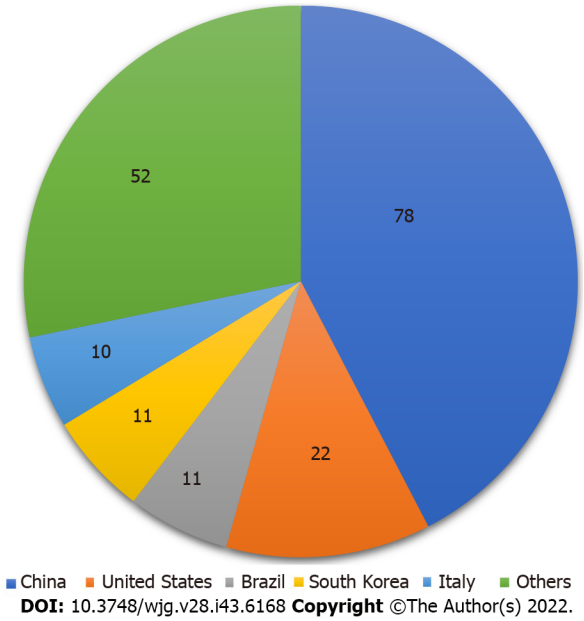


Figure 7 Country sources and number of articles published in *World Journal of Stem Cells* in 2019-2020.

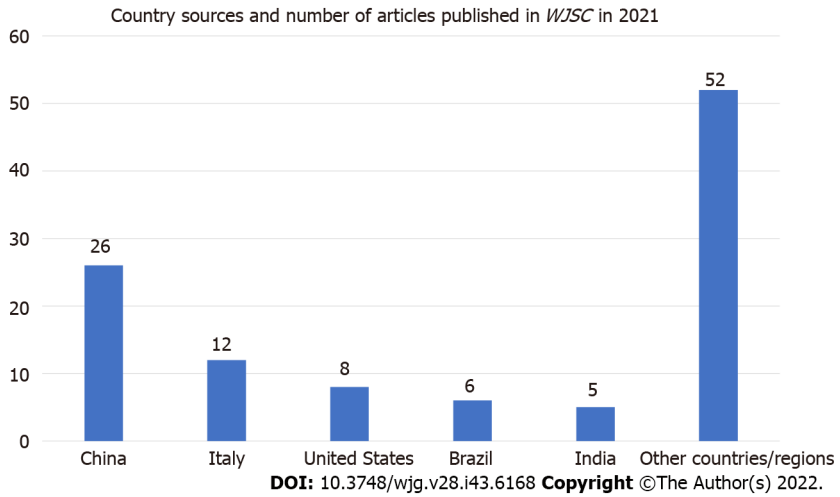


Figure 8 Country sources and number of articles published in *World Journal of Stem Cells* in 2021. *WJSC: World Journal of Stem Cells.*

Number of webpage visits and downloads received by *World Journal of Diabetes* in 2019-2021: From 2019 to 2021, *WJD* website received a total of 129630 visits in 2019, 189456 in 2020 (an increase of 46.2% compared with that in 2019), and 264148 in 2021 (an increase of 39.4% compared with that in 2020), with visits from more than 200 countries and regions worldwide (Figure 13). The number of downloads was 76163 in 2019, 137218 in 2020 (an increase of 80.2% compared with that in 2019), and 239021 in 2021 (an increase of 74.2% compared with that in 2020), with downloads from more than 170 countries and regions worldwide.

The 2021 JIF, number of received and published articles, citations, and number of webpage visits and downloads for World Journal of Psychiatry in 2019-2021

The 2021 JIF, number of received and published articles, and citations for *World Journal of Psychiatry* in 2019-2021: According to the *JCR* 2022, the 2021 JIF of *World Journal of Psychiatry* (*WJP*) is 3.500, and the 5-year JIF is 7.380, ranking 89th among 155 journals in the field of psychiatry, located in Q3 (Figure 14). The 2021 JIF of *WJP* decreased by 1.071 from its 2020 JIF (4.571).

According to the *RCA* database, *WJP* has a 2022 *JAII* of 15.402, ranking 93rd out of 222 journals in the field of psychiatry in the *RCA*, with 6238 total citations (165/222) and 405 total articles (193/222).

From 2019 to 2020, *WJP* received a total of 112 articles (average: 56 articles/year), among which 60 (53.6%) were invited and 52 (46.4%) were freely submitted; the acceptance rate was 31.3%. During the

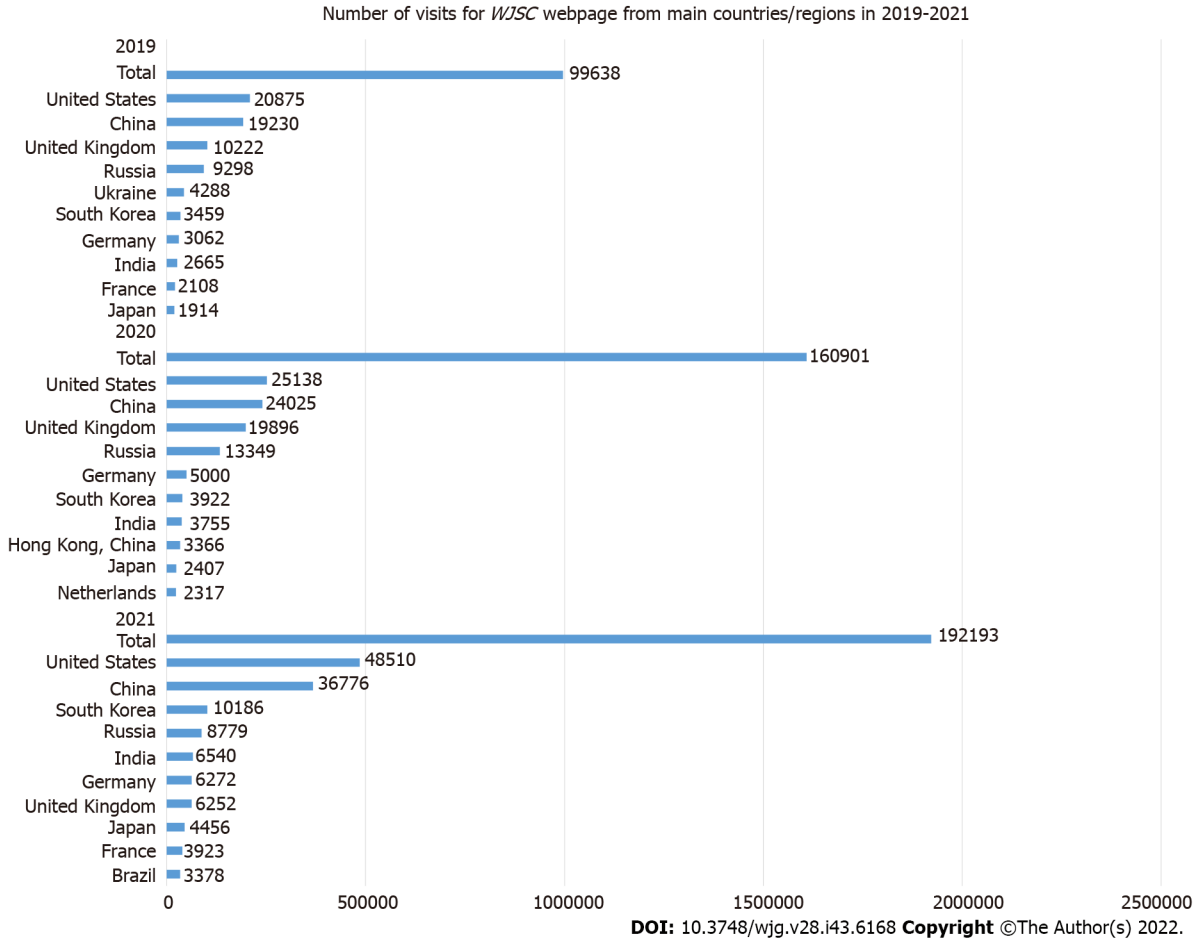


Figure 9 Number of visits to the *World Journal of Stem Cells* webpage from main countries/regions in 2019-2021. *WJSC*: *World Journal of Stem Cells*.

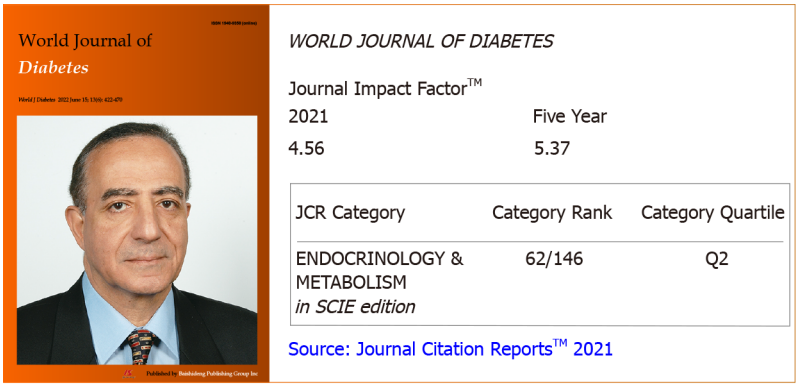


Figure 10 The 2021 Journal Impact Factor and category rank of *World Journal of Diabetes*. *JCR*: *Journal Citation Report*™; SCIE: Science Citation Index Expanded™.

same period, *WJP* published 35 articles (average: 18 articles/year), among which 25 (71.4%) were invited and 10 (28.6%) were freely submitted. As of June 28, 2022, the articles published in *WJP* received a total of 219 citations (without self-citations: 217) by 214 articles (without self-citations: 212), yielding a self-citation rate of 0.91%; there were a total of 121 citations in 2021. After excluding self-citations, the 212 articles that cited the *WJP*-published articles were from 76 journals (data from Web of Science, Table 5); among these journals, 9 (11.8%) had a JIF of > 10 (data from Web of Science), accounting for 1.4% of the 660 total journals that had received a JIF of > 10 in the *JCR* 2022. Moreover, the journals citing the *WJP*-published articles include renowned academic journals in the field of psychiatry such as *World Psychiatry* (2021 JIF 79.683, record count: 1), *Asian Journal of Psychiatry* (2021 JIF 13.890, record count: 2), and *Molecular Psychiatry* (2021 JIF 13.437, record count: 1).

Table 5 Rank and record count of journals that published articles that cited the 35 articles published in *World Journal of Psychiatry* in 2019-2020, *n* (%)

Rank	Publication title	Record count
1	*Frontiers in Psychiatry*	9 (4.245)
2	*Journal of Affective Disorders*	7 (3.302)
3	*Australian & New Zealand Journal of Psychiatry*	6 (1.415)
4	*BMC Psychiatry*	6 (2.830)
5	*International Journal of Environmental Research and Public Health*	6 (2.830)
6	*International Journal of Advanced Computer Science and Applications*	5 (2.358)
7	*European Psychiatry*	4 (1.887)
8	*Brain and Behavior*	3 (1.415)
9	*Journal of Clinical Medicine*	3 (1.415)
10	*Journal of Personalized Medicine*	3 (1.415)
11	*Psychiatria Danubina*	3 (1.415)
12	*Advances in Medical Education and Practice*	2 (0.943)
13	*Asian Journal of Psychiatry*	2 (0.943)
14	*Brain Sciences*	2 (0.943)
15	*Clinical Neuropsychiatry*	2 (0.943)
16	*European Archives of Psychiatry and Clinical Neuroscience*	2 (0.943)
17	*European Neuropsychopharmacology*	2 (0.943)
18	*Frontiers in Neuroscience*	2 (0.943)
19	*Frontiers in Psychology*	2 (0.943)
20	*Indian Journal of Psychiatry*	2 (0.943)
21	*Indian Journal of Psychological Medicine*	2 (0.943)
22	*Journal of Autism and Developmental Disorders*	2 (0.943)
23	Other journals, *n* = 54	135 (63.679)

From 2019 to 2020, the authors of the 35 articles published in *WJP* were from 22 countries/regions, represented by 10 articles (28.6%) from the United States, 8 (22.9%) from China, 3 (8.6%) from Australia, and 14 (40.0%) from other countries/regions (data from Web of Science, Figure 15). Of note, the article entitled "How to construct neuroscience-informed psychiatric classification? Towards nomothetic networks psychiatry", which was contributed by Professor Stoyanov and Maes *et al*[12] from Medical University of Plovdiv, Bulgaria in 2020, is the article with the highest number of citations among the articles published in *WJP*. This article mainly reviews how to construct neuroscience-informed psychiatric classification from the perspective of nomothetic networks psychiatry. As of June 28, 2022, this article has been cited 19 times.

In 2021, *WJP* received a total of 265 manuscripts, of which 222 (83.8%) were invited and 43 (16.2%) were freely submitted; the acceptance rate was 38.9%, which was 373.2% higher than that in 2019-2020 (average: 56/year). A total of 103 articles were published in *WJP* in 2021, including 93 (90.3%) invited and 10 (9.7%) freely submitted. As of June 28, 2022, the number of total citations was 146. The number of articles published in *WJP* in 2021 was 472.2% higher than that in 2019-2020 (18 articles/year on average). The authors were from 28 countries/regions, represented by 11 articles (10.7%) from China, 10 (9.7%) from Italy, 8 (7.8%) from the United States, 5 (4.9%) from Spain, 4 (3.9%) from Austria, 4 (3.9%) from Brazil, and 61 (59.2%) from other countries/regions (Figure 16).

Number of webpage visits and downloads received by *WJP* in 2019-2021: From 2019 to 2021, *WJP* received a total of 47123 visits in 2019, 82197 in 2020 (an increase of 74.4% compared with that in 2019), and 130151 in 2021 (an increase of 58.3% compared with that in 2020), with visits from more than 180 countries and regions worldwide (Figure 17). The number of downloads was 28838 in 2019, 59532 in 2020 (an increase of 106.4% compared with that in 2019), and 113939 in 2021 (an increase of 91.4% compared with that in 2020), with downloads from more than 130 countries and regions worldwide.

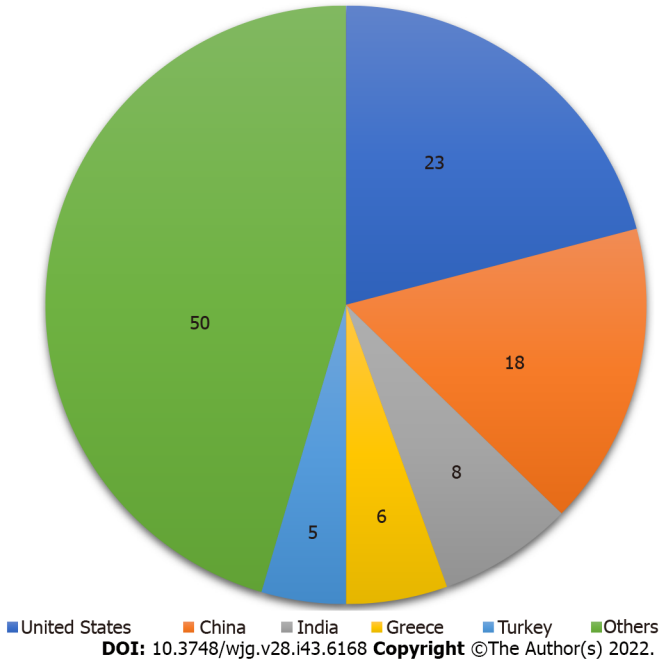


Figure 11 Country sources and number of articles published in *World Journal of Diabetes* in 2019-2020.

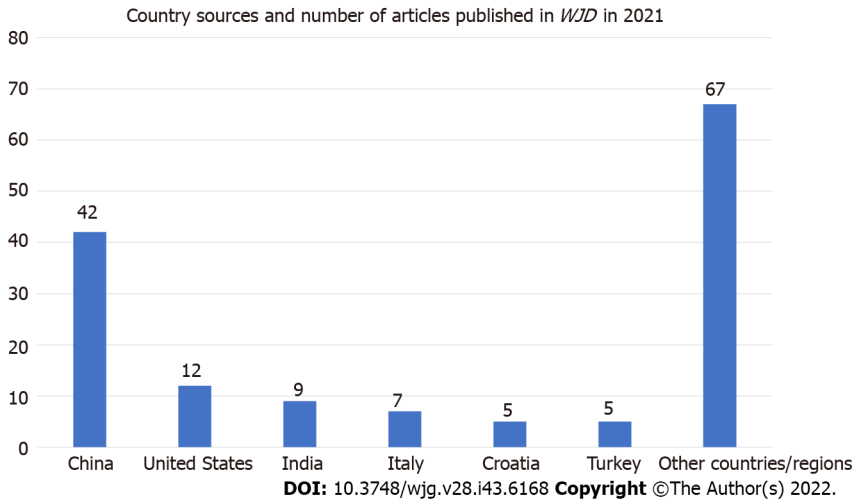


Figure 12 Country sources and number of articles published in *World Journal of Diabetes* in 2021. *WJD: World Journal of Diabetes.*

The 2021 JIF, number of received and published articles, citations, and number of webpage visits and downloads for World Journal of Gastrointestinal Oncology in 2019-2021

The 2021 JIF, number of received and published articles, and citations for *World Journal of Gastrointestinal Oncology* in 2019-2021: According to the *JCR* 2022, the 2021 JIF of *World Journal of Gastrointestinal Oncology* (*WJGO*) is 3.404, and the 5-year JIF is 3.250, ranking 162nd among 245 journals in the field of oncology, and ranking 59th among 93 journals in the field of cell biology, located in Q3 (Figure 18). The 2021 JIF of *WJGO* increased by 0.011 from its 2020 JIF (3.393).

According to the *RCA* database, *WJGO* has a 2022 *JAII* of 11.592, ranking 57th out of 102 journals in the field of gastroenterology & hepatology in the *RCA*, with 11627 total citations (63/102) and 1003 total articles (74/102).

From 2019 to 2020, *WJGO* received a total of 476 articles (average: 238 articles/year), among which 200 articles (42.0%) were invited and 276 (58.0%) were freely submitted; the acceptance rate was 45.8%. During the same period, *WJGO* published 218 articles (average: 109 articles/year), among which 81 (37.2%) were invited and 137 (62.8%) were freely submitted. As of June 28, 2022, the articles published in *WJGO* received a total of 1543 citations (without self-citations: 1533) by 1495 articles (without self-citations: 1486), yielding a self-citation rate of 0.65%; there were a total of 780 citations in 2021. After excluding self-citations, the 1486 articles that cited the *WJGO*-published articles were from 791 journals

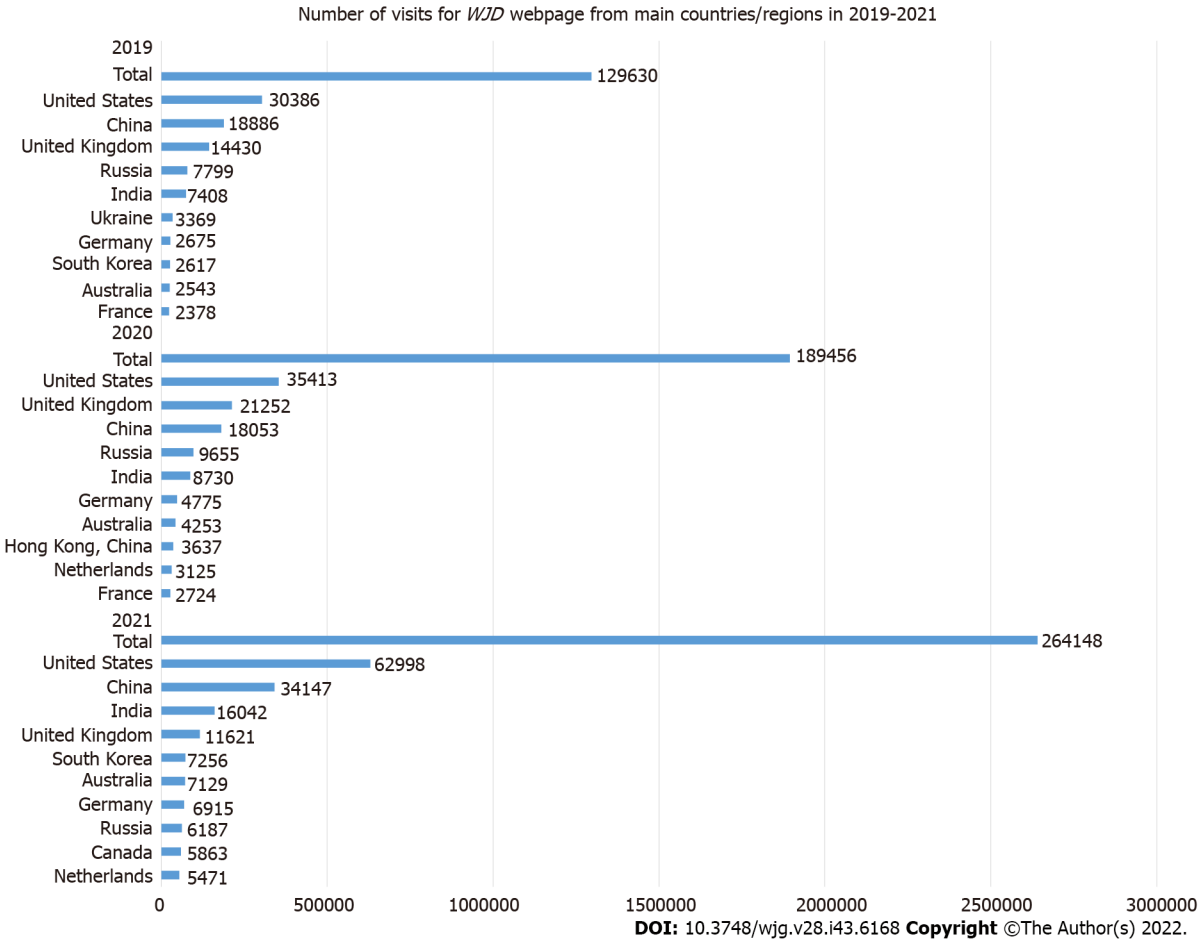


Figure 13 Number of visits to the *World Journal of Diabetes* webpage from main countries/regions in 2019-2021. *WJD*: *World Journal of Diabetes*.

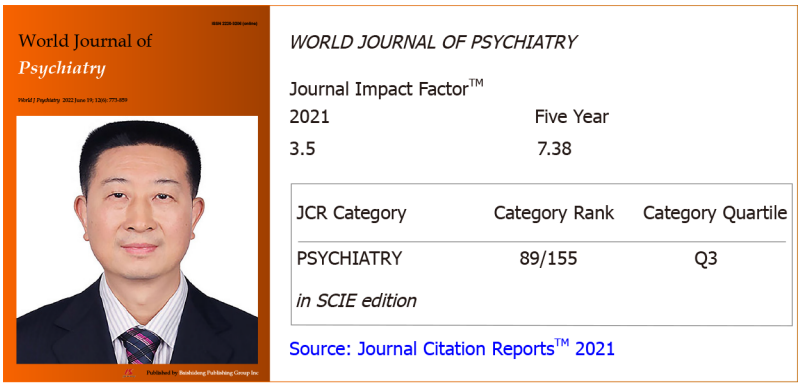


Figure 14 The 2021 Journal Impact Factor and category rank of *World Journal of Psychiatry*. *JCR*: *Journal Citation Report*™; SCIE: Science Citation Index Expanded™.

(data from Web of Science, Table 6); among these journals, 45 (5.7%) had a JIF of > 10 (data from Web of Science), accounting for 6.8% of the 660 total journals that had received a JIF of > 10 in the *JCR* 2022. Moreover, the journals citing the *WJGO*-published articles include renowned academic journals in the field of oncology such as *CA-A Cancer Journal for Clinicians* (2021 JIF 286.130, record count: 1), *Nature Reviews Cancer* (2021 JIF 69.800, record count: 1), and *Nature Reviews Clinical Oncology* (2021 JIF 65.011, record count: 1). From 2019 to 2020, the authors of the 218 articles published in *WJGO* were from 31 countries/regions, represented by 112 articles (51.4%) from China, 29 (13.3%) from the United States, 16 (7.3%) from Japan, 10 (4.6%) from Italy, 8 (3.7%) from South Korea, and 43 (19.7%) from other countries/regions (data from Web of Science, Figure 19). Of note, the article entitled "Pancreatic ductal adenocarcinoma: Treatment hurdles, tumor microenvironment and immunotherapy", which was

Table 6 Rank and record count of journals that published articles that cited the 218 articles published in *World Journal of Gastrointestinal Oncology* in 2019-2020, *n* (%)

Rank	Publication title	Record count
1	*Cancers*	70 (4.711)
2	*Frontiers in Oncology*	64 (4.307)
3	*International Journal of Molecular Sciences*	32 (2.153)
4	*Cells*	21 (1.413)
5	*BMC Cancer*	20 (1.346)
6	*Cancer Management and Research*	17 (1.144)
7	*Aging*	16 (1.077)
8	*Aging US*	16 (1.077)
9	*Biomedicines*	16 (1.077)
10	*Scientific Reports*	16 (1.077)
11	*Frontiers in Cell and Developmental Biology*	15 (1.009)
12	*Journal of Clinical Medicine*	15 (1.009)
13	*World Journal of Clinical Cases*	14 (0.942)
14	*Biomed Research International*	13 (0.875)
15	*European Review for Medical and Pharmacological Sciences*	13 (0.875)
16	*World Journal of Gastroenterology*	13 (0.875)
17	*Journal of Gastrointestinal Oncology*	12 (0.808)
18	*Frontiers in Genetics*	11 (0.740)
19	*Annals of Surgical Oncology*	10 (0.673)
20	*Frontiers in Pharmacology*	10 (0.673)
21	*Oncology Letters*	10 (0.673)
22	*PLOS One*	10 (0.673)
23	*Surgical Endoscopy*	10 (0.673)
24	Other journals, *n* = 768	1042 (70.121)

contributed by Sarantis *et al*[13] from National and Kapodistrian University of Athens, Greece in 2020, is the article with the highest number of citations among the articles published in *WJGO*. This article mainly reviews the latest research advances in the treatment barriers, tumor microenvironment, and immunotherapy for pancreatic ductal adenocarcinoma. As of June 28, 2022, this article has been cited 50 times.

In 2021, *WJGO* received a total of 424 manuscripts, of which 254 (59.9%) were invited and 170 (40.1%) were freely submitted; the acceptance rate was 37.0%, which was 78.2% higher than that in 2019-2020 (average: 238 articles/year). A total of 157 articles were published in *WJGO* in 2021, including 115 (73.2%) invited and 42 (26.8%) freely submitted. As of June 28, 2022, the number of total citations was 156. The number of articles published in *WJGO* in 2021 was 44.0% higher than that in 2019-2020 (109 articles/year on average). The authors were from 33 countries/regions, represented by 62 articles (39.5%) from China, 15 (9.6%) from Italy, 14 (8.9%) from the United States, 10 (6.4%) from Japan, 5 (3.2%) from South Korea, and 51 (32.5%) from other countries/regions (Figure 20).

Number of webpage visits and downloads received by *WJGO* in 2019-2021: From 2019 to 2021, the *WJGO* webpage received a total of 122230 visits in 2019, 179298 in 2020 (an increase of 46.7% compared with that in 2019), and 197781 in 2021 (an increase of 10.3% compared with that in 2020), with visits from more than 180 countries and regions worldwide (Figure 21). The number of downloads was 77505 in 2019, 165794 in 2020 (an increase of 113.9% compared with that in 2019), and 266672 in 2021 (an increase of 60.8% compared with that in 2020), with downloads from more than 130 countries and regions worldwide.

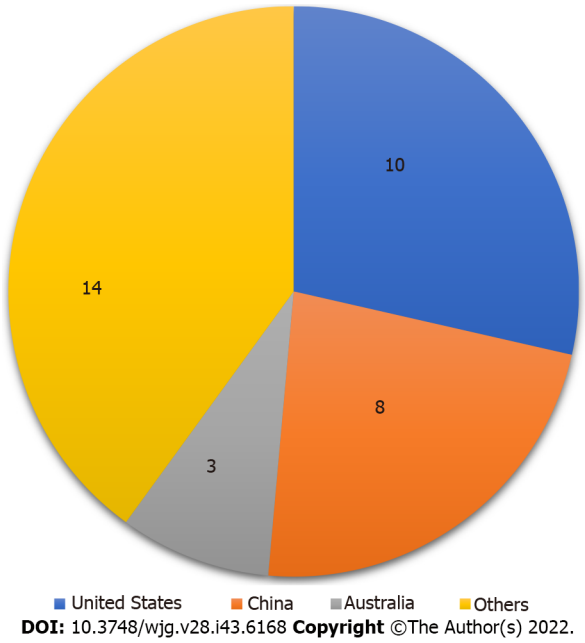


Figure 15 Country sources and number of articles published in *World Journal of Psychiatry* in 2019-2020.

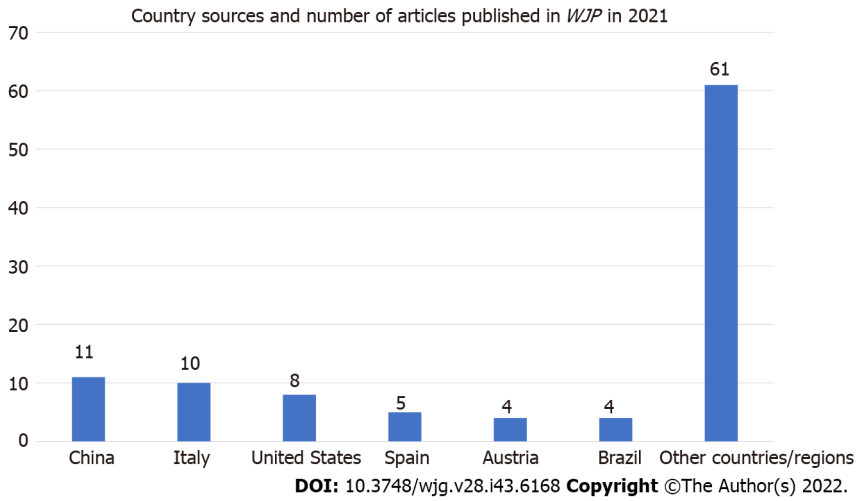


Figure 16 Country sources and number of articles published in *World Journal of Psychiatry* in 2021. *WJP*: *World Journal of Psychiatry*.

The 2021 JIF, number of received and published articles, citations, and number of webpage visits and downloads for World Journal of Gastrointestinal Surgery in 2019-2021

The 2021 JIF, number of received and published articles, and citations for *World Journal of Gastrointestinal Surgery* in 2019-2021: According to the *JCR* 2022, the 2021 JIF of *World Journal of Gastrointestinal Surgery* (*WJGS*) is 2.505, and the 5-year JIF is 3.099, ranking 104th among 211 journals in the field of surgery, located in Q2, and ranking 81st among 93 journals in the field of gastroenterology & hepatology, located in Q4 (Figure 22). The 2021 JIF of *WJGS* decreased by 0.077 from its 2020 JIF (2.582).

According to the *RCA* database, *WJGS* has a 2022 *JAII* of 10.615, ranking 62nd out of 102 journals in the field of gastroenterology & hepatology in the *RCA*, with 8694 total citations (71/102) and 819 total articles (80/102).

From 2019 to 2020, *WJGS* received a total of 297 articles (average: 149 articles/year), among which 113 (38.0%) were invited and 184 (62.0%) were freely submitted; the acceptance rate was 33.7%. During the same period, *WJGS* published 100 articles (average: 50 articles/year), among which 50 (50.0%) were invited and 50 (50.0%) were freely submitted. As of June 28, 2022, the articles published in *WJGS* received a total of 483 citations (without self-citations: 482) by 473 articles (without self-citations: 472), yielding a self-citation rate of 0.21%; there were a total of 242 citations in 2021. After excluding self-citations, the 472 articles that cited the *WJGS*-published articles were from 341 journals (data from Web of Science, Table 7); among these journals, 11 (3.2%) had a JIF of > 10 (data from Web of Science),

Table 7 Rank and record count of journals that published articles that cited the 100 articles published in *World Journal of Gastrointestinal Surgery* in 2019-2020, *n* (%)

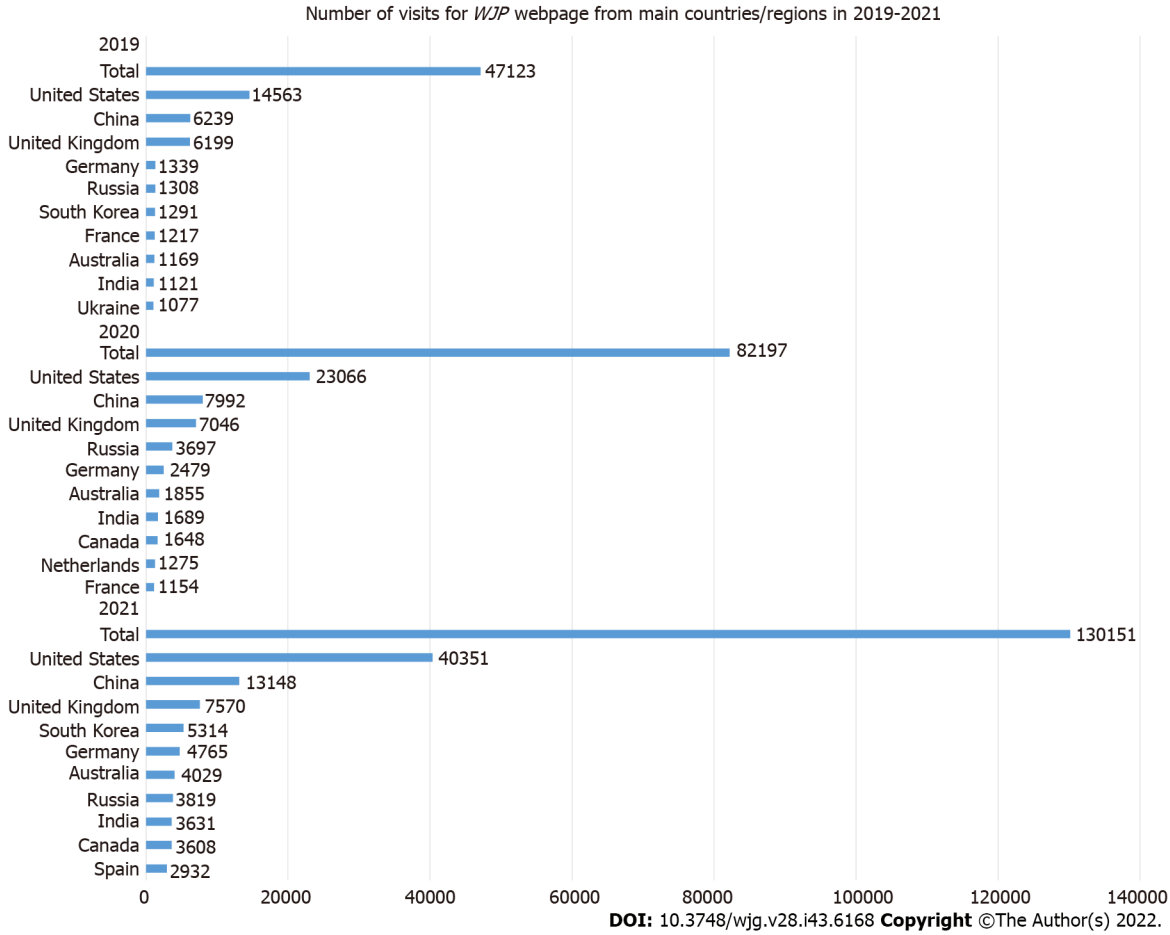
Rank	Publication title	Record count
1	*Frontiers in Oncology*	13 (2.754)
2	*Surgical Endoscopy*	13 (2.754)
3	*Surgical Endoscopy and Other Interventional Techniques*	13 (2.754)
4	*Cancers*	12 (2.542)
5	*Cureus*	11 (2.331)
6	*HPB*	10 (2.119)
7	*Journal of Gastrointestinal Surgery*	10 (2.119)
8	*Langenbeck's Archives of Surgery*	10 (2.119)
9	*Journal of Clinical Medicine*	9 (1.907)
10	*Diseases of the Esophagus*	8 (1.695)
11	*Updates in Surgery*	8 (1.695)
12	*World Journal of Clinical Cases*	7 (1.483)
13	*BMJ Case Reports*	6 (1.271)
14	*Frontiers in Surgery*	6 (1.271)
15	*Annals of Coloproctology*	5 (1.059)
16	*BMC Surgery*	5 (1.059)
17	*Egyptian Journal of Surgery*	5 (1.059)
18	*Indian Journal of Surgery*	5 (1.059)
19	*Scientific Reports*	5 (1.059)
20	*Annals of Coloproctology*	4 (0.847)
21	*Cureus Journal of Medical Science*	4 (0.847)
22	Other journals, *n* = 320	303 (64.195)

accounting for 1.7% of the 660 total journals that had received a JIF of > 10 in the *JCR* 2022. Moreover, the journals citing the *WJGS*-published articles include renowned academic journals in the field of surgery such as *JAMA Surgery* (2021 JIF 16.681, record count: 1) and *British Journal of Surgery* (2021 JIF 11.122, record count: 1).

From 2019 to 2020, the authors of the 100 articles published in *WJGS* were from 31 countries/regions, represented by 18 articles (18.0%) from the United States, 12 (12.0%) from China, 8 (8.0%) from Japan, 7 (7.0%) from Greece, 7 (7.0%) from Italy, and 48 (48.0%) from other countries/regions (data from Web of Science, Figure 23). Of note, the article entitled "Carbohydrate antigen 19-9 - tumor marker: Past, present, and future", which was contributed by Dr. Lee *et al*[14] from Nanyang Technological University, Singapore in 2020, is the article with the highest number of citations among the articles published in *WJGS*. This article mainly reviews the related research on the tumor marker carbohydrate antigen 19-9. As of June 28, 2022, this article has been cited 31 times.

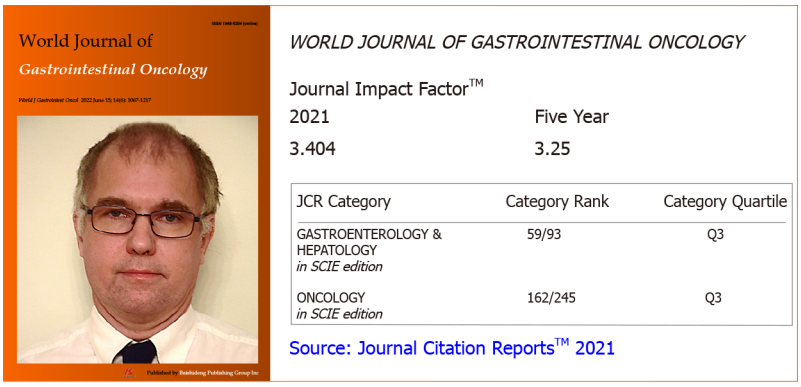
In 2021, *WJGS* received a total of 274 manuscripts, of which 141 (51.5%) were invited and 133 (48.5%) were freely submitted; the acceptance rate was 50.4%, which was 83.9% higher than that in 2019-2020 (average: 149 articles/year). A total of 138 articles were published in *WJGS* in 2021, including 86 (62.3%) invited and 52 (37.7%) freely submitted. As of June 28, 2022, the number of total citations was 119. The number of articles published in *WJGS* in 2021 was 176.0% higher than that in 2019-2020 (50 articles/year on average). The authors were from 30 countries/regions, represented by 47 articles (34.1%) from China, 16 (11.6%) from Italy, 12 (8.7%) from Japan, 8 (5.8%) from the United States, 5 (3.6%) from Singapore, 5 (3.6%) from Turkey, and 45 (32.6%) from other countries/regions (Figure 24).

Number of webpage visits and downloads received by *WJGS* in 2019-2021: From 2019 to 2021, the *WJGS* webpage received a total of 110712 visits in 2019, 137115 in 2020 (an increase of 23.8% compared with that in 2019), and 178025 in 2021 (an increase of 29.8% compared with that in 2020), with visits from more than 190 countries and regions worldwide (Figure 25). The number of downloads was 60397 in 2019, 111880 in 2020 (an increase of 85.2% compared with that in 2019), and 188632 in 2021 (an increase of 68.6% compared with that in 2020), with downloads from more than 150 countries and



DOI: 10.3748/wjg.v28.i43.6168 **Copyright** ©The Author(s) 2022.

Figure 17 Number of visits to the *World Journal of Psychiatry* webpage from main countries/regions in 2019-2021. *WJP*: *World Journal of Psychiatry*.



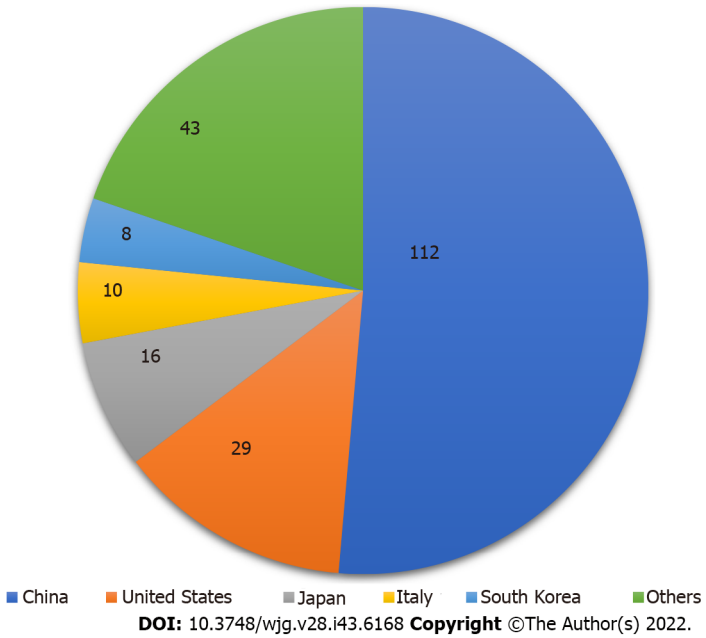
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Figure 18 The 2021 Journal Impact Factor and category rank of *World Journal of Gastrointestinal Oncology*. *JCR*: *Journal Citation Report*™; SCIE: Science Citation Index Expanded™.

regions worldwide.

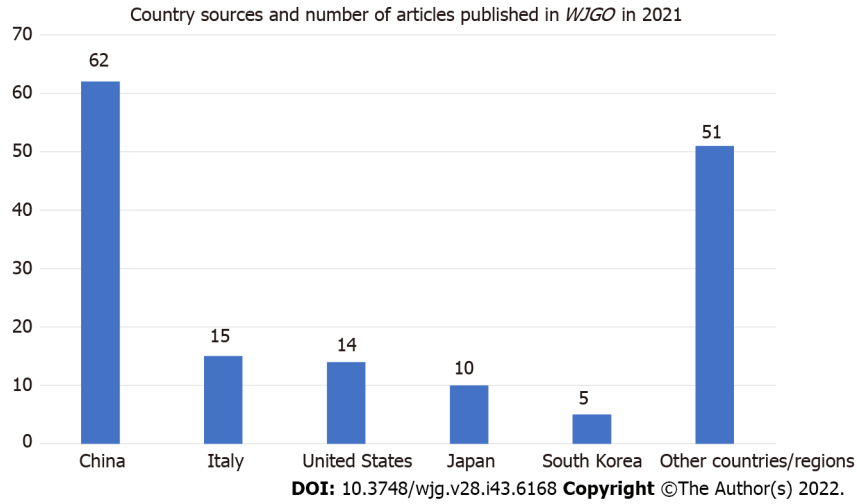
The 2021 JIF, number of received and published articles, citations, and number of webpage visits and downloads for World Journal of Clinical Cases in 2019-2021

The 2021 JIF, number of received and published articles, and citations for *World Journal of Clinical Cases* in 2019-2021: According to the *JCR* 2022, the 2021 JIF of *World Journal of Clinical Cases* (*WJCC*) is 1.534, and the 5-year JIF is 1.599, ranking 135th among 172 journals in the field of general & internal medicine, located in Q4 (Figure 26). The 2021 JIF of *WJCC* increased by 0.197 from its 2020 JIF (1.337).



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Figure 19 Country sources and number of articles published in *World Journal of Gastrointestinal Oncology* in 2019-2020.



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Figure 20 Country sources and number of articles published in *World Journal of Gastrointestinal Oncology* in 2021. *WJGO*: *World Journal of Gastrointestinal Oncology*.

According to the *RCA* database, *WJCC* has a 2022 *JAII* of 3.046, ranking 174th out of 177 journals in the field of general & internal medicine in the *RCA*, with 12852 total citations (91/177) and 4219 total articles (52/177).

From 2019 to 2020, *WJCC* received a total of 3820 articles (average: 1910 articles/year), among which 485 (38.0%) were invited and 3335 (62.0%) were freely submitted; the acceptance rate was 33.1%. During that same period, *WJCC* published 1263 articles (average: 632 articles/year), among which 228 (18.1%) were invited and 1035 (81.9%) were freely submitted. As of June 28, 2022, the articles published in *WJCC* received a total of 3966 citations (without self-citations: 3933) by 3842 articles (without self-citations: 3815), yielding a self-citation rate of 0.83%; there were a total of 2112 citations in 2021. After excluding self-citations, the 3815 articles that cited the *WJCC*-published articles were from 2298 journals (data from Web of Science, Table 8); among these journals, 92 (4.0%) had a JIF of > 10 (data from Web of Science), accounting for 13.9% of the 660 total journals that had received a JIF of > 10 in the *JCR* 2022. Moreover, the journals citing the *WJCC*-published articles include renowned academic journals in the field of general & internal medicine such as *New England Journal of Medicine* (2021 JIF 176.079, record count: 1), *Annals of Internal Medicine* (2021 JIF 51.598, record count: 1), and *JAMA Internal Medicine* (2021 JIF 44.409, record count: 2).

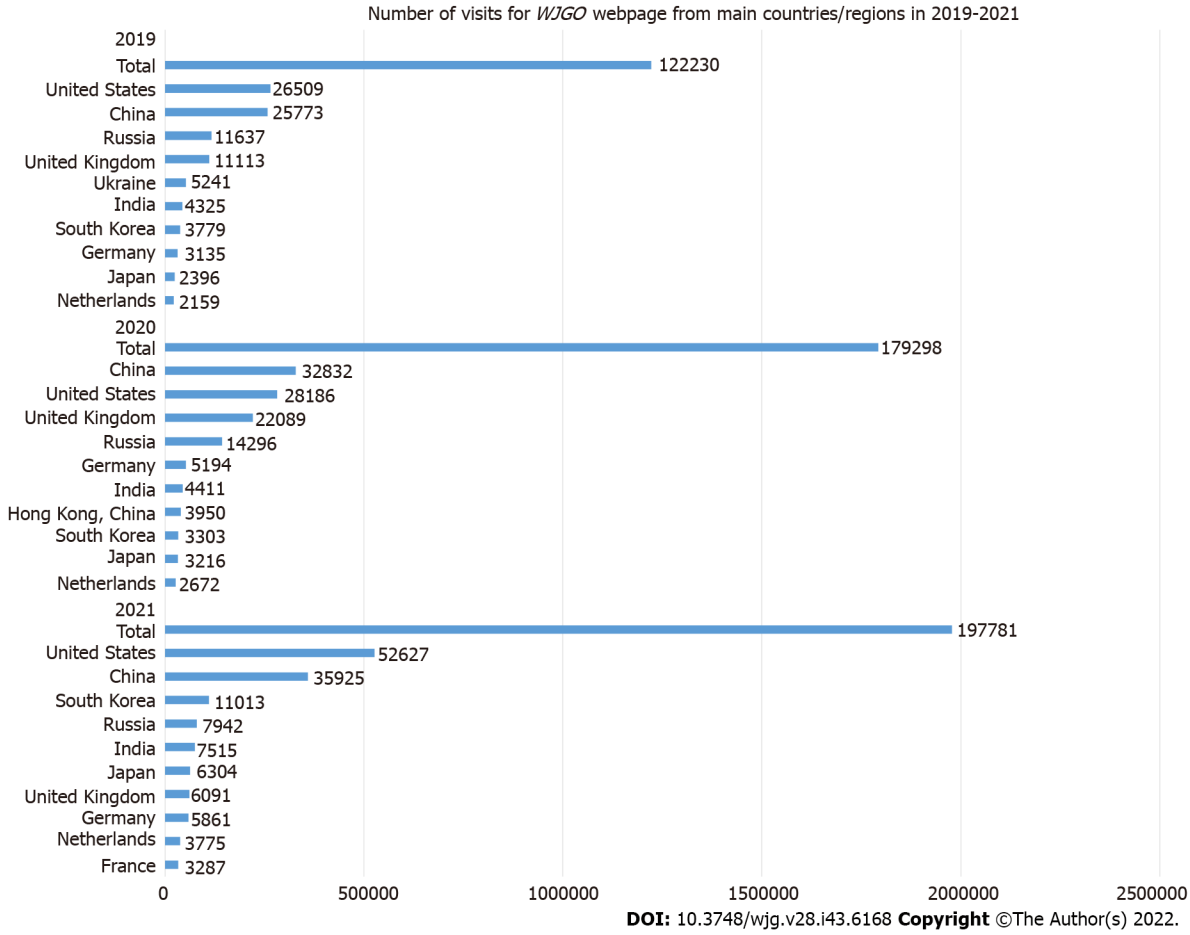
Table 8 Rank and record count of journals that published articles that cited the 1263 articles published in *World Journal of Clinical Cases* in 2019-2020, *n* (%)

Rank	Publication title	Record count
1	*Cureus*	63 (1.651)
2	*International Journal of Molecular Sciences*	54 (1.415)
3	*Journal of Clinical Medicine*	44 (1.153)
4	*Medicine*	39 (1.022)
5	*Medicine Hagerstown*	39 (1.022)
6	*Frontiers in Oncology*	36 (0.944)
7	*Cancers*	31 (0.813)
8	*World Journal of Gastroenterology*	30 (0.786)
9	*Cureus Journal of Medical Science*	29 (0.760)
10	*International Journal of Environmental Research and Public Health*	29 (0.760)
11	*Scientific Reports*	29 (0.760)
12	*BMJ Case Reports*	24 (0.629)
13	*Diagnostics*	24 (0.629)
14	*Diagnostics Basel Switzerland*	24 (0.629)
15	*Nutrients*	23 (0.603)
16	*PLOS One*	22 (0.577)
17	*BMC Gastroenterology*	20 (0.524)
18	*Frontiers in Medicine*	20 (0.524)
19	*American Journal of Translational Research*	19 (0.498)
20	*Abdominal Radiology*	18 (0.472)
21	*Abdominal Radiology New York*	18 (0.472)
22	*Biomedicines*	16 (0.419)
23	*Cells*	16 (0.419)
24	*Frontiers in Immunology*	16 (0.419)
25	Other journals, *n* = 2274	3132 (82.097)

From 2019 to 2020, the authors of the 1263 articles published in *WJCC* were from 59 countries/regions, represented by 905 articles (71.7%) from China, 69 (5.5%) from South Korea, 55 (4.4%) from the United States, 49 (3.9%) from Japan, 43 (3.4%) from Italy, and 142 (11.2%) from other countries/regions (data from Web of Science, Figure 27). Of note, the article entitled "Fear can be more harmful than the severe acute respiratory syndrome coronavirus 2 in controlling the corona virus disease 2019 epidemic", which was contributed by Chief Physician Ren *et al*[15] from Beijing Aerospace General Hospital, China in 2020, is the article with the highest number of citations among the articles published in *WJCC*. This article mainly reviews the discrimination and prejudice caused by the fear of disease or misinformation after the outbreak of COVID-19. As of June 28, 2022, this article has been cited 146 times.

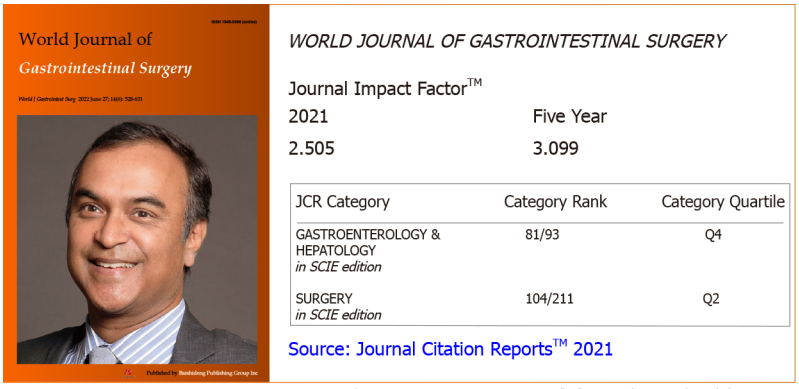
In 2021, *WJCC* received a total of 3650 manuscripts, of which 233 (6.4%) were invited and 3417 (93.6%) were freely submitted; the acceptance rate was 35.5%, which was 91.1% higher than that in 2019-2020 (average: 1910 articles/year). A total of 1296 articles were published in *WJCC* in 2021, including 136 (10.5%) invited and 1160 (89.5%) freely submitted. As of June 28, 2022, the number of total citations was 563. The number of articles published in *WJCC* in 2021 was 105.1% higher than that in 2019-2020 (632 articles/year on average). The authors were from 45 countries/regions, represented by 982 articles (75.8%) from China, 80 (6.2%) from South Korea, 38 (2.9%) from Japan, 32 (2.5%) from Taiwan, 20 (1.5%) from the United States, and 144 (11.1%) from other countries/regions (Figure 28).

Number of webpage visits and downloads received by *WJCC* in 2019-2021: From 2019 to 2021, the *WJCC* webpage received a total of 363959 visits in 2019, 712451 in 2020 (an increase of 95.8% compared with that in 2019), and 908002 in 2021 (an increase of 27.4% compared with that in 2020), with visits from more than 210 countries and regions worldwide (Figure 29). The number of downloads was 174808



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Figure 21 Number of visits to the *World Journal of Gastrointestinal Oncology* webpage from main countries/regions in 2019-2021. *WJGO*: *World Journal of Gastrointestinal Oncology*.



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Figure 22 The 2021 Journal Impact Factor and category rank of *World Journal of Gastrointestinal Surgery*. *JCR*: *Journal Citation Report*™; SCIE: Science Citation Index Expanded™.

in 2019, 442635 in 2020 (an increase of 153.2% compared with that in 2019), and 128.1% in 2021 (an increase of 52.6% compared with that in 2020), with downloads from more than 180 countries and regions worldwide.

Number of non-conforming submissions identified in the preview of submissions to the seven journals in 2019-2021

From 2019 to 2021, a total of 16618 articles were submitted by the authors to Baishideng's seven SCIE-indexed journals. After preview by the Baishideng editorial office, 856 manuscripts (5.2%) were found not to meet the requirements of Baishideng for receiving manuscripts, and the authors failed to modify

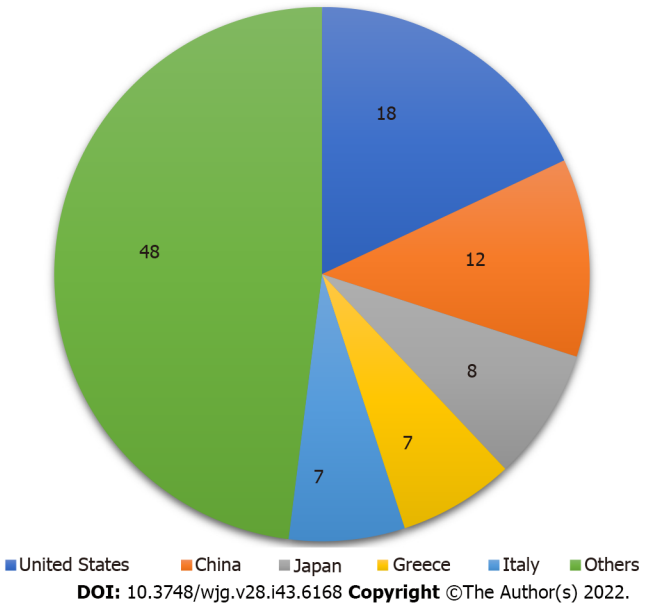


Figure 23 Country sources and number of articles published in *World Journal of Gastrointestinal Surgery* in 2019-2020.

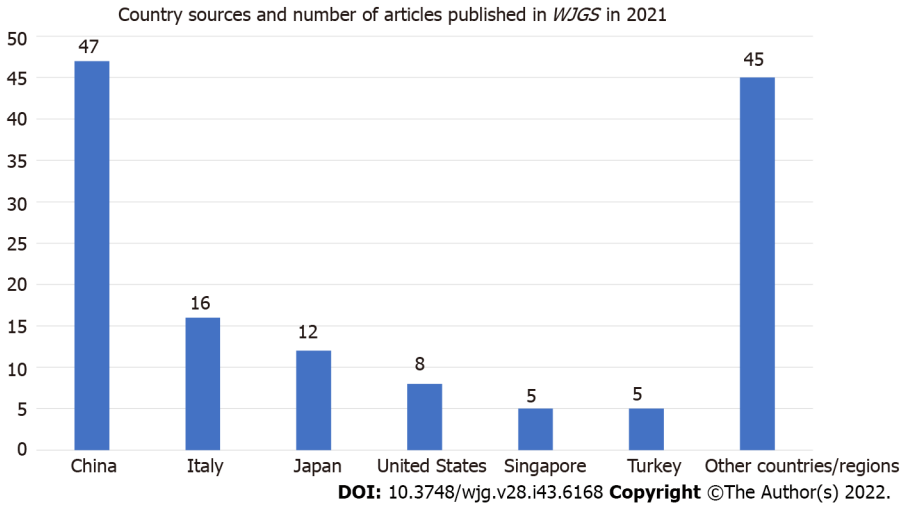


Figure 24 Country sources and number of articles published in *World Journal of Gastrointestinal Surgery* in 2021. *WJGS*: *World Journal of Gastrointestinal Surgery*.

and supplement the relevant documents within the prescribed time. As a result, these manuscripts failed to pass the submission preview and were not sent for peer review.

Number of peer reviewers and editorial board members involved in peer review of manuscripts submitted to the seven journals in 2019-2021

From 2019 to 2021, a total of 15762 manuscripts submitted to Baishideng's seven SCIE-indexed journals passed manuscript preview, and a total of 463297 peer reviews were performed by invited peer reviewers or editorial board members. Among these individuals, 30866 (6.7%) completed the peer review successfully, 66836 (14.4%) rejected the invitation to conduct peer review, 17976 (3.9%) agreed to conduct the peer review but failed to submit the peer review report on time, and 347619 (75.0%) failed to respond to the invitation. The success rate of manuscript peer review was low. We found that some of the peer reviewers' comments on the manuscript were only one-sentence in length, with no explanation for their suggestion of acceptance of the manuscript and no changes to the manuscript, and comments providing no guiding value in improving the quality of the manuscript (*e.g.*, "This is an interesting study. Recommend an acceptance.", "This manuscript is very well written.", "I recommend reject the study.", *etc*). Baishideng acknowledged all the peer reviewers and editorial board members who completed the manuscript peer review online, on the publisher's home page. For more details, please visit: https://www.f6publishing.com/highlyinfluentialpeerreviewers.

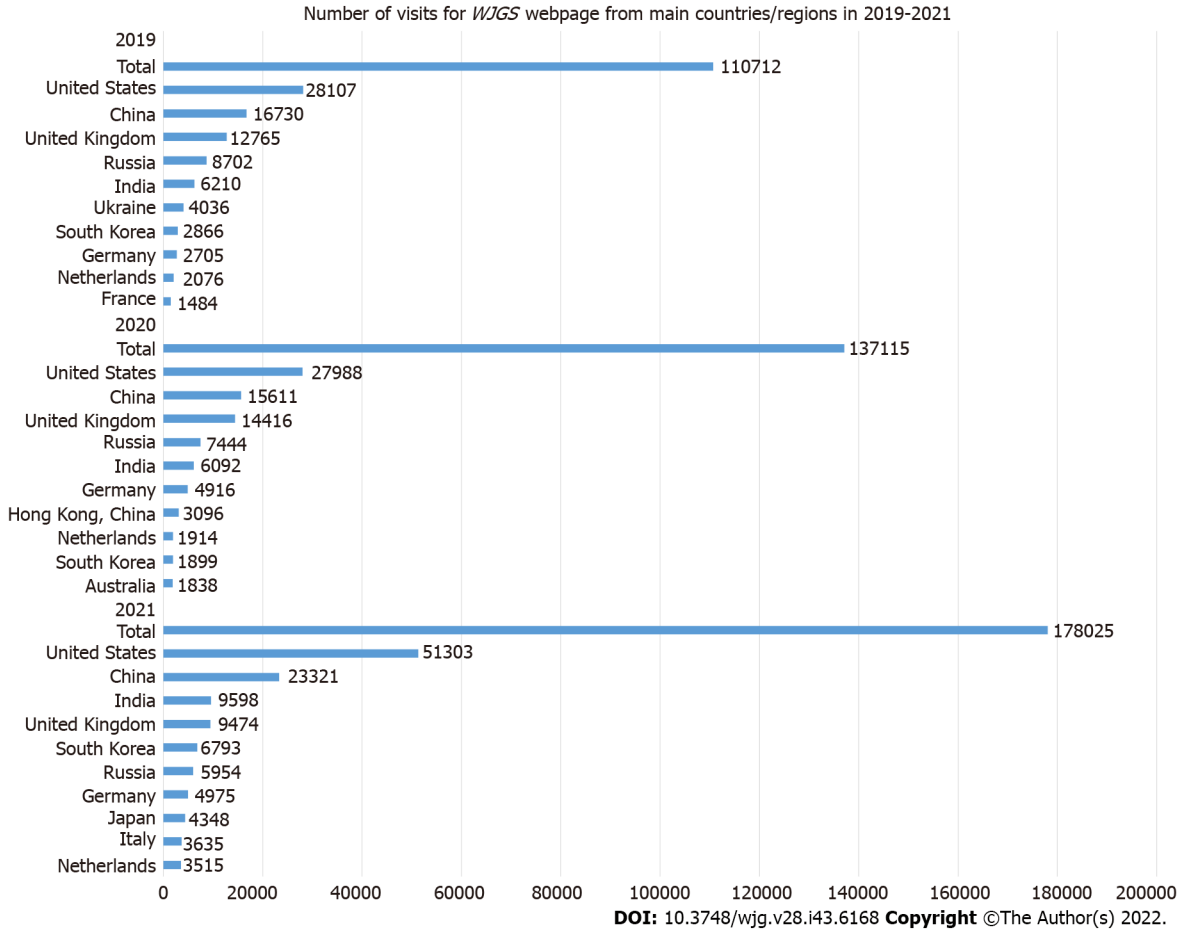


Figure 25 Number of visits to the *World Journal of Gastrointestinal Surgery* webpage from main countries/regions in 2019-2021. *WJGS*: *World Journal of Gastrointestinal Surgery*.



Figure 26 The 2021 Journal Impact Factor and category rank of *World Journal of Clinical Cases*. *JCR*: *Journal Citation Report*™; SCIE: Science Citation Index Expanded™.

Number of manuscripts linguistically polished in the seven journals in 2019-2021

From 2019 to 2021, a total of 5543 manuscripts were accepted and published online in Baishideng's seven SCIE-indexed journals. The grammatical content of all manuscripts submitted by non-native English speakers was verified based on Baishideng's manuscript language editing policy (https://www.wjgnet.com/bpg/gerinfo/240). Among them, 3406 (61.4%) were polished by Baishideng's language editors.

All seven journals adopt an OA publishing model

All seven of Baishideng's SCIE-indexed journals adopt the OA publishing model, that is, authors pay to

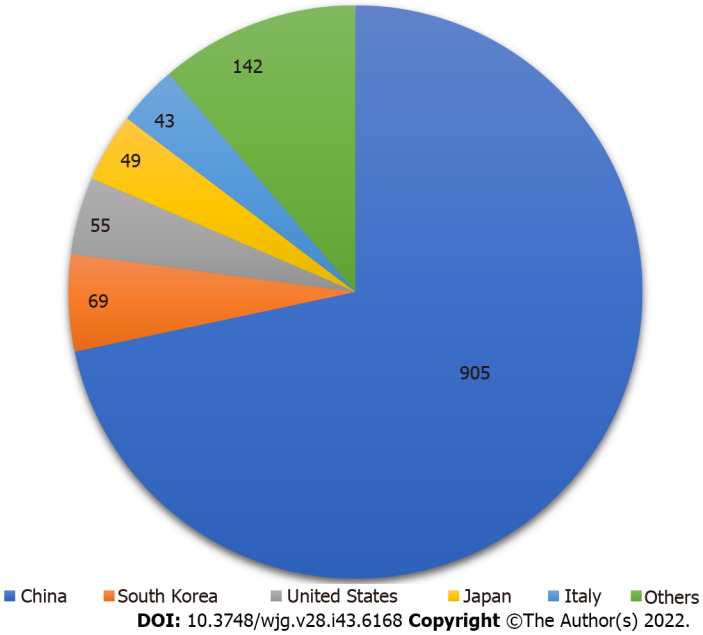


Figure 27 Country sources and number of articles published in *World Journal of Clinical Cases* in 2019-2020.

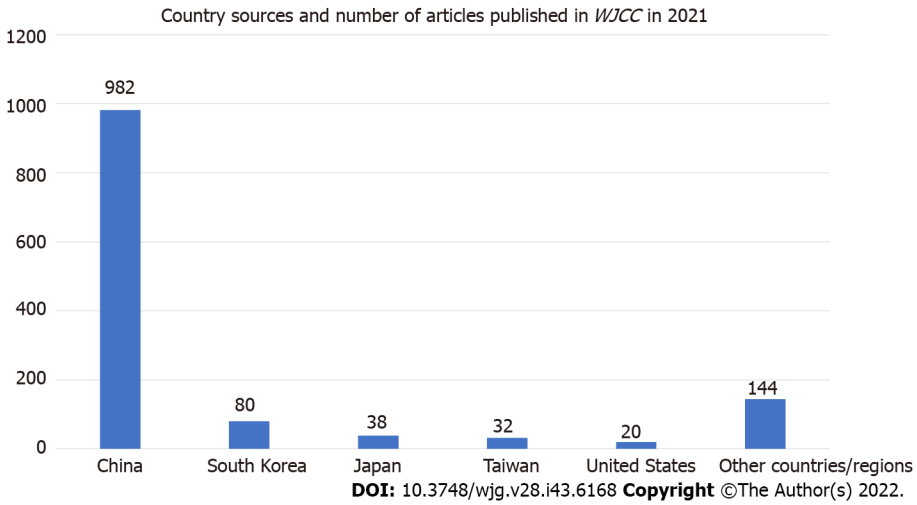


Figure 28 Country sources and number of articles published in *World Journal of Clinical Cases* in 2021. *WJCC*: *World Journal of Clinical Cases*.

publish and readers read for free. A total of 5543 articles were published in the seven journals from 2019 to 2021, all of which are OA articles with unlimited length, unlimited number of figures and tables, and unlimited number of references and citations, available for all readers to access and download online. Of these, 2083 (37.6%) articles were invited and published free of article processing charge (APC). At the same time, Baishideng has an APC discount policy, in which the APC will be reduced by 10% if the free contribution meets one of the following conditions: (1) Supported by a fund of national level or above; (2) Having a corresponding author who is a member of an association of national level or above; (3) Having a first author who is a young scholar under 45 years old; or (4) Having a corresponding author who is a member of the editorial board or who serves as a peer reviewer.

Quality tracking for articles published in the seven journals in 2019-2021

As of June 28, 2022, Baishideng has received 3903 quality-tracking evaluation reports from members of the Baishideng journal editorial boards for 5543 articles published in the seven journals from 2019 to 2021. The Baishideng editorial office verified and replied to every article tracking evaluation report received, and dealt with the important issues found and feedback by the editorial board members in a timely manner. Among them, 3765 reports (96.5%) gave positive comments or did not provide comments, 105 (2.7%) proposed revision opinions or pointed out article limitations, and 33 (0.8%) proposed editing and production quality problems. The Baishideng editorial office has asked the

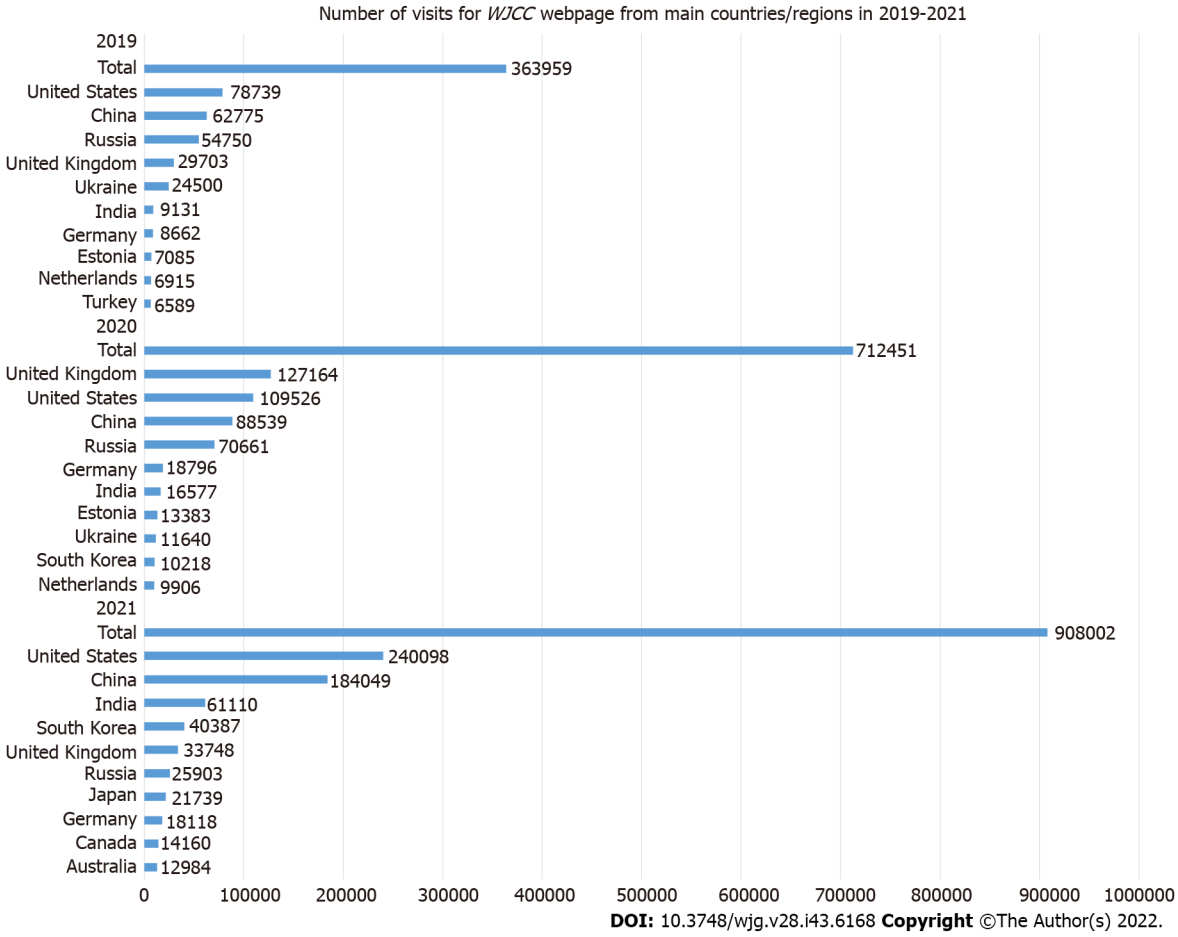


Figure 29 Number of visits to the *World Journal of Clinical Cases* webpage from main countries/regions in 2019-2021. *WJCC*: *World Journal of Clinical Cases*.

authors to clarify the problems raised by reviewers or write readers' letters for further discussion. The production department has verified and consequently revised the highlighted problems in the production process of articles.

Author evaluation for articles published in the seven journals in 2019-2021

As of June 28, 2022, Baishideng has received evaluation and feedback from the authors for 4655 (84.0%) of the 5543 articles published in the seven journals from 2019 to 2021. The Baishideng editorial office promptly verified and replied to the authors' comments received for each article. The vast majority of authors commented positively on Baishideng's peer review, as well as the editing and publishing process. As for the problems pointed out by some authors, the editorial office has verified each one-by-one and dealt with them accordingly. As for the suggestions given by the authors, we consequently organized discussions, reasonably optimized the publishing process of manuscripts, and improved the guidelines for authors.

Reader evaluation of articles published in the seven journals in 2019-2021

Since the launch of the reader evaluation system on March 25, 2021 to June 28, 2022, Baishideng's seven SCIE-indexed journals have received a total of 424 reader evaluations and 229 manuscripts of letters from readers. The Baishideng editorial office carefully read and responded to every reader comment received. For online reader comments with academic value, the Baishideng editorial office invited the commentors to write them as formal reader letters (Letters to the Editor) and submit them online through the F6Publishing system. In 2021, a total of 38 peer-reviewed Letters to the Editor were published online in Baishideng's journals.

DISCUSSION

JIF is still one of the most commonly used criterion for judging the quality of academic journals, although it has faced controversies since its inception. Regardless, increasing the JIF of academic

journals is an effective way of attracting high-quality contributions from scholars. How to improve the JIF of academic journals remains the primary issue of concern for academic journal publishers. Among the various factors influencing the JIF of academic journals, whether an academic journal is an OA journal and the integrity of its editing and publishing process exert substantive impacts on the number of articles it receives and subsequently publishes, the number of webpage visits and downloads it receives, and thus its JIF and academic influence in the overall discipline and topical field. The complete editing and publishing process of Baishideng's seven journals is introduced and discussed in detail in the following subsections.

Manuscript preview

For most academic journals worldwide, manuscripts are firstly previewed by the internal editors. It is the internal editors who usually decide to directly reject the manuscript or send it to the external reviewers. However, Baishideng journals are previewed in a different way. When the editorial office receives an author's contribution, the in-house assistant editor in the editorial office will verify whether the online submitted manuscript meets the requirements for Baishideng's formal receipt of a manuscript, including verification of whether the content of the manuscript falls within the scope of the journal, whether the basic information on the authors is correct (including the names, E-mail addresses, and ORCID information of all authors), and whether the manuscript document and the documents on ethics and academic rules and norms for the column that the manuscript belongs to are correctly provided [*i.e.*, (1) Institutional Review Board Approval Form or Document; (2) Institutional Animal Care and Use Committee Approval Form or Document; (3) Biostatistics Review Certificate; (4) Signed Informed Consent Form(s) or Document(s); and (5) Non-Native Speakers of English Editing Certificate]. If any of the above items do not meet the requirements, the manuscript will be returned to the authors for revision and resubmission; if the authors do not resubmit the manuscript within 7 d and do not reply with a reason, the manuscript will be rejected. If the manuscript meets the requirements, the F6Publishing artificial intelligence system will search the Baishideng database to identify relevant editorial board members and peer reviewers based on the keywords of the manuscript, and then invite five of the journal's editorial board members and 15 external peer reviewers to perform a single-blind peer review of the manuscript. At the same time, the in-house science editor will invite 3-5 members of the journal's editorial board to conduct a single-blind peer review of the manuscript.

Journals should have a policy for safeguarding research data submitted to them and to detect research misconduct and ensure the accuracy and reliability of the information published[16]. The main purpose of this step is to intercept submissions at risk of academic misconduct, prior to peer review. From 2019 to 2021, 5.2% of the submissions to Baishideng's seven SCIE-indexed journals were rejected at this stage, thus excluding the research that does not conform to the academic rules and norms, and effectively reducing the risk of publishing articles that may have academic misconduct. Compared with the figures in 2019-2020, the number of received articles by the seven journals in 2021 all increased (by 5.8% for *WJG*, 8.6% for *WJSC*, 104.1% for *WJD*, 373.2% for *WJP*, 78.2% for *WJGO*, 83.9% for *WJGS*, and 91.1% for *WJCC*). This increase in the number of articles received provides a solid foundation for the selection of high-quality articles published in the journals.

Manuscript peer review and first decision

Peer reviewers, in accordance with ethical norms for peer review, conduct a single-blind peer review of manuscripts submitted to Baishideng's journals to assess their scientific and linguistic quality. It is of critical importance to prevent citation manipulation by peer reviewers, and as such they are requested to cautiously recommend authors to cite their own published papers. If a peer reviewer recommends the author to cite his/her own papers, he/she must include a specific rationale as to why their inclusion is merited, so that the author can decide rationally on whether or not to cite the paper. If the peer reviewer cannot provide sufficient reasons for the request, their opinion will be deemed invalid, the editorial office will delete this comment and close the peer reviewer's account. In addition, the manuscript will be sent to other peer reviewers to review.

The peer reviewers' evaluation criteria for the scientific quality and language quality of the manuscript are as follows: (1) Scientific quality. Peer reviewers classify the scientific quality of the manuscript as Grade A (Excellent), Grade B (Very good), Grade C (Good), Grade D (Fair), or Grade E (Do not publish); (2) Language quality. Peer reviewers classify the language quality of the manuscript as Grade A (Priority publishing), Grade B (Minor language polishing), Grade C (A great deal of language polishing), or Grade D (Rejection); and (3) Conclusion. The conclusions and recommendations of peer reviewers on the manuscript are (A) Accept (High priority), (B) Accept (General priority), (C) Minor revision, (D) Major revision, and (E) Rejection. The reviewer will also state whether they would like to re-review the manuscript once it has been revised by the authors.

After a manuscript is peer reviewed by at least two peer-reviewer(s) and/or editorial board member(s), the first decision will be made by an Academic Editor (external), and an evaluation report of the first decision will be provided to the authors. The main task of this work is to verify and evaluate the academic and language quality of manuscripts, and suggest a first decision. The Academic Editors (external) are advised to consider the peer-review report; however, the comments by reviewers represent only a part of the information they will consider in making a first decision. The Academic

Editors (external) should also evaluate the manuscript based on the following: (1) Their own knowledge of the topic; (2) The manuscript's theme; (3) The main content of the manuscript; (4) The resolution of the figures and readability of the tables; (5) The appropriateness of the reference citations and the authors self-citations; (6) The language quality; (7) The medical ethics of the study; and (8) The study's adherence to academic norms. Then, the Academic Editors (external) will independently decide to accept or reject the manuscript, or suggest to the authors a transfer of the manuscript to another of Baishideng's journals that is more appropriate in scope and/or topic. They will also provide a summary of this decision, in order to help the authors and the journal to further improve the academic quality of the manuscript.

If the Academic Editor (external) decides to accept the manuscript, the Company Editor-in-Chief (internal) will review whether the manuscript conforms to academic ethics, rules and norms, and make the first decision to accept or reject the manuscript.

The peer review process is probably the most important process for a journal to ensure that it publishes original and high-quality research or commentary[17]. Peer review underpins the quality and integrity of scientific publishing[18]. Although author-suggested reviewers are not necessarily, as commonly assumed, less neutral than reviewers not suggested by the authors[19], in order to avoid academic misconduct arising from manipulation of manuscript peer review by authors or third parties, Baishideng does not accept author-suggested reviewers. In addition, Baishideng selects peer reviewers from a different country than the authors to review the manuscript in order to prevent potential conflicts of interest and other improper practices.

From 2019 to 2021, each manuscript submitted to Baishideng's seven SCIE-indexed journals was peer-reviewed by an average of 1.96 peer reviewers or editorial board members. The strict adherence to peer review processes kept the acceptance rates of these journals at a low level. From 2019 to 2020, the acceptance rates of *WJG*, *WJSC, WJD, WJP, WJGO, WJGS*, and *WJCC* were 27.6%, 46.5%, 37.4%, 31.3%, 45.8%, 33.7%, and 33.1%, respectively; the corresponding percentages in 2021 were 27.5%, 50.7%, 49.0%, 38.9%, 37.0%, 50.4%, and 35.5%. This practice has ensured not only the academic and language quality of the manuscripts but also the authenticity and standardization of the research involved in each manuscript, thus providing a guarantee for Baishideng journals to publish high-quality articles.

Second decision

After receiving the revised manuscript from the authors, the in-house science editor first checks whether the authors have made careful revisions to the manuscript according to the requirements and have answered the comments raised by the peer-reviewers, academic editor, and Company Editor-in-Chief, based on the peer review report, comments from the academic editor and Company Editor-in-Chief, Answering Reviewers document (submitted by the authors), and guidelines and requirements for revision of the manuscript. Then, the in-house science editor verifies whether all other manuscript-related documents submitted by the authors meet the journal's publication requirements. After the manuscript has been edited by the in-house science editor, it is sent to the Editor-in-Chief for a second decision, and then the Company Editor-in-Chief reviews the manuscript and makes the final decision on whether to accept or reject the manuscript.

Verification of documents related to the revised manuscript: The in-house science editor verifies the documents related to the revised manuscript based on the manuscript type. Different documents are required for different types of manuscripts, with some overlap. All manuscripts should include: (1) Revised manuscript file; (2) Answering Reviewers document - the authors should make a point-to-point response to the reviewers' comments and revise the manuscript accordingly; (3) Conflict-of-interest disclosure form; (4) Copyright license agreement; (5) Audio core tip; and (6) Bing Check Report. In the process of revising the manuscript, the authors' response to the reviewers' comments can help to greatly improve the quality of the manuscript[20]; therefore, it is particularly important for the science editor to verify the authors' revised manuscript.

After the document verification is completed and the revised manuscript passes duplication detection, the in-house science editor edits the manuscript and then sends it back to the authors for proofreading according to the guidelines and requirements for revision of the manuscript and the Science Editor Working Checklist; the latter (entitled, "Checklist of Responsibilities for Scientific Editors of the Baishideng Publishing Group Journals," and publicly available on the publisher's website) outlines the primary responsibilities of Baishideng's scientific editors. Then, the in-house science editor further edits and revises the authors' proofread manuscript. As Katz[21] said: "Anything published in professional literature is going to go through an editing process, and this is where dedicated experts get to make our work look better than it perhaps started off as". The editing and processing of manuscripts by science editors are important parts of the publishing process.

Language polishing at second decision: In order to strictly control and ensure the language quality of the manuscript, the in-house science editor verifies whether the manuscript needs to be sent to a Baishideng language editor for further language polishing according to Baishideng's English language publishing standard for manuscripts at the second decision. The purpose of language polishing is to improve the readability of the manuscript by modifying, polishing, and improving the language of the

full text from the aspects of grammar and spelling, syntax, articles/prepositions/conjunctions, punctuation, professional language, definitions and consistency of use of abbreviations, and so on without affecting the academic content or the original meaning of the manuscript.

Baishideng formulated the following rules for sending manuscripts to their language editors for language polishing according to whether the authors' native language is English or not: (1) For native English-speaking authors, the revised manuscripts submitted no longer need to be sent to a Baishideng language editor to polish the language; (2) For non-native English-speaking authors who have provided a language certificate (written affirmation by a professional agency attesting to the high quality of the manuscript's language) or those from countries/regions with relatively good repute for English proficiency, such as those from Japan, Korea, Greece, Turkey, India, Brazil, Taiwan, and Hong Kong, the manuscripts do not need to be sent to a Baishideng language editor to polish the language if the peer reviewers had rated the revised manuscript's language as Grade A or B and the authors have provided the language certificate; (3) For non-native English-speaking authors who are from countries/regions without a solid repute for English proficiency, such as China, if the revised manuscript's language was rated Grade A or B by the peer reviewers and the authors have provided a language certificate issued by any of the professional language editing companies that have been vetted and are recommended by Baishideng, they do not need to be sent to a Baishideng language editor to polish the language. The following language editing companies are vetted and deemed acceptable by Baishideng: Filipodia Publishing, MedE Editing Group, American Journal Experts, Nature Publishing Group Language Editing, Elsevier, Wiley, Medjaden, Editage, Enago, and Charlesworth; (4) For non-native English-speaking authors who provide a language certificate from an individual or other editing service provider not listed above, such manuscripts must be linguistically polished by a Baishideng language editor before they are accepted for publication; and (5) Finally, for authors from non-native English-speaking countries/regions who do not provide a language certificate, if their country/region has a solid repute for overall high level of English proficiency, the revised manuscript does not need to be sent to a Baishideng language editor to polish the language if the manuscript's language has been rated as Grade A by at least two peer reviewers.

At Baishideng, we believe that the feature of high-quality language in a manuscript can greatly improve the readability and hence understanding of its academic content. From 2019 to 2021, 61.4% of the articles published in Baishideng's seven SCIE-indexed journals were polished by professional language editors, which effectively improved the language quality of the articles.

XML and PDF document production: After the manuscript is proofread by the authors, further edited by the in-house science editor, and polished by the language editor, the in-house science editor regenerates the Microsoft Word document of the manuscript through the TINY Technologies editor built into the F6Publishing system. The Word document, including the complete textual content and corresponding tables of the manuscript, is then automatically converted into the XML simple text-based format through the F6Publishing system. After redrawing the figure(s) of the manuscript, the in-house science editor converts the image file(s) and the XML document into a PDF document through the F6Publishing system, and sends both the Word document and PDF document to the authors for verification of column type, titles (main and short), authors, affiliated institutions, abstract, text, and the figures and tables. If the authors find any errors in spelling or grammar, they will revise the manuscript (excluding images) in the Microsoft Word format with the Track Changes software feature enabled.

In order to prevent potential academic misconduct caused, in this proofreading process, by changes made by the authors, such as modifications that affect the content of the manuscript or drastic revision of the references, the following rules apply without exception: (1) Baishideng does not allow the manuscript title to be changed; (2) Baishideng does not allow the order of authors to be changed, the authors and corresponding author to be changed or deleted, or for any new authors to be added; (3) Baishideng does not allow any funding agency or grant number to be added or deleted; (4) Baishideng does not allow the manuscript text to be added to or deleted; (5) Baishideng does not allow any figures or tables to be added/added to or deleted; and (6) Baishideng does not allow manuscript references to be added or deleted.

Second decision: The in-house science editor proofreads and revises the manuscript and related documents, which culminates with submission of all the documents to the Director of Science Editor Development Department for review through the F6Publishing system. After verification and confirmation that the relevant documents of the manuscript are complete, the Director of Science Editor Development Department forwards the manuscript to the external Editor-in-Chief for the second decision. The external Editor-in-Chief makes a final assessment of the scientific and linguistic quality of the manuscript, based on all documents related to the manuscript submitted by the in-house science editor, and makes a second decision to reject or accept the manuscript. If the decision of the external Editor-in-Chief is to accept the manuscript, the in-house Editor-in-Chief double-checks and confirms whether the manuscript complies with the ethics and academic rules and norms or not, and makes the final decision to accept or reject the manuscript.

OA online publishing

After the manuscript is finally accepted, the production department publishes the electronic version (PDF) and the web version (HTML) of the article online. For this, the production director first determines the list of articles to be published in the current issue according to the journal publication plan, and sorts all manuscripts to be published in the current issue according to column type. After this, the in-house production editor adds the publication date, page numbers, website address, DOI, and other information to the articles on the basis of the XML and PDF documents of the manuscript submitted by the in-house science editor, to generate the electronic version (PDF) and the web version (HTML) of the article for online release. In addition, the production director reviews the current electronic version and the web version to check whether the page numbers are continuous, whether the figures and tables are complete, whether the picture quality meets the publishing requirements, and whether the numbers, units, and letter case in the articles conform to the publishing standards. Finally, the article is officially published online by the in-house production editor through the F6publishing system, and the authors are invited to submit the author evaluation by informing them of the publication information *via* email.

The Baishideng's seven SCIE-indexed journals involved in this study all adopt the OA publishing model. OA is a form of publishing model in which a publisher makes all articles and related content associated with a certain journal available immediately and for no-cost on the journal's website in perpetuity; these features are also the basis of the JCR award of a 'Gold OA' label. In this model, authors are often asked to bear the cost of publication, typically through an APC[22]. Among the 21430 journals listed in the *JCR* 2022, 5300 (24.7%) hold the Gold OA designation. Wassef *et al*[23] pointed out that OA articles are associated with a higher number of citations than subscription access articles. OA plays a vital role in improving the quality, efficiency, transparency, and integrity of academic journal publishing. Therefore, more and more academic journals are adopting the OA publishing model.

For Baishideng's seven SCIE-indexed journals, the number of published articles was higher in 2021 compared to the numbers in 2019-2020 (specifically, the increases were 5.4% for *WJG*, 18.5% for *WJSC*, 167.2% for *WJD*, 472.2% for *WJP*, 44.0% for *WJGO*, 176.0% *WJGS*, and 105.1% for *WJCC*). Throughout, the authors of these published articles are from many countries and regions around the world and an expansive trend is indicated; for example, in 2019 to 2020, the authors of articles published in *WJG* were from 71 countries/regions, and in 2021 alone the authors were from 56 countries/regions. According to a global cities research report by Elsevier in collaboration with the Administrative Center of Shanghai R&D Public Service Platform, Beijing (China), London (United Kingdom), Boston (United States), Tokyo (Japan), and Seoul (South Korea) had the highest number of active researchers between 2014 and 2018 [24]. Most of the Chinese cities in the study were in the top 10, reflecting China's increased efforts to cultivate, support, and introduce talent year after year. There may be a certain relationship between the author group and the regionality of the journal. The *WJG*, the first Baishideng English journal, was launched in China in 1995; thus, Baishideng journals may attract more authors from China. Moreover, the 3012 total articles published in the seven SCIE-indexed journals in 2019 to 2020 were cited by more than 20000 articles from approximately 8000 academic journals; among them, many articles were from journals with a JIF of > 10 (*WJG*, 171; *WJSC*, 61; *WJD*, 30; *WJP*, 9; *WJGO*, 45; *WJGS*, 11; *WJCC*, 92), including internationally renowned academic journals such as *CA-A Cancer Journal for Clinicians* (2021 JIF 286.130), *Lancet* (2021 JIF 202.731), *New England Journal of Medicine* (2021 JIF 176.079), *Nature Reviews Molecular Cell Biology* (2021 JIF 113.915), and *Nature Reviews Immunology* (2021 JIF 108.555). This suggests that the academic quality of articles published in Baishideng journals has been widely recognized.

Verification of ethics and academic rules and norms by Baishideng

Many scholars have found that most of the retracted articles in academic journals have academic misconduct[25-27]. In order to guarantee the academic ethics of articles and avoid academic misconduct, Baishideng formulated specific guidelines for each manuscript type that are customized for related ethics and documents relevant to the standards and norms for academic publication. First, for all manuscripts involving human studies and/or animal experiments, author(s) must submit the related formal ethics documents that were reviewed and approved by their local ethical review committee. This is mandatory and is one of the determining factors as to whether or not the manuscript will be sent for peer review or finally accepted. If the human and animal studies had received a waiver of the approval requirement from their ethics committee, the author(s) must provide an official statement to this effect made by their ethics committee. For the full compendium of guidelines for manuscript type and their related ethics and relevant documents/statements, please visit https://www.wjgnet.com/bpg/gerinfo/287. When authors return a revised manuscript during the peer review process, the in-house science editor rechecks whether the documents of academic rules and norms continue to correspond to the manuscript column and whether the non-native speakers of English language certificate is authentic; this is invariably followed by a new check of the full manuscript with iThenticate (CrossCheck) for duplication detection.

In order to make the process of manuscript peer review and publication more open and transparent, Baishideng publishes each article online along with the relevant documents such as those related to ethics and the international academic rules and regulations, including the peer-review report,

answering reviewers letter, conflict-of-interest statement, copyright license agreement, language certificate, institutional review board statement, informed consent statement, *etc*. Of particular importance, the authors are asked to provide the primary version (PDF) of the Institutional Review Board's official approval in the official language of the authors' country to the F6Publishing system; for example, authors from China should upload the Chinese version of the document. This plays an important role in eliminating academic misconduct in ethics approval documents.

For the 543 articles published from 2019 to 2021 in Baishideng's seven SCIE-indexed journals involved in this study, all were subject to and passed review of the above documents of ethics and academic standards and norms and other relevant documents; all of which are also published online, alongside the corresponding manuscript. Public disclosure of these ethics approval document(s) and the other relevant documents plays a supervisory role on its own, as it can effectively deter and dissuade academic misconduct. The ultimate outcome will be of benefit to the reputation of the academic journals, further promoting their ability to attract more high-quality manuscripts for peer review and publication and subsequently improving their JIF.

Baishideng's article quality tracking system

In order to continually improve the quality of its published articles, Baishideng not only conducts rigorous peer review of each manuscript but also provides an article quality tracking system for focused post-publication assessment. After an accepted article is published online, Baishideng, through the F6Publishing system, invites some of the journal editorial board members to re-evaluate the quality of the article, including its objectivity, credibility, and scientificity. These evaluation findings by the editorial board members are submitted online as tracking reports, which are immediately passed along to the authors and the Baishideng editorial office for verification and reference.

Post-publication peer review has been increasingly recognized as an important activity by which academic journals can control their academic quality[28-30]. As a newly released means of post-publication peer review (in 2018), the Baishideng article quality tracking system has been used to track 2.7% of the articles published between 2019 and 2021 in the seven SCIE-indexed journals. This activity has allowed editorial board members to put forth productive suggestions for refining the editing and production processes, bolstered by evidentiary explanations of limitations and quality problems that may have been overlooked. For example, an editorial board member (ID: 00182114) raised the following comments on an article reporting a retrospective study on survival and outcomes for co-infection of chronic hepatitis C with and without cirrhosis and COVID-19, which was contributed by Afify *et al*[31]: "Authors concluded that male gender, diabetes mellitus, and liver cirrhosis were the independent factors affecting mortality in COVID-19 patients. I agree to authors' opinion. … Please comment about the relationship between HCV and COVID-19". For the complete comments, please visit: https://www.wjgnet.com/1007-9327/full/v27/i42/7362.htm. In this way, the authors were able to address an additional important feature of their findings that may have been beyond the scope of their manuscript's focused content for the initial publication.

The launch of the article quality tracking system has proven to be greatly beneficial to Baishideng journals. It not only improves the academic quality of the published articles and the publishing standards but also has emerged as a useful platform for productive communication among the members of the editorial board, the authors, and the editorial office. In addition, the establishment of this system has benefited the editorial board members, improving their own academic influence, and the authors, facilitating rapid and wide dissemination of their articles.

Baishideng's author evaluation system

Authors are the foundation of academic journals. Without authors' writing and submitting articles for peer-reviewed publication, there would be no academic journals. Therefore, in order to better serve and understand the needs of its authors, Baishideng established and launched an author evaluation system in 2018. In this system, after the online publication of an article in any of Baishideng's journals, Baishideng invites the authors to personally evaluate the entire publication process of their articles *via* F6Publishing; their assessment involves a broad span of the process, including the manuscript submission system itself, peer review time, quality of peer review reports, manuscript editing quality, manuscript duplication detection system, quality of PDF and web version production, quality of figure and table production, the editorial office's ethical and academic regulatory requirements for the manuscript, and their overall satisfaction with the publication cycle of the article. For example, Patrone *et al*[32] provided a comment that then prompted an internal refinement of Baishideng's process: "The submission and editing process was fair. …The manuscript was sent in January 2021 and was finally accepted after several months. …during the year spent in the publishing process and therefore the value of the manuscript is somehow limited". For the complete author's comments, please visit: https://www.wjgnet.com/1948-5204/full/v13/i12/2203.htm.

Approximately 84.0% of the articles published from 2019 to 2021 in the seven SCIE-indexed journals underwent evaluation by and received feedback from the authors. Through the timely feedback of these authors, Baishideng has been able to gain a better understanding of author needs and has continued to provide better publishing services for them; ultimately, this has strengthened Baishideng's reputation among authors.

Baishideng's reader evaluation system

Journals have become deeply embedded in academic infrastructure[33]. As the vehicle of knowledge dissemination, academic journals are published for the purpose of presenting high-quality academic papers to readers. In order to better serve readers and share high-quality articles published in Baishideng journals in a timely manner, for readers to read and evaluate, Baishideng has established and launched a reader evaluation system. In this system, after the online publication of an article in any of Baishideng's journals, Baishideng will recommend and share the articles to relevant global scholars through the F6Publishing system. The accuracy of the target recipients of these recommendations is established by consideration of the article's subject area and research keywords. The invitation to scholars to read the full text of the articles (including title, abstract, research background, research methods, research results, discussion, figures, statistical methods, references, academic ethical statements, *etc*), is accompanied by the opportunity to provide an online evaluation of the scientific and linguistic quality of the articles from the perspective of readers. Scholars can make further comments on the research highlights of the article, the rationality of the research design, the integrity of the data, and the limitations and shortcomings of the study, and make suggestions for future research directions. For example, a reader (ID: 01714826) gave the following valuable advice on an article reporting a retrospective study on preoperative hemoglobin to albumin ratio for the short-term survival of gastric cancer patients, which was contributed by Hu *et al*[34]: "In this paper the authors used HAR as yet another measure to assess the prognosis of gastric cancer. … However, it would have been better if the authors included Hb and Albumin as independent parameters to predict prognosis along with the HAR data they have analyzed. Additionally, it would have been nice to mention whether other factors like UGI bleeding resulted in a low Hb…". For the complete reader's comments, please visit: https://www.wjgnet.com/1948-9366/coretip/v14/i6/580.htm.

After readers' online comments are posted, they are automatically sent to the authors for verification and reference. At the same time, readers can also write a formal letter about an article that they have read and submit it to us online for consideration of traditional publication. After the reader's letter has been peer-reviewed and finally accepted for publication, it will be published online. From 2019 to 2021, the articles published in the seven SCIE-indexed journals were read and downloaded by readers from all over the world; moreover, webpage visits and downloads are showing an increasing trend year by year for each. For example, the number of total visits received by the *WJG* webpage increased by 17.4% in 2020 compared with that in 2019, and by 14.4% in 2021 compared with that in 2020; the number of downloads increased by 120.1% in 2020 compared with that in 2019, and by 52.6% in 2021 compared with that in 2020. As a result, the academic influence of the seven SCIE-indexed journals was improved greatly. The reader evaluation system continues to be of great benefit, particularly as it has improved the readership volume of published articles, expanding the academic influence of each study's findings, improving citation counts, and enhancing the authors' influence among readers in their field and beyond.

CONCLUSION

This paper introduces the complete editing and publishing process of Baishideng's journals, providing a unique perspective on the publication of academic journals. In our experience, the integrity of the editing and publishing process of academic journals has a great impact on the number of articles received and published, and the number of webpage visits and downloads received by the journals, and thus is invaluable to expanding the influence of academic journals and improving their JIFs.

In our focused analyses of seven SCIE-indexed journals, we found that the JIFs increased for three from 2020 to 2021; for the other four, the JIFs decreased only slightly due to the influence of factors such as the increased numbers of published articles. Overall, the JIFs of these journals have steadily increased over time. Further findings were that *WJG* has the highest 2021 JIF (5.374) and *WJCC* has the lowest (1.534), while *WJSC* has the highest self-citation rate (1.43%) and *WJGS* has the lowest (0.21%). In Baishideng's *RCA* database, all seven of the SCIE-indexed journals received a 2022 *JAII*. For example, *WJG* has a 2022 *JAII* of 22.048, ranking 18th among 102 journals in the field of gastroenterology & hepatology included in the *RCA*; its total citations rank 6th and total articles rank 5th. Both the number of articles received and published by the seven SCIE-indexed journals increased. For example, compared with the figures in 2019-2020, the number of articles received by *WJCC* increased by 91.1% and the number of articles published increased by 105.1% in 2021. The articles published in the seven SCIE-indexed journals in 2019-2020 were cited by more than 20000 articles from approximately 8000 academic journals, including internationally renewed journals such as *CA-A Cancer Journal for Clinicians*, *Lancet*, and *New England Journal of Medicine*.

The webpage visits and downloads received by these journals have also increased year by year. For example, the total number of visits received by the *WJG* webpage was 1974052 in 2019, 2317835 in 2020 (an increase of 17.4% compared with that in 2019), and 2652555 in 2021 (an increase of 14.4% compared with that in 2020). The visitors were from more than 220 countries and regions worldwide, such as the United States, China, and the United Kingdom. A total of 5543 OA articles were published in the seven

SCIE-indexed journals from 2019 to 2021. OA plays a crucial role in improving the quality, efficiency, transparency, and integrity of academic journal publishing.

Following the online publication of all articles, through Baishideng's article quality tracking system, 96.5% of the article quality-tracking evaluation reports gave positive comments or did not put forward an opinion, providing further affirmation of the quality of the articles. Through the author evaluation system, 84.0% of the articles received author evaluations and feedback, and most of the authors gave positive evaluations. Through the reader evaluation system, the seven SCIE-indexed journals received a total of 424 reader evaluations and 229 letters from readers, both of which improved the readership volume and influence of the articles and their authors.

Baishideng's continual improvements to its journal editing and publishing process have greatly improved the academic influence of its journals and their publications, as demonstrated by its seven SCIE-indexed journals. Other publishers and academic journals will similarly benefit from actively tracking and refining the integrity of their editing and publishing process. The collective outcome could benefit all of science worldwide, as greater transparency and demonstrated adherence to ethical standards and norms will strengthen trust in scientific studies and their findings and in collaborative efforts worldwide. Moreover, open communication between editors, authors and readers will provide useful information on current and ongoing limitations to their editing and publication process, thereby providing insights through which they can improve their process and their JIFs.

In the face of all this potential benefit, however, many challenges still exist; these include: How to address the low success rate of peer review; how to improve the quality of manuscript peer review; how to avoid peer reviewers' misconduct more effectively, including attempts to force authors to cite the peer reviewer's own published articles, plagiarizing the author's articles, *etc*; how to avoid falsified ethical approval documents and language certificates more effectively; how to effectively avoid improper authorship or falsified signature on copyright license agreements; and, how to effectively train academic editors and science editors. These unresolved issues highlight the importance of collaborative support from authors, journal editors-in-chief, editorial board members, peer reviewers, academic editors, editors, readers, and publishers. Academic journals should always prioritize publishing high-quality innovative research, compliance with the international editing and publishing ethics standards and norms, establishment of an efficient article quality tracking system, author evaluation system, and reader evaluation system, and maintenance of a rigorous attitude towards ensuring a high-quality and ethical editing and publishing process and a high standard of service to create a solid academic exchange platform for the dissemination of researchers' academic achievements.

ARTICLE HIGHLIGHTS

Research background

Journal Impact Factor™ (JIF) is often used to evaluate the relative reputation and quality of academic journals in their respective fields. It can greatly influence the quality and scope of subsequent manuscript submissions. Few studies have focused on the impact of the integrity of the editing and publishing process on improvement of the JIF of academic journals.

Research motivation

This study introduces and discusses the editing and publishing processes of Baishideng's journals in their entirety, as they form the basis for our objective of safeguarding and bolstering integrity in academic publication.

Research objectives

This study aimed to explore the importance of the integrity of the editorial and publication process in improving the academic influence of academic journals and JIF of academic journals.

Research methods

In this paper, we describe our statistical analysis of bibliometric factors, such as the 2021 JIFs released in the *Journal Citation Report*™ 2022, discipline rankings, received and published articles in 2019-2021, and webpage visits and downloads for seven journals published by Baishideng Publishing Group (Baishideng) and indexed in Science Citation Index Expanded™. The editing and publishing processes of Baishideng's journals in their entirety were introduced and discussed, as they form the basis of safeguarding and bolstering integrity in academic publication.

Research results

For the seven journals assessed, their 2021 JIFs were basically unchanged from 2020. The 3012 articles published in 2019-2020 were cited by more than 20000 articles from approximately 8000 academic journals, including many journals with JIFs > 10. Baishideng's journals have been widely recognized for their academic quality. The numbers of manuscripts received and published in 2021 are both higher

than those in 2019-2020. The numbers of webpage visits and downloads received by the seven journals have increased year by year. The visitors were from all around the world. From 2019 to 2021, a total of 5543 open access articles were published in the seven journals, of which 2083 (37.6%) were invited and published free-of-charge. In addition, the quality of the articles was further evaluated through Baishideng's article quality, author evaluation tracking and reader evaluation systems.

Research conclusions

The findings from these bibliometric assessments indicate that establishing, promoting and actively practicing processes that safeguard and bolster the integrity of the editing and publication process also help to improve the academic influence of academic journals, which itself is the cornerstone for improving JIF.

Research perspectives

How to address the low success rate of peer review; how to improve the quality of manuscript peer review; how to avoid peer reviewers' misconduct more effectively, including attempts to force authors to cite the peer reviewer's own published articles, plagiarizing the author's articles, *etc*; how to avoid falsified ethical approval documents and language certificates more effectively; how to effectively avoid improper authorship or falsified signature on copyright license agreements; and, how to effectively train academic editors and science editors are still challenges and require further research in the future.

FOOTNOTES

Author contributions: Ma LS conceptualized and designed the study; Wang JL organized the study materials, performed the data collection and analysis, and wrote the first draft of the manuscript; and all authors reviewed and commented on each iterative version of the manuscript, and read and approved the final manuscript.

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CORRECTION

Correction to "MicroRNA-596 acts as a tumor suppressor in gastric cancer and is upregulated by promotor demethylation"

Zhen Zhang, Dong-Qiu Dai

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Abstract

Correction to "Zhang Z, Dai DQ. MicroRNA-596 acts as a tumor suppressor in gastric cancer and is upregulated by promotor demethylation. *World J Gastroenterol* 2019; 25: 1224-1237 [PMID: 30886505 DOI: 10.3748/ wjg.v25.i10.1224]". In this article, we found the following errors in Figure 4: Three images of the NC and miR-NC groups in the MGC-803 cell wound healing assay were misapplied during the preparation of submission; the mimcs and miR-NC icons were incorrectly edited in the image of the statistical chart. According to the reviewer's comments, we have re-analyzed the images of the wound-healing assay and revised the charts depicting the analyzed results. The corrected Figure is given in this correction.

Key Words: Correction; MicroRNA-596; Gastric cancer; Figure; Errors

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Core Tip: This manuscript is to correct the images in Figure 4 of "Zhang Z, Dai DQ. MicroRNA-596 acts as a tumor suppressor in gastric cancer and is upregulated by promotor demethylation. *World J Gastroenterol* 2019; 25: 1224-1237 [PMID: 30886505 DOI: 10.3748/wjg.v25.i10.1224]".

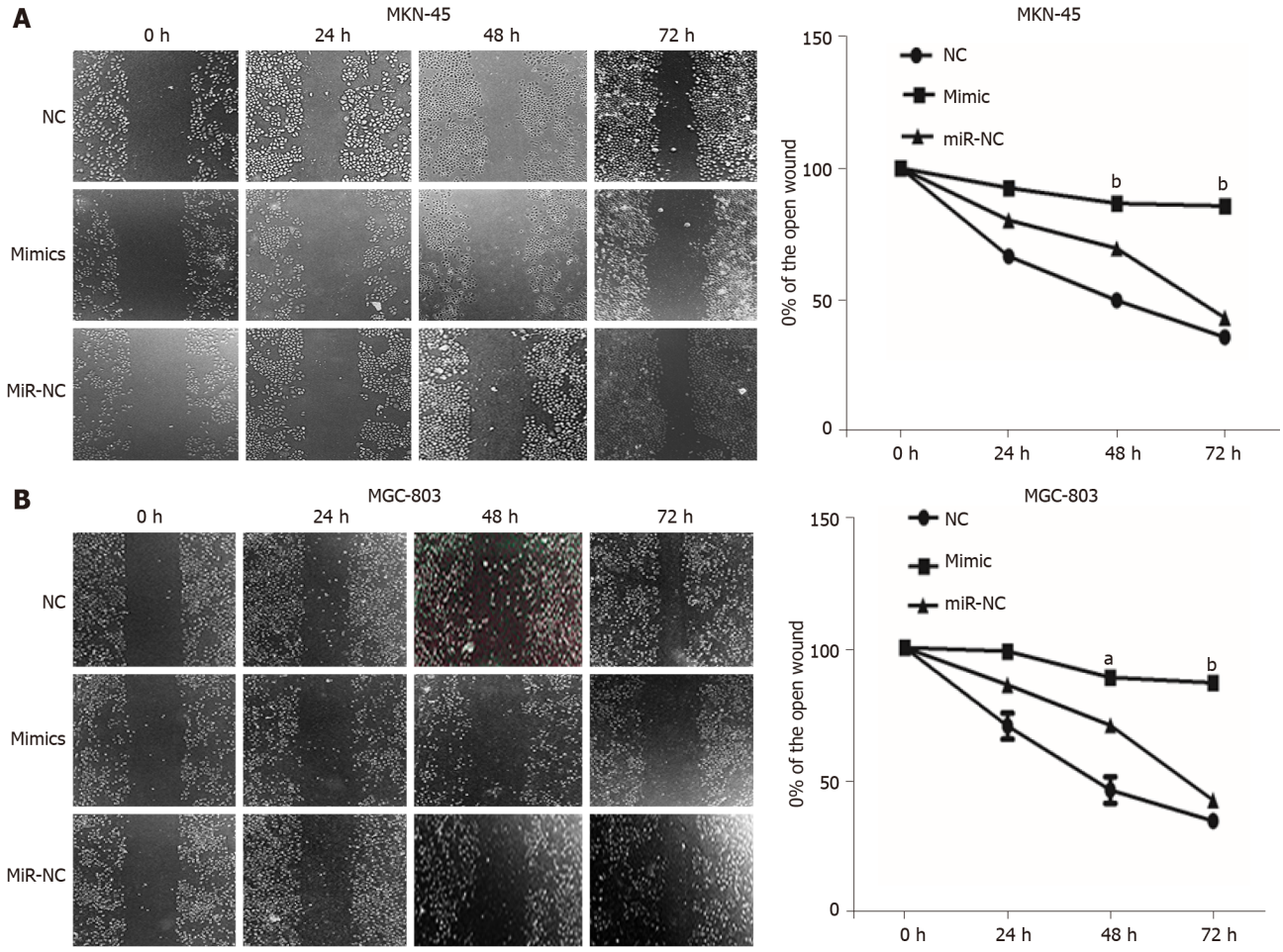
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TO THE EDITOR

Correction

Correction to: Zhang Z, Dai DQ. MicroRNA-596 acts as a tumor suppressor in gastric cancer and is upregulated by promotor demethylation. *World J Gastroenterol* 2019; 25: 1224-1237 [PMID: 30886505 DOI: 10.3748/wjg.v25.i10.1224].

In the original publication of the article[1], we found the following errors in Figures 4A and 4B (in this manuscript marked as Figure 1): Three images of the NC and miR-NC groups in the MGC-803 cell wound healing assay were misapplied during the preparation of submission; the mimcs and miR-NC icons were incorrectly edited in the image of the statistical chart. According to the reviewer's comments, we have re-analyzed the images of the wound-healing assay and revised the charts depicting the analyzed results. The corrected Figure is given in this correction. This correction will have no influence on the interpretation of the entire results and conclusion in this study. We apologize for any inconvenience this may cause.



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Figure 1 Wound healing assay for detecting cell migration in MKN-45 and MGC-803 cells transfected with miR-NC or microRNA-596 mimic.
A: MKN-45 cells; B: MGC-803 cells. [a]*P* < 0.05; [b]*P* < 0.01 *vs* miR-NC.

FOOTNOTES

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ABOUT COVER

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The primary aim of *World Journal of Gastroenterology* (*WJG*, *World J Gastroenterol*) is to provide scholars and readers from various fields of gastroenterology and hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJG* mainly publishes articles reporting research results and findings obtained in the field of gastroenterology and hepatology and covering a wide range of topics including gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, gastrointestinal oncology, and pediatric gastroenterology.

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EDITORIAL

Medical imaging for pancreatic diseases: Prediction of severe acute pancreatitis complicated with acute respiratory distress syndrome

Ling-Ji Song, Bo Xiao

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Abstract

In this editorial we comment on the article published in the recent issue of the *World Journal of Gastroenterology* [2022; 28 (19): 2123-2136]. We pay attention to how to construct a simpler and more reliable new clinical predictive model to early identify patients at high risk of acute respiratory distress syndrome (ARDS) associated with severe acute pancreatitis (SAP), and to early predict the severity of organ failure from chest computed tomography (CT) findings in SAP patients. As we all know, SAP has a sudden onset, is a rapidly changing condition, and can be complicated with ARDS and even multiple organ dysfunction syndrome, and its mortality rate has remained high. At present, there are many clinical scoring systems for AP, including the bedside index for severity in AP, acute physiology and chronic health evaluation II, systemic inflammatory response syndrome, Japanese severe score, quick sepsis-related organ failure assessment, *etc.* However, some of these scoring systems are complex and require multiple and difficult clinical parameters for risk stratification. Although the aforementioned biomarkers are readily available, their ability to predict ARDS varies. Accordingly, it is extremely necessary to establish a simple and valuable novel model to predict the development of ARDS in AP. In addition, the extra-pancreatic manifestations of AP patients often involve the chest, among which pleural effusion and pulmonary consolidation are the more common complications. Therefore, by measuring the semi-quantitative indexes of chest CT in AP patients, such as the amount of pleural effusion and the number of lobes involved as pulmonary consolidation, it has important reference value for the early diagnosis of SAP complicated with ARDS and is expected to provide a basis for the early treatment of ARDS.

Key Words: Severe acute pancreatitis; Acute respiratory distress syndrome; Clinical scoring system; Prediction model; Semi-quantitative

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Core Tip: Respiratory failure has been confirmed to be the most common type of organ failure in acute pancreatitis (AP) and is closely related to high mortality. Acute respiratory distress syndrome (ARDS) is one of the most common patterns of respiratory failure in AP and is still a little-known disease. Although some studies have shown that it is promising to predict the results of AP-related ARDS, the preventive strategies for ARDS development are still in their infancy. For this reason, we need to establish a simple and valuable new prediction model, combined with chest computed tomography findings, to early identify high-risk patients with severe AP and ARDS and help clinicians take timely intervention measures to prevent disease progression.

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INTRODUCTION

Acute pancreatitis (AP) is a common digestive disease that causes acute abdominal pain. A meta-analysis showed that the annual morbidity and mortality of AP all over the world were 33.74/100000 and 1.60/100000, respectively[1]. According to statistics, AP was the second leading cause of the total hospitalization rate, the largest contributor to the total cost, and the fifth leading cause of in-hospital mortality[2]. Many reports point out that the disease is increasing year by year, which is related to the incidence of biliary disease and the increase of alcoholism[3]. The clinical manifestations of AP vary greatly. Most of the patients who can recover after general treatment are considered to have mild AP, which is called a "self-limited disease"; some patients with transient organ failure (duration < 48 h) are considered to have moderately severe AP[4], with an approximately 20% developing severe AP (SAP). SAP has an aggressive onset with organ failure (duration > 48 h) and presence or absence of pancreatic or peripancreatic tissue necrosis, with a mortality rate of 20%-40%[5].

Acute respiratory distress syndrome (ARDS) is a life-threatening form of respiratory failure. Its main clinical manifestations are shortness of breath and refractory hypoxemia. ARDS is caused by ischemia and hypoxia injury secondary to pulmonary pathological changes under the influence of a variety of intrapulmonary and extrapulmonary factors, resulting in impaired lung function and serious condition of the life of patients[6]. The PaO_2/FiO_2 ratio (oxygenation index) is a component of the assessment of patients with ARDS[7], and refers to the ratio of the partial pressure of arterial oxygen to the concentration of oxygen in the inhaled air, with a normal value of 400-500 mmHg. The Berlin definition of ARDS is the most widely accepted criterion, which defines the specific time from clinical injury to the onset of new respiratory symptoms, specific chest X-ray findings, and the severity of ARDS based on the PaO_2/FiO_2 ratio. ARDS is defined as positive end-expiratory pressure ≥ 5 cm H_2O, the ratio of atrial oxygen partial pressure to inhaled oxygen fraction ≤ 300 mmHg, and bilateral pulmonary infiltrative lesions that cannot be explained completely by fluid overload or heart failure. The Berlin definition also emphasizes that ARDS can be divided into three categories based on the severity of hypoxemia[8]: Mild (PaO_2/FiO_2 200-300), moderate (PaO_2/FiO_2 100-200), and severe (PaO_2/FiO_2 ≤ 100).

SAP is a clinical critical disease with rapid progression, many complications, and high mortality. In addition to causing local disorders, it can also cause damage to other organs, and its serious complications are the main factors leading to poor prognosis[5]. Previous studies have confirmed that the lung is the first damaged target organ in the early induction of systemic inflammatory response syndrome (SIRS) or multiple organ dysfunction syndrome by SAP, and respiratory failure is the most common organ failure in SAP[9-11]. ARDS is considered to be an important type of respiratory failure with high mortality[12]. It has been reported that 4%-15% of patients with AP have concomitant ARDS[13] , while this percentage may be up to a third in SAP[14]. ARDS is the most dangerous complication of AP, which usually occurs between 2 and 7 d after the onset of pancreatic inflammation. According to the literature data, SAP-related ARDS accounted for 60% of all deaths in SAP patients in the first week of the disease [15]. These data indicate that the more serious the condition of SAP, the worse the progression of the AP-related ARDS, which may further suggest that the severity of SAP is negatively correlated with PaO_2/FiO_2. If the occurrence of ARDS is not predicted early, the patient's lung function will drop sharply, which may even lead to death during the acute reaction period. Thus, there is an urgent need for a simple and accurate new clinical prediction model combined with chest computed tomography (CT) findings of AP patients to diagnose and predict SAP-related ARDS at an early stage. And timely diagnosis and treatment can greatly improve the survival rate of SAP patients.

New prediction model compared with clinical scoring systems

In the recent issue of the *World Journal of Gastroenterology*, Li *et al*[16] published an interesting paper

entitled "Development and external validation of models to predict acute respiratory distress syndrome related to severe acute pancreatitis". This study constructed and validated a new simple and accurate prediction model for SAP-related ARDS. In this multicenter retrospective study, 597 patients diagnosed with AP from four hospitals in different regions of China from 2017 to 2021 were divided into two cohorts: The derivation cohort (n = 407) and the validation cohort (n = 190). Of these, 139 were diagnosed with SAP and 99 were diagnosed with ARDS. Multivariate logistic regression showed that four identical variables of SAP and ARDS were identified as independent risk factors, including heart rate, respiratory rate, serum calcium concentration, and blood urea nitrogen. In the derivation and validation cohorts, the area under the operating characteristic curve (AUC) for predicting SAP was 0.879 and 0.898, respectively, and that value for ARDS was 0.892 and 0.833, respectively. In the derivation cohort for SAP prediction, an AUC value of the new model was significantly better than that of the SIRS (AUC = 0.808) or quick sepsis-related organ failure assessment (qSOFA) (AUC = 0.730); with respect to ARDS prediction, a corresponding AUC value was better than that of the SIRS (AUC = 0.815) or qSOFA (AUC = 0.742). The study developed a novel predictive model for SAP-related ARDS in patients with AP. Furthermore, the results of the new model indicated that patients with AP who exhibited higher respiratory rate, heart rate, and blood urea nitrogen (BUN) concentration, and lower serum calcium concentration on admission might develop SAP and ARDS with a higher risk.

In another study by Ding *et al*[17], 779 AP patients were randomly assigned to the primary cohort (n = 560) and the validation cohort (n = 219), and AP patients in each cohort were further divided into an ARDS group and a non-ARDS group. The heart rate, BUN, and serum calcium in the ARDS group were higher than those in the non-ARDS group, and the heart rate was significantly different between the two groups. Comparing variables in the primary cohort, the heart rate and serum calcium were statistically significantly different between the two groups. Zhang *et al*[18] also divided SAP patients into ARDS and non-ARDS groups, and their results showed statistically significant differences in respiratory rate and heart rate between two groups. Respiratory rate > 30 /min (odds ratio = 2.405) was an independent risk factor for ARDS in patients with SAP. As far as we know, the BUN level has not been used as a direct predictor of ARDS. However, the marker can be used as a predictor of pathogenesis associated with other risk factors, such as AP[6]. BUN not only is selected in the new prediction model of Li *et al*[16], but also is participated in other prediction models of SAP, such as bedside index for severity in AP (BISAP). The results of Dai *et al*[19] showed that the only independent risk factor correlated with 30-d all-cause mortality was BUN level in AP patients. In addition, the validity of BUN as a prognostic marker was further verified using a receiver operating characteristic curve with an AUC of 0.803 for BUN and an optimal cut-off value of 12.01 mmol/L (sensitivity = 0.714, specificity = 0.810). Another study has shown that elevated BUN at admission and within 24 h after admission can predict AP mortality[20]. According to our knowledge, the study of Li *et al*[16] presented the first model to use serum calcium concentration to predict ARDS in SAP. Ye *et al*[21] showed that BISAP and serum calcium were independent predictors of AP severity. The results of the study showed that the model established by the combination of BISAP and serum calcium was remarkably better than that established by BISAP and serum calcium alone. Additionally, the study also found that serum calcium concentration was negatively correlated with the severity of AP, while BISAP was positively correlated with AP severity [21]. It is further suggested that there may be some relationship between serum calcium levels and SAP-related ARDS in patients with AP. A previous study showed that hypocalcemia was an independent risk factor for respiratory failure in SAP[22]. They believed that serum calcium concentration was a valuable tool for evaluating rapidly persistent organ failure in AP patients. Further studies need to be done in this respect.

Because of the high mortality rate of SAP patients, it is necessary to quickly identify patients with a more severe disease state and a higher risk of death at an early stage. For risk stratification, several pancreas-specific or general scoring systems have been established in the past. BISAP is a potential prognostic scoring system for identifying AP patients at high risk of death in hospital, with high specificity[23], but it needs to evaluate pleural exudation. And most hospitalized patients do not undergo the examination within 24 h, so the score cannot be completed[24]. Multi-parameter scores such as the acute physiology and chronic health evaluation II (APACHE II) and BISAP are very important for clinical trials, but the APACHE II score is not specific for AP. In addition, the method is too complex and time-consuming to calculate, and its application in practical clinical work is limited [25]. A clinical study has confirmed that SIRS can be used as an "early warning device" of the severity of AP[26]. At present, the commonly used AP scoring systems, such as the BISAP and the Japanese severe score systems, contain the relevant content of SIRS. The qSOFA score is also rapid and easy to obtain and can be used for rapid evaluation of preclinical patients or emergency patients, but its effect on the prognosis of the disease is limited[27]. Therefore, a simple model with a small number of parameters will be more practical. The novel prediction model reported by Li *et al*[16] involves only four routine parameters for SAP and ARDS prediction. This study confirms that the new model is not inferior to BISAP in predicting SAP-related ARDS, allowing early identification of patients at high risk of SAP and ARDS. It has clinical value for improving the prognosis of AP.

Chest CT findings of AP for early prediction of ARDS

Based on clinical observations, we can evaluate SAP and related ARDS not only from the predictive new model but also from chest CT presentations of AP at the time of initial diagnosis. Up to 55% of AP patients may have abnormal chest CT findings, including pleural effusion, pulmonary atelectasis, pulmonary consolidation, and ARDS-related pulmonary edema[28]. Pulmonary consolidation and pleural effusion are common complications in patients with AP, which are closely related to the severity of AP. The imaging manifestations of pulmonary consolidation are patchy, segmental, and diffuse pulmonary changes, which may contain bronchial inflation signs. It is reported that the incidence of pleural effusion in AP was about 3%-17% in the earlier literature. But recent reports showed that, according to CT scan, the incidence was as high as 46%-50%[29,30]. The chest CT findings of SAP complicated with ARDS mainly include bilateral or left-sided pleural effusion[28,31], and solid changes in the basal segments of lower lobes of both lungs[32] (Figure 1).

Accordingly, we can semi-quantitatively evaluate the pleural effusion volume (PEV) and pulmonary consolidation score (based on the number of involved lobes) in patients with AP. A recent study showed that PEV and pulmonary consolidation lobes can provide early predictions of SAP and organ failure [32]. Peng *et al*[32] reported that PEV was strongly correlated with BISAP score and CT severity index (CTSI) score, but was weakly correlated with the length of hospital stay and APACHE II score. The lung consolidation score was moderately correlated with the BISAP score, CTSI score, and APACHE II score. On the contrary, Yan *et al*[33] showed that there was a strong correlation between PEV and length of hospitalization or APACHE II score. In addition, Peng *et al*[32] described that the accuracy of PEV in predicting SAP was similar to that of the BISAP score, APACHE II score, and CTSI score. As for predicting organ failure, the accuracy of PEV was also similar to that of the BISAP score, APACHE II score, and CTSI score. While Yan *et al*[33] predicted SAP, the accuracy of PEV was distinctly higher than that of the BISAP score and CTSI score, but was significantly lower than that of the APACHE II score. In the prediction of organ failure, the accuracy of PEV was distinctly higher than that of CTSI score, and its accuracy was similar to that from BISAP score or APACHE II score. Peng *et al*[32] also described that the accuracy of the lung consolidation score was similar to that of the BISAP score, APACHE II score, and CTSI score in predicting SAP and organ failure. Although some results of Peng *et al*[32] and Yan *et al*[33] were different, they both confirmed that chest imaging findings of AP patients could indicate the severity of AP and organ failure to some extent.

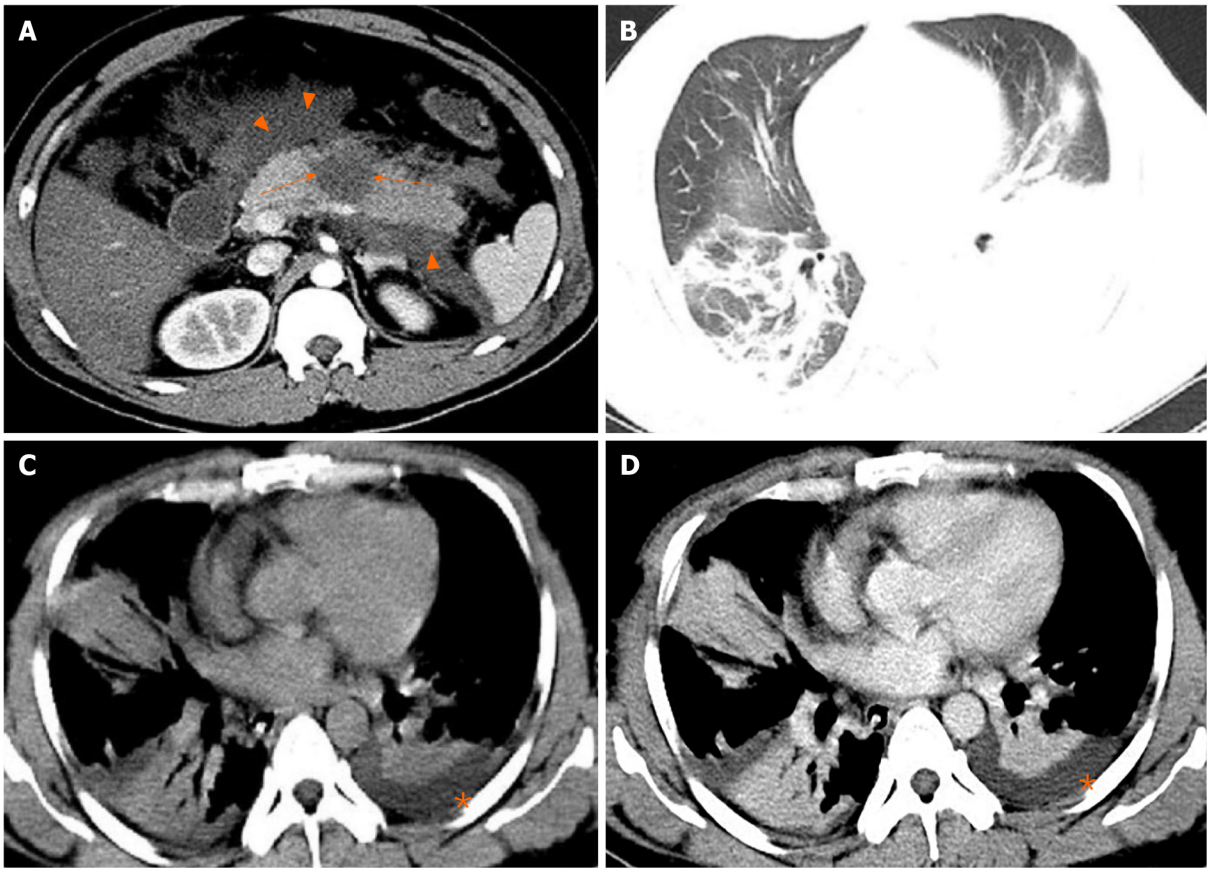
Previous studies have confirmed that respiratory failure is the most common type of organ failure in AP. Schepers *et al*[11] reported that the proportion of AP patients with respiratory failure was 92% (221/240). It was also found that this was the most common type of organ failure in the early and late stages of AP. Moreover, the distribution of different types of organ failure was different in this study, and the median duration of respiratory failure was 19 d. The mortality rate of respiratory failure was 37%, and the mortality rates of renal failure and circulatory failure was 47% and 40%, respectively. Raghu *et al*[28] claimed that the development of pulmonary consolidation in patients with AP was related to the occurrence of respiratory failure. As mentioned above, the incidence of ARDS was associated with respiratory failure. They also observed that pleural effusion was significantly associated with the severity of AP, the occurrence of respiratory failure, and poor prognosis. To sum up, chest CT findings of AP patients are potentially valuable for early prediction of possible subsequent respiratory failure in our daily clinical work.

Future trends and prospects

The 21st century is the era of big databases. Radiomics is an emerging technology that can extract a large number of parameters from images that are difficult for human eyes to observe and distinguish, and transform image data into high-dimensional and minable data through a variety of algorithms. Therefore, it can carry out a comprehensive quantitative analysis of the heterogeneity of the disease, and assist in clinical diagnosis, treatment, and other work[34]. Lin *et al*[35] first reported a radiomics model based on contrast-enhanced magnetic resonance imaging (MRI) to predict the clinical severity of AP. Their study showed that the prediction accuracy of the portal venous-phase MRI imaging radiomics model reached 85.6% and 81.0% in the training cohort and validation cohort, respectively. These results suggest that the portal venous-phase MRI radiomics model may be more accurate in early predicting the clinical severity of AP, and these findings may have a broad application prospect in the classification of AP severity. We believe that there is also great potential for predicting SAP-related ARDS in the future.

CONCLUSION

In summary, the essence of ARDS in AP is the embodiment of AP complicated with multiple organ dysfunction, with rapid progression and poor prognosis. Clinically, it is necessary to establish a novel predictive model and accurately observe the imaging features of SAP in chest CT. It is conducive to early prediction of the risk of AP patients complicated with ARDS. After that, timely intervention and active treatment of the primary disease will ultimately improve the survival rate of SAP patients.



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Figure 1 A 31-year-old man with severe acute pancreatitis complicated with acute respiratory distress syndrome. A: Axial contrast-enhanced computed tomography image in the arterial phase shows flake parenchymal necrosis (arrows) in the region of body of the pancreas, as well as extensive heterogeneous collections (acute necrotic collections) in the peripancreatic and the left pararenal anterior spaces (arrowheads); B: Lung window; C: Mediastinal window; D: Chest axial contrast-enhanced venous phase image. The three images show partial pulmonary consolidation in the middle and lower lobes of the right lung, in which bronchial inflation signs can be seen, and partial consolidation with partial atelectasis in the lower lobe of the left lung caused by external pressure of pleural effusion (asterisks).

FOOTNOTES

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REVIEW

Role of intestinal flora in primary sclerosing cholangitis and its potential therapeutic value

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Abstract

Primary sclerosing cholangitis (PSC) is an autoimmune disease characterized by chronic cholestasis, a persistent inflammation of the bile ducts that leads to sclerotic occlusion and cholestasis. Gut microbes, consisting of microorganisms colonized in the human gut, play an important role in nutrient intake, metabolic homeostasis, immune regulation, and immune regulation; however, their presence might aid PSC development. Studies have found that gut-liver axis interactions also play an important role in the pathogenesis of PSC. Patients with PSC have considerably reduced intestinal flora diversity and increased abundance of potentially pathogenic bacteria. Dysbiosis of the intestinal flora leads to increased intestinal permeability, homing of intestinal lymphocytes, entry of bacteria and their associated metabolites, such as bile acids, into the liver, stimulation of hepatic immune activation, and promotion of PSC. Currently, PSC effective treatment is lacking. However, a number of studies have recently investigated the targeted modulation of gut microbes for the treatment of various liver diseases (alcoholic liver disease, metabolic fatty liver, cirrhosis, and autoimmune liver disease). In addition, antibiotics, fecal microbiota transplantation, and probiotics have been reported as successful PSC therapies as well as for the treatment of gut dysbiosis, suggesting their effectiveness for PSC treatment. Therefore, this review briefly summarizes the role of intestinal flora in PSC with the aim of providing new insights into PSC treatment.

Key Words: Primary sclerosing cholangitis; Intestinal flora; Antibiotics; Fecal microbiota transplantation; Probiotics; Bile acids

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Core Tip: Primary sclerosing cholangitis (PSC) is an autoimmune disease that currently lacks treatment. The intestinal flora comprises microorganisms that colonize the human gut and play essential roles in nutrient intake, metabolic homeostasis, immune regulation, and PSC development. Thus, the intestinal flora may be a potential therapeutic target for PSC, and many recent studies have attempted to regulate it. In this review, we have reviewed the role of the intestinal flora in PSC. We believe that our study makes a significant contribution to the literature because our paper demonstrated the great potential of the gut flora as a therapeutic target for PSC treatment.

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is an autoimmune-mediated chronic cholestatic liver disease characterized by progressive bile duct inflammation, leading to intra- and extrahepatic bile duct stenosis and occlusion and cholestatic cirrhosis[1]. Patients with PSC have a greatly increased risk of developing cancers (cholangiocarcinoma, gallbladder cancer, hepatocellular carcinoma, and colorectal cancer); approximately half of patients with PSC develop cancer, ultimately leading to death[2]. Although the etiology of PSC remains unclear, it is generally accepted that interactions between genetics and the environment determine PSC development[3]. Owing to the close anatomical and physiological connection between the intestine and the liver, the intestinal flora is closely related to liver disease[4]. Several studies have suggested that the intestinal flora is involved in PSC development through the gut-liver axis[5,6]. Moreover, patients with PSC have significantly reduced intestinal flora diversity and an increased abundance of potentially pathogenic bacteria[7,8]. Intestinal flora dysbiosis leads to increased intestinal permeability, intestinal lymphocyte homing, entry of bacteria and their associated metabolites [*e.g.* bile acids (BAs)] into the liver, activation of the hepatic immune response, and bile duct inflammation and fibrosis[9].

The incidence of PSC is increasing, but an effective treatment does not exist currently. PSC can eventually progress to cirrhosis or liver failure, but these conditions are still symptomatically treated[10,11]. For patients with end-stage PSC, liver transplantation is the sole option; however, transplantations are not universally available owing to high costs and potential transplant rejection. Furthermore, approximately 30%-50% of patients experience PSC recurrence within 10 years of transplantation[12].

Many studies have reported that the gut flora is a promising therapeutic target for PSC, and that antimicrobial therapy based on gut flora modulation, fecal microbiota transplantation (FMT), and probiotics is an emerging therapeutic options[13,14]. Thus, in this review, we discuss the latest research findings on the role of intestinal flora in PSC and provide important insights into potential microbe-altering interventions.

PSC PATHOPHYSIOLOGY AND THE GUT-LIVER AXIS

PSC is a rare disease with an incidence of 0.91-1.30/100000. The incidence of small bile duct PSC is approximately 0.15/100000, with the highest prevalence in the Nordic countries, reaching an incidence of 16.2/100000[15]. PSC mostly occurs in men aged 40-50 years, with age of diagnosis at 30-40 years and a male to female ratio of approximately 2:1[1]. The pathogenesis of PSC is complex, but it is currently believed that interactions between genetic susceptibility factors and environmental promoters plays a role in the occurrence and development of PSC[16]. Human leukocyte antigen is strongly associated with PSC pathogenesis[17]. However, the risk ratio for first-degree relatives is approximately 11, suggesting that environmental factors play a more critical role in the pathogenesis of PSC[18]. In addition, the geographic distribution of PSC pathogenesis may indicate that the disease is influenced by the environment[19]. Interactions of the gut-liver axis, such as damage to the intestinal mucosal barrier, dysbiosis, and immune interactions, also play a role in the pathogenesis of PSC[1].

The gut-liver axis refers to the bidirectional relationship between the intestine, its microbiota, and the liver, resulting from the interaction of dietary, genetic, and environmental factors[20]. The close association between the intestine and the liver begins during embryonic development, with both organs originating together in the ventral foregut endoderm. From a physiological point of view, the gut-liver

axis is one of the most important links between the intestinal flora and the liver[21]. The liver, which receives approximately 70% of its blood from the portal vein, is a receiver and filter of nutrients and bacterially produced toxins and their metabolites. The secretion of substances such as BAs and antibodies into the intestine acts as a feedback mechanism that affects intestinal homeostasis[22].

Approximately 70% of patients with PSC have concomitant inflammatory bowel disease (IBD) and more commonly ulcerative colitis (UC)[23-25]. Patients with PSC have a reduced risk of death after a colectomy, and after receiving liver transplantation, colectomy significantly reduces the risk of PSC recurrence. This close association between PSC and IBD suggests that intestinal flora may play a key role in the pathogenesis of PSC[26,27] through the gut-liver axis[28].

PATIENTS WITH PSC AND THEIR DYSBIOSIS OF INTESTINAL FIORA

Intestinal flora dysbiosis

Under normal physiological conditions, the human body contains a large and diverse community of intestinal microorganisms, reaching up to 10^{14} organisms that comprise more than 1000 species; this is collectively known as the intestinal flora[29]. A normal intestinal flora is primarily composed of *Firmicutes, Bacteroidetes, Proteobacteria*, and *Actinobacteria*, which promote nutrient digestion and absorption, defend against foreign pathogens, regulate immunity, and participate in metabolic processes[30].

In healthy populations, the intestinal microenvironment is in homeostasis due to the mutual regulation of various flora. Intestinal flora dysbiosis is a disruption of the dynamic balance between intestinal flora when various factors cause disturbances in the human body environment, and changes in the number, species, and ratio of favorable, conditionally pathogenic, and harmful bacteria[31,32]. The manifestations of intestinal flora dysbiosis include intestinal flora translocation (transfer of the original colonizing bacteria from the intestine to the deep mucosa or from the intestine to other sites) and intestinal flora imbalance (decrease in the abundance of the original beneficial intestinal flora and increase in the abundance of pathogenic flora). Dysbiosis of the intestinal flora leads to impairment of the intestinal barrier, increased endotoxemia, and a breakdown of the liver's immune tolerance to the intestinal flora and its metabolites, which in turn causes a strong immune response in the liver[33].

The intestinal flora of patients with PSC

It was found that patients with PSC have a marked dysbiosis of the intestinal flora compared with the healthy population. Rossen *et al*[34] performed the first 16S rRNA analysis of the microbiota of the intestinal mucosa in the ileocecal region of patients with PSC and found that, at the genus level, the relative abundance of uncultured *Clostridium II* was notably lower in patients with PSC compared with patients with UC and non-inflammatory controls. In addition, the mucosal adherent microbiota at the level of the ileocecal region in patients with PSC showed considerably reduced diversity and abundance. Torres *et al*[8], using bacterial 16S rRNA next-generation sequencing, reported that patients with PCS had similar overall microbiome characteristics at different locations in the gut, exhibiting enrichment in *Blautia* and *Barnesiellaceae spp*. A more in-depth taxon analysis at the operational taxonomic unit (OTU) level revealed the most significant changes in *Clostridiales*, with 3 decreases and 66 OTU enrichments. Sabino's study found that species richness (defined as the number of different OTUs observed in the samples) was reduced in patients with PSC compared with healthy controls, that *Enterococcus, Clostridium, Lactobacillus,* and *Streptococcus* were enriched, and that an operational taxonomic unit belonging to the *Enterococcus* genus is positively correlated with the levels of alkaline phosphatase (ALP) levels (a marker of disease severity)[35]. In addition, PSC has its own unique gut microbial profile that is not associated with IBD co-morbidity. Subsequently, a study by Kummen *et al*[36] also confirmed the unique gut microbiota of PSC independent of the receipt of antibiotics and ursodeoxycholic acid (UDCA) treatment, with a marked reduction in bacterial diversity in patients with PSC. Furthermore, Quraishi *et al*[37] explored the intestinal mucosal flora of PSC-IBD patients, further complementing the study by Kummen *et al*[36] *Escherichia, Lachnospiraceae,* and *Megasphera* were markedly increased, whereas *Prevotella* and *Roseburia* (butyrate producers) were decreased in abundance in PSC-IBD patients compared with controls. They hypothesized that intestinal microecological dysregulation in patients with PSC may prompt dysregulation associated with mucosal immunity by modulating abnormal homing of intestinal-specific lymphocytes and intestinal permeability. This is consistent with the hypothesis of Kummen *et al*[36], Rühlemann *et al*[38,39] also showed that PSC itself drives the observed changes in fecal microbiota and that the findings of Kummen *et al*[36] regarding microbiota as a diagnostic marker are promising.

In the last two years, studies on the PSC gut flora have gained more interest. Quraishi *et al*[40] tried to unravel the PSC disease mechanism by integrating mucosal transcriptomics, immunophenotyping, and mucosal microbial analysis; their study reported that PSC-IBD patients had considerably higher abundance of *Bacteroides fragilis, Roseburia spp., Shewanella sp.,* and *Clostridium ramosum* species, which was associated with changes in the BA metabolic pathway. In addition, the amine oxidase-expressing bacterium *Sphingomonas sp*. is upregulated in PSC-IBD. Amine oxidase is associated with abnormal

homing of intestinal lymphocytes to the liver[41]. The upper gastrointestinal tract and bile ducts of patients with PSC are equally affected by microbial ecological dysbiosis. Liwinski *et al*[42] showed that the biliary microbiome of patients with PSC exhibited the most extensive changes, including reduced biodiversity and expansion of pathogenic bacteria, with the marked increase of *Enterococcus spp.* directly causing epithelial barrier damage and mucosal inflammation. Lapidot *et al*[43] found that microbial alterations in PSC were consistent in saliva and gut, with 27 bacterial species present in both the salivary and gut microbiomes, including *Clostridium perfringens XlVa, Veillonella, Lachnospiraceae, Streptococcus,* and *Blautia*. Of these, *Lactobacillus, Ruminococcus gnavus,* and *Streptococcus salivarius* were extensively enriched. The study by Lemoinne *et al*[44] confirmed previous findings of altered bacterial microbiota composition in patients with PSC, such as *Faecalibacterium* and *Ruminococcus* in reduced proportions, and reported for the first time the occurrence of fungal ecological dysbiosis in patients with PSC. PSC-associated fungal ecological dysbiosis is characterized by increased biodiversity (alpha diversity) and altered composition compared with in healthy subjects or patients with IBD, including a marked increase in the abundance of *Exophiala spp*. In some patients. Kummen *et al*[45] provided a detailed functional analysis of microbial genes encoding enzymes and metabolic pathways by using metagenomic shotgun sequencing. *Clostridium spp.* increased in the intestinal flora of patients with PSC, while *Eubacterium spp.* and *Ruminococcus obeum* decreased. Targeted metabolomics revealed reduced concentrations of vitamin B6 and branched-chain amino acids in PSC. Microbial metabolism of essential nutrients and circulating metabolites associated with the disease process were considerably altered in patients with PSC compared with in healthy individuals, suggesting that microbial function may be related to the disease process in PSC.

Most of these studies used 16S gene sequencing to examine the microbiota in the intestinal mucosa and feces of patients with PSC. Although these studies came from all over the world, some of them had relatively small sample sizes, and dietary and lifestyle habits varied between the samples of each study, possibly affecting the final gut floral composition of patients with PSC and limiting the generalization of the results. However, these studies also yielded some common findings that reveal to some extent the gut microbiota characteristics of patients with PSC[46]. Patients with PSC suffer intestinal dysbiosis, which has its own unique biological characteristics, as evidenced by a decrease in gut bacterial α-diversity (average species diversity of the ecosystem) and marked changes in β-diversity (spatial variation in species composition), a decrease in specialized anaerobic bacteria, and an increase in the abundance of potentially pathogenic bacteria (Table 1)[7,35-39,42-44,47]. Among which, *Veillonella, Enterococcus, Streptococcus, Clostridium,* and *Lactobacillus spp.* were markedly elevated[36,38,42-44]. An increase in *Veillonella* species, a potential pathogen in humans, can serve as a biomarker of the severity of certain diseases, such as autoimmune liver disease and cirrhosis[48,49].

INTESTINAL FLORA AND PSC-MECHANISTIC INSIGHTS

The leaky gut hypothesis was first proposed by Bjarnason *et al*[50] in 1984, providing theoretical support for the involvement of the intestinal flora in the development of PSC. Normal intestinal flora plays the role of maintaining the balance of the intestinal environment and preventing pathogenic bacteria and toxins from entering the blood circulation[51]. The germ-free (GF) multidrug resistance 2 knockout (Mdr2-/-) mice is a well-studied PSC model that shows a lack of microbial regulation, which is direct evidence that intestinal flora has a key role in PSC development[52]. Intestinal flora dysbiosis damages the intestinal barrier in patients with PSC, allowing bacteria and enteric-derived endotoxins to enter the liver *via* the portal vein, triggering an immune response[53]. Simultaneously, when liver function is impaired, Kupffer cells cannot inactivate endotoxins as efficiently, impairing bile excretion. Furthermore, this increases intestinal permeability, intestinal lymphocyte nesting, and the entry of bacteria and their metabolites [*i.e.* pathogen-associated molecular patterns (PAMPs)] into the liver, impairs normal BA metabolism, and promotes bile duct inflammation and fibrosis (Figure 1)[54,55].

Intestinal flora dysbiosis activates liver immunity

Intestinal flora dysbiosis damages the intestinal barrier: Lapidot *et al*[43] found that in patients with PSC, a decrease in the relative abundance of commensal bacteria in the intestinal flora, including *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii*, and bacterial diversity led to decreased short-chain fatty acids (SCFAs) with anti-inflammatory effects, such as acetate and butyric acid. This decrease caused intestinal barrier dysfunction and lack of antimicrobial peptides, exacerbating the leaky gut syndrome. When intestinal flora dysregulation occurs in patients with PSC, PAMPs in the gut bind to Toll-like receptors (TLRs) and NOD-like receptors (NLRPs) on the surface of dendritic cells. This event activates the cytoplasmic downstream nuclear transcription factor κB (NF-κB), causing the production and secretion of inflammatory cytokines and chemokines. Disruption of intestinal epithelium tight junctions and the normal intestinal barrier leads to increased intestinal permeability[31,56]. Furthermore, *Enterococcus faecalis* (*E. faecalis*), which increased the most in the intestinal flora of patients with PSC, produces gelatinase, which damages the intestinal epithelium and causes impaired intestinal barrier function[35]. Nakamoto *et al*[57] also found that increased *Klebsiella pneumoniae* during PSC

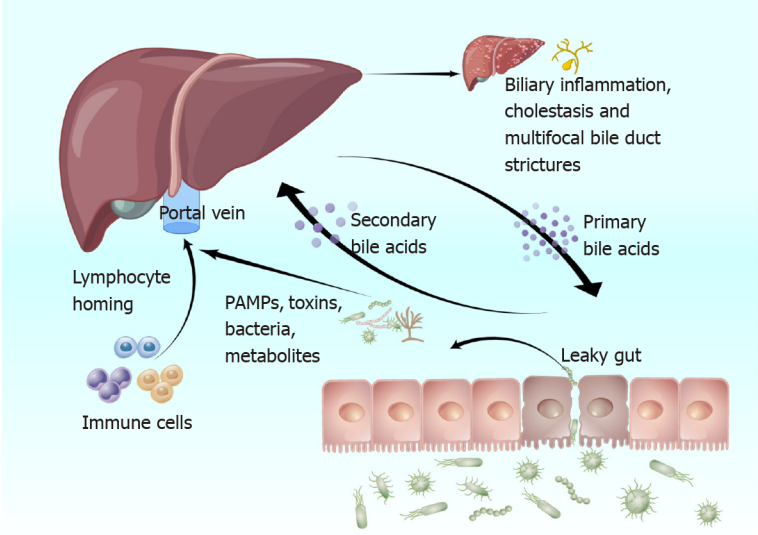
Table 1 Changes in the intestinal flora of patients with primary sclerosing cholangitis

Methods	Increased	Decreased	Ref.
16S RNA gene sequencing: ileum, colon, and rectal samples	*Actinobacillus*, *Bifidobacterium*, *Fusobacterium*, *Haemophilus*, *Roseburia*	*Bacteroides*	[135]
qPCR: fecal samples	*Enterobacteriaceae*	*Bifidobacterium*, *Lactobacillus*	[136]
16S RNA gene sequencing: fecal samples	*Clostridium*, *Lactobacillus*, *Ruminococcus gnavus*, *Veillonella*	*Coprococcus*, *Faecalibacterium*, *Phascolarctobacterium*, *Ruminococcus*	[137]
16S RNA gene sequencing: duodenal fluid, saliva, duodenal mucosa, and bile samples	Duodenal mucosa biopsy: *Escherichia coli*, *Veillonella dispar*; Bile fluid: *Enterococcus*, *Neisseria*, *Proteobacteria*, *Staphylococcus*, *Veillonella dispar*		[42]
Metagenomic shotgun sequencing	*Clostridiales*	*Eubacterium*, *Ruminococcus obeum*	[45]
16S RNA gene sequencing: fecal and saliva samples	*Bacteroides fragilis*, *Blautia*, *Clostridium spp. Enterococcus*, *Enterobacteriaceae*, *Lactobacillus*, *Ruminococcus gnavus*, *Streptococcus salivarius Veillonella dispar*	*Bacteroides thetaiotaomicron*, *Faecalibacterium prausnitzii*	[43]
Sigmoid mucosal biopsies and 16S RNA gene sequencing	*Haemophilus parainfluenzae*, *Pseudomonas*, *Streptococcus*	*Lachnospiraceae*	[40]
16S RNA gene sequencing: fecal samples	*Veillonella*	*Blautia*, *Faecalibacterium*, *Lachnoclostridium*, *Ruminococcus*	[44]
16S RNA gene sequencing: fecal samples	*Enterococcus*, *Lactobacillus*, *Streptococcus*, *Veillonella*	*Clostridium cluster IV*, *Coprococcus*, *Desulfovibrio*, *Faecalibacterium*, *Holdemanella*	[38]
16S RNA gene sequencing: fecal samples	*Veillonella*	*Coprococcus*, *Desulfovibrio*, *Phascolarctobacterium*, *Succinivibrio*	[36]
16S RNA gene sequencing: fecal samples	*Clostridium*, *Enterococcus*, *Haemophilus*, *Rothia*, *Streptococcus*, *Veillonella*	*Adlercreutzia equolifaciens*, *Coprococcus catus*, *Faecalibacterium prausnitzii*, *Prevotella copri*, *Ruminococcus gnavus*	[47]
Colonic mucosal biopsies and 16S RNA gene sequencing	*Escherichia*, *Lachnospiraceae*, *Megasphaera*	*Bacteroides*, *Prevotella*, *Roseburia*	[37]
16S RNA gene sequencing: fecal samples	*Veillonella*		[39]
16S RNA gene sequencing: fecal samples	*Enterococcus*, *Streptococcus*, *Veillonella*		[7]
16S RNA gene sequencing: fecal samples	*Bacteroidetes*, *Enterococcus*, *Fusobacterium*, *Lactobacillus*, *Morganella*, *Streptococcus*, *Veillonella*	*Anaerostipes*	[35]
Colonic mucosal biopsies and 16S RNA gene sequencing: ileal samples	*Barnesiellaceae*, *Blautia*		[8]
Mucosal biopsy of the ileocecal region and 16S RNA gene sequencing	*Clostridium clusters IVand XIVa*, *Akkermansia sp.* (*Verrucomicrobia*), *Uncultured Clostridiales II*		[34]

forms pores by disrupting the intestinal epithelium, leading to increased intestinal permeability, thus prompting other bacteria (*e.g. Proteus mirabilis* and *Enterococcus gallinarum*) to cross the intestinal barrier. In turn, a Th17 cell-mediated inflammatory response initiates in the liver. Finally, Manfredo *et al*[58] demonstrated that *Enterococcus gallinarum* could reach several organs, such as the mesentery, mesenteric lymph nodes, liver, and spleen, after crossing the damaged intestinal epithelium, causing autoimmune diseases such as PSC.

In addition, PSC recurrence in patients who had undergone liver transplantation was associated with specific intestinal flora changes before transplantation. The rate of PSC recurrence was decreased in patients with a higher abundance of *Shigella spp*. in the intestinal flora before transplantation, suggesting that *Shigella spp*. may reduce bacterial translocation and endotoxemia by improving the intestinal mucus layer function and repairing the intestinal barrier[59].

Intestinal bacterial translocation induces liver inflammation: Secondary bacterial overgrowth in the small intestine of rats, achieved by using a blind jejunal loop, led to the translocation of intestinal flora and its metabolite. Consequently, the intestines exhibited characteristic pathological changes of PSC, such as irregular dilatation and bead-like changes in the intra- and extrahepatic bile ducts[60]. Furthermore, Tedesco *et al*[61] found elevated serum interleukin (IL)-17 levels in PSC mice; enriched *Lactobacillus gasseri*, peribiliary collagen deposition, and periportal fibrosis; and increased numbers of IL-17A+ and γδTCR+ cells in mouse liver tissues, which are characteristic inflammatory responses.



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Figure 1 The effect of intestinal flora dysbiosis in patients with primary sclerosing cholangitis. Intestinal flora dysbiosis causes increased intestinal permeability, intestinal lymphocyte homing, and entry of bacteria and their metabolites (*i.e.* pathogen-associated molecular patterns) into the liver. It also impairs normal bile acid metabolism and promotes bile duct inflammation and fibrosis. PAMPs: Pathogen-associated molecular patterns. By Figdraw, www.figdraw.com.

Additionally, Liao *et al*[62] used Mdr2-/- mice to investigate the role of intestinal flora in PSC, reporting that Mdr2-/- mice had intestinal flora dysbiosis. This caused the NLRP3-mediated innate immune response in the liver, amplified by intestinal barrier failure and enhanced bacterial translocation. Finally, Dhillon *et al*[63] compared the serum soluble cluster of differentiation 14 (sCD14) and lipopolysaccharide-binding protein (LBP) levels of patients with PSC and healthy controls, finding that patients with PSC had elevated levels of sCD14 and LBP. The sCD14 and LBP bind to lipopolysaccharides (typical bacterial translocation markers in humans) in response to significant intestinal flora translocation in patients with PSC[64].

The liver contains many immune cells, including Kupffer cells, natural killer (NK) cells, NK T cells, T cells, and B cells, and is a vital immune organ. In healthy individuals, only a few translocated bacterial products make it to the liver. The liver immune system tolerates these bacterial products to avoid harmful reactions, known as hepatic immune tolerance[65]. The intestinal flora dysbiosis in PSC impairs the intestinal barrier function, allowing bacteria and their products to enter the liver continuously. Thus, the hepatic immune tolerance breaks, inducing local inflammation and immune responses by activating TLR-based pattern recognition receptors on hepatic immune cells. Gram-positive bacteria mainly mediate TLR2 activation, endotoxins mediate TLR4 activation, bacterial flagella mediate TLR5 activation, and unmethylated CpG DNA mediates TLR9 activation[66]. TLR activation promotes a downstream inflammatory cascade that activates the MyD88-mediated NF-κB pathway to induce liver fibrosis[67]. Simultaneously, inflammatory cytokines and chemokines [*e.g.* IL-6 and tumor necrosis factor-α (TNF-α)] are overexpressed, inflammatory cells infiltrate, and oxidative stress and endoplasmic reticulum stress occur in the bile duct epithelium. Eventually, bile duct sclerosis and occlusion, cholestasis, and bile duct fibrosis develop[54,68].

Intestinal lymphocyte homing exacerbates liver inflammation

Up to 70% of patients with PSC also develop IBD, suggesting a correlation between the intestine and the liver in patients with PSC and IBD. The discovery of reciprocal transport pathways of lymphocytes to target tissues, as well as the expression of gut-specific adhesion molecules and chemokines in the liver, suggest the homing of intestinal lymphocytes as a contributing factor to PSC pathogenesis[69,70]. Endothelial cells in the hepatic sinusoids of patients with PSC overexpress mucosal vascular addressin cell adhesion molecule 1 (an endothelial adhesion molecule) and C-C motif chemokine ligand 25 (a chemokine), which bind to α4β7 integrin and C-C motif chemokine receptor expressed by intestinal mucosal lymphocytes. This event prompts the recruitment of lymphocytes of an intestinal origin into the liver, which then recognizes the corresponding antigen and triggers an autoimmune response, causing liver injury[71,72]. Trivedi *et al*[41] suggested that this mechanism is associated with hepatic vascular adhesion protein-1 (VAP-1) overexpression. Increased *Veillonella* in the gut of patients with PSC results in primary amine metabolism, which participates in VAP-1 synthesis (as a VAP-1 substrate). Furthermore, hepatic interstitial cells express VAP-1, which recruits intestine-derived T cells to the liver, promoting liver inflammation and fibrosis[73]. Moro-Sibilot *et al*[74] found that elevated levels of sVAP-

1 were associated with poor disease outcomes in PSC. High sVAP-1 Levels correlate with the expression of mucosal addressin cell adhesion molecule 1 in the liver, which contributes to the homing of intestinally activated T cells to the hepatobiliary tract[75]. Meanwhile, sVAP-1 triggers oxidative stress in hepatocytes and aggravates liver injury[76]. B cells in the liver are also derived from intestine-associated lymphoid tissue. B cells are activated by intestinal bacteria and enter the liver, producing antibacterial molecules, such as immunoglobin A, that aggravate liver damage.

Intestinal flora affects PSC through BAs metabolism

It has been established that several intestinal bacterial genera produce BA hydrolases, such as *Lactobacillus, Clostridium, Enterococcus,* and *Bifidobacterium*. Normal microbial metabolism increases BA diversity as well as hydrophobicity, which facilitates BA excretion[77,78]. Intestinal flora plays a key role in the pathogenesis of PSC by mediating BA biosynthesis and farnesol X receptor (FXR) signaling. FXR regulates BA synthesis through a negative feedback loop thereby affecting the intestinal flora[79]. BAs can directly damage intestinal bacterial cell membranes and indirectly affect the intestinal flora composition by binding to FXR and enhancing the action of antimicrobial peptides. Intestinal flora can also alter BA metabolism by affecting the ab initio synthesis of BAs and enterohepatic circulation[80]. Liwinski *et al*[42] found that patients with PSC had increased taurolithocholic acid concentrations in their bile, which causes inflammation; the levels were closely related to the abundance of *Enterococcus.* BA hydrolase expression, which catalyzes the conversion of primary BAs to secondary BAs, is highest when the human intestinal flora contains *E. faecalis*. Thus, a significant increase in *E. faecalis* in the bile of patients with PSC may affect BA metabolism and cause excessive accumulation of secondary BAs in the body, exacerbating PSC[7,42,81]. Tabibian *et al*[82] found that Mdr2-/- mice produced similar biochemical and histological features of PSC (confirmed by liver pathology and hydroxyproline assays) compared to conventionally reared Mdr2-/- mice; these mice were deficient in secondary BAs due to lack of intestinal flora. Further studies showed that GF-Mdr2-/- mice and antibiotic-induced specific pathogen-free Mdr2-/- mice showed imbalance in BA homeostasis, increased BA reuptake, and accelerated accumulation of harmful BAs in the liver due to dysregulation of intestinal microecology [83].

A recent study showed that *Prevotella copri* in the human gut regulates BA metabolism and transport pathways through gut microbiota interactions, especially the FXR signaling pathway, significantly improving chlorosis and liver fibrosis in 3,5-diethoxy-carbonyl-1,4-dihydropyridine-induced PSC mice [84]. Another study showed that intestinal flora attenuates liver damage by promoting UDCA production. The mechanism of UDCA, which has antioxidant, immunomodulatory, hepatocyte-protective, and membrane-maintaining functions, includes re-establishing the intestinal flora, and is widely used to treat PSC[85]. Lee *et al*[86] found that *Ruminococcus gnavus N53* and *Collinsella aerofaciens* in normal human intestinal flora catalyze the conversion of goose deoxycholic acid to UDCA by expressing the 7β-hydroxysteroid dehydrogenase gene, which increases UDCA acid, thereby reducing liver damage in pathological conditions.

TARGETED INTESTINAL FLORA MODULATION FOR PSC TREATMENT

There are no clear and effective options for treating PSC. Pharmacological and endoscopic treatments exist; however, these treatments primarily target the symptoms, and the only effective treatment for end-stage PSC is liver transplantation[16]. In recent years, the incidence of PSC has increased, but intestinal flora research has also expanded, resulting in antimicrobial therapy based on intestinal flora modulation and FMT as potential PSC treatment options[87]. Studies have found that antibiotics, probiotics, and FMT improve intestinal flora disorders, thereby treating PSC (Table 2)[88,89].

Antibiotics

Studies have shown that patients with PSC treated with vancomycin had significant reductions in their serum ALP and bilirubin levels and Mayo PSC risk scores (MRSs) and significant improvements in clinical symptoms, such as fatigue and pruritus[90,91]. An open-label prospective therapeutic clinical trial study showed that oral vancomycin was well tolerated in patients with PSC, with peripheral blood γ-gamma-glutamyl transpeptidase (GGT), alanine aminotransferase (ALT) concentrations, white blood cell counts, and neutrophil counts returning to normal from pre-treatment elevated levels within 3 mo of oral administration. Cholangiography, histological, and liver stiffness assessment at the end of follow-up showed improved results, and the trial also showed that that peripheral blood levels of CD4 + CD25hiCD127 Lo and CD4 + FoxP3 + regulatory T cells were also elevated in PSC-IBD patients treated with oral vancomycin[92,93]. Furthermore, Britto *et al*[94] found fewer potentially pathogenic bacteria, such as *Fusobacterium, Haemophilus,* and *Neisseria*, in the intestinal flora of patients with PSC after oral vancomycin treatment. A significant recovery in flora diversity was also observed, suggesting that vancomycin treatment indirectly leads to a secondary increase in bacterial diversity by prompting the intestinal flora to suppress mucosal inflammation. The efficacy of vancomycin for PSC may be related to its selectivity for *Clostridium perfringens*[95]. Shah *et al*[96] reported that vancomycin has a relatively

Table 2 Intestinal flora regulation in primary sclerosing cholangitis treatment

Treatment			Results	Ref.
Oral Antibiotics	Vancomycin	125 mg four times daily for 90 d	Fecal calprotectin and serum GGT levels returned to normal	[94]
		125 mg four times daily for 12 wk	Significantly decreased ALP, MRS, ESR, and GGT levels. Fatigue, pruritus, diarrhea, and anorexia significantly improved	[91]
		50 mg/kg/d for 30 to 118 mo	Decreased ALT, AST, GGT, and ESR levels. Jaundice improved. The overall rate of positive serum autoantibodies decreased after 3.5 mo	[138]
	Vancomycin and metronidazole	Vancomycin: 125 mg or 250 mg four times daily for 12 wk; Metronidazole: 250 mg or 500 mg three times daily for 12 wk	Decreased ALP and MRS levels. The decrease in ALP level was more pronounced following vancomycin administration	[90]
	Metronidazole with ursodeoxycholic acid	Taken together for 36 mo	Significantly decreased ALP and MRS levels	[98]
	Azithromycin	500 mg three days per week for 6 wk	Decreased ALP and TBIL levels and cholestasis-related symptoms. The urine was turned dark in color again	[139]
	Rifaximin	550 mg twice daily for 12 wk	Decreased GGT and CRP levels; Improved pruritus symptoms	[95]
	Minocycline	100 mg twice daily for one year	Significantly decreased ALP and MRS levels	[100]
Fecal microbiota transplantation at 24 wk			Significantly decreased ALP levels; Reduced AST levels (by at least 30%)	[107]
Probiotics	*Lactobacilli* and *Bifidobacteria*	Three months of: *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus salivarius*, and *Lactococcus lactis*, *Bifidobacterium bifidum*, *Bifidobacterium*	Reduced ALP levels (by 15%)	[113]
	Combined	Prednisolone (30 mg/d), salazosulfapyridine (3000 mg/d), and *Lactobacillus casei* Shirota (3 g/d) for 2 wk	Decreased ALP, ALT, AST, and GGT levels	[114]

ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MRS: Mayo primary sclerosing cholangitis risk score; ESR: Erythrocyte sedimentation rate; GGT: Gamma-glutamyl transpeptidase; CRP: C-reactive protein; TBIL: Total bilirubin.

narrow antibiotic spectrum and specifically targets *Clostridiales*. Consequently, vancomycin affects the abundance of *Clostridiales* in the intestinal flora of the distal small intestine and colon by reducing primary BA dehydroxylation and preventing excessive secondary BA accumulation, thereby reducing PSC activity. In addition, Davies *et al*[97] demonstrated that vancomycin directly attenuates the inflammatory response to periportal inflammation and liver injury during PSC.

Studies in animal models have demonstrated that metronidazole also has a therapeutic effect on liver injury in PSC[60]. For example, Karvonen *et al*[98] found that treating patients with PSC with both UDCA and metronidazole significantly reduces the serum glutamyl transpeptidase and ALP levels, and significantly improves the MRS and pathological staging compared with those treated with only UDCA. Furthermore, Krehmeier *et al*[99] reported that metronidazole reduced intestinal permeability, decreased bacterial endotoxin entry into the blood, inhibited endotoxin-induced TNF-α production, inhibited hepatic Kupffer cells and macrophage activation, reduced chemokine and cytokine secretion by biliary epithelial cells, attenuated liver inflammation, and prevented PSC-like bead-like liver injury. Finally, Silveira *et al*[100] showed that minocycline is a safe and effective PSC treatment, significantly reducing the ALP level and MRS after one year of oral minocycline administration.

FMT

FMT is the transplantation of fecal flora from healthy individuals into a patient's intestine to replenish or restore normal intestinal flora. This procedure aims to reverse intestinal dysbiosis, regulate product metabolism, and improve clinical symptoms to treat the disease (*Clostridium* difficile infection, IBD, diabetes mellitus, cancer, liver cirrhosis, gut-brain disease and others)[101,102]. FMT restores the health of the intestinal flora, further reducing the transport of harmful metabolites, such as endotoxins to the liver, and reducing the damage caused by metabolites to the liver[103]. FMT uses the principle of bacterial therapy to restore the health of the intestinal flora. The transplanted beneficial bacteria (*Bifidobacteria*, *etc*.) are involved in the conversion of polysaccharides to monosaccharides, producing SCFAs such as acetate, propionate, and butyrate[104]. These metabolites regulate normalization of the intestinal flora and reduce intestinal permeability in patients with liver disease, to further reduce the transport of metabolites such as endogenous ethanol and endotoxins to the liver, thus, reducing the

damage to the liver[103,105,106]. Studies have shown intestinal flora normalization, a significant improvement in intestinal flora diversity, reduced cholestasis, and decreased ALP levels in PSC patients after FMT. Allegretti *et al*[107] performed the first human FMT trial in ten patients with PSC who had ALP levels more than three times the normal upper limit. After FMT, 30% of the patients had decreased ALP levels by 50%, and 70% had a 30% reduction in the levels of serum liver transaminases (ALT and aspartate aminotransferase). One week after FMT, the recipients' intestinal flora diversities were higher than the baseline level of all patients and continued increasing for 24 wk. Furthermore, Philips *et al*[108] found that fecal flora diversity improved in patients with PSC after FMT, with a decrease in the relative abundance of *Proteobacteria* and an increase in the abundances of *Bacteroidetes* and *Firmicutes*; this intestinal flora composition was more similar to that of healthy individuals. The blood biochemistry and total BA indicators also significantly improved.

Probiotics

Probiotic is a general term for a group of active microorganisms that have beneficial roles by regulating intestinal flora growth and improving the host's intestinal microecology. They regulate the intestinal microenvironment metabolism, increase SCFAs production, and reduce the permeability of the intestinal barrier[109,110]. Additionally, probiotics upregulate intestinal epithelial tight junction protein expression, improve intestinal motility[110,111], increase adhesion and colonization of intestinal flora, reduce TNF-α production, and maintain tissue homeostasis[112]. One study demonstrated that oral administration of probiotic preparations (consisting of six strains of viable and freeze-dried bacteria: *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus salivarius, Lactococcus lactis, Bifidobacterium bifidum,* and *Bifidobacterium lactis*) decreased the serum alkaline phosphatase level by 15 % in patients with PSC compared to healthy individuals[113]. Furthermore, Shimizu *et al*[114] treated a patient with PSC with a combination of prednisolone, salazosulfapyridine, and probiotics, and reported that the patient's symptoms and tests improved after two weeks. Additionally, repeat pathological biopsies at 30 mo showed significant improvements in liver inflammatory cell infiltration and peribiliary fibrosis. *Lactobacillus plantarum Lp2* has the potential to ameliorate liver injury by inhibiting the activation of LPS-induced inflammatory pathways in the liver, reducing inflammation, and decreasing oxidative damage and apoptosis[115]. Therefore, probiotics have a therapeutic effect on PSC by suppressing intestinal inflammation and maintaining intestinal flora homeostasis.

BAs and other metabolites

Compared to conventional mice, germ-free mice show higher concentrations of BA in the plasma and significantly reduced concentrations in the feces. Additionally, FXR signaling is significantly inhibited, resulting in reduced BA synthesis in germ-free mice[116,117]. Colonization of germ-free mice with human feces activates the expression of FXR target genes and increases the levels of BAs in the liver and ileal tissue[118]. FXR agonists inhibit cholesterol 7α-hydroxylase activity and, thus, intracellular BA synthesis. These agonists can activate transcription of the bile salt export pump on the hepatocyte membrane, enhancing the transport of BAs from hepatocytes to bile ducts and promoting BA excretion. Simultaneously, These agonists can inhibit the expression of extracellular matrix proteins in hepatic astrocytes and, thus, prevent the transformation of liver fibrosis in patients with PSC[119]. Obeticholic acid (OCA) is one of FXR agonists representative drugs that alleviates the cholestatic symptoms of PSC by reducing the BA pool[120]. OCA is also approved for the treatment of PSC[121,122]. In fact, there are phase II clinical trials demonstrating the efficacy and safety of OCA in patients with PSC[123].

Relevant clinical trials

In addition to the above-mentioned studies, there are currently several relevant clinical trials demonstrating the efficacy of treatments targeting intestinal flora and its metabolites in PSC (Table 3). From these clinical studies, we found that oral vancomycin is the most established for the treatment of PSC, and all phase IV clinical trials using vancomycin have been completed. Vancomycin can significantly reduce biochemical indexes such as ALP and ALT and reduce MRS in patients with PSC [92,124]. One case study also described a decrease in serum γ-GGT, which reached normal levels at 195 d, in pediatric patients with PSC-UC who were administered vancomycin[94]. *Fusobacterium, Haemophilus,* and *Neisseria*, which generally have a significantly high abundance in PSC, showed decreased abundance in the saliva and feces of these patients[40,42,43,47]. Results of meta-analyses have also shown vancomycin to be beneficial in patients with PSC[96]. Currently, there are clinical guidelines recommending the use of antimicrobial agents and FXR agonists for the treatment of PSC[125,126]. Clinical trials of UDCA for PSC are also well established[127]. UDCA is a hydrophilic dihydroxy BA, and pharmacological studies have confirmed that UDCA has a strong affinity in bile, promoting bile secretion, protecting bile duct cells from the cytotoxicity of hydrophobic BAs, and protecting hepatocytes from BA-induced apoptosis[128]. It promotes the formation of liquid cholesterol crystal complexes, accelerates cholesterol excretion and clearance to the intestine, acts as a cholagogue, and competitively inhibits endogenous hepatic BA absorption in the small intestine, reducing serum BA levels[129]. 24-norUDCA is a side chain shortened congener of C23UDCA, which makes a bile hepatic shunt possible. Based on its pharmacological properties of relative amidation resistance and reduced

Table 3 Clinical trials related to primary sclerosing cholangitis treatment

Study Phase	Study title	ClinicalTrials.gov identifier	Actual enrollment	Status	Interventions
Phase 1	A Pilot Study of Vancomycin or Metronidazole in Patients with Primary Sclerosing Cholangitis (PSC)	NCT01085760	35 participants	Completed	Drug: vancomycin; Drug: metronidazole
	Minocycline in Primary Sclerosing Cholangitis (PSC)	NCT00630942	16 participants	Completed	Drug: minocycline
	Treating Primary Sclerosing Cholangitis and Biliary Atresia with Vancomycin	NCT02137668	200 participants	Recruiting	Drug: oral vancomycin
	A Pilot Study to Characterize Bile Acid Metabolism and Dysbiosis in Primary Sclerosing Cholangitis	NCT02464020	8 participants	Completed	Drug: vancomycin
	Gastrointestinal Microbiota in Primary Sclerosing Cholangitis and Biliary Atresia with Vancomycin (PSC)	NCT01322386	32 participants	Completed	Drug: vancomycin
	Fecal Microbiota Transplantation for the Treatment of Primary Sclerosing Cholangitis.	NCT02424175	10 participants	Completed	Biological: Fecal microbiota transplantation
Phase 2	Norursodeoxycholic Acid in the Treatment of Primary Sclerosing Cholangitis (NUC-3)	NCT01755507	159 participants	Completed	Drug: norursodeoxycholic acid; Drug: placebo
	Obeticholic Acid (OCA) in Primary Sclerosing Cholangitis (PSC) (AESOP)	NCT02177136	77 participants	Completed	Drug: OCA; Drug: placebo
	Vancomycin for Primary Sclerosing Cholangitis	NCT03710122	102 participants	Recruiting	Drug: vancomycin; Other: placebo
	Trial of High-dose Urso in Primary Sclerosing Cholangitis	NCT00059202	150 participants	Completed	Drug: ursodeoxycholic acid; Drug: placebo
	Fecal Microbiota Transplantation for the Treatment of Primary Sclerosing Cholangitis.	NCT02424175	10 participants	Completed	Biological: fecal microbiota transplantation
Phase 3	Primary Sclerosing Cholangitis with Oral Vancomycin by the Study of Its Antimicrobial and Immunomodulating Effects (PSC)	NCT01802073	34 participants	Completed	Drug: oral vancomycin
	Probiotics in Patients with Primary Sclerosing Cholangitis	NCT00161148	12 participants	Unknown[1]	Drug: probiotics
	Norursodeoxycholic Acid *vs* Placebo in PSC	NCT03872921	300 participants	Recruiting	Drug: norursodeoxycholic acid
	Vancomycin for Primary Sclerosing Cholangitis	NCT03710122	102 participants	Recruiting	Drug: vancomycin; Other: placebo
	Trial of High-dose Urso in Primary Sclerosing Cholangitis	NCT00059202	150 participants	Completed	Drug: ursodeoxycholic acid; Drug: placebo
Phase 4	Effect and Safety of Oral Vancomycin in Primary Sclerosing Cholangitis Patients	NCT02605213	30 participants	Unknown[1]	Drug: vancomycin; Drug: placebo

[1]Study has passed its completion date and status has not been verified in more than two years.

PSC: Primary sclerosing cholangitis; OCA: Obeticholic Acid; AESOP: Aetiology and Ethnicity in Schizophrenia and Other Psychoses.

secondary BA production, norUDCA is a promising drug for a range of cholestatic liver and bile duct diseases[130]. Some clinical trials have shown that norUDCA improved cholestasis and significantly reduced serum alkaline phosphatase levels in patients after 12 wk in a dose-dependent manner. Importantly, norUDCA treatment has shown a good safety profile[131]. OCA is a potent FXR agonist that affects the hepatic transport of conjugated BAs in humans and reduces duration of hepatocyte exposure to potentially cytotoxic BAs[132,133]. Clinical trials have demonstrated the efficacy and safety of OCA in patients with PSC; Treatment with OCA 5-10 mg resulted in a significant reduction in ALP in patients with PSC after 24 wk[123]. In addition, clinical studies of probiotics, FMT, and other approaches targeting intestinal flora for the treatment of PSC are ongoing to highlight their efficacy and safety in PSC and demonstrate their therapeutic potential[108,134].

CONCLUSION

PSC is a chronic progressive autoimmune disease that can develop into cirrhosis or liver failure, thereby severely affecting the patient's quality of life if not actively and effectively treated. Intestinal flora dysbiosis is crucial in the occurrence and development of PSC, as it destroys the intestinal barrier and

prompts intestinal lymphocyte homing and translocation of bacteria and their metabolites, thus aggravating the immune damage to the liver. The intestinal flora also interacts with BAs and participates in PSC development.

Our understanding of the gut flora has expanded with the development of genomics, metabolomics, and high-throughput sequencing technologies. These research approaches help elucidate the complex role of the gut flora in diseases, such as PSC. Technological advances have also provided individualized treatment options for patients with PSC that target the intestinal flora with good clinical results. Treatments, including antibiotics, FMT, and probiotics, have offered new ideas for managing PSC. More precise therapies, such as probiotics, synbiotics, and phages, have shown promising results in PSC patients. However, there remain some challenges in the use of intestinal flora for PSC treatment. The intestinal flora regulation mechanisms for PSC are not fully understood, and the optimal method and timing have not been standardized. Future prospective studies with a large sample size or multi-center studies are warranted to provide direct evidence of the role of the intestinal flora in PSC and establish a therapeutic protocol for the use of the intestinal flora. If these issues are resolved, targeted regulation of the intestinal flora will become a new option for PSC treatment.

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REVIEW

Machine learning insights concerning inflammatory and liver-related risk comorbidities in non-communicable and viral diseases

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Abstract

The liver is a key organ involved in a wide range of functions, whose damage can lead to chronic liver disease (CLD). CLD accounts for more than two million deaths worldwide, becoming a social and economic burden for most countries. Among the different factors that can cause CLD, alcohol abuse, viruses, drug treatments, and unhealthy dietary patterns top the list. These conditions prompt and perpetuate an inflammatory environment and oxidative stress imbalance that favor the development of hepatic fibrogenesis. High stages of fibrosis can eventually lead to cirrhosis or hepatocellular carcinoma (HCC). Despite the advances achieved in this field, new approaches are needed for the prevention, diagnosis, treatment, and prognosis of CLD. In this context, the scientific community is using machine learning (ML) algorithms to integrate and process vast amounts of data with unprecedented performance. ML techniques allow the integration of anthropometric, genetic, clinical, biochemical, dietary, lifestyle and omics data, giving new insights to tackle CLD and bringing personalized medicine a step closer. This review summarizes the investigations where ML

techniques have been applied to study new approaches that could be used in inflammatory-related, hepatitis viruses-induced, and coronavirus disease 2019-induced liver damage and enlighten the factors involved in CLD development.

Key Words: Machine learning; Liver inflammation; Liver disease; Viral diseases; Comorbidity

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Core Tip: Chronic liver disease has become a global burden, and new approaches need to be explored to tackle this disease. In this context, machine learning techniques bring a whole new set of opportunities to study novel approaches and biomarkers for prevention, diagnosis, treatment, and prognosis of inflammatory and virus-related liver diseases. The application of machine learning algorithms constitutes a pivotal piece of personalized medicine, allowing the integration of different phenotypical and genotypical data for a precision outcome concerning inflammatory liver comorbidities in non-communicable and viral diseases.

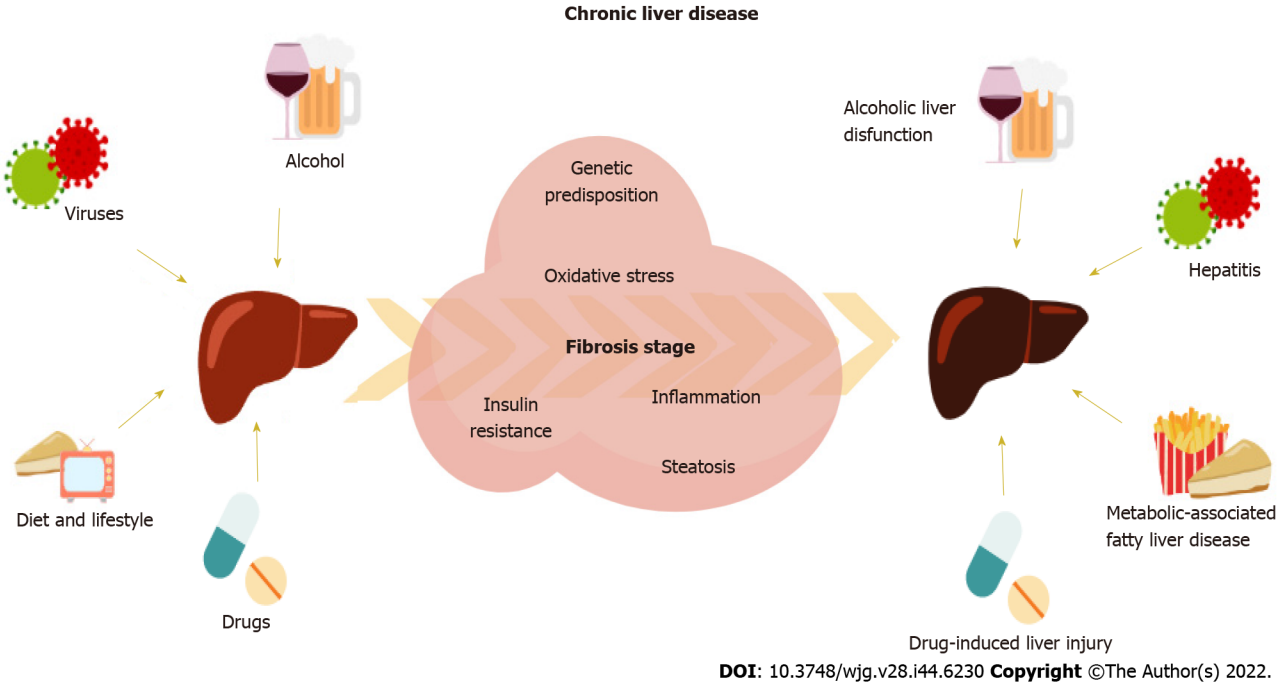
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INTRODUCTION

The liver is a key organ involved in relevant homeostatic metabolic and detoxifying human functions [1]. Thus, the liver is the epicenter of an organ-organ network weaving a series of complex interactions in the organism, which makes liver damage an underlying adverse condition in a whole set of diseases. Chronic liver disease (CLD) can be caused mainly by alcoholic liver-related dysfunctions, hepatitis B virus (HBV), hepatitis C virus (HCV), drug treatments, or non-alcoholic fatty liver disease (NAFLD), as recently updated to the term metabolic-associated FLD (Figure 1)[2,3]. Patients with liver-related diseases need frequent follow-ups and careful monitoring since CLD can eventually lead to cirrhosis or hepatocellular carcinoma (HCC) if not diagnosed on time for treatment or surgery. These CLD-related conditions have become a global burden, whose mortality associated rates have increased over the years reaching more than 2 million deaths worldwide[4].

CLD is usually accompanied by an unhealthy inflammatory environment[5]. The immune response is a fundamental process to maintain homeostasis within the organism defense machinery and is characterized by the secretion of proinflammatory cytokines, like interleukin (IL)-1, tumor necrosis factor-α (TNF-α), and prostaglandin E2, in an acute manner in order to resolve sudden damage[5]. However, if sustained over time, these abnormal levels of inflammatory cytokines cause low-grade inflammation (LGI). LGI is a silent condition that predisposes to the development of metabolic and infectious diseases that has become a worldwide health issue[6]. Patients with CLD, such as non-alcoholic steatohepatitis (NASH), present impaired immune function, dysbiosis, insulin resistance (IR) and LGI, all of which can aggravate infectious disease progression and perpetuate excess of adipose tissue, are characterized by overstimulation of the production of adipose-derived inflammatory molecules[5,7-9].

The liver also secretes important hepatokines that act as signaling proteins modulating functions in other organs and are involved in a wide range of conditions, such as IR and adipogenesis[1]. For instance, fibroblast growth factor-21 (FGF-21) is a mediator participating in glucose metabolism mainly secreted by the liver that modulates adipogenesis, while fetuins, liver-derived plasma proteins, are participating in metabolic impairment and inflammation[1]. A dysregulation in systemic cytokines prompts fat accumulation in hepatocytes, which in turn promotes local secretion of proinflammatory hepatokines, leading to liver steatosis and IR. In addition, immune cells also find difficulty in this inflammatory environment to exert their role appropriately. Persistent inflammatory signals over time also abnormally activate immune cells, impairing the body's ability to fight infection, repair tissue damage, or recover from possible poisoning. Inflammation comes hand in hand with an increase in oxidative stress, a state characterized by an imbalance in favoring the accumulation of higher reactive oxygen (ROS) and nitrogen species. These molecules in unusual concentrations damage the cell and environmental milieu by promoting the expression of proinflammatory genes, resulting in a vicious cycle. Thus, CLD presents an oxidative atmosphere, probably linked to the proinflammatory state[10,11]. This environment is the perfect setting for the fibrogenic process to unfold, an underlying condition of CLD that is characterized by progressive accumulation of fibrillar extracellular matrix in the liver



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Figure 1 Factors involved in the development of chronic liver disease triggering associated processes that lead to increased fibrosis stage.

[12]. The stage of hepatic fibrosis has been associated with the risk of mortality and liver-related morbidity in patients with NAFLD[13], virus-induced hepatitis[14,15], and alcoholic-derived liver disease[16], eventually leading to HCC.

In this context, infection by human hepatitis viruses (HHVs) is the most common cause of hepatitis, leading to the activation of the immune system, and the subsequent inflammatory response[17]. HBV and HCV acute infections can be resolved with antiviral and immune therapy. However, in a significant percentage they can progress to chronic hepatitis. This persistent infection can lead to comorbidities outside the liver, like arthritis, vasculitis, myalgia, and peripheral neuropathies[18]. Moreover, another new infectious disease appeared in late 2019 that can cause liver damage: Coronavirus disease 2019 (COVID-19). COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, and it has become a global health issue since its outbreak in 2020 was declared a pandemic. Beyond lung function, COVID-19 can affect a wide variety of tissues, like the gastrointestinal tract, kidneys, and liver, with an underlying adverse inflammatory environment[19]. This inflammatory-related condition has been strongly associated to metabolic status and worsening diseases like obesity, diabetes, and hypertension[7,20-22]. For instance, COVID-19 can increase hepatic lipid accumulation by mitochondrial and endoplasmic reticulum (ER) dysfunction or worsen NAFLD if it was already present. A recent systematic review depicted that the parameters normally used for liver impairment screening were significantly increased in COVID-19 patients[23], placing CLD as a risk factor for progressive and severe COVID-19[24,25].

CLD is a global health problem, and new methods are needed to tackle this life-threatening condition. In this line, this review aims to explore machine learning (ML)-based approaches to manage CLD and develop biomarkers for diagnosis and prognosis. Its goal is to shed light on the factors involved in CLD to help health professionals in clinical management with the support of ML and identify new targets that can define therapeutic care lines in viral infections and non-communicable diseases (NCD), with an impact on liver functions with an inflammatory component. This includes the new disease, COVID-19.

MECHANISMS BY WHICH NCD AND INFLAMMATORY/IR PHENOMENA CAN AFFECT LIVER FUNCTION

The incidence of NCD, such as cardiovascular diseases and diabetes, has skyrocketed in the last decades, pressing authorities to establish developmental goals to achieve in the near future in terms of decreasing NCD-caused mortality[26]. Some of the risk factors that contribute to the development of NCD are excess of adipose tissue and high levels of glycemia. In this context, adipose tissue plays a key role in the development of FLD by secreting adipokines and other molecules, like free fatty acids (FFA) [8].

An energy excess prompts fat accumulation in the organism and the subsequent dysregulation of this tissue. This is of relevance since an inflamed adipose tissue results in increased levels of FFA and pro-inflammatory cytokines, IR, and infiltration of macrophages in the liver by the activation of Th1 and Th17 cells[8]. FFA enter the liver through the portal vein and trigger a series of reactions. For instance, they serve as ligands to toll-like receptor-4 complex, stimulating the production of TNF-α through the activation of nuclear factor-kappa B, favoring an inflammatory environment. Moreover, the excess of fat drives the polarization state of this increased number of macrophages from anti-inflammatory M2 to proinflammatory M1 macrophages and prompts fat accumulation in the liver and IR[8]. Adipose-derived macrophages also secrete inflammatory molecules, like TNF-α and IL-6, and adipokines, such as visfatin [also named nicotinamide phosphoribosyl transferase (NAMPT)]. NAMPT has gained relevance as a pivotal molecule linking adipose tissue and FLD. NAMPT is a pleiotropic molecule that can be found in an extracellular (eNAMPT) or an intracellular (iNAMP) form. Studies indicate that eNAMPT has enzyme and cytokine-like activity, stimulating the release of proinflammatory cytokines. Meanwhile, iNAMPT catalyzes the rate-limiting step in nicotinamide adenine dinucleotide (NAD+) formation. Because of this NAD+ boosting property, levels of iNAMPT have been proposed as beneficial for the homeostasis of the cell due to influencing the activity of NAD-dependent enzymes, such as sirtuins (SIRT). Remarkably, SIRT1 plays a key role in the liver by modulating the acetylation status of target molecules in lipid metabolism[27].

Furthermore, IR is characterized by hyperglycemia and the subsequent hyperinsulinemia to counteract high glucose levels, being a risk factor for NCDs, particularly type 2 diabetes, where it has been closely linked to oxidative stress[28]. A normal insulin signaling pathway starts with the activation of the insulin receptor so that it can bind to phosphoinositide 3-kinase to ultimately activate protein kinase B (Akt). Activated Akt drives glucose entry into the cell by promoting GLUT4 expression and glycogen synthesis[29]. Oxidative stress impairs this signal transduction through many different mechanisms, like inhibiting the transcription factors insulin promoter factor 1 and peroxisome proliferator-activated receptor gamma, which mediate insulin and GLUT-4 expression, respectively. Moreover, under hyperglycemic conditions, fetuin A hepatokine inhibits the insulin receptor and promotes inflammation, while FGF-21 inhibits lipid accumulation and increases insulin sensitivity. Dysregulation of this hormones, together with oxidative stress imbalance, lead to impaired insulin signaling[30].

The metabolic conditions underlying the development of NCD are complex, and they often reinforce each other, perpetuating an inflammatory environment and oxidative stress imbalance. As the orchestrating organ, these processes converge in the liver, affecting metabolic functions and setting the basis for the onset of the fibrogenic process characteristic of CLD.

MECHANISMS BY WHICH VIRAL INFECTIONS AND INFLAMMATORY/IR PHENOMENA CAN AFFECT LIVER FUNCTION

Persistent virus-associated liver damage can progress to CLD, which pressures health systems with a big social and economic burden. Although lots of resources have been invested to study the molecular mechanisms that mediate this process, results are diverse and still under investigation by the scientific community. HHVs directly infect hepatocytes, and the internalization into the cell is believed to happen by endocytosis, requiring the interaction with several host cell factors[17]. However, viral entry of HBV and HCV within hepatocytes is unclear, and further research is needed to elucidate this question. Sodium taurocholate co-transporting polypeptide was recently identified as an HBV receptor that would mediate HBV cell entry[31]. In the case of HCV, specific intercellular adhesion molecules appear key to cell adhesion and subsequent internalization[32].

Regarding HBV and HCV replication, it has been found that liver X receptor-α (LXR-α) plays a key role. LXR-α is a transcription factor whose activation triggers the expression of different genes that directly or indirectly modulate these viruses' replication as well as the lipid and inflammatory alterations associated to CLD[33]. This inflammation is also mediated by the nucleotide-binding oligomerization domain-like receptor protein 3, which is activated by the abnormal production of ROS after a viral infection occurs in the liver. This ROS increase is associated with a decreased expression of nuclear factor-e2-related factor-2, a transcription factor that regulates ROS/recepteur d'origine nantais balance by maintaining redox homeostasis. These alterations compromise the normal state of the cell, laying the foundations on which the fibrotic process of CLD begins[11].

In the case of COVID-19, the mechanisms by which liver damage can occur are more unclear, but it is widely accepted that inflammation plays a huge role. This infection can trigger an exaggerated immune response leading to an uncontrolled cytokine release, also known as a "cytokine storm". It is characterized by abnormal levels of IL-6, IL-1, C-C motif chemokine ligand (CCL)-5, chemokine (C-X-C motif) ligand (CXCL)-8, CXCL-1, and TNF-α among others[19]. This inflammatory cascade affects bile duct function since cytokines like TNF-α, IL-1, and IL-6, can induce hepatocellular cholestasis by downregulating hepatobiliary uptake and excretory systems[34].

Furthermore, the presence of this inflammatory environment can upregulate the expression of angiotensin converting enzyme 2 (ACE2) receptor in different tissues, like the adipose tissue and the liver[35-39]. This is of relevance since ACE2 receptors are the main cell entrance of the SARS-CoV-2 virus, and they are present in different tissues. Particularly in the liver, the cholangiocytes (characteristic cells of the bile duct)[40], as well as liver vascular endothelial cells[41], express ACE2 receptors. Hepatocytes and cholangiocytes are permissive to the SARS-CoV-2 virus, mediating subsequent entrance into the liver[42]. Several studies have found that *ACE2* expression in hepatocytes is increased under hypoxia[43], a frequent condition in COVID-19 patients, and fibrotic conditions[44]. Besides ACE2 receptors, transmembrane serine protease 2 (TMPRSS2) and paired basic amino acid cleaving enzyme (FURIN) have been noted as significant for infection in the liver[45,46]. In this context, *ACE2* expression is increased in patients in HCV-related cirrhosis[44], whereas *TMPRSS2* and *FURIN* expression are upregulated in patients with obesity and NAFLD[47]. Moreover, infection by SARS-CoV-2 increases glucose-regulated protein 78 and 94, two biomarkers of ER stress[48,49], and impairs mitochondrial function[50]. This process is of interest since this state has been associated with *de novo* lipogenesis in hepatocytes[51], which could eventually lead to steatosis in these patients.

The use of therapeutic drugs can be another underlying cause of liver damage[3]. Because of detoxifying functions, the liver is subject to drug-induced damage coming from a wide range of approved drugs. Oncology drugs account for most hepatotoxicity cases, followed by those used for infectious diseases[3]. Since the beginning of the COVID-19 pandemic, a wide range of different treatments (antivirals, antibiotics, antimalaria, or corticosteroids) have been used in the absence of an efficient drug to treat severe infections. This pharmacological administration could explain that drug-induced liver injury appears in nearly 25% of COVID-19 patients[23], a consequence to consider when addressing liver damage in this disease.

ML APPROACHES IN INFLAMMATORY AND LIVER-RELATED COMORBIDITIES IN NON-COMMUNICABLE AND VIRAL DISEASES

Despite all the advances in the mechanisms driving the onset of these diseases, new techniques to detect innovative biomarkers for diagnosis and prognosis as well as to discover novel drugs are needed, for example artificial intelligence (AI). AI seeks to mimic human behavior, and within this science, ML is the most common approach[52]. The advances in computational science in the last decades have permitted the development of powerful algorithms based on this science. ML algorithms are particularly relevant for biological research because they allow the processing and integration of the huge amount of data that the latest advances in this field have brought by applying statistical methods to enable machines to improve experiences. This methodological approach can be categorized into two big groups: Supervised and unsupervised learning. In supervised algorithms, data is tagged in order to train the algorithm and fit it appropriately, whereas if it is unsupervised, the algorithm learns patterns from unlabeled data[53]. ML algorithms are generally assessed by simple methodologies like sensitivity, specificity, and accuracy. While sensitivity evaluates the proportion of true positives correctly identified, specificity evaluates the proportion of true negatives. Meanwhile, the accuracy value indicates the number of times the model is correct[54].

Supervised algorithms can be divided into two categories depending on the purpose: Prediction, in which the algorithm is fed and trained predictive models to data; or classification, which consists in clustering data within explanatory groups[55,56]. Predictive algorithms are based on regression models, and the most used are linear and logistic regression (LR), support vector machine (SVM), support vector regression (SVR), extra tree regression (ETR), artificial neural networks (ANN), and decision trees (DT). Regression models analyze the influence of one or multiple variables on a nominal or ordinal categorical outcome. ANN are more complex mathematical models (deep learning algorithms) that mimic the brain neural network, like the convolutional neural network (CNN), in which an input is fed through a hidden layer of many different well connected and structured nodes to produce a final output. In deep neuronal network (DNN) models, a great number of hidden successive layers use the output from the previous layer as input in a more complex algorithm. DT can also classify data, like random forest (RF) or gradient boosting (GB) models. Instead of minimizing error, these models determine thresholds derived from input data, assigning weight values to variables. Other models of classification are the Ada-Boost, Bayesian network (BN), Naïve Bayes (NB), K-Nearest Neighbors (KNN), and linear discriminant analysis (LDA) that group data into clusters[55,56]. All these models can shed light into biological questions and are normally used indistinctively to obtain the best performance with the same dataset. For instance, Mijwil and Aggarwal[57] analyzed and compared 7 ML algorithms to predict appendix illness in the same dataset, revealing that certain models performed better than others, allowing for higher accuracy and results.

In FLD, the common techniques used in diagnostics are based on techniques like ultrasonography and magnetic resonance imagining (MRI). These methods are subjective, and the informed outcome mainly relies on the interpretation of the professional carrying out the procedure. Several investigations have studied the implementation of ML in order to classify FLD and other liver diseases by using

images from ultrasounds, computed tomography (CT), and MRI[58,59]. However, the downside of this approach is that the quality of the images differs from one another because of several factors, such as equipment precision and interpersonal differences, for instance. Therefore, there is a need for ML approaches to help in image segmentation, and some authors have already implemented this technique to improve clinical practice[60,61].

Moreover, ML can help with the integration of more complex information beyond imaging to study and diagnose liver diseases since patients with CLD in the developmental phase require frequent follow-ups to check the progress of the disease and early detection changes in the diagnosis[58]. For example, patients with HHV-induced CLD are normally on antivirals. However, there is no consensus or guidelines about when to stop antiviral therapy or even if quitting these drugs will increase HCC risk. Therefore, new approaches need to be established to classify and prevent the development of more severe illnesses, like cirrhosis or cancer. In this line, ML approaches can be used to measure liver fibrosis, optimize diagnosis, and predict disease progression of CLD[62]. Table 1 summarizes selected studies that have used ML for these purposes, which have been collected for this review, and Table 2 summarizes the most repeated inputs from all compiled ML models along with the most repeated predictive results for the main four inflammation-related liver conditions.

ML in inflammation-related liver disease

In recent years, promising results have been found when applying ML approaches in CLD. Regarding prevention, Fialoke *et al*[63] screened 108139 patients to identify those diagnosed with benign steatosis and NASH, a type of NAFLD, train ML classifiers for NASH and healthy (non-NASH) populations, and predict NASH disease status on patients diagnosed with NAFLD according to aspartate transaminase (AST), alanine transaminase (ALT), and platelet (PLT) levels. In this line, another study detected body mass index (BMI), triglycerides (TG), gamma-glutamyl transpeptidase (GGT), ALT, and uric acid as the top 5 features contributing to NAFLD, with the BN model performing the best[64]. Accordingly, Yip *et al*[65] selected TG, ALT, white blood cell count, high-density lipoprotein cholesterol (HDL-c), glycated hemoglobin A1c (HbA1c), and the presence of hypertension as the six variables to build ML models, of which Ada-Boost outperformed the others individually and described the NAFLD status in 922 subjects.

More recently, Pei *et al*[66] designed a ML model that integrated medical records as a clinical variable to classify FLD. Concretely, they selected the variables of age, height, BMI, hemoglobin, AST, glucose, uric acid, low-density lipoprotein cholesterol (LDL-c), alpha-fetoprotein, TG, HLD-c, and carcinoembryonic antigen. They tested six different ML models in 3419 participants, of which 845 were diagnosed with FLD: LR, RF, ANN, KNN, extreme gradient boosting (XGBoost) (a type of GB model), and LDA. Results from these authors showed that the XGBoost model had the highest performance, followed by LR and ANN, to predict the risk of FLD. BMI, uric acid, and TG levels were the top three variables associated to FLD risk across the six analyzed models.

When it comes to diagnosis and treatment, several ML models have been tested for different purposes obtaining good specificity, sensitivity, and accuracy values[62]. For example, to determine the stage of liver fibrosis, some authors have used CT images processed by segmentation algorithms. Choi *et al*[67] used CNN upon CT images, whereas Chen *et al*[68] employed RF, KNN, SVM, and the NB classifiers with real-time tissue elastography imaging, age, and sex as feeding variables. In both cases, the ML approach outperformed the classical methods. Regarding treatment, different ML models have been used to define the best therapy for liver diseases such as carcinomas and virus-induced hepatitis. Jeong *et al*[69] used DNN to classify intrahepatic cholangiocarcinoma susceptible to adjuvant therapy following resection according to laboratory and clinicopathological markers and found it more accurate than the commonly used staging system.

Wübbolding *et al*[70] studied the prediction of early virological relapse analyzing soluble immune markers using supervised ML approaches like KNN, RF, and LR. This study showed that IL-2, monokine induced by interferon γ/CCL9, RANTES/CCL5, stem cell factor, and TNF-related apoptosis-inducing ligand in combination were more reliable in predicting virological relapse than viral antigens. In the same way, researchers have used ML classifiers to explore new methods able to better predict prognosis of liver diseases[71-74]. The weighted variables are usually CT images and/or biochemical parameters that involved invasive and costly methods. However, researchers have recently proposed volatile organic compounds as new biomarkers for progression and prognosis of liver disease. These researchers monitored isoprene, limonene, and dimethyl sulfide concentrations from a breath sample in liver patients compared to healthy subjects. They used regression ML models (LR, ETR, SVR, and RF) to demonstrate that these approaches together with breath profile data can predict clinical scores of liver disease[75]. These findings are promising and open the way for new, safe, and non-invasive approaches to study liver function and for diagnosis purposes.

ML methods have been employed when studying the comorbidities of liver-related diseases, like obesity, diabetes, and cardiovascular diseases[53,55,76]. For example, ML algorithms have been built to study the risk factors associated to overweight and obesity development, showing that BMI, age, dietary pattern, blood test results, socioeconomic status, and sedentarism were key factors when studying excess of adipose tissue[77]. In this line, further research has revealed by ML techniques that the minutes devoted to physical activity in one week[78], as well as specific species of gut microbiota[79], are also crucial for obesity prediction. ML algorithms have also elucidated the risk factors of childhood

Table 1 Summary of machine learning articles studying virus and inflammatory-related liver damage

Ref.	Objective	Subjects	Variables	ML model	Performance	Observations/remarks
Fialoke *et al* [63]	To predict NASH in NAFLD patients	*n* = 108139, NASH and healthy (non-NASH) populations	Demographic data, type 2 diabetes status, and blood biomarkers	RF, XGBoosting, DT, LR	AUROC of 88% by XGBoosting	The average and maximum value of ALT appeared was the most important variable
Ma *et al*[64]	To predict NAFLD in the general population	*n* = 10508, Subjects who attended a health examination	Age, blood biomarkers, and anthropometric data	LR, RF, SVM, baggin, DT, LR, KNN, BN, hidden NB, AdaBoosting, AODE	83% accuracy, 0.878 specificity, 0.675 sensitivity, and 0.655 F-measure score by BN	BMI, TG, GGT, ALT and uric acid were the top five predictors
Yip *et al*[65]	To detect NAFLD for the general population	*n* = 500, involving NAFLD patients and healthy subjects	Demographic, clinical data and blood biomarkers	LR, RIDGE regression, AdaBoosting, DT	AUROC of 90% by AdaBoosting	ALT, HDL-c, TG, HbA1c and white blood cells to predictors
Pei *et al*[66]	To identify FLD in general patients	*n* = 3419, patients of which 845 had FLD	Age, anthropo-metric, and blood biomarkers	RF, ANN, KNN, XGBoosting, LDA	0.9415 accuracy, 0.9306 AUC, and 0.9091 sensitivity by XGBoosting	Uric acid, BMI, and TG were the top three risk factors
Choi *et al*[67]	To stage liver fibrosis	*n* = 7461, patients with pathologically confirmed liver fibrosis	Age, sex, clinical data, CT images, and liver fibrosis stage	CNN	Overall staging accuracy of 79.4% and an AUROC of 0.96, 0.97, and 0.95 for diagnosing significant and advanced fibrosis, and cirrhosis, respectively	The model outperformed the radiologist's interpretation, APRI, and FIB-4 index
Chen *et al*[68]	To stage liver fibrosis in patients with CHB	*n* = 513, patients with confirmed liver fibrosis	Age, sex, CT liver images	RF, KNN, SVM, NB	0.8118-0.9125 accuracy by RF for all stages	The adopted classifiers significantly outperformed the liver fibrosis index method
Jeong *et al*[69]	To classify susceptible individuals for adjuvant treatment in patients with ICC after resection	*n* = 1421, ICC patients	Age, sex, clinical data, and blood biomarkers	DNN	AUC of 0.78	The model was found to be more accurate than the traditional AJCC stage classifier
Wübbolding *et al*[70]	To identify immune profiles for the prediction of early virological relapse	*n* = 284, patients with CHB and treated with NA antivirals	Age, sex, and analytical and blood biomarkers	KNN, RF, LR	AUC of 0.89	The combination of IL-2, MIG/CCL9, RANTES/CCL5, SCF, and TRAIL was reliable in predicting viral relapse
Hong *et al*[71]	To predict esophageal varices in patients with HBV related cirrhosis	*n* = 197, patients with HBV-related cirrhosis	PLT count, spleen width, and portal vein diameter	ANN	Sensitivity of 96.5%, specificity of 60.4%, accuracy of 86.8%	The model obtained a positive predictive value of 90.00%; and a negative predictive value of 80.85%
Zhong *et al*[72]	To compare the prognostic performance of ALBI and CTP grades for HCC treated with TACE combined with sorafenib as an initial treatment	*n* = 504, HCC patients	ALBI and CTP grades BCLC stage, clinical data and plasma α-fetoprotein	ANN	-	The ALBI grade had higher importance in survival prediction compared to the CTP one
Shi *et al*[73]	To predict in-hospital mortality after primary liver cancer surgery	*n* = 22926, HCC surgery patients	Age, sex, clinical, and hospital data	ANN, LR	97.28% of accuracy and 84.67 % of AUROC by ANN	ANN model had higher overall performance indices and accurately predicted in-hospital mortality
Shi *et al*[74]	To predict 5-yr mortality after surgery for HCC	*n* = 22926, HCC surgery patients	Age, sex, clinical, and hospital data	ANN, LR	96.57 % of accuracy and 88.51 % of AUROC by ANN	Surgeon volume was the top predictor parameter
Patnaik *et al*	To predict liver	*n* = 28, healthy patients	Age, anthropo-	LR, RF, SVR,	R^2 values of 0.78,	Isoprene, limonene and

[75]	function-related scores (MELD, APRI, CTP) using breath biomarkers	compared to *n* = 17, liver patients	metric data, blood biomarkers, breath analysis	ETR	0.82, and 0.85 for CTP score, APRI score, and MELD, respectively, by ETR	dimethyl sulfide can be potential biomarkers for liver disease
Butt *et al*[85]	To diagnose the stage of hepatitis C	*n* = 968, patients with HCV	Age, anthropo-metric data, blood biomarkers, and histological staging	ANN, RF, SVM, XGBoosting	98.89% precision by ANN	The model performed better than previously presented models by other authors
Wei *et al*[87]	To predict HBV and HCV-related hepatic fibrosis	*n* = 490, HBV patients; *n* = 254, and 230 HCV patients	Age, BMI, analytical data (FIB-4 score), and liver biopsy	GB, DT, RF	AUROC of 0.918 by GB	GB outperformed the FIB-4 predictive score
Barakat *et al* [89]	To predict and stage hepatic fibrosis in children with HCV	*n* = 166, children with CHC	Analytical data (APRI and FIB-4 scores)	RF	AUCs of 0.903 for any type of fibrosis	RF outperformed FIB-4 and APRI predictive score
Konerman *et al* [88]	To predict progression of HCV	*n* = 72683, veterans with CHC	Age, BMI, demographic, and blood biomarkers (APRI score)	CS and LGT Cox and boosting	AUROC of 0.830 and 0.77 sensitivity by LGT boosting model for 1 yr follow-up	APRI and PLT count were top predictors in the LGT boosting model
Wong *et al*[86]	To predict HCC in patients with CVH	*n* = 86804, CHV patients, of which 6821 with HCC	Age, sex, clinical data, and blood biomarkers	LR, RIDGE regression, AdaBoosting, RF, DT	AUROC of 0.992 and 0.837 by RF in training and validation cohort, respectively	ML models obtained better AUROCs than HCC traditional risk scores
Feldman *et al* [91]	To predict DAA therapy duration in hepatitis C	*n* = 3943, HCV patients with sofosbuvir/ledipasvir as the first course of DAA, of which *n* = 240, received the prolonged DAA treatment	Age, sex, and clinical data (including hepatitis C record data)	XGBoosting, RF, SVM	AUC of 0.745 by XGBoosting	Results showed age, comorbidity burden, and type 2 diabetes status as new predictors for DAA therapy duration
Kamboj *et al* [92]	To predict repurposed drugs for HCV	*n* = 17968 HCV molecular fingerprints	Experimentally validated small molecules from the ChEMBL database with bioactivity against HCV NS3, NS3/A4, NS5A and NS5B proteins	SVM, ANN, KNN, RF	R^2 value of 0.92 by SVM	Results identified more than 8 repurposed treatments anti-HCV
Tian *et al*[93]	To predict HBsAg seroclearance	*n* = 2235, patients with CHB, of which 106 achieved HBsAg seroclearance	Age, BMI, demographic and clinical data, and blood biomarkers	LR, RF, DT, XGBoosting	AUC of 0.891 by XGBoosting	Level of HBsAg followed by age and HBV DNA were the top predictors
Chen *et al*[94]	To predict HBV-induced HCC using quasispecies patterns of HBV	*n* = 307, CHB patients; *n* = 237, HBV-related HCC patients	*rt* nucleic acid and *rt*/s amino acid sequences	SVM, RF, KNN, LR	AUC of 0.96, and accuracy of 0.90 by RF	HBV *rt* gene features can efficiently discriminate HCC from CHB
Mueller-Breckenridge *et al*[95]	To classify HBeAg status in HBV patients using virus full-length genome quasispecies	*n* = 352, CHB untreated patients	Matrix of allele frequencies (0.1-0.99) and the associated HBeAg status	RF	Range balanced accuracy of 0.8-1	n1896GA, n1934AT, n1753TC mutants were the highest-ranking variables
Kayvanjoo *et al*[96]	To predict HCV interferon/ribavirin therapy outcome based on viral nucleotide attributes	*n* = 76, gene attributes	HCV nucleotide attributes	DT, SVM, NB, DNN	Accuracy of 84.17% by SVM in responder *vs* relapser of subtype 1b sequences	Dinucleotides UA and UU were top predictors in the combination treatment outcome
Li *et al*[98]	To distinguish influenza from COVID-19 patients	*n* = 398, COVID-19 and influenza cases	Age, sex, blood biomarkers, clinical data, and CT and X-ray scans	XGBoosting, RF, and LASSO and RIDGE regression models	AUC of 0.990, sensitivity of 92.5% and a specificity of 97.9% by XGBoosting	Age, CT scan result, and temperature were top three predictors
Bhargava *et al*	To detect novel	*n* = 31454, images	CT or X-ray scans	KNN, SRC,	99.14 of accuracy	SVM model classified with

[99]	COVID-19 and discriminate between pneumonia	acquired from nine distinct datasets of COVID-19 patients		ANN, SVM	by SVM	the highest recognition rate the images as normal, pneumonia, and COVID-19 positive
Bennett *et al* [97]	To predict early severity and clinically characterize COVID-19 patients	*n* = 174568, patients with a positive lab test for COVID-19	Age, sex, demographic, anthropometric and clinical data, and blood biomarkers	RF, LR, XGBoosting	AUROC of 0.87 by XGBoosting	Age, oxygen respiratory rate, and blood urea nitrogen were ranked as top predictor for severity outcome
Günster *et al* [100]	To identify independent risk factors for 180-d all-cause mortality in COVID-19 patients	*n* = 8679, hospitalized COVID-19 patients	Age, sex, BMI, and clinical data	LR	AUC of 0.81	A high BMI and age were strong risk factors for 180-d all-cause mortality, while female sex was protective
Deng *et al*[101]	To identify clinical indicators for COVID-19	*n* = 379, patients, 62 with COVID-19 and 317 with pneumonia	Age, sex, demographic and clinical data, and blood biomarkers	EBM	AUC of 0.948	Variables grouped under liver function was top the predictor category for COVID-19 prediction
Lipták *et al* [102]	To identify gastrointestinal predictors for the risk of COVID-19-related hospitalization	*n* = 680, patients	Age, sex, clinical data, and blood biomarkers	RF	AUC of 0.799	AST was top predictor for hospitalization
Elemam *et al* [103]	To identify immuno-logical and clinical predictors of COVID-19 severity and sequelae	*n* = 37, COVID-19 patients; *n* = 40, controls	Age, sex, BMI, clinical data, and blood biomarkers	Stepwise linear regression	AUC of 0.93 for cytokines as predictors. AUC of 0.98 for biochemical markers as predictors	IL-6 and granzyme B were top potential predictors of liver injury in COVID-19 patients
Mashraqi *et al* [104]	To predict adverse effects on liver functions of COVID-19 ICU patients	*n* = 140, COVID-19 patients admitted to ICU	Blood biomarkers and existence of liver damage	SVM, KNN, ANN, NB, DT	AUC of 0.857 and precision of 0.95 by SVM	AST and ALT were top predictors of liver damage in these patients
Soltan *et al* [106]	To evaluate a laboratory-free COVID-19 triage for emergency care	*n* = 114957, emergency presentations prior to the global COVID-19 pandemic and *n* = 437, COVID-19 positive	Blood biomarkers, blood gas, and vital signs	LR, XGBoosting, RF	AUROC range of 0.9-0.94 by XGBoosting for datasets	The model could effectively triage patients presenting to hospital for COVID-19 without lab results
Gao *et al*[111]	To predict mortality in patients with alcoholic hepatitis	*n* = 210, alcoholic hepatitis patients	Age, clinical data, blood biomarkers, and omics data sets (metage-nomics, lipidomics, and metabolomics)	GB, LR, SVM, RF	AUC of 0.87 by GB for 30-d mortality prediction using the dataset combining clinical data, bacteria and MetaCyc pathways and for and 90-d mortality prediction using the fungi dataset	The model performed better than the currently used MELD score

NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease; CHB: Chronic hepatitis B virus infection; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; CHC: Chronic hepatitis C virus infection; CVH: Chronic viral hepatitis; RF: Random forest; DT: Decision trees; LR: Logistic regression; SVM: Support vector machine; KNN: K-nearest neighbors; BN: Bayesian network; NB: Naïve Bayes; AODE: Aggregating one-dependence estimators; FLD: Fatty liver disease; ANN: Artificial neural networks; LDA: Linear discriminant analysis; CNN: Convolutional neural network; DNN: Deep neuronal network; SRC: Sparse representative classifier; EBM: Explainable boosting machine; CS: Cross-sectional; LGT: Longitudinal; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B virus e antigen; BMI: Body mass index; ALT: Alanine transaminase; AST: Aspartate transaminase; APRI: Aspartate transaminase/platelet ratio index; COVID-19: Coronavirus disease 2019; CT: Computed tomography; GB: Gradient Boosting; AUC: Area under the curve; AUROC: Area under the receiver operating characteristic curve; ICU: Intensive care unit; IL-6: Interleukin 6; DAA: Direct-acting antiviral; MELD: Model for end-stage liver disease; TG: Triglycerides; HbA1c: Glycated hemoglobin A1c; ICC: Intrahepatic cholangiocarcinoma; ML: Machine learning; ETR: Extra tree regression; AJCC: American Joint Committee on Cancer; CXCL: chemokine (C-X-C motif) ligand; CCL: C-C motif chemokine ligand; SVR: Support vector regression; MIG: Monokine induced by interferon γ; SCF: Stem cell factor; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; PLT: Platelet; GGT: Gamma-glutamyl transpeptidase; HDL-c: High density lipoprotein cholesterol; FIB-4: Fibrosis-4; HBV: Hepatitis B virus; TACE: Transarterial chemoembolization; BCLC: Barcelona Clinic Liver Cancer.

Table 2 Summary of the most repeated inputs of the machine learning models with the most repeated predictor outcomes for the four main inflammatory-related liver conditions

Inflammatory-related liver condition	Inputs	Most repeated predictors
FLD	Age, sex, blood biomarkers, and demographic, anthropometric, and clinical data	BMI, uric acid, TG, and ALT levels
Liver fibrosis	Age, sex, and CT images	Better diagnosis compared to classical methods like APRI and FIB-4 indexes
Virus-induced hepatitis	Age, sex, blood biomarkers, and demographic, anthropometric, and clinical data	AST, PLT levels, APRI index, and age
COVID-19	Age, sex, blood biomarkers, CT images, and demographic, anthropometric, and clinical data	Age, BMI, CT images, oxygen rate, AST, and ALT levels

FLD: Fatty liver disease; CT: Computed tomography; BMI: Body mass index; TG: Triglycerides; ALT: Alanine transaminase; AST: Aspartate transaminase; PLT: Platelet; APRI: Aspartate transaminase/platelet ratio index; COVID-19: Coronavirus disease 2019; FIB-4: Fibrosis-4.

obesity, of which parental BMI and the upbringing environment play a huge role[80-82]. Furthermore, researchers have observed by training a multivariate LR model with a dataset of 3634 children and adolescents' vitamin intake that vitamins A, D, B1, B2, and B12 were associated in a negative manner with obesity in this cohort[83]. These results are of interest since new insights are needed to discover novel targets to tackle comorbidities that affect liver function.

ML in hepatitis virus-induced liver damage

HBV and HCV infections can dangerously become chronic if not treated early and with the right treatment[84]. While scientists are still relentlessly working on an effective vaccine against HCV, a good and efficient diagnosis is key to prevent chronic HCV infection (CHC), and ML algorithms have been elucidated for this purpose. Thus, Butt *et al*[85] designed an ANN model and trained it with a dataset of 19 variables, among which age, sex, BMI, transaminase, and PLT count levels were included. The algorithm was able to better identify the stage of hepatitis C compared to other XGBoost, RF, and SVM models tested by other researchers with a higher precision rate and a decreased miss rate.

ML algorithms have been applied and compared to traditional methods used to follow HHV-induced advanced liver disease[86-88]. For instance, Wei *et al*[87] used a GB model trained with the same variables that the formula fibrosis-4 (FIB-4) uses, which are age, AST, ALT and PLT levels in a cohort of 490 HBV patients, and two cohorts of HCV patients (n = 240 each). The GB model outperformed FIB-4 score in classifying hepatic fibrosis and the existence of cirrhosis. Barakat *et al*[89] designed an RF model that also outperformed the FIB-4 score, as well as the AST/PLT ratio index (APRI), for prediction and staging of fibrosis in children with hepatitis C. In this line, data of 72683 veterans with CHC were used to predict the progression of the disease. GB models were used and compared with cross-sectional or linear models fed with variables like transaminases levels, alkaline phosphatase (ALP), PLT, AST, APRI, albumin, bilirubin, glucose, white blood cells, and BMI were included in the dataset. Results showed that APRI, PLT, AST, albumin, and AST/ALT ratio were the best predictors for featuring CHC progression[88].

Regarding therapy, CHC can be effectively treated with direct-acting antiviral (DAA) therapy, a novel treatment that targets viral non-structural proteins. Although it has null side effects compared to standard treatment, it has some downsides. Treatment failure in a low percentage of the cases, a very high cost, and no treatment duration established[90]. New methods to define this therapy duration are needed to optimize adherence and success. Feldman *et al*[91] studied the prediction of DAA treatment duration in hepatitis C patients using XGBoost, RF, and SVM models. They used the dataset of 240 patients with prolonged first course of DAA against another dataset of 3478 patients on standard duration. Age, sex, comorbidities, and previous hepatitis C treatment record were considered. The predictive model constructed with XGBoost obtained the best performance in predicting prolonged DAA treatment, in which the presence of cirrhosis, type 2 diabetes, age, HCC, and previous standard treatment were the most determining variables. Meanwhile, Kamboj *et al*[92] used ML approaches in the search of repurposed drugs that could target non-structural proteins, developing regression-based algorithms able to identify inhibitors of these proteins, and proposing new drugs to test in CHC.

A huge milestone when treating chronic HBV infection (CHB) is seroclearance of HBV surface antigen (HBsAg)[84]. It has been demonstrated that seroclearance of HBsAg is associated to a better prognosis in CHB. Some authors used ML models to predict HBsAg seroclearance in a cohort of 2235 patients, of which 106 achieved it. They used XGBoost, RF, and LR, among other models, and tested a total of 30 categorical and continuous variables, including sex, drinking history, initial diagnosis and treatment, age, BMI, and serum and radiological indicators. Results revealed that the XGBoost model showed the best predictive performance, indicating that HBsAg levels were the best predictor for

HbsAg seroclearance, followed by age, and the DNA level of HBV[93].

Interestingly, ML has also contributed to personalized medicine in this field. HHVs evolve and adapt to different cellular environments in order to escape immune responses and drugs to survive. These adaptations rely on high mutagenetic activity, especially within the target genes of antivirals. Regarding HBV, Chen *et al*[94] used ML to identify patients with HCC or CHB based solely on genetic differences and found that the RF model impressively discriminated both cases based on the *rt* gene sequence of HBV. Moreover, Mueller-Breckenridge *et al*[95] ultra-deep sequenced 400 HBV samples and used an RF model to classify the status of a particular HBsAg according to the novel viral variants encountered. Results showed five genotypes that could benefit from personalized healthcare. In the case of HCV, Kayvanjoo *et al*[96] built several ML algorithms and trained them with two datasets of responders *vs* non-responders of antiviral therapy in HCV infection caused by two different strains. These investigations reported novel genetic markers that could predict therapy response with high accuracy. These results are very promising since they contribute to bringing personalized medicine to the public system.

ML in COVID-19-induced liver damage

A recent systematic review depicted that the parameters normally used for liver impairment screening were significantly increased in COVID-19 patients[23]. Particularly, several studies showed that levels of AST and/or ALT can increase in these patients up to 20%, bilirubin up to 14%, ALP up to 6%, and GGT levels up to 21%. Prothrombin is a protein synthesized in the liver that results in thrombin, a protein with a critical role in coagulation function. Prolonged prothrombin is a symptom of decreased production of coagulation factors, characteristic of liver disease. For this reason, the prolonged prothrombin time (PT) is another parameter usually checked when screening for liver injury, and it has been described that COVID-19 patients present nearly a 10% increase in PT[23]. Besides biochemical alterations, COVID-19 illness can lead to hypoxemia, impaired cardiac function, and secondary damage due to multiple organ dysfunction, which can result in liver injury in patients with or without prior liver disease. Therefore, new insights of the relationship between this recent infectious illness and liver disease are expected.

The use of ML approaches has been encouraged by the National COVID Cohort Collaborative Consortium for early detection, prediction, and follow-up of severe COVID-19 cases since the pandemic started[97]. For instance, some researchers used the XGBoost approach and found that age, CT scan result, body temperature, lymphocyte levels, fever, and coughing can classify influenza patients from COVID-19 patients[98]. Bhargava *et al*[99] tried different ML approaches to detect novel COVID-19 and discriminate between pneumonia using CT and *X*-ray scans as inputs. These authors pre-processed the images by normalization and then segmented them by fuzzy c-means clustering. Results showed that the SVM model was the one that better classified patients in COVID-19 positive, pneumonia, and healthy groups, obtaining a very high accuracy.

In this same line, obesity and liver disease were identified as risk factors for higher clinical severity in a cohort of 174568 adults with severe acute respiratory syndrome associated with SARS-CoV-2 infection by a multivariable LR model[97]. Interestingly, a German study of 8679 patients used an LR model and identified liver disease and BMI as determinant risk factors for 180-d all-cause mortality in hospitalized COVID-19 patients[100]. A case-control study with COVID-19 patients compared to patients with community-acquired pneumonia showed how, by applying a GB model, the category of liver function appeared as one of the top systematic predictors for COVID-19 risk factors, with albumin, total bilirubin, and ALT among the most important input variables[101]. Furthermore, a study with 710 enrolled patients diagnosed with COVID-19 identified AST levels as the top predictor for COVID-19-related hospitalization based on an RF algorithm, followed by age and diabetes mellitus[102].

A stepwise linear regression model identified IL-6 and granzyme B as potential predictors of liver dysfunction, characterized by an elevation in the levels of ALT and/or AST[103]. Other authors designed a model for detecting liver damage testing different ML approaches with laboratory parameters as the input variables. SVM was the model with the best accuracy, and AST and ALT levels were the variables with the best predictive scores[104]. In this context, the newest version of the CURIAL model was developed to identify COVID-19 patients using vital signs, blood gas, and laboratory blood tests. It showed greater sensitivity, making this model a potential emergency workflow [105,106]. All these ML-based methods would dramatically improve the time to diagnosis, free hospital laboratories and rooms of potential positive subjects, and reduce costs if implemented in the public health system.

AI has also been employed to discover potential efficient new drugs to tackle SARS-CoV-2 infection [107]. Baricitinib is a drug initially approved for rheumatoid arthritis that was selected by ML as a potential drug to treat COVID-19. Researchers proved the anti-inflammatory and antiviral properties of this drug in human liver spheroids infected with live SARS-CoV-2 to check any potential drug-induced liver injury[107]. Due to the good results, researchers moved on to a clinical trial where they tested baricitinib in a few COVID-19 patients. Levels of liver enzymes were not altered, except for a transient increase in liver aminotransferases in all patients that remitted in the following 72 h without interrupting treatment. The authors stated that this might be reflective of disease severity rather than a drug-induced injury, showing overall good tolerance and results in this pilot study[108]. In summary, ML approaches support liver biochemistry as a prognostic tool in COVID-19 disease.

PERSONALIZED MEDICINE IN LIVER-RELATED DISEASES SUPPORTED BY ML

In the early 21st century, the Human Genome Project started the genomic era in which new disciplines like precision medicine appeared. Precision medicine aims to deliver targeted treatments based on a group of individual factors that greatly influence the onset and progression of a disease, like omics sciences. This approach covers a great number of patients, overcoming potential adverse effects and ensuring effectiveness of the treatment. In this context, computational advances have greatly contributed to the escalation of this science by lowering the costs of omics analysis and allowing the processing and integration of an enormous amount of data based on ML algorithms (Figure 2).

ML has permitted the development of diagnostics and therapeutics based on the integration of omics data (genomics, epigenomic, transcriptomics, proteomics, metabolomics, and metagenomics) with clinical data. The ultimate goal is to bridge these omics data with the phenotype to bring molecular accuracy to the diagnosis, treatment, prognosis, and recurrence process of a pathological condition. This methodology has been used in a wide range of diseases in the search for more efficient and effective approaches, like heart and liver diseases[109,110]. For example, ML algorithms fed with omics data have been able to predict mortality in patients with alcoholic hepatitis. In this study, routine clinical variables of 210 patients with this disease were used to build six different datasets to assess mortality at 30 d and 90 d. Five different ML models were tested, obtaining the best performance in predicting 30-d mortality with a GB model using bacteria, MetaCyc pathways, and clinical data, as well as LR using viral and clinical data[111].

In hepatitis B, it has been found that ML algorithms can be very useful in assessing HBV-associated HCC progression. Ye *et al*[112] analyzed 67 HBV-positive HCC samples with or without intrahepatic metastases and discovered key genes for metastatic progression and survival training ML models. The majority of them were inflammatory or related to the inflammation process, like IL-2 receptor and osteopontin, which encodes an extracellular cytokine ligand whose overexpression favors metastasis. These authors were able for the first time to draw a molecular signature useful to classify metastatic HBV-HCC patients, opening the way for early detection and new treatments to increase patient survival. In hepatitis C, the CC and CT genotypes of the rs12979860 polymorphism in the *IL28B* gene have been associated with liver fibrosis progression, being able to predict antiviral treatment effectiveness[113].

Moreover, ML algorithms have allowed the diagnosis of advanced liver fibrosis according to the rs12979860 genotype with higher performance compared to APRI and FIB-4 scores[114]. In this study, patients were divided into two groups according to HCV-related liver fibrosis stage: None to moderate fibrosis (n = 204); or with advanced fibrosis (n = 223). ML algorithms revealed the *IL28B* genotype as the first predictor, while the second predictor depended on the mentioned genotype. For instance, in CT patients, PLT, albumin, and age were the determining variables, while for patients with the TT genotype, white blood cell count was the decisive feature to assess advanced fibrosis probability.

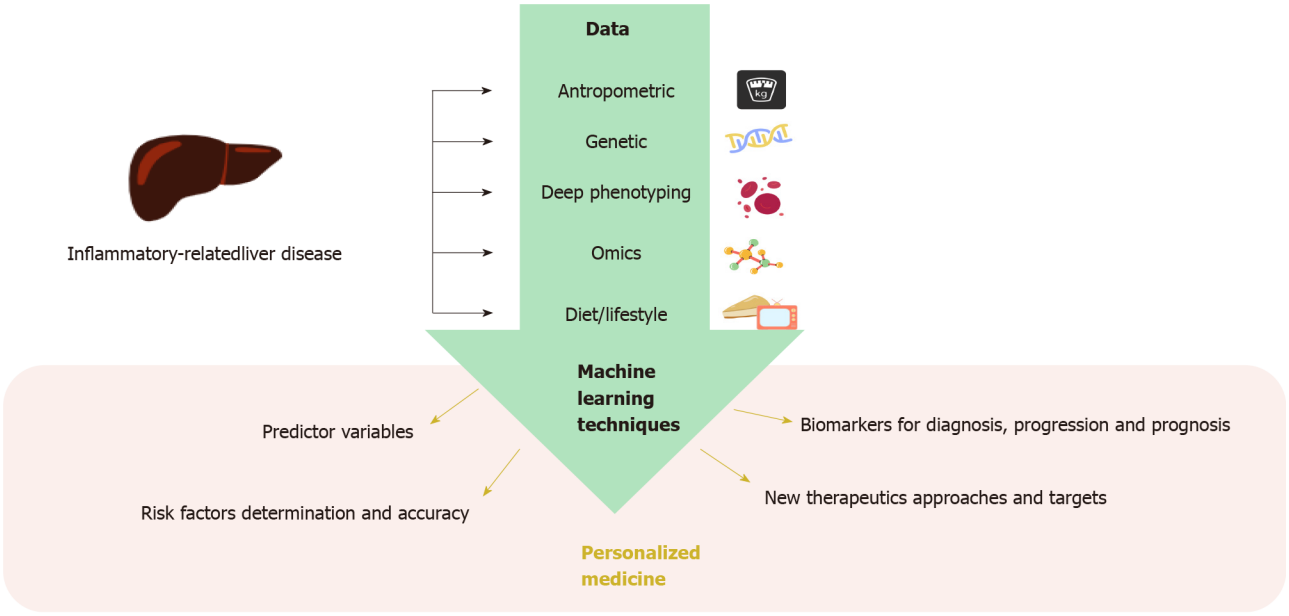
ML approaches have also helped to categorize obesity in different subtypes based on metabolic status [115-117]. For example, Masi *et al*[115] studied a cohort of 2567 subjects suffering from obesity and made clusters of metabolically healthy or metabolically unhealthy patients based on clinical and biochemical variables using two ML models. The first model showed that IR, body fat, HbA1c, red blood cells, age, ALT, uric acid, white blood cells, insulin growth factor-1, and GGT were the top predictors of a metabolically healthy obesity, revealing the importance of liver function.

Other authors have also used ML models to classify 882 obese patients in subtypes of obesity according to glucose, insulin, and uric acid levels[116]. Results showed four stable metabolic clusters in this cohort, which were characterized by a healthy metabolic status, or by hyperuricemia, hyperinsulinemia, and hyperglycemia, respectively. Furthermore, Lee *et al*[117] explored three-way interactions between genome, epigenome, and dietary/lifestyle factors using GB and RF models in a subset (n = 394) of the exam 8 of the Framingham Offspring Study cohort. Interestingly, GB obtained the best performance, revealing 21 single nucleotide polymorphisms, 230 methylation sites in relevant genes (like *CPT1A*, *ABCG1*, and *SREBF1*), and 26 dietary factors as top predictors for obesity. Intake of processed meat, artificially sweetened beverages, French fries, and alcohol intake, among other dietary factors, were highly associated with overweight/obesity.

Personalized and precision medicine aims to harmonize the greatest number of factors so that diagnosis, prognosis, and treatment are based on the greatest number of decision elements. Much remains to be investigated to establish guidelines in the context of personalized medicine. However, it is safe to say that precision medicine will drive modern medicine, combining the most classic variables with the newest digital variables. Health professionals must be prepared to understand and implement these new technologies in the near future.

CONCLUSION

In summary, ML science can process and integrate a vast amount of different data with unprecedented outstanding performance. The objective of this article was to collect the information derived from ML



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Figure 2 Data implicated in the onset of inflammatory-related liver diseases can be used to train machine learning algorithms for prediction, diagnosis, treatment, and prognosis of chronic liver disease, leading the way to personalized medicine.

techniques in liver damage induced by inflammatory conditions, including the new disease COVID-19. The main role of ML in liver pathologies is to help identify high risk patients for referral to specialized centers. Results show that the use of ML models have brought new insights into biology and medicine questions that can be very useful in determining the next directions for research in diagnosis, prognosis, and treatment of inflammatory and virus-related liver diseases, leading the way to personalized medicine. Also inflammation/IR biomarkers related to liver disease can be boosted by ML strategies. This review clarified and compiled the importance of the different factors involved in CLD and analyzed by ML algorithms, which can be useful information for clinicians, like endocrinologists and gastroenterologists, and other healthcare professionals with a focus on hepatology and bioinformatics.

FOOTNOTES

Author contributions: Martínez JA and Alonso-Bernáldez M contributed equally to this work as first co-authors. Martínez JA and Ramos-Lopez O conceived and designed the study; Martínez JA, Alonso-Bernáldez M, and Ramos-Lopez O performed the search of articles and wrote the draft of the manuscript; Martínez-Urbistondo D, Vargas-Nuñez JA, Dávalos A, and Ramos-Lopez O contributed to the analysis and critical interpretation of the data; and all authors read and approved the final manuscript.

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MINIREVIEWS

Development of Epstein-Barr virus-associated gastric cancer: Infection, inflammation, and oncogenesis

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Abstract

Epstein-Barr virus (EBV)-associated gastric cancer (EBVaGC) cells originate from a single-cell clone infected with EBV. However, more than 95% of patients with gastric cancer have a history of *Helicobacter pylori* (*H. pylori*) infection, and *H. pylori* is a major causative agent of gastric cancer. Therefore, it has long been argued that *H. pylori* infection may affect the development of EBVaGC, a subtype of gastric cancer. Atrophic gastrointestinal inflammation, a symptom of *H. pylori* infection, is observed in the gastric mucosa of EBVaGC. Therefore, it remains unclear whether *H. pylori* infection is a cofactor for gastric carcinogenesis caused by EBV infection or whether *H. pylori* and EBV infections act independently on gastric cancer formation. It has been reported that EBV infection assists in the oncogenesis of gastric cancer caused by *H. pylori* infection. In contrast, several studies have reported that *H. pylori* infection accelerates tumorigenesis initiated by EBV infection. By reviewing both clinical epidemiological and experimental data, we reorganized the role of *H. pylori* and EBV infections in gastric cancer formation.

Key Words: *Helicobacter pylori*; Epstein-Barr virus; Epstein-Barr virus-associated gastric cancer; Coreceptor; Inflammation; Oncogenesis

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Core Tip: Epstein-Barr virus (EBV)-associated gastric cancer (EBVaGC) tumor cells originate from a single cell clone infected with EBV. In contrast, it is reported that more than 95% of patients with gastric cancer have a history of *Helicobacter pylori* (*H. pylori*) infection. Accordingly, it has long been argued that *H. pylori* infection may have some effect on the development of EBVaGC, a subtype of gastric cancer. It is also a mystery that the number of gastric cancer patients is higher in Asia, South America, and the Middle East. We will reorganize the role of *H. pylori* and EBV infections in gastric cancer formation.

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INTRODUCTION

Epstein-Barr virus (EBV)-associated gastric cancer (EBVaGC) accounts for 10% of all gastric cancers. At the same time, more than 95% of patients with gastric cancer have a history of *Helicobacter pylori* (*H. pylori*) infection. Thus, the question arises as to whether *H. pylori* and EBV infections promote gastric cancer formation in a dependent or independent manner. The high prevalence of gastric cancer in Asia, South America, and the Middle East is also intriguing.

EBV IS AN ONCOGENIC HUMAN HERPESVIRUS

EBV infects B lymphocytes and epithelial cells and is an oncogenic virus that assists in the proliferation of latently infected cells, resulting in the development of Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma, and EBVaGC[1].

More than 90% of adults are latently infected with EBV; however, cytotoxic T lymphocytes that recognize EBV antigens suppress the proliferation of viral antigen-positive cells. When the local or systemic immune function is compromised, EBV-positive cells begin to proliferate. B lymphocytes that migrate to local areas where immune surveillance is weak often transition to lytic infection, resulting in viral production. Under such conditions, EBV appears to be transmitted to and infects gastric epithelial cells. The expression of EBV genes causes epithelial cells to acquire proliferative properties and resist apoptosis, and cells that escape immunological elimination may begin proliferating[2].

EBV-ASSOCIATED GASTRIC CANCER

Molecular features

Classification of gastric cancer by molecular mechanism was performed through an exhaustive analysis of next-generation sequencing data from numerous cases. The results divided gastric cancer into the following four molecular subtypes: Microsatellite instability (MSI), chromosomal instability (CIN), genomically stable (GS), and EBV[3]. These classifications have facilitated the identification of specific therapeutic candidates for each subtype of gastric cancer, and have revealed that each of these four subtypes is driven by a specific developmental mechanism that needs to be clarified individually. In particular, the molecular biology of EBVaGC is characterized by frequent and extensive methylation of the promoter regions of tumor cell genes[4]. *De novo* EBV infection induces DNA methylation in more than 3000 gene promoter regions within 4 wk[4]. However, methylation of the promoter of the mismatch repair gene *MLH1*, which is frequently observed in MSI, is not observed in EBVaGC[5]. In addition to inactivation by DNA methylation, the EBV genome binds to heterochromatin, a region of inactivation that causes aberrant activation of the region (enhancer infestation) and increases the expression of surrounding proto-oncogenes[6].

Clinical features

In EBVaGC, which accounts for 5%–10% of all gastric cancers, all tumor cells are infected with EBV. Endoscopy is the most informative method for diagnosing gastric cancer. EBVaGC is observed as a superficial depressed lesion in the upper part of the stomach. Using endoscopic biopsy specimens, EBV-

encoded RNA *in situ* hybridization (EBER-ISH), stains all gastric cancer cells positive for EBER, even in the intramucosal cancer stage[7]. The histological hallmark of EBVaGC is lymphoepithelioma-like carcinoma, in which a diffuse lymphocytic infiltrate is observed around EBER-positive epithelial tumor cells[8]. Furthermore, EBVaGC tumor cells are derived from the proliferation of a single EBV-infected epithelial cell[8,9].

Many studies have shown a male predominance (2-fold) of EBVaGC, suggesting that the risk may exist in male lifestyle and occupational factors[10]. The percentage of patients with EBVaGC to those with total gastric cancer is higher in younger patients. In men, the proportion of EBVaGC decreases with increasing age, especially in patients with pyloric gastric cancer. In women, the decrease in the proportion of EBVaGC with increasing age is unclear. Consumption of salty foods that cause mechanical damage to the gastric epithelium as well as exposure to wood and iron filings are associated with a higher EBVaGC risk[11].

EBVaGC is a gastric cancer with a relatively good prognosis. A Dutch study reported that EBVaGC is characterized by fewer lymph node metastases, less residual disease, and younger patient age, which results in longer disease-free survival[12]. Cohort study data from TCGA also reported that EBVaGC has the best recurrence-free period and overall survival compared to MSI, GS, and CIN subtypes[13].

EBVaGC tumors are frequently found in non-antral parts of the stomach[10,14]. In contrast, *H. pylori*-associated gastric cancer mostly occurs in the antral region[10]. Because moderate to severe atrophic gastric mucosa due to *H. pylori* infection was characteristically observed surrounding early gastric cancers, gastritis may play an important role in the tumorigenesis of EBVaGC[14]. Development of gastric cancer is supposed to follow the "infection, inflammation, and carcinogenesis" route, which consists of *H. pylori* infection followed by chronic gastritis, intestinal metaplasia, and cancer. In contrast, in the case of EBVaGC, it is controversial whether tumor formation is initiated by EBV-infected normal mucosal cells or promoted by EBV-infected cells in precancerous lesions[15]. Abe *et al*[16] performed EBER-ISH on 1110 sections of non-neoplastic gastric mucosal tissue from 300 cases and found 2 (0.18%) ductal-level EBER-positive lesions.

The mutual contribution of EBV and *H. pylori* in the carcinogenesis will be discussed later in the chapter “Inflammation and carcinogenesis”.

EBV INFECTION OF EPITHELIAL CELLS

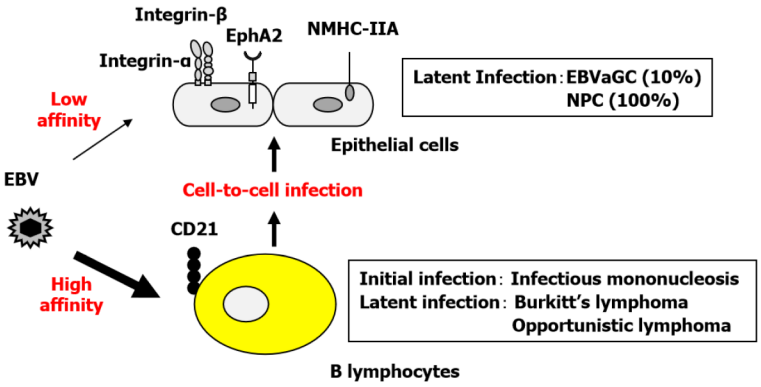
EBV infects B lymphocytes through the binding of the viral glycoprotein gp350 to the high-affinity receptor CD21, followed by binding of gp42 to HLA class II molecules, resulting in membrane fusion [17]. In contrast, when low-affinity co-receptors are used to infect CD21-negative epithelial cells, the infection efficiency is extremely low (Figure 1).

The CD21-independent routes of epithelial cell infection include the following: (1) The viral envelope glycoprotein gp350/220 binds to CD35; (2) Integrins αVβ5, αVβ6, and αVβ8 interact with the viral envelope glycoprotein gH/gL complex to fuse the viral envelope with the epithelial cell membrane; (3) The BMRF2 membrane protein expressed during EBV lytic infection binds to α3, α5, αV, and β1 integrins; and (4) EphA2 and NMHC-IIA bind to gH/gL produced by many herpesviruses and enhance infection efficiency.

A previous study reported that a boy with X-linked agammaglobulinemia who did not have mature B lymphocytes due to a genetic enzymatic deficiency did not develop an EBV infection[18]. EBV infection of epithelial cells was considered to occur after EBV infection of B lymphocytes because the epithelial cells of the affected boy were intact. EBV-infected B lymphocytes are believed to carry and deliver EBV to the epithelial cells *via* cell-to-cell transfer. In the case of CD21-independent infection, the efficiency of epithelial cell infection by cell-to-cell transfer is more than 1000 times higher than that of direct epithelial cell infection by EBV particles[19]. It is speculated that infection of epithelial cells *via* B lymphocytes is promoted when viral activation and lymphocyte infiltration are accompanied by inflammation (Figure 1).

EBV-associated gastric cancer-derived cell lines

In EBVaGC, all tumor cells are infected with EBV. However, cell lines established from gastric cancer tissues, similar to those in nasopharyngeal carcinoma, are almost entirely EBV-negative[20]. The EBV genome in EBVaGC tumor cells exists as a plasmid-like episome that does not integrate into the host chromosomes. However, the presence of the virus does not appear to favor cell growth *in vitro*. Rather, it may be more convenient for *in vitro* cell growth to avoid the use of extra energy to maintain the episomes. Alternatively, the expression of viral genes such as microRNAs may be crucial for tumor cells to evade elimination by the *in vivo* immune system. In fact, EBV-positive KT cells established from EBVaGC can only be passaged by transplantation into SCID mice and cannot be expanded in an *in vitro* culture system[21]. SNU-719, YCCEL1, and NCC-24 are rare cells established from EBVaGC and can be propagated *in vitro*. These cell lines appear to be unique because the presence of EBV episomes is essential for their growth. Experiments with hydroxyurea and EBNA1 siRNAs were not successful in shedding the EBV episome from SNU-719 cells[22].



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Figure 1 Epstein-Barr virus infects B lymphocytes and epithelial cells to form tumors. Epstein-Barr virus (EBV) infects B lymphocytes and epithelial cells *via* the CD21 receptor and co-receptors, respectively. Although the efficiency of epithelial cell infection is extremely low, approximately 1000000 times lower than that of B lymphocytes, cell-to-cell EBV infection by B lymphocytes increased the efficiency of EBV infection by more than 1000-fold. The squares show the EBV infection status and disease names that are established differently depending on the cell type. EBV: Epstein-Barr virus; EBVaGC: Epstein-Barr virus-associated gastric cancer; NPC: Nasopharyngeal carcinoma.

Gastric epithelial cell lines infected with recombinant EBV

We established gastric epithelial cells infected with recombinant EBV, where a drug-resistant gene was inserted into the nonessential *BXLF1* (thymidine kinase) gene (Figure 2). It is possible to elucidate the oncogenic molecular mechanism of EBV-infected epithelial cells by comparing EBV-positive cells with EBV-negative cells. EBV infection markedly promotes the proliferation of gastric epithelial cells[23].

EBV-infected gastric epithelial cells also exhibit type I latent infection that expresses EBNA1 and LMP2A, similar to that in EBVaGC *in vivo*. EBNA1 promotes tumorigenesis *via* p53 ubiquitination, suppresses transforming growth factor-β signaling, and enhances the transcription of the anti-apoptotic protein survivin[24]. In contrast, LMP2A activates PI3K/Akt signaling similar to that activated by B-cell receptor stimulation, increases survivin expression, and resists apoptosis[25]. LMP2A also induces DNA methyltransferases, resulting in epigenetic changes in infected cells[26]. *BARF1* is strongly expressed as a latent gene in EBV-associated epithelial tumors[27]. Nasopharyngeal carcinoma-derived cells infected with recombinant EBV constitutively expressing *BARF1* exhibit resistance to apoptosis[28].

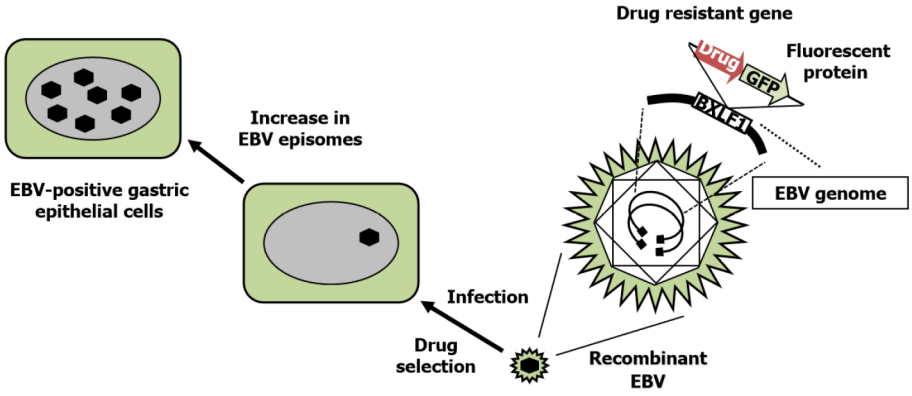
In addition to the oncogenic activity of EBV proteins expressed in type I latent infections, non-coding RNAs (miRNAs and EBERs) that are not translated into proteins have been investigated. Multiple BART miRNAs cooperatively repress lytic replication[29]. BART miRNAs also downregulate pro- and anti-apoptotic mediators such as caspase 3[30]. EBERs bind to protein kinase R and disrupt innate immune function[31]. Elimination of EBER2 from the EBV genome reduces the efficiency of B lymphocyte transformation[32].

INFLAMMATION AND CARCINOGENESIS

Clinical observation

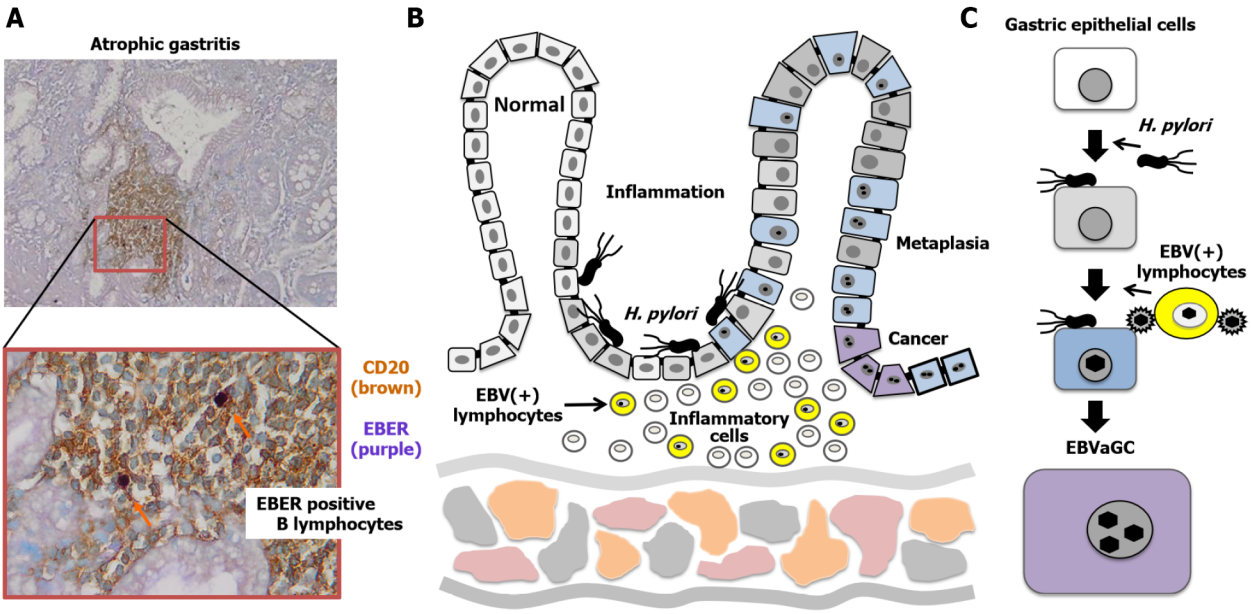
It is very difficult to collect EBVaGC cases without *H. pylori* infection, because most patients with gastric cancer are infected with *H. pylori*[33,34]. However, a clinical study was conducted to investigate the relationship between EBV infection, *H. pylori* infection, and atrophic gastritis in 468 patients with chronic gastritis[35]. This study confirmed that patients who were EBV-positive had a lower pepsinogen I/pepsinogen II ratio than patients who were EBV-negative. EBV infection significantly increases the risk of atrophic gastritis, especially in *H. pylori*-negative patients. However, a report from Mexico mentioned that EBER1 *in situ* hybridization showed that EBV infection of epithelial cells could be detected in gastric cancers as well as in one-third of non-atrophic gastritis samples[36]. This study showed that EBV infection affected early cancer precursor lesions. However, it is difficult to determine whether EBV causes cancer directly or indirectly by triggering inflammation.

Inflammation and initiation of innate immune mechanisms promote EBV activation, although it is difficult to assess the extent to which these mechanisms are involved in tumorigenesis of EBV-infected cells (Figure 3). EBV proliferation occurs at the early stage of EBVaGC formation because early antigens-immunoglobulin G (IgG) and viral capsid antigen-IgG antibodies against early viral antigens and capsids are elevated in the sera of patients with EBVaGC. In addition, while the incidence of EBVaGC is approximately 10% worldwide, the incidence of gastric cancer after surgical invasion by gastric anastomosis increases by three times (30%)[8].



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Figure 2 Isolation of recombinant Epstein-Barr virus-infected gastric epithelial cells. A drug resistant gene and fluorescent protein gene were inserted into the viral genome (*BXLF1*) using gene recombination technique. *BXLF1* is an *EBV* gene that does not affect viral production or infectivity by disruption through the insertion of marker genes. It is possible to isolate only recombinant virus-infected cells by infecting cells with a recombinant virus and selecting them for a drug. When recombinant virus-infected cells are cultured in the presence of the drug, the viral plasmid copy number in the nucleus of infected cells increases[52]. EBV: Epstein-Barr virus; GFP: Green fluorescent protein.



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Figure 3 Interaction of *Helicobacter pylori* and Epstein-Barr virus in the formation of Epstein-Barr virus-associated gastric cancer. A: Infiltration of Epstein-Barr virus (EBV)-infected B lymphocytes in non-tumor areas of EBV-associated gastric cancer. Numerous CD20-positive B lymphocytes infiltrate the submucosal lesions of atrophic gastritis. CD20 is stained brown and EBV-encoded RNA (EBER) is stained purple. EBER-positive B lymphocytes are indicated by arrows. Chronic gastritis in the background is counterstained by Hematoxylin-Eosin staining; B: Induction of inflammatory cytokine production by bacterial adhesion to epithelial cells and tumorigenesis of EBV-infected epithelial cells. *Helicobacter pylori* adhesion induces production of inflammatory cytokines from gastric epithelial cells. Inflammation of gastric mucosa leads to an accumulation of various immune cells. EBV-positive B lymphocytes localized in the submucosa are activated by inflammation and transition from latent to lytic EBV infection. The viral particles produced are transferred to gastric epithelial cells, and the infected epithelial cells eventually form tumors; C: EBV transfer infection and tumorigenesis of epithelial cells *via* activated EBV-positive B lymphocytes. Infectious viral particles produced during inflammation adhere to CD21-positive B lymphocytes and transferred to epithelial cells expressing EBV coreceptors. Thus, gastric epithelial cells infected with EBV form Epstein-Barr virus-associated gastric cancers over time. EBV: Epstein-Barr virus; EBVaGC: Epstein-Barr virus-associated gastric cancer; *Helicobacter pylori*: *H. pylori*; EBER: EBV-encoded RNA.

Here, we investigated the relationship between *H. pylori*-associated gastritis and EBV propagation in the stomach. Gastric biopsy specimens were collected from patients with chronic atrophic gastritis and categorized into three histopathological stages: Mild, moderate, and severe. The specimens were subjected to DNA extraction and quantitative polymerase chain reaction to quantify EBV genome copy numbers[37]. More than 900 copies of the EBV genome have been frequently detected in patients with moderate atrophic gastritis. In other words, EBV frequently activates proliferation in patients with *H. pylori* infection with moderate chronic atrophic gastritis and strong histological inflammation.

In contrast, EBVaGC is significantly associated with marked mucosal atrophy and moderate to marked lymphocytic infiltration, but there is no direct association with intestinal metaplasia[7]. Although this appears to indicate that EBVaGC is not directly associated with *H. pylori* infection, this result is consistent with our findings. This is because the intestinal metaplastic epithelium resulting from prolonged gastritis is an unsuitable mucosal environment for the growth of both *H. pylori* and EBV [38].

Experimental observation

Several studies have been investigated the interaction between EBV and *H. pylori* in gastric epithelial cell lines. Because it is difficult to infect the epithelial cells with the two microorganisms simultaneously, experiments have been conducted on sequential infection with EBV first and *H. pylori* second, or vice versa.

Persistent infection of the gastric mucosa by CagA-positive *H. pylori* strains causes gastric cancer. This is because the tyrosine-phosphorylated CagA protein binds to the tyrosine phosphatase SHP2 in gastric epithelial cells, activating *Ras* oncogene. In contrast, SHP1, which competes with SHP2 weakens the oncogenic activity of SHP2. Saju *et al*[39] showed that EBV infection of gastric epithelial cells activates host cell promoter methylation and decreases SHP1 expression[39]. In other words, SHP2 activity is relatively higher and EBV infection promotes carcinogenesis of *H. pylori* associated gastric carcinoma. The induction of DNA methylase by EBV infection in gastric epithelial cells also decreases the expression of tumor suppressor genes such as *APC*, *breast cancer susceptibility gene 1*, and *phosphatase and tensin homolog deleted from chromosome 10* (*PTEN*)[40].

Furthermore, activation of innate immune signals by *H. pylori* attachment enhances the expression of the EBV co-receptor EPHA2 in gastric epithelial cells, thereby increasing the frequency of EBV infection in epithelial cells[41]. Another study demonstrated that organoids derived from gastric cancer cells were infected with EBV but did not infect those derived from the normal gastric epithelium[42]. The probable reason for this is that gastric organoids maintain cell polarity and express EPHA2 only between cells. Therefore, the localization of EPHA2 might change due to gastric epithelial cell injury caused by *H. pylori* infection or by a prior gene mutation, which subsequently facilitates EBV infection.

TUMORIGENIC MECHANISM OF EBV-INFECTED EPITHELIAL CELLS

At present, it is difficult to infect primary gastric epithelial cells with EBV and immortalize them. Instead, gastric epithelial cell lines persistently infected with EBV have been used to elucidate the tumorigenic mechanisms of EBV genes during latent infections.

EBV genes that encode untranslational RNA

The EBV genome contains two miRNA clusters, consisting of four BHRF1 miRNAs and 40 BART miRNAs. Although BHRF1 miRNA is poorly expressed in epithelial cells, BART miRNAs are highly expressed in latently infected epithelial cells and play a substantial role in tumorigenesis[43].

Epigenetic changes of gene expression in EBV-infected epithelial cells

Modification of gene expression *via* methylation is frequently observed in patients with EBVaGC. Tumor suppressor genes, such as *p14*, *p16*, *p73*, *PTEN*, *APC*, *RASSF1A*, and *CXXC4*, are repressed by promoter methylation. And the expression of molecules important for cell invasion, including THBS1, E-cadherin (CDH1), and TIMP2, is also repressed by promoter methylation. The decreased expression of these molecules may be involved in carcinogenic processes[44].

Multiple EBV episomal DNAs have been shown to approach enhancer sites in the genome, alter the surrounding chromatin structure (enhancer infestation), and activate genes such as transcription factors [6]. Although epigenetic analyses have been conducted to understand tumorigenesis, the overall mechanism remains unclear.

Model of tumorigenesis for epithelial EBV infection

Viral gene products transcribed in cells latently infected with EBV confer resistance to apoptosis. *EBV* gene products also accumulate mutations in the genes of the infected cells. Genetic changes in infected cells further affect *EBV* gene expression and alter intercellular communication, including the cross-talk between EBV-infected epithelial cells and immune cells[45] or epithelial-mesenchymal transition[46]. In other words, changes induced by persistent EBV infection in host cell signaling and host immune responses advance the tumorigenic stage[47].

FUTURE PROSPECTS

With the progress in research on EBER, miRNA, and long non-coding RNA, the functions of these

molecules in latent EBV-infected cells are being elucidated. A highly tumorigenic B81 EBV strain was isolated from a patient with nasopharyngeal carcinoma[48]; however, an EBV strain unique to gastric cancer has not yet been isolated.

Host gene mutations frequently observed in EBVaGC, including changes in *PIK3CA*, *ARID1A*, *PD-L1*, and *PD-L2*[3] are considered to affect histological characteristics, clinical course, and response to treatment. EBV-induced tumorigenesis is believed to be affected by environmental factors such as previous infections; however, the molecular basis that characterizes EBVaGC remains to be elucidated.

Considering that EBVaGC most strongly expresses *PD-L1* and *PD-L2* among the four molecular subtypes of gastric cancer, immune checkpoint inhibitors are expected to be effective therapeutic agents for EBVaGC[49,50]. *PIK3CA* mutations and *JAK2* amplification are frequently observed in EBVaGC. Therefore, PI3K and JAK2 inhibitors may be effective. Other EBNA-1 inhibitors are also expected to be EBV-specific therapeutic agents[51].

CONCLUSION

Several clinical and experimental data support the etiological role of *H. pylori* in EBV-associated gastric cancer.

FOOTNOTES

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MINIREVIEWS

Glucagon-like peptide-2 analogues for Crohn's disease patients with short bowel syndrome and intestinal failure

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Abstract

Short bowel syndrome (SBS) with intestinal failure (IF) is a rare but severe complication of Crohn's disease (CD), which is the most frequent benign condition that leads to SBS after repeated surgical resections, even in the era of biologics and small molecules. Glucagon-like peptide-2 analogues have been deeply studied recently for the treatment of SBS-IF. These drugs have a significant intestinotrophic effect and the potential to reduce the chronic dependence of SBS-IF patients on parenteral support or nutrition. Teduglutide has been approved for the treatment of SBS-IF, and apraglutide is currently in clinical development. The use of these drugs was examined with a focus on their use in CD patients.

Key Words: Short bowel syndrome; Intestinal failure; Crohn's disease; Glucagon-like peptide-2 analogues; Teduglutide; Apraglutide; Glepaglutide

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Core Tip: Short bowel syndrome with intestinal failure and chronic dependency on parenteral support are rare but severe complications of Crohn's disease (CD) after repeated intestinal resections. New therapeutic options are available, including glucagon-like peptide-2 analogues. Their use in CD appears safe and efficacious, but more data from specifically designed studies are needed.

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INTRODUCTION

Short bowel syndrome (SBS) in adults is a condition in which the normal length of the bowel (which ranges from 3 to 8 metres) is reduced to less than 2 metres[1]. It is classified anatomically into: (1) Type 1: End-jejunostomy; (2) Type 2: Jejuno-colonic anastomosis; and (3) Type 3: Jejuno-ileal anastomosis[2]. The onset of SBS may lead to intestinal failure (IF), which is defined according to the European Society for Clinical Nutrition and Metabolism as the reduction of gut function below the minimum necessary for the absorption of macronutrients and/or water and electrolytes, and intravenous supplementation is required to maintain health and/or growth. A reduction in gut absorptive function that does not require intravenous supplementation to maintain health and/or growth is considered intestinal deficiency. IF is categorised according to temporal and functional evolution into: (1) Type 1 - acute, short term and self-limiting; (2) Type 2 - a prolonged acute condition, often in metabolically unstable patients, that requires complex multidisciplinary care and intravenous supplementation over periods of weeks or months; and (3) Type 3 - a chronic condition in stable patients[3].

However, not all patients with SBS develop IF, and not all patients with IF have underlying SBS[1]. The anatomical classification of SBS is mirrored by different clinical presentations: (1) Type 1 (end-jejunostomy with no colon in continuity) shows a higher risk of dehydration and electrolyte imbalance, especially immediately after surgical resection; (2) Type 2 (jejuno-colonic anastomosis) patients may develop malnutrition in a long-term setting (months to years); and (3) Type 3 (jejuno-ileal anastomosis with ileo-caecal valve conservation) has a lower risk of malnutrition[4].

The causes of SBS-IF in 2919 patients with benign chronic IF were CD (22.4%), mesenteric ischaemia (17.7%), surgical complications (15.8%), primary chronic pseudo-obstruction (9.75%) and radiation enteritis (7.3%). The pathogenetic mechanisms of IF were heterogeneous in the same population, with most patients presenting (38.6%) with end jejunostomy, and approximately 20% of patients presenting with jejuno-colonic anastomosis[5]. The remainder of the cohort was divided into intestinal dysmotility (17.5%), fistulas (7%), mucosal disease (6.8%), jejuno-ileal anastomosis (5.9%), and mechanical obstruction (4.4%)[5]. However, other reports, such as the United States intestinal transplant (IT) registry, describe mesenteric ischaemia as the first cause of IT (24%) and CD as the second most frequent cause (11%)[6].

Following a large bowel resection, humans undergo a wide range of functional and anatomical modifications due to the reduction of the intestinal area dedicated to the absorption of nutrients[7]. Structural and anatomical changes include crypt hyperplasia, angiogenesis, bowel dilation and bowel elongation. Functional changes include accelerated crypt differentiation, a slower transit time, an increase in the number of transporters and a consequent increase in nutrient absorption[8]. After an early phase immediately following surgery, the adaptation phase starts 48 h after resection and lasts at least 1-2 years. Most of the intestinal adaptation described above occurs in this phase. Nutritional homeostasis may be achieved in the maintenance phase *via* oral autonomy or with parenteral support (PS)[3].

The clinical consequences of SBS are very heterogeneous and may considerably impact the patient's quality of life. One frequent symptom is diarrhoea due to accelerated intestinal transit, intestinal and gastric increased secretion[9], intestinal bacterial overgrowth[10], and the malabsorption of fats and bile salts[11,12]. One common consequence of SBS is the formation of stones. Asymptomatic gallbladder stones were reported in a population of SBS patients[13]. Most of these stones consist of calcium bilirubinate. The formation of gallbladder stones is favored by altered enterohepatic circulation[14], gallbladder hypomobility[15], and the reduced secretion of cholecystokinin after meals[16]. The formation of calcium oxalate kidney stones is also common, especially in patients with SBS and colon in continuity[17]. Within the colon, unabsorbed long-chain fatty acids compete with oxalate for available luminal calcium, and a larger amount of free oxalic acid is absorbed *via* passive diffusion and ultimately excreted by the kidney[13]. Other mechanisms may be involved, such as regional differences in oxalate absorption[17].

A relatively rare but underestimated complication of patients with SBS, especially patients with colon preservation, is so-called D-lactic acidosis. When a carbohydrate meal is consumed, colonic bacteria metabolise non-absorbed carbohydrates to short-chain-fatty acid and lactate. Lactate lowers the intraluminal pH, which leads to the overgrowth of D-lactate-producing bacteria, *Bifidobacterium* and *Lactobacillum*. D-lactate is absorbed into the systemic circulation and is responsible for the onset of neurological symptoms[18]. From a clinical point of view, this condition is characterized by neurological symptoms, such as ataraxia, movement disorders and altered mental status[19].

The prevalence of IF due to benign disease ranges from 5 to 80 cases of patients on chronic home parenteral nutrition (HPN) per million population in Europe[3]. Benign chronic IF and secondary SBS are in the directory of rare diseases ORPHANET (ORPHA:294422 and ORPHA:95427).

SBS-IF IN CROHN'S DISEASE

A survey in 64 centres from 22 countries enrolling 2919 patients on HPN described CD as the most frequent benign cause of SBS-IF[5]. However, a long-term study from a single IF centre in the United Kingdom described a change in the causes of IF over time, and the prevalence of CD decreased from 44% in 1978-1988 to 22% in 2006-2012[20].

Surgery remains a common treatment for CD during the course of the disease[21]. The indications for small intestinal resection in CD range from complications, such as stenosis, fistulas and abscesses, to the treatment of medically refractory disease[22]. Surgery rates in CD declined in recent decades due to multifactorial reasons, including earlier diagnosis and treatment, the use of biological agents, a decline in smoking rates, and improved patient education[23,24].

Although a United States population study demonstrated that biological agents reduced the proportion of CD patients undergoing resection, this reduction was not observed in patients with SBS-IF, which likely represents a subgroup of CD patients characterized by a more severe disease course and resistance to treatments[25]. Predictors of SBS-IF in CD were reviewed and roughly correspond to predictors of severe disease course. Notably, the CD phenotype characterized by an ileocolonic location is associated with a greater risk, and the absence of the ileocecal valve increases the risk of HPN dependence. Patients with perianal disease at diagnosis have a higher risk of disabling disease course, including bowel resections, and patients with penetrating disease have longer bowel resections and more frequently depend on HPN. Patients younger than 40 years at the time of CD diagnosis were more likely to have a "very short bowel" (< 100 cm), and an older age at first surgery was associated with decreased odds of IF. Patients who had ever smoked were more likely to develop IF. A family history of inflammatory bowel disease (IBD), frequent corticosteroid use for flares, and the number of bowel resections and complications of surgery were also identified as risk factors for SBS-IF[26].

A recent case-control study of 410 CD patients (41 with SBS) at a single centre demonstrated that subjects with SBS underwent significantly more bowel resections than controls. Patients treated with IV steroids were at higher risk of SBS, and Montreal B1 (inflammatory) behavior and treatment with budesonide characterized patients at a lower risk of SBS[27].

Another study of 2456 IBD patients identified 25 patients who required long-term HPN (1%, 24 CD). They described that HPN use was significantly associated with smoking, narcotic use, IBD-related surgeries, and lower quality-of-life scores. They found that these refractory patients had a 15-fold increase in annual median health care charges compared to control IBD patients[28].

MEDICAL TREATMENT OF SBS-IF AND COMPLICATIONS

The management of SBS-IF is complex, expensive and requires a multidisciplinary approach. A multidisciplinary team consisting of at least gastroenterologists, nutritionists, surgeons, radiologists, stoma therapists, care managers, pharmacists, and home care nurses is useful to provide patients with the best management possible[29]. The process of intestinal rehabilitation may be improved with medical management and includes spontaneous adaptation (dietary intervention and oral rehydrating solutions) and pharmacological therapies to improve symptoms. Gastric hypersecretion may be reduced with Proton-pump inhibitors. The accelerated transit time may be slowed with loperamide. The reduced reabsorption of bile acids may be targeted with cholestyramine, and bacterial overgrowth may be modulated with antibiotics, particularly rifaximin. Other drugs commonly used in the symptomatic management of diarrhoea are diphenoxylate, atropine, codeine, and antihistamines (*e.g.*, ranitidine and cimetidine).

Although symptom management provides an initial benefit in quality of life and the patient's perception of disease, it does not provide any improvement in prognosis[30]. The cornerstone in the treatment of SBS-IF is HPN. HPN ensures the correct intake of micro- and macronutrients and provides a significant improvement in prognosis. HPN is a life-saving treatment for these patients, but it significantly lowers patients' quality of life and is not free of complications[31].

HPN is generally administered in a single dose *via* a nutrition bag and infused over 10 to 12 h, typically overnight. Patients are encouraged to continue oral feeding with HPN[32]. Catheter infections [also called catheter-related blood stream infections (CRBSIs)] and central venous catheter (CVC) thrombosis are the most feared complications of parenteral nutrition. The rates of CRBSI range from 0 to 11.89 episodes per 1000 catheter days in systematic reviews and meta-analyses[33]. No significant difference was shown in catheter infection rates between peripherally inserted central catheters (PICCs) and tunneled catheters. However, PICCs showed lower CRBSI rates than ports[34]. The more frequently implicated pathogens are Gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus species*, and *Strepto-*

coccus species), Gram-negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter cloacae*), and fungi (*Candida parapsilosis*, *Candida albicans*, and *Candida glabrata*)[35]. Antibiotic therapy is the mainstay of CRBSI management. An empiric regimen with broad-spectrum drugs is generally used until the responsible agents are identified[36]. Bacterial infections are generally treated with the catheter in place, but fungal infections are treated by removing the catheter[37]. Locking the catheter with ethanol[38,39], taurolidine[40] or antibiotics[41] has been proposed as a strategy for the prevention of CRBSI. However, the results from randomized controlled trials (RCTs) are nonunivocal, and no clear guidelines have been generated on this topic[42].

The incidence of CVC thrombosis is approximately 0.12 events/1000 catheter days[43]. The management of catheter-related thrombotic events is based on the administration of low-molecular-weight heparins (LMWH)[44] or thrombolytic agents, such as streptokinase[45]. Flushing the CVC with a saline solution or LMWH has been proposed as a measure to prevent the occurrence of thrombotic events[46,47].

One possible complication of HPN is progressive steatohepatitis and liver damage, also known as "IF-associated Liver Disease" (IFALD). Up to half of the patients receiving total parenteral nutrition develop severe liver disease after 5 years[48,49], and higher rates of incidence and prevalence are observed in children[50]. IFALD manifests as steatosis, steatohepatitis or a cholestatic pattern. It may lead to progressive liver damage with fibrosis and cirrhosis in some cases[51]. Therapeutic strategies include fish oil-based lipid emulsions[52], ursodeoxycholic acid[53], and lecithin administration[54].

Malabsorption-related anaemia may develop into microcytic anaemia due to iron deficiency or macrocytic anaemia due to malabsorption of vitamin B12 and folate, which lead to the need for iron and vitamin supplementation[3,55]. The occurrence of metabolic bone disease must be monitored[56]. Bone densitometry, the biochemical dosing of vitamin D and calcium profiles play a role in preventing the insurgency of metabolic bone disease[57]. Manganese toxicity must be noted. Manganese is a central element of HPN, but its requirement is low. This low requirement leads to an increase in blood concentration and accumulation in the central nervous system, which is detectable using magnetic resonance imaging. Manganese toxicity presents with neurological symptoms that are similar, but not identical, to Parkinson's disease[58].

NEW THERAPIES: GLUCAGON-LIKE PEPTIDE-2 ANALOGUES

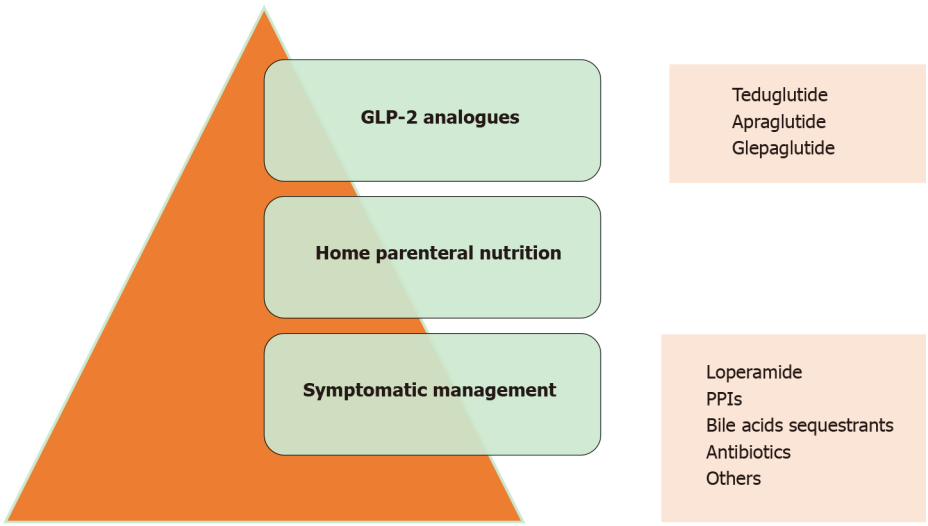
Hormonal manipulation may also increase intestinal absorption[59] (Figure 1). Only two molecules have been approved for SBS-IF therapy, growth hormone in the United States and the glucagon-like peptide-2 (GLP-2) analogue teduglutide (TED) in the United States and Europe[60,61]. TED is a recombinant analogue of GLP-2 that is resistant to degradation by DPD4. The elimination half-life of TED is 2 to 6 h, which allows administration as a daily subcutaneous injection. Notably, the elimination half-life of endogenous GLP-2 is approximately 7 min[62].

The mechanism of action of TED is complex and involves direct and indirect effects of interaction with a GLP-2 receptor and includes the following most relevant factors: Crypt cell proliferation, increase in bowel weight and villous growth, enhancement of intestinal barrier function, inhibition of motility of the gastrointestinal tract and gastric acid secretion, and increase of intestinal blood flow[60]. The effect of TED on intestinal epithelial stem cells is of particular relevance in the possible risk of the drug being tumorigenic[63].

The first open-label trial on TED was performed and published in 2005. Sixteen patients with end-jejunostomy or colon in continuity received s.c. TED for 21 d. Patients treated with TED showed an increased absorption of nutrients, urine output and sodium excretion. TED significantly increased villus height, crypt depth and mitotic index in the small intestines of patients with end-jejunostomy. Leg oedema and enlargement of the stoma nipple were the most common, but not severe, side effects[64].

The most significant RCT of TED effectiveness was performed from 2008 to 2011 and published in 2012 (STEPS study). Eighty-six patients receiving PS were enrolled: 43 patients underwent management with TED, and 43 were treated with placebo (PBO) for 24 wk. In the TED group, 63% of patients were responders who reduced their PS by at least 20% compared to 30% of patients in the PBO group (P = 0.02). The secondary outcomes of this study were the reduction of parenteral nutrition volume at week 24 (2.1 litres per week difference between the TED group and PBO, $P < 0.05$) and the number of patients achieving at least 1 d off parenteral nutrition per week (21/39 in the TED group *vs* 9/39 in the PBO group)[65].

The STEPS study was followed by STEPS-2, which was a 2-year, open-label extension of the prior study. The enrolled patients completed 24 wk of TED (TED/TED) or PBO (PBO/TED) in the initial PBO-controlled study or qualified for the original study but were not treated (NT/TED) because of full enrolment. Patients received 0.05 mg/kg/d subcutaneous TED for up to 24 mo (NT/TED and PBO/TED) or up to 30 mo (TED/TED). In patients who completed the study, clinical response (as defined in the STEPS study) was achieved in 28/30 (93%) TED/TED, 16/29 (55%) PBO/TED, and 4/6 (67%) NT/TED patients. The mean PS volume reductions from baseline were 7.6 (66%), 3.1 (28%), and 4.0 (39%) litres/week in the TED/TED, PBO/TED, and NT/TED groups, respectively. Thirteen patients



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Figure 1 Step-up approach in the management of short bowel syndrome-intestinal failure. GLP-2: Glucagon-like peptide-2; PPIs: Proton-pump inhibitors.

achieved full enteral autonomy. This trial demonstrated the long-term effectiveness and safety of TED [66].

A further extension of STEPS and STEPS-2 studies is the STEPS-3 study. Long-term treatment with TED in this 1-year, open-label extension trial showed a safety profile consistent with previous studies, with prolonged effectiveness and a further reduction in HPN necessity[67]. A post hoc analysis determined factors associated with the response to TED. Notably, the remaining bowel anatomy, rather than the remaining bowel length, primarily affected the outcome of TED therapy. Patients with jejunostomy or ileostomy (SBS-IF type 1 according to the anatomical classification) experienced the most pronounced benefit from TED compared to patients with colon continuity. The same study outlined how patients with higher baseline PS volume had higher reductions in PS, which means that SBS–IF patients with more severe malabsorption benefit the most from therapy with TED[68].

Safety data from clinical trials of TED reported abdominal distension and gastrointestinal stoma complications as the most frequent adverse events (AEs), and the AE frequency was higher early in the course of treatment and declined thereafter[69]. The capacity of GLP-2 to accelerate the growth of experimental intestinal tumors was known before the introduction of TED[70,71]. A post hoc analysis of STEPS studies found that 50 of 65 patients with remnant colon (77%) underwent a protocol-mandated postexposure colonoscopy. Colon polyps were reported in 12% (9/73) of patients at baseline and 18% (9/50) of patients postexposure. Two patients had polyps at baseline and postexposure. Histology was available for 7 patients: 5 had adenomas (1 serrated, 4 tubular), and none had malignancies or high-grade dysplasia[72].

A large population analysis characterizing 170 patients treated with TED from the United States from 2015 to 2020 found that an overall 5.9% of patients developed posttreatment polyps of the large intestine. Twenty events (12%) of catheter infection were described in the same study within 1 year from the start of administration. Other documented adverse effects were abdominal pain (70 cases, 41.2%), nausea (40 cases, 23.5%), intestinal obstruction (30 cases, 17.6%) and stoma complications (20 cases, 11.8%). Ten patients (6%) developed colon polyps[73].

A French real-life study reported a population of 54 patients with SBS-IF treated with TED. The effectiveness and safety were fully confirmed. Twenty-four weeks after the start of treatment, 85% of patients experienced a reduction in PS of at least 20%, and 24% of patients were weaned off PS[74]. Another real-life cohort of 18 patients treated with TED for a median of 3.2 years was reported. Twelve of these patients achieved a clinical response at 12 mo, which was maintained in most of these patients at 36 mo. Five patients were able to wean off PS, but no predictor was identified due to the small sample[75].

New GLP-2 analogue molecules were studied recently. Apraglutide (TA799-007), which is characterized by high protein binding, resistance to DPP4 cleavage, low clearance and slow absorption after subcutaneous administration, has been highlighted[76]. These features make apraglutide clinically appealing, which suggests the possibility of more delayed dose administration[77].

Apraglutide showed efficacy in studies of animal models of SBS-IF in terms of increases in intestinal length and weight, crypt weight and villous height and lower faecal fat and energy losses[78,79]. The effects on intestinal length and mucosal hypertrophy after discontinuation of TED and apraglutide were also studied in piglets. These two molecules apparently showed similar results in this case: Intestinal growth appeared to be a lasting outcome of treatment with long-acting GLP-2 and persisted for at least 7 d after discontinuation. In contrast, mucosal hypertrophy appeared to regress 7 d after the end of

treatment with both agents[80]. However, these results must be interpreted with caution and need further confirmation. The once weekly administration of apraglutide to neonatal piglets showed superior intestinotrophic effects than a single daily dose of TED and led to increased bowel length and villus height[81].

A recent PBO-controlled, randomized, phase II clinical trial treated eight adults with SBS-IF with once-weekly 5-mg apraglutide or PBO for 4 wk, followed by once-weekly 10-mg apraglutide doses for 4 wk, with a washout period of 6-10 wk between treatments. No severe AEs related to the drug were detected, and comparable safety profiles were observed between the low-dose and high-dose regimens. The main AE detected was an increase in urine output, and the following less frequent AEs were reported in decreasing order: Stoma complications, decreased thirst, oedema, increased weight, and injection site reactions[82]. A phase 3 study in SBS-IF patients treated with apraglutide *vs* PBO is currently ongoing[83].

Glepaglutide is another long-acting analogue of GLP-2 that chemically differs from native GLP-2 by 9 amino acid substitutions and a C-terminal tail consisting of six lysine residues. It has a half-life of approximately 50 h and has been evaluated in a phase II study, where it demonstrated good tolerance and the ability to improve intestinal absorption in SBS patients treated with 1 mg and 10 mg daily glepaglutide[84]. A phase 3 study comparing weekly or twice weekly glepaglutide and PBO administration to SBS patients is currently ongoing[85].

Other molecules are in the preclinical phase of their development. Elsiglutide is a GLP-2 analogue with a long half-life that reduced diarrhoea induced by lapatinib, a tyrosine kinase inhibitor, in a rat model[86]. Dapiglutide is a dual GLP-1 and GLP-2 agonist that showed beneficial effects in a rat model of SBS by improving body weight, promoting intestinal growth, increasing villous height and intestinal length, and reducing watery stool losses[87].

For the duration of therapy with GLP-2 analogues in patients with SBS-IF, the available data indicate that these drugs must be administered over a long life because reversal of the previous need for PS occurs if TED is discontinued[64]. A recent report described 13 patients (one with CD and one with ulcerative colitis) who discontinued TED after a successful clinical outcome. The volume of PS remained stable in the first 4 years but later increased in 12/13 patients up to 9 years after withdrawal[88]. These data support further studies exploring the possibility of the periodic use of GLP-2 analogues for selected SBS-IF patients.

GLP-2 ANALOGUES IN CROHN'S DISEASE

Knowledge of GLP-2 analogue use in patients with CD is limited. No RCTs specifically address the efficacy and safety of GLP-2 in CD patients with SBS-IF. However, 18 patients with CD were included in the 86 subjects of the STEPS study, 10 in the TED arm and 8 in the PBO arm[65]. Sixteen CD patients were included in the STEPS-2 long-term study[66]. The CD patients in these studies were in the inactive phase of their inflammatory disease, and the concomitant use of immunosuppressants and biologics were exclusion criteria. The subsequent post hoc analysis of the STEPS studies analysing factors associated with the response to TED in patients with SBS-IF found that the effects of TED on PS volume reduction were more pronounced in patients with jejunostomy/ileostomy than in patients with partial colon continuity. Only 10.5% of CD patients had colon in continuity in this study. Therefore, CD patients were likely among the best responders according to this analysis[68].

Subsequent data from real-life studies reported the experiences of TED treatment for CD patients treated with immunosuppressants and biologics and patients with active inflammation. A retrospective cohort study published in 2017 analysed 13 patients with CD and SBS-IF treated with TED for an average of 1 year. Nine of these patients were under parenteral nutrition before beginning TED therapy, and only 1 of them needed parenteral nutrition after TED. All of these patients required intravenous fluids before TED, but only 7 of them required this supplementation after TED. Eight of these patients were treated with biological therapies and/or immunosuppressants. TED was well tolerated in most of these CD patients, with the exception of one patient who intermittently presented with obstructive symptoms that required the cessation of therapy[89].

Two other active CD cases treated with TED were described. The first case was a 38-year-old patient with relapsing perianastomotic disease treated with ustekinumab. This patient received PN daily for SBS and was weaned off after 7 mo of TED. The second patient was 39 years old and had received PN daily since 2003. This patient was treated with vedolizumab and 6-mercaptopurine. After multiple resections, he was left with a jejunocolic anastomosis and 60 cm of residual small bowel length. PN was reduced to 1 night per week after TED[90].

One case of long-term TED use (54 mo) was reported in a patient with multidrug-resistant CD, and beneficial effects on nutritional status, a significant anti-inflammatory effect and reduction in CD clinical activity were observed[91]. A recent real-life cohort of 52 patients successfully treated with TED found that 16 had CD[74]. Another real-life series of 18 patients on TED found that 10 patients had SBS-IF caused by CD, and the 5 who were able to withdraw PS were all CD patients[75]. The findings of these studies are presented in Table 1.

Table 1 Main studies and case reports on glucagon-like peptide-2 analogues in Crohn's disease with short bowel syndrome

Ref.	Molecule tested	Number of patients	Number of CD patients	Study type	Main results	Adverse events
Jeppesen *et al*[64], 2005	Teduglutide	16	12 (in clinical remission)	Pilot open label, phase II	Increased wet weight absorption; decreased urine weight and urine sodium excretion; decreased fecal wet weight and fecal energy content; increased villus height, crypt depth and mitotic index in end-jejunostomy patients	Enlargement of the stoma nipple; mild lower leg oedema; severe AE (dehydration, sepsis, CS) in 4/16, not judged to be related to the drug
Jeppesen *et al*[65], 2012	Teduglutide	43 (+ 43 PBO)	10 (in clinical remission)	Multicenter, randomized, double blind, PBO-controlled phase III	63% of TED patients had a ≥ 20% reduction of PS volume at week 24 (significant versus 30% of the PBO group); increased serum citrulline (index of intestinal mucosa mass)	Mostly mild GI symptoms (abdominal pain, nausea, stoma complication, or abdominal distension); 7/43 CS; not different from PBO
Schwartz *et al*[66], 2016	Teduglutide	88	16	2 yr open label extension study	Clinical response (≥ 20% reduction of PS volume) in 28/30 (93%) and 66% of PS volume reduction in TED/TED group; 13 reached enteral autonomy	34%abdominal pain; 25% episodes of weight decrease; 39% infections (SAE); 2 CD exacerbations (12% of CD, SAE)
Kochar *et al* [89], 2017	Teduglutide	13	13 (8 on biologics and/or IS)	Retrospective cohort study (median duration 1 yr)	9 patients on PN at the beginning of therapy; 1 patient still on PN at the end of therapy; PS reduced from median 9000 mL/wk to 3100 mL/wk, 6 patients no PS at the end	Among non-immunosuppressed (5) only 2 minor AE and 1 CS; among immunosuppressed (8) minor AE, 3 CS and 2 pancreatitis
Barberio *et al*[94], 2019	Teduglutide	1	1 (on EN and adalimumab)	Case report	EN reduction of 50% after 24 wk of TED; EN suspension after 72 wk of treatment	Transient nausea and mild abdominal pain and nausea
Al Draiweesh *et al*[90], 2019	Teduglutide	2	2 (on biologics)	Case report	Weaning off from PS after 7 mo of TED; improvement in oral intake, reduced stool output, and weight gain; reduction of PN to 1 night/wk	Any reported
Naimi *et al* [84], 2019	Glepaglutide	16	8	Double-blind randomised phase II trial	1 mg daily glepaglutide reduces the fecal output by 592 mg/d; 10 mg daily glepaglutide reduces the fecal output by 833 mg/d	Stoma complications (73%); injection site reactions (61%); peripheral edema (56%); nausea and abdominal pain (44%); SAEs (stoma obstruction, sepsis) in 4 patients
Joly *et al* [74], 2020	Teduglutide	54	16	Retrospective multicenter real life cohort	85% of patients were responders (PS reduction ≥ 20%) at week 24; 24% weaned off PS at week 24	Not specifically collected
Borghini *et al*[91], 2020	Teduglutide	1	1 (clinically active)	Case report	At least 20% reduction in PS; daily Kcal intake reduction of 15%; reduction in CD activity and severity	Two mild CVC-related infections
Mouillot *et al*[93], 2020	Teduglutide	1	1	Case report	Reduction of PS and increase of nutrients absorption; hypertrophy of villi at capsule endoscopy; increase in the size of intestinal villi and crypts assessed by biopsy	Not specifically assessed
Puello *et al* [75], 2021	Teduglutide	18	10	Retrospective single center real life cohort	Response (reduction of > 20% PS) in: 16 (75%) patients at 12 mo; 10 of 13 (76.9%) patients at 24 mo; 7 of 10 (70%) patients at 36 mo; 3 of 3 (100%) patients at 60 mo; 5 patients (all CD) weaned off PS	Abdominal pain or cramping (3); volume overload (1); taste loss (1); legs edema (2); increase in ostomy size (2)
Eliasson *et al*[82], 2022	Apraglutide	8	3 (in clinical remission)	Open label phase I-II trial	Weekly 5 mg apraglutide increases urinary output by 714 mL/d; weekly 10 mg apraglutide increases urinary output by 795 mL/d	Polyuria (7/8); any stoma complication (6/8); thirst decrease (4/8) and appetite decrease (3/8); edema (4/8); no SAE related to the drug

CD: Crohn's disease; PN: Parenteral nutrition; PS: Parenteral support; EN: Enteral nutrition; TED: Teduglutide; PBO: Placebo; GI: Gastrointestinal; SAEs: Severe adverse events; CS: Catheter-related sepsis; CVC: Central venous catheter; IS: Immunosuppressives; AE: Adverse events.

Other retrospective and prospective cohorts of SBS-IF patients treated with TED (including a variable proportion of CD patients) were described and recently summarized in a meta-analysis that confirmed the efficacy of TED in adult SBS-IF patients[92]. This analysis suggested a non-significant trend of CD aetiology of SBS-IF as a positive predictor of response and weaning from PS.

A case of intestinal adaptation induced by TED in CD was recently described. The case was a 40-year-old female patient suffering from SBS-IF due to CD since 2001. The patient underwent terminal jejunostomy, with 100 cm of jejunum remaining. SBS and IF resulted, and the woman needed continuous PS. She was recommended to start treatment with TED, and her intestinal status and disease activity were studied at baseline. No active CD was detected, and the intestinal villi appeared normal. After 1 year of continued TED treatment, a new biopsy of intestinal villi was performed and showed an increase of 33% in the length of villi[93].

The efficacy of off-label TED use in a 65-year-old CD patient treated with adalimumab with SBS and enteral nutrition was also described, and the patient was weaned off enteral nutrition after 72 wk[94]. Concerning TED discontinuation, a report described 2 CD patients on chronic TED treatment who were not able to tolerate even a few days withdrawal of the drug[95].

One line of research attempted to determine whether TED had anti-inflammatory activity in CD based on the results of the use of glucagon-like peptides in animal models of colitis[96]. An RCT was performed on 100 CD patients with moderately to severely active disease who were treated with TED (0.05, 0.10, or 0.20 mg/kg) daily for 8 wk or PBO. Seventy-one patients completed the study, and the results showed numerically higher response and remission rates in all treatment groups compared to PBO, but these differences were not statistically significant. The results were more substantial in the group treated with the higher dose (44% response and 32% remission *vs* 32% response and 20% remission in the PBO group). It is questionable whether the clinical disease activity indices modifications described in this study were mostly due to the effect of TED on diarrhoea because no significant modification of C-reactive protein was detected. AEs were not different between the PBO and treatment groups[97]. This last result is particularly relevant, because it came from a controlled study on a relevant number of patients with active CD, although not affected by SBS-IF. The good safety profile in this setting justifies the use of GLP-2 analogues in patients with SBS-IF and active CD, but more data on this specific population are needed. Data on the use of the new GLP-2 analogues (apraglutide and glepaglutide) in CD are lacking.

CONCLUSION

SBS-IF is a rare condition that affects patients who undergo several intestinal resections due to CD and other gastrointestinal conditions. Although surgery rates in CD have declined in recent decades due to improved diagnostic and pharmacological modalities, the rate of CD patients developing SBS-IF (albeit low) has not been reduced. Several mechanisms are adopted by organisms to adapt to intestinal functional reduction. However, these adaptations are often not sufficient to avoid the clinical impact and long-term complications of SBS-IF, and several patients require HPN. The main therapeutic approaches consist of PS and symptomatic therapy. Yet, PS is associated with several AEs that complicate patient management, such as catheter-related complications, liver and metabolic disease, iron deficiency anaemia, and manganese toxicity.

New molecules and therapeutic approaches have been studied in recent years. The GLP-2 analogue TED demonstrated a relevant clinical utility for SBS-IF patients, and it significantly reduced HPN volume and/or days of infusion, allowing enteral autonomy in some patients. However, because this therapy is likely life-long or of long duration, more data are needed on the long-term safety and cost-effectiveness. Recent further consideration has been given to new GLP-2 analogues that have undergone phase I and II studies, including apraglutide and glepaglutide. These agents showed low clearance and slow absorption after subcutaneous administration, which allowed for a single weekly administration. Relatively scant data are available on the use of GLP-2 analogues in CD patients with SBS-IF. However, signals from RCTs and real-life observations indicate that TED is efficacious and well tolerated by CD patients, even if they are being treated with immunosuppressants and/or biological agents. More data are needed on the use of GLP-2 analogues in patients with active CD to clarify their safety and efficacy in this setting. This new pharmacological approach may improve the quality of life of patients with CD and SBS-IF and reduce their dependence on artificial nutrition. Clinical studies specifically addressing this peculiar population are warranted.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Postoperative outcomes and recurrence patterns of intermediate-stage hepatocellular carcinoma dictated by the sum of tumor size and number

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Abstract

BACKGROUND

The selection criteria for Barcelona Clinic Liver Cancer (BCLC) intermediate-stage hepatocellular carcinoma (HCC) patients who would truly benefit from liver resection (LR) remain undefined.

AIM

To identify BCLC-B HCC patients more suitable for LR.

METHODS

We included patients undergoing curative LR for BCLC stage A or B multinodular HCC (MNHCC) and stratified BCLC-B patients by the sum of tumor size and number (N + S). Overall survival (OS), recurrence-free survival (RFS), recurrence-to-death survival (RTDS), recurrence patterns, and treatments after recurrence in BCLC-B patients in each subgroup were compared with those in BCLC-A patients.

RESULTS

In total, 143 patients who underwent curative LR for MNHCC with BCLC-A (n = 25) or BCLC-B (n = 118) were retrospectively analyzed. According to the N + S, patients with BCLC-B HCC were divided into two subgroups: BCLC-B1 (N + S ≤ 10, n = 83) and BCLC-B2 (N + S > 10, n = 35). Compared with BCLC-B2 patients, those with BCLC-B1 had a better OS (5-year OS rate: 67.4% *vs* 33.6%; P < 0.001), which was comparable to that in BCLC-A patients (5-year OS rate: 67.4% *vs* 74.1%; P = 0.250), and a better RFS (median RFS: 19 mo *vs* 7 mo; P < 0.001), which was worse than that in BCLC-A patients (median RFS: 19 mo *vs* 48 mo; P = 0.022). Further analysis of patients who developed recurrence showed that both BCLC-B1 and BCLC-A patients had better RTDS (median RTDS: Not reached *vs* 49 mo; P =

0.599), while the RTDS in BCLC-B2 patients was worse (median RTDS: 16 mo *vs* not reached, $P < 0.001$; 16 mo *vs* 49 mo, $P = 0.042$). The recurrence patterns were similar between BCLC-B1 and BCLC-A patients, but BCLC-B2 patients had a shorter recurrence time and a higher proportion of patients had recurrence with macrovascular invasion and/or extrahepatic metastasis, both of which were independent risk factors for RTDS.

CONCLUSION

BCLC-B HCC patients undergoing hepatectomy with N + S ≤ 10 had mild recurrence patterns and excellent OS similar to those in BCLC-A MNHCC patients, and LR should be considered in these patients.

Key Words: Hepatocellular carcinoma; Multinodular; Intermediate-stage; Liver resection; Recurrence pattern; Prognosis

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Core Tip: Subgroups of Barcelona Clinic Liver Cancer (BCLC) intermediate-stage hepatocellular carcinoma (HCC) patients who would truly benefit from liver resection (LR) remain undefined. We demonstrated that the sum of tumor size and number (N + S) can predict not only prognosis in BCLC-B patients undergoing LR, but also the recurrence patterns and recurrence-to-death survival (RTDS) in these patients. In addition, we indicated that BCLC-B patients undergoing hepatectomy with N + S ≤ 10 had mild recurrence patterns, good RTDS and excellent overall survival similar to those in BCLC-A multinodular HCC patients. The results of this study are helpful in selecting BCLC-B patients more suitable for LR.

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INTRODUCTION

As the sixth most common cancer globally, primary liver cancer accounted for 906,000 newly confirmed cancer cases and 830,000 cancer-related deaths worldwide in 2020, of which 75%-85% were hepatocellular carcinoma (HCC)[1].

Barcelona Clinic Liver Cancer (BCLC) staging system, which was proposed in 1999, has been widely used to guide treatment decisions in patients with HCC[2,3]. The 2022 version of the BCLC strategy recommends liver transplantation (LT), transarterial chemoembolization (TACE), and systemic therapy, respectively, for BCLC intermediate-stage HCC patients based on their expected survival time[4].

In addition, emerging studies have suggested that liver resection (LR) may also be a good treatment option for BCLC-B HCC patients[5,6]. Nevertheless, the subgroups of BCLC-B HCC patients who would truly benefit from LR have yet to be defined. Several previous studies found that some BCLC-B HCC patients undergoing LR had favorable long-term overall survival (OS) rates (5-year OS rates: 50%-75%); however, these selected patients still had high postoperative recurrence rates (2-year recurrence rate: ≥ 50%), which means that many of these patients had good recurrence-to-death survival (RTDS)[7,8]. Both recurrence patterns and treatments after recurrence can affect the RTDS of HCC patients who develop recurrence after LR[9-11]. However, previous studies did not analyze the main reasons why these selected patients had good RTDS, which may affect the judgment of the role of LR in these patients[7,8].

In this study, we retrospectively included patients undergoing curative LR for BCLC stage A or B multinodular HCC (MNHCC) and stratified the BCLC-B patients by the sum of tumor size and number (N + S), which combines the two main prognostic factors of BCLC-B patients into a continuous variable [7,8]. BCLC-B patients more suitable for LR were identified by comparing the outcomes, recurrence patterns, and treatments after recurrence in BCLC-B patients in each subgroup with those in BCLC-A patients.

MATERIALS AND METHODS

Patients

We enrolled BCLC stage A or B MNHCC patients who underwent curative LR in Tongji Hospital from January 2010 to May 2018. The inclusion criteria were: (1) MNHCC pathologically diagnosed with two or more nodules, in which lesions less than 1 cm in diameter and less than 2 cm away from the main nodule were defined as satellite nodules[12]; (2) Curative resection, defined as complete macroscopic removal of all tumors with negative histologic resection margins for the tumors (R0 resection)[13,14]; and (3) No preoperative anticancer treatment other than TACE. The exclusion criteria were: (1) Recurrent HCC or combined HCC and cholangiocarcinoma; and (2) Complicated with other malignancies.

Data collection

Patient data at the time of initial hepatectomy including sex, age, body mass index, hepatitis B antigen status, liver function, tumor characteristics, surgical procedure, and preoperative treatment were recorded. Liver function in this study was classified by the albumin-bilirubin score[15]. Maximum tumor size was defined as the maximum diameter of the largest tumor. Microvascular invasion was defined as tumor within a vascular space lined by endothelium that was visible only on microscopy[16].

In addition, the recurrence patterns, which consisted of recurrence time and tumor characteristics at the time of recurrence, and treatments after recurrence in those patients who developed recurrence during follow-up were also recorded. Recurrence time was defined as the time between initial LR and the first recurrence.

Initial hepatectomy

In our center, we routinely estimated the residual liver volume in MNHCC patients before hepatectomy, and only patients with residual liver volume of more than 40% of the standard liver volume (for patients with liver cirrhosis) or more than 30% (for patients without liver cirrhosis) would receive LR [17,18]. The decision to perform anatomical or non-anatomical hepatectomy depended largely on the tumor distribution, and major resection was defined as the resection of three or more Couinaud liver segments[19]. Intraoperative ultrasound was routinely used to locate the tumor and screen the nodules. All nodules were completely resected intraoperatively and negative margin was determined according to postoperative pathology.

Follow-up

Patients were followed every month with measurement of serum alpha-fetoprotein (AFP), chest radiography and ultrasound or computed tomography (CT) or magnetic resonance imaging (MRI) in the first 6 mo after discharge from hospital, and every 3-6 mo thereafter. When HCC recurrence was confirmed by CT or MRI, patients were treated with repeated hepatectomy, ablation, TACE or systemic therapy. Follow-up was terminated on May 15, 2022.

Recurrence-free survival (RFS) was calculated from the date of hepatectomy until recurrence or last follow-up. OS was defined as the time from LR to death or last follow-up, and RTDS was defined as the time from recurrence to death or last follow-up.

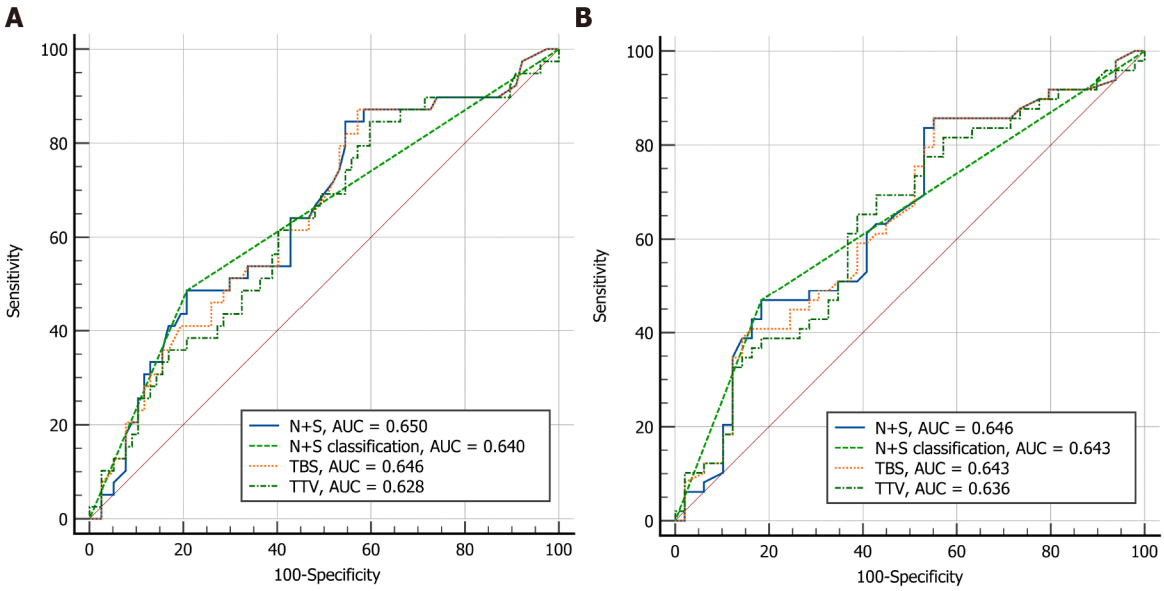
Statistical analysis

Continuous variables were presented as mean ± SD or median (interquartile range; IQR). Categorical variables were described by frequency and percentage. In the comparison of different subgroups, continuous variables were compared using the Student's t or Mann-Whitney U test, and categorical variables using the χ^2 or Fisher's exact test, as appropriate. Survival was analyzed by the Kaplan-Meier method, and survival curves were compared by the log-rank test. Univariate and multivariate analyses were based on the Cox proportional analysis. Variables with P values less than 0.1 identified by the univariate analysis were included in multivariate analysis. The cutoff value of N + S was determined by X-tile, a bioinformatics tool produced by Camp and colleagues[20]. The area under receiver operating characteristic (ROC) curve (AUC) was compared using DeLong test[21]. $P < 0.05$ was considered to indicate statistical significance. Both SPSS (version 23.0, SPSS, Inc., Chicago, IL, United States) and MedCalc software (version 20.115, MedCalc Software, Ostend, Belgium) were used for the analysis.

RESULTS

Variables and outcomes of the entire cohort

A total of 143 patients who underwent curative LR for BCLC stage A or B MNHCC were enrolled. Their mean age was 52.1 years and most patients were male (n = 134, 93.7%) and were hepatitis B surface antigen positive (n = 131, 91.6%). Median maximum tumor size in the entire cohort was 5.6 cm (IQR: 3.4–7.6) and tumor number in the vast majority of patients was ≤ 3 (n = 136, 95.1%). Overall, 17.5% (n = 25) of patients had BCLC-A MNHCC, and 82.5% (n = 118) had BCLC-B MNHCC (Table 1).



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Figure 1 The receiver operating characteristic analysis of the sum of tumor size and number, the classification of the sum of tumor size and number, tumor burden score and total tumor volume in intermediate-stage hepatocellular carcinoma patients. A: 3-year overall survival (OS); B: 5-year OS. N + S: The sum of tumor size and number; TBS: Tumor burden score; TTV: Total tumor volume; AUC: Area under receiver operating characteristic curve.

After a median follow-up of 54 mo (IQR 27–79), 5-year OS and RFS after R0 resection in all patients were 60.2% and 23.2%, respectively. Of note, BCLC-B patients had worse 5-year OS (57.2% *vs* 74.1%, P = 0.028, Supplementary Figure 1A) and RFS (19.4% *vs* 41.6%, P = 0.002, Supplementary Figure 1B).

Stratification of BCLC-B patients based on N + S

Among patients undergoing LR for BCLC-B HCC, the median maximum tumor size was 6.2 cm (IQR: 4.1–8.4) and tumor number in 111 (94.1%) patients was ≤ 3. Of note, 43.2% (n = 51) of patients had bilateral disease and 14.4% (n = 17) of patients underwent TACE before initial LR (Table 1).

Using the X-tile program[20], patients with BCLC-B HCC were divided into two groups by N + S: BCLC-B1 (≤ 10, n = 83, 70.3%), BCLC-B2 (> 10, n = 35, 29.7%) (Supplementary Figure 2).

The prognostic ability of N + S and the rationality of the cut-off value of 10 were then verified by time-dependent ROC curves and Cox-regression analysis. The AUCs for 3-year and 5-year OS in BCLC-B patients were 0.650 and 0.646, respectively, for N + S, and 0.640 and 0.643, respectively, for stratification according to N + S (Figure 1). Multivariate analysis showed that N + S > 10 was an independent risk factor for OS [hazard ratio (HR) 2.996, 1.779 to 5.045; P < 0.001] (Table 2) and RFS (HR 1.657, 1.057 to 2.596; P = 0.028) (Table 3) in BCLC-B patients.

In addition, we compared the predictive accuracy of N + S with those of tumor burden score (TBS) and total tumor volume (TTV), both of which were previous prognostic models based on tumor size and number of HCC patients[22,23]. The results showed that the AUCs of N + S at 3 and 5 years were both similar to those of TBS (3-year AUC, 0.650 *vs* 0.646, P = 0.552; 5-year AUC, 0.646 *vs* 0.643, P = 0.762) and TTV (3-year AUC, 0.650 *vs* 0.628, P = 0.171; 5-year AUC, 0.646 *vs* 0.636, P = 0.535) (Figure 1).

Comparison of the clinical characteristics, OS, and RFS among BCLC-A, BCLC-B1 and BCLC-B2 patients

Clinical characteristics, OS and RFS of patients with BCLC-B1, BCLC-B2, and BCLC-A were compared (Figure 2, Supplementary Table 1). The results showed that BCLC-B2 patients had a higher serum AFP level and a larger proportion of bilateral tumor distribution, compared to patients with BCLC-A and BCLC-B1. With an increase in N + S, the maximum tumor size gradually increased, and a larger proportion of patients underwent major resection (Supplementary Table 1).

Both BCLC-A patients and BCLC-B1 patients had good 5-year OS (74.1% *vs* 67.4%, P = 0.250), which was better than that in BCLC-B2 patients (74.1% *vs* 33.6%, P < 0.001; 67.4% *vs* 33.6%, P < 0.001) (Figure 2A). Compared with BCLC-A patients, BCLC-B1 patients had a worse RFS (median RFS: 19 mo *vs* 48 mo; P = 0.022), which was better than that in BCLC-B2 patients (median RFS: 19 mo *vs* 7 mo; P < 0.001) (Figure 2B).

Table 1 Characteristics of patients with Barcelona Clinic Liver Cancer stage A or B multinodular hepatocellular carcinoma, *n* (%)

Variables	Total (*n* = 143)	BCLC-A (*n* = 25)	BCLC-B (*n* = 118)	*P* value
Male gender	134 (93.7)	25 (100)	109 (92.4)	0.330
Age (yr)	52.1 ± 12.7	50.5 ± 14.5	52.4 ± 12.4	0.490
BMI	22.97 ± 3.15	23.05 ± 3.35	22.96 ± 3.12	0.895
HBs-Ag positive	131 (91.6)	24 (96)	107 (90.7)	0.635
Albumin (g/L)	38.89 ± 4.51	39.95 ± 5.15	38.65 ± 4.34	0.194
Bilirubin (μmol/L)	13.8 (9.9, 18)	12.9 (10.2, 20.9)	13.9 (9.7, 17.8)	0.568
ALBI grade				0.680
1	69 (48.3)	13 (52)	56 (47.5)	
2/3	74 (51.7)	12/0 (48/0)	62/0 (52.5/0)	
AFP (μg/L)	239 (13, 2338)	74 (6, 390)	483 (16, 2944)	0.011
Maximum tumor size (cm)	5.6 (3.4, 7.6)	2.5 (2.1, 2.9)	6.2 (4.1, 8.4)	< 0.001
Tumor number				0.460
≤ 3	136 (95.1)	25 (100)	111 (94.1)	
> 3	7 (4.9)	0	7 (5.9)	
Tumor distribution				0.506
Unilateral	83 (58)	16 (64)	67 (56.8)	
Bilateral	60 (42)	9 (36)	51 (43.2)	
Presence of microvascular invasion	15 (10.5)	1 (4)	14 (11.9)	0.420
Edmondson-Steiner grade				0.337
I-II	85 (59.4)	17 (68)	68 (57.6)	
III-IV	58 (40.6)	8 (32)	50 (42.4)	
Major resection	64 (44.8)	4 (16)	60 (50.8)	0.001
Anatomical hepatectomy	22 (15.4)	4 (16)	18 (15.3)	1.000
Preoperative TACE				0.690
No	121 (84.6)	20 (80)	101 (85.6)	
Yes	22 (15.4)	5 (20)	17 (14.4)	

BMI: Body mass index; HBs-Ag: Hepatitis B surface antigen; ALBI: Albumin-bilirubin; AFP: Alpha-fetoprotein; IQR: Interquartile range; TACE: Transarterial chemoembolization.

Comparison of recurrence patterns, treatments after recurrence, and RTDS in BCLC-A, BCLC-B1 and BCLC-B2 patients

During follow-up, 14 (56%) BCLC-A, 66 (79.5%) BCLC-B1 and 34 (97.1%) BCLC-B2 patients developed recurrences ($P < 0.001$). Nine BCLC-B1 and 4 BCLC-B2 patients who lacked information on tumor characteristics at the time of recurrence and treatments after recurrence were excluded from the analysis. Ultimately, 14 BCLC-A, 57 BCLC-B1 and 30 BCLC-B2 patients with recurrence were included in the analysis. The recurrence patterns and treatments after recurrence in these patients are summarized in Supplementary Table 2.

Compared with BCLC-A and BCLC-B1 patients, BCLC-B2 patients had a shorter recurrence time and a higher proportion of recurrence with macrovascular invasion and/or extrahepatic metastasis. However, there were no significant statistical differences in recurrence patterns and treatment after recurrence between BCLC-B1 and BCLC-A patients. Fewer BCLC-B2 patients underwent curative treatments after recurrence than BCLC-A patients, but the treatment after recurrence was similar between BCLC-B2 patients and BCLC-B1 patients (Supplementary Table 2).

Both BCLC-B1 and BCLC-A patients had good RTDS (median RTDS: Not reached, *vs* 49 mo for BCLC-B1 and BCLC-A patients, respectively; $P = 0.599$), while BCLC-B2 patients had a worse RTDS (16 mo *vs* not reached, $P < 0.001$; 16 mo *vs* 49 mo, $P = 0.042$) (Figure 3).

Table 2 Univariate and multivariate analysis of overall survival in patients with Barcelona Clinic Liver Cancer stage B hepatocellular carcinoma

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Gender (male)	1.128 (0.409-3.117)	0.816		
Age (> 65 yr)	0.668 (0.285-1.562)	0.352		
BMI > 25	0.954 (0.513-1.772)	0.880		
HBs-Ag positive	1.084 (0.466-2.525)	0.851		
ALBI grade				
1	1.00 (Reference)		1.00 (Reference)	
2	1.891 (1.112-3.217)	0.019	2.279 (1.236-4.201)	0.008
AFP > 400 ng/mL	1.969 (1.165-3.327)	0.011		
Maximum tumor size > 5 cm	2.510 (1.354-4.651)	0.003		
Tumor number > 3	3.806 (1.716-8.444)	0.001	5.519 (2.207-13.803)	< 0.001
N + S > 10	3.403 (2.031-5.702)	< 0.001	2.996 (1.779-5.045)	< 0.001
Bilateral tumor distribution	2.201 (1.312-3.694)	0.003		
Presence of MVI	1.816 (0.855-3.858)	0.120		
Edmondson-Steiner III-IV	2.084 (1.248-3.480)	0.005	2.051 (1.219-3.449)	0.007
Major resection	1.886 (1.115-3.191)	0.018		
NAH	0.905 (0.458-1.788)	0.775		
Preoperative TACE				
No	1.00 (Reference)			
Yes	0.494 (0.198-1.238)	0.132		

BMI: Body mass index; HBs-Ag: Hepatitis B surface antigen; ALBI: Albumin-bilirubin; AFP: Alpha-fetoprotein; N + S: The sum of tumor size and number; MVI: Microvascular invasion; NAH: Non-anatomical hepatectomy; TACE: Transarterial chemoembolization.

Independent risk factors for RTDS

We further conducted a multivariate analysis of the factors affecting RTDS of BCLC stage A or B MNHCC patients undergoing LR. Multivariate analysis showed that initial tumor with BCLC-B2 (N + S > 10) (HR 2.696, 1.468 to 4.953; $P = 0.001$), recurrence within 2-year (HR 4.353, 1.024 to 18.503; $P = 0.046$), recurrent tumor number > 3 (HR 3.247, 1.629 to 6.474; $P = 0.001$), recurrence with macrovascular invasion and/or extrahepatic spread (HR 2.894, 1.458 to 5.746; $P = 0.002$) and noncurative treatments after recurrence (HR 2.423, 1.209 to 4.854; $P = 0.013$) were independent risk factors for RTDS (Supplementary Table 3).

DISCUSSION

The role of LR in BCLC-B HCC patients is unclear. Although the latest BCLC staging system still does not recommend LR for BCLC-B patients, the results of emerging studies have indicated that LR resulted in a good 5-year OS for BCLC-B HCC patients[4-6]. In this study, patients who underwent LR for BCLC-B HCC had an overall 5-year OS rate of 57.2%, which demonstrated that LR was a good treatment option in these patients.

To select BCLC-B patients more suitable for LR, we stratified these patients according to N + S, which has been used to select HCC patients who are more suitable for LT and for TACE[24,25]. In fact, Matsukuma *et al*[26] suggested that N + S was a good prognostic factor for BCLC-B HCC patients undergoing hepatectomy. The present study increased the cutoff point of N + S from 8 to 10, which may be related to different study cohorts and different calculation methods used for the cutoff value[26]. Nevertheless, the results of this study and in the study by Matsukuma *et al*[26] demonstrated that N + S could predict the recurrence and OS of BCLC-B HCC patients undergoing hepatectomy. In addition, the present study showed that N + S had a predictive accuracy similar to TBS and TTV in predicting OS in

Table 3 Univariate and multivariate analysis of recurrence-free survival in patients with Barcelona Clinic Liver Cancer stage B hepatocellular carcinoma

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Gender (male)	1.135 (0.526-2.450)	0.747		
Age (> 65 yr)	0.881 (0.514-1.511)	0.646		
BMI > 25	1.157 (0.729-1.836)	0.536		
HBs-Ag positive	1.109 (0.558-2.202)	0.769		
ALBI grade				
1	1.00 (Reference)			
2	1.474 (0.988-2.198)	0.057		
AFP > 400 ng/mL	1.458 (0.984-2.162)	0.060		
Maximum tumor size > 5 cm	1.253 (0.830-1.891)	0.283		
Tumor number > 3	2.449 (1.123-5.343)	0.024		
N + S > 10	2.113 (1.385-3.224)	0.001	1.657 (1.057-2.596)	0.028
Bilateral tumor distribution	2.104 (1.409-3.140)	< 0.001	1.820 (1.187-2.791)	0.006
Presence of MVI	1.757 (0.973-3.171)	0.062		
Edmondson-Steiner III-IV	1.709 (1.151-2.539)	0.008	1.676 (1.127-2.493)	0.011
Major resection	1.285 (0.867-1.904)	0.211		
NAH	1.126 (0.650-1.950)	0.673		
Preoperative TACE				
No	1.00 (Reference)			
Yes	0.784 (0.437-1.405)	0.414		

BMI: Body mass index; HBs-Ag: Hepatitis B surface antigen; ALBI: Albumin-bilirubin; AFP: Alpha-fetoprotein; N + S: The sum of tumor size and number; MVI: Microvascular invasion; NAH: Non-anatomical hepatectomy; TACE: Transarterial chemoembolization.

BCLC-B patients. However, compared with the complicated calculation of TBS and TTV, the calculation of N + S is simpler and more suitable for clinical application.

Previous studies have focused on the OS when selecting BCLC-B patients for LR, and ignored that those selected patients still had a high postoperative recurrence rate[7,8]. In order to demonstrate that the selected BCLC-B HCC patients truly benefit from LR rather than remedial treatments after recurrence, and to better understand the tumor characteristics of the selected patients, we compared not only the OS and RFS, but also the RTDS, recurrence patterns, and treatments after recurrence.

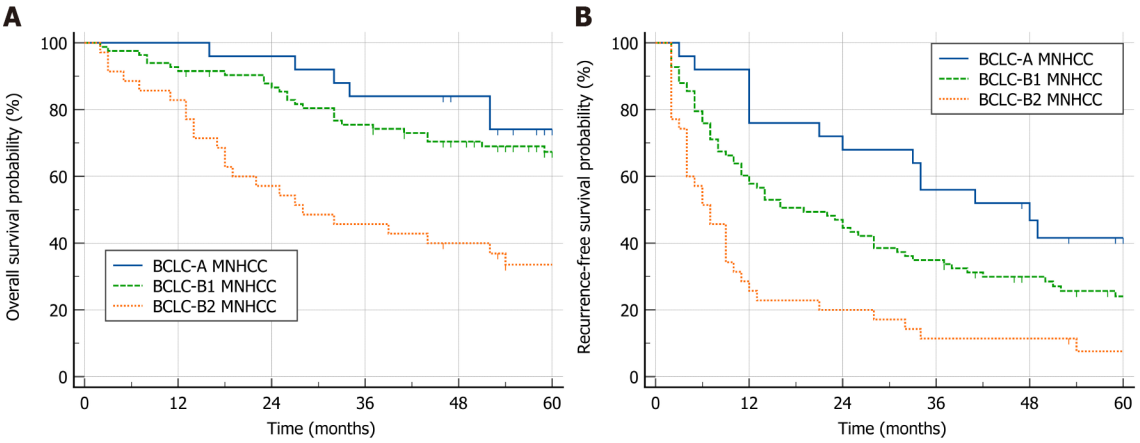
In the present study, BCLC-B1 (BCLC-B with N + S ≤ 10) HCC patients were considered as BCLC-B HCC patients who likely benefitted most from LR. Although BCLC-B1 HCC patients were still more likely to develop recurrence after LR than BCLC-A MNHCC patients, these BCLC-B1 patients had mild recurrence pattern, good RTDS and excellent OS similar to BCLC-A MNHCC patients.

However, BCLC-B2 (BCLC-B with N + S > 10) HCC patients not only had a high postoperative recurrence rate, but also an aggressive recurrence pattern. Although the treatment after recurrence was similar between BCLC-B2 patients and BCLC-B1 patients, the BCLC-B2 patients still had a worse RTDS. The long-term OS of BCLC-B2 patients undergoing LR is not satisfactory.

To the best of our knowledge, this study is the first to demonstrate that N + S could predict not only prognosis in BCLC-B HCC patients, but also the recurrence patterns and RTDS in these patients.

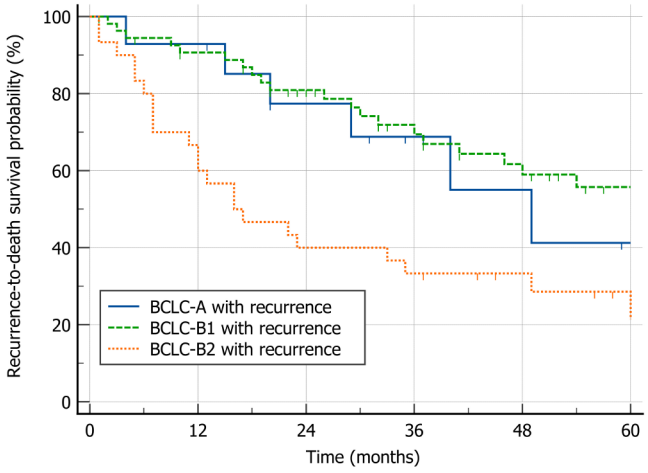
In addition, it is interesting to note that patients with BCLC-B1 HCC had worse RFS but comparable OS than patients with BCLC-A MNHCC in this study. In fact, previous studies comparing LT *vs* LR in HCC patients found a similar phenomenon. These studies showed that although patients receiving LR had a higher rate of postoperative recurrence, the 5-year OS between LR and LT was comparable[27,28]. Previous studies have suggested that the reasons for this phenomenon may be related to noncancerous death in the LT group and treatment after recurrence in the LR group, and our results suggest that it may also be related to the recurrence patterns after LR.

As a single-center retrospective study, the present study has some limitations. Firstly, the sample size was small, which may have affected the accuracy of the results. Secondly, there was a lack of



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Figure 2 Comparison of overall survival and recurrence-free survival in multinodular hepatocellular carcinoma patients with Barcelona Clinic Liver Cancer stage A, B1 and B2. A: overall survival; B: recurrence-free survival. BCLC: Barcelona Clinic Liver Cancer; MNHCC: Multinodular hepatocellular carcinoma.



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Figure 3 Comparison of recurrence-to-death survival in Barcelona Clinic Liver Cancer stage A, B1 and B2 multinodular hepatocellular carcinoma patients with recurrence. BCLC: Barcelona Clinic Liver Cancer.

comparison of treatment options other than LR. Some patients with BCLC-B HCC and N + S ≤ 10 would meet the 'Extended Liver Transplant criteria', and the best treatment option for these patients remains to be explored[29]. Finally, the results of this study need to be verified by an external cohort.

CONCLUSION

N + S is a good measure that could predict the OS, RFS, RTDS and recurrence patterns in BCLC-B HCC patients undergoing LR. In particular, BCLC-B patients with N + S ≤ 10 had survivals similar to those of BCLC-A MNHCC patients. Given the computational simplicity of N + S, it is worth exploring the application of N + S to guide decision-making in the treatment of BCLC-B patients.

ARTICLE HIGHLIGHTS

Research background

Emerging studies have shown that Barcelona Clinic Liver Cancer (BCLC) intermediate-stage hepatocellular carcinoma (HCC) patients had a good prognosis after liver resection (LR), but the subgroups of BCLC-B patients more suitable for LR have yet to be defined.

Research motivation

There is a lack of studies on whether the sum of tumor size and number (N + S) can be used to select BCLC-B patients who are more suitable for LR. The effect of recurrence patterns on long-term survival in BCLC-B patients undergoing LR is also poorly explored.

Research objectives

The present study aimed to identify BCLC-B patients more suitable for LR and to further analyze the reasons why these patients could benefit from LR.

Research methods

BCLC stage A or B multinodular HCC (MNHCC) patients undergoing curative hepatectomy were enrolled. Overall survival (OS), recurrence-free survival (RFS), recurrence-to-death survival (RTDS), recurrence patterns, and treatments after recurrence in BCLC-B patients in each subgroup according to N + S were compared with those in BCLC-A patients.

Research results

N + S could predict not only the OS and RFS in BCLC-B HCC patients undergoing hepatectomy, but also the recurrence patterns and RTDS in these patients. BCLC-B patients with N + S ≤ 10 had mild recurrence patterns, good RTDS and excellent OS similar to those in BCLC-A MNHCC patients.

Research conclusions

N + S can be used to select BCLC-B HCC patients who are more suitable for LR, and LR should be considered in BCLC-B patients with N + S ≤ 10.

Research perspectives

As a measure that can be easily obtained and calculated in clinical practice, N + S can help with the clinical decision-making in the treatment of BCLC-B HCC patients.

FOOTNOTES

Author contributions: Hu XS performed the research and wrote the paper; Yang HY performed the follow-up; Leng C designed the research and supervised the report; Zhang ZW provided clinical advice and supervised the report; and all authors read and approved the final version.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology (Approval No. TJ-IRB20210918).

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: All the authors report no relevant conflicts of interest for this article.

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ORIGINAL ARTICLE

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Virological and histological evaluation of intestinal samples in COVID-19 patients

Dajana Cuicchi, Liliana Gabrielli, Maria Lucia Tardio, Giada Rossini, Antonietta D'Errico, Pierluigi Viale, Tiziana Lazzarotto, Gilberto Poggioli

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Abstract

BACKGROUND

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the pathogen responsible for pandemic coronavirus disease 2019 (COVID-19). It is a highly contagious virus which primarily affects the respiratory tract, nevertheless, the lungs are not the only target organs of the virus. The intestinal tract could represent an additional tropism site for SARS-CoV-2. Several observations have collectively suggested that enteric infections can occur in COVID-19 patients. However, the detection of viral RNA in gastrointestinal (GI) tissue samples has not been adequately investigated and results are conflicting.

AIM
To detect the presence of SARS-CoV-2 RNA in intestinal mucosa samples and to evaluate histological features.

METHODS
The COVID-19 patients hospitalized at an Italian tertiary hospital from April 2020 to March 2021 were evaluated for enrollment in an observational, monocentric trial. The study population was composed of two groups of adult patients. In the first group (biopsy group, 30 patients), patients were eligible for inclusion if they had mild to moderate disease and if they agreed to have a rectal biopsy; in the second group (surgical specimen group, 6 patients), patients were eligible for inclusion if they underwent intestinal resection during index hospitalization. Fifty-nine intestinal mucosal samples were analyzed.

RESULTS
Viral RNA was not detectable in any of the rectal biopsies performed (0/53). Histological examination showed no enterocyte damage, but slight edema of the lamina propria with mild inflammatory lymphoplasmacytic infiltration. There was no difference in inflammatory infiltrates in patients with and without GI symptoms. SARS-CoV-2 RNA was detected in fecal samples in 6 cases out of 14 cases examined (42.9%). In the surgical specimen group, all patients underwent emergency intestinal resection. Viral RNA was detected in 2 surgical specimens of the 6 examined, both of which were from patients with active neoplastic disease. Histological examination also pointed out abundant macrophages, granulocytes and plasma cells infiltrating the muscular layer and adipose tissue, and focal vasculitis.

CONCLUSION
Mild-moderate COVID-19 may not be associated with rectal infection by the virus. More comprehensive autopsies or surgical specimens are needed to provide histological evidence of intestinal infection.

Key Words: COVID-19; SARS-CoV-2; Intestinal infection; Intestinal samples; Intestinal tropism; Rectal samples

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Core Tip: The detection of viral RNA in gastrointestinal tissue samples has not been adequately investigated. In this trial, 53 rectal biopsies and 6 surgical specimens from coronavirus disease 2019 patients were analyzed, with the primary objective of detecting severe acute respiratory syndrome coronavirus-2 RNA, using real time reverse transcriptase-polymerase chain reaction, and evaluating the histological features. Viral RNA was not detectable in any of the rectal biopsies performed (0/53). Histological examination of rectal biopsies showed no enterocyte damage, but mild inflammatory infiltration. Viral RNA was detected in 2 surgical specimens of the 6 examined, both of which were from patients with active neoplastic disease. Histological examination also pointed out mild inflammatory infiltration and focal vasculitis.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the pathogen responsible for pandemic coronavirus disease 2019 (COVID-19). It is a highly contagious virus which primarily affects the respiratory tract, typically causing symptoms, such as fever, dry cough and dyspnea, up to respiratory failure and death[1]. Nevertheless, the lungs are not the only target organs of the virus. Several studies have suggested that the intestinal tract could represent an additional tropism site for SARS-CoV-2[2]. Gastrointestinal (GI) symptoms, including diarrhea, nausea, vomiting, anorexia and abdominal pain are present in a substantial number of COVID-19 patients; the incidence can vary from

10% to 55%[3-6]. Many studies have reported that viral nucleic acids are detected in stool samples of COVID-19 patients with rates varying from 15.3% to 66.7%[7-11]. Viral receptor angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine-type 2 are highly expressed in the epithelial cells of the intestinal mucosa[7,12]. Moreover, some studies have demonstrated that SARS-CoV-2 virus can actively infect and replicate in human enteroids and *in vitro* models of human intestinal epithelium[13, 14]. These observations have collectively suggested that enteric infection can occur in patients with COVID-19. However, more robust evidence is limited. The detection of viral RNA in GI tissue samples or the isolation of the virus in stool samples have been reported in studies which enrolled only a limited number of cases[15-18]. In the present study, a greater number of intestinal mucosal samples from COVID-19 patients (as compared to previous studies) were analyzed, with the primary objective of detecting SARS-CoV-2 RNA and evaluating histological features.

MATERIALS AND METHODS

The COVID-19 patients hospitalized at IRCCS Sant'Orsola Hospital, University of Bologna, Bologna, Italy from June 2020 to March 2021 were evaluated for enrollment in a monocentric trial. SARS-CoV-2 infection was diagnosed at admission using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) on pharyngeal swab specimens. The study was approved by the local hospital ethics committee and informed consent was obtained from all of the patients.

Inclusion/exclusion criteria

The study population was composed of two groups of adult patients (≥ 18 years of age) hospitalized for COVID-19. In the first group (biopsy group), patients were eligible for inclusion if they had mild to moderate disease (mild: Only mild symptoms with no radiological signs; moderate: Characterized by fever, respiratory symptoms and radiological signs of pneumonia)[19] and if they agreed to have a rectal biopsy; in the second group (surgical specimen group), patients were eligible for inclusion if they underwent intestinal resection during index hospitalization.

In the biopsy group, patients who had severe (dyspnea, respiratory frequency ≥ 30 breaths per minute, blood oxygen saturation ≤ 93%, partial pressure of arterial oxygen to fraction of inspired oxygen ratio < 300, and/or lung infiltrates > 50% within 24-48 h) and critical (respiratory failure, septic shock, and/or multiple organ dysfunction or failure) disease[19], contraindications to rectal biopsies (anticoagulant therapy with the exception of therapy with low molecular weight heparin and antiplatelet therapy), rectal disease (*e.g.*, chronic inflammatory disease and proctitis), previous abdominoperineal resection, recent anal surgery, anal stenosis or anal pain were excluded. In the surgical specimen group, those patients undergoing surgical treatment, cases without intestinal resection were excluded.

Interventions and design overview

Patients enrolled in the biopsy group were asked to collect a stool sample and undergo anoscopy with biopsy during their hospital stay. The anoscopy was performed at the bedside with the patient in the left lateral decubitus position using a disposable anoscope 18 mm diameter, lubricated with an anesthetic gel. During each anoscopy, 2 biopsies were performed at different sites of the rectal mucosa for viral RNA detection and stored, one in RNA Preservation Medium (RNAlater™Stabilization Solution, Thermo Fisher Scientific, United States) and then fresh frozen, and one in 10% buffered formalin. The biopsy specimen fixed in formalin was subsequently paraffin embedded, cut into 4-μm-thick sections and then stained with hematoxylin and eosin. Multiple sections were obtained to assess the extent of the inflammation. Serial paraffin sections, 3-μm-thick, mounted on precoated slides were processed using standardized automated procedures with prediluted anti-CD68 antibody (clone PG-M1; DBS, Pleasanton, CA). The stool samples and all the biopsies were tested for the detection of SARS-CoV-2 RNA using real-time RT-PCR. The extraction of nucleic acids, reverse transcription reaction and real-time PCR amplification were performed using a SARS-CoV-2 ELITe MGB® Kit (ELITechGroup, Italy) on an ELITeInGenius® instrument. The assay detects the RNA of two SARS-CoV-2 specific genomic regions: RdRp gene and ORF8 gene. The tissue viral load was reported as number of copies/microgram RNA. The limit of detection of the test is 2 copies/reaction (10 copies/microgram RNA). Positive results below the lower limit of quantification (5 copies/reaction) were reported as < 25 copies/microgram RNA. Qualitative data were provided for the fecal samples.

Patients enrolled in the surgical specimen group were asked for consent in order to take a tissue sample from the surgical specimen to identify the presence of both inflammatory cells infiltrated and virus SARS-CoV-2 RNA using the same methodology described above. For each patient, data were collected regarding sex, age, comorbidities, disease severity, symptoms on admission, radiological features and clinical outcomes.

Outcomes

The primary aim was to evaluate the presence of SARS-CoV-2 RNA in intestinal mucosa samples using real-time RT-PCR. The secondary aims were to detect both SARS-CoV-2 RNA in stool samples using

RT-PCR and the inflammatory state in all tissue samples.

Statistical analysis

Sample size analysis was not carried out due to the exploratory nature of the study. Statistical analysis was carried out using SPSS 20 software (SPSS Inc, Chicago, IL). Continuous data were expressed as means ± SDs (for data with normal distribution), or median and range (for data with nonnormal distribution); discrete data were expressed as percentages. The descriptive analyses were carried out using parametric methods, depending on the distribution of the variables under examination. Variables between the 2 groups were compared using the following tests as appropriate: *t*-test and Fisher's test. The differences were considered statistically significant for values of < 0.05.

RESULTS

From June 2020 to March 2021, 957 patients hospitalized for confirmed COVID-19 were screened. The diagram in Figure 1 shows the flow of participants in the trial. The biopsy group consisted of 30 patients and the surgical specimen group had 6 patients. The clinical characteristics of the patients in both groups are shown in Tables 1 and 2. In the biopsy group, GI symptoms were present in 19 patients (63.3%). Diarrhea was the most common GI symptom; it was reported in 14 cases (73.7%), anorexia in 8 cases (42.1%), nausea and vomiting in 7 cases (36.8%) and abdominal pain in 4 cases (21.0%). There was no statistically significant difference in the general demographics or clinical outcomes between patients with and without GI symptoms (Table 1); SARS-CoV-2 RNA was detected in the stool in 36.4% of the patients with GI symptoms and in 66.7% of those without GI symptoms. Nevertheless, the number of positive fecal cases did not show significant difference between the two groups. Considering only patients who had diarrhea on admission, viral RNA was found in half of the fecal samples examined. Overall, SARS-CoV-2 RNA was detected in fecal samples in 6 cases out of 14 cases examined (42.9%). The greatest number of positive cases was found in the fecal samples collected in the 2nd wk after the first positive nasopharyngeal swab (NPS) (3/3, 100%), 2 cases in the 1st wk (2/5, 40%) and the remaining case in the 3rd wk (1/4, 25%). There was no significant difference in time interval between sampling and the first positive NPS in positive and negative viral RNA fecal samples (9.0 ± 4.6 *vs* 16.4 ± 14.7 respectively, $P = 0.26$). Viral RNA was not detectable in any of the 53 rectal biopsies performed. The histological examination of the rectal samples showed that the mucosal epithelium of the rectum did not have any major damage in patients with and without GI symptoms. The glandular architecture was always normal. In no case was there any enterocyte damage. Moreover, microscopy revealed slight expansion of the lamina propria by moderate edema in 26 cases out of 30 (86.7%) and an inflammatory lymphoplasmacytic infiltration in the lamina propria, varying from mild to moderate, in 28 and 2 cases (93.3% and 6.7%), respectively. There was no difference in inflammatory infiltrates in patients with and without GI symptoms (Table 1). Rare eosinophilic and neutrophil granulocytes were identified in the lamina propria in 20 and 2 cases (66.7% and 6.7%), respectively.

In the surgical specimen group, all patients underwent emergency intestinal resection (Table 2). Three patients were hospitalized for COVID-19 symptoms and, 8-20 d after the first positive NPS, underwent bowel resection for iatrogenic perforation of the rectum, diverticular perforation and ischemic colitis, respectively. One patient was hospitalized for intestinal obstruction secondary to bridles and underwent segmental resection of the small intestine within 1 d after a positive pharyngeal swab for SARS-CoV-2. One patient with plurimetastic colon cancer (liver, lung, peritoneum and brain), during hospitalization for a spontaneous pneumothorax developed symptoms of COVID-19 and, soon after, had an intestinal perforation for which the patient underwent a right hemicolectomy. The remaining patient affected by acute myeloid leukemia developed COVID-19 symptoms while hospitalized for chemotherapy; a week later, the patient underwent an appendectomy and colonic resection for peritonitis secondary to an inflammatory mass englobing the appendix and the sigmoid colon. All the patients died during hospitalization. SARS-CoV-2 RNA was detected in 2 cases out of 6 (33.3%). In both cases, the virus RNA was positive in the colonic tissue of the 2 patients with active neoplastic disease. In both cases, the viral load was very low. In one intestinal specimen, the viral load was less than the limit of quantification of the test (< 25 copies/microg RNA) and, in the second, it was 29 copies/microgr RNA. Histological examination of the apparently healthy tissue of all the cases showed normal glandular architecture, no enterocyte damage, a slight expansion of the lamina propria by edema and inflammatory lymphoplasmacytic infiltration in the lamina propria varying from mild to moderate. However, in the two cases positive for viral RNA, histological examination also pointed out abundant macrophages, granulocytes and plasma cells infiltrating the muscular layer, and adipose tissue, focal vasculitis and some macrophages in the vascular lumen (Figures 2A and B).

Table 1 Baseline characteristics of the biopsy group

Characteristic	All patients, *n* = 30	Patients with GI symptoms at onset, *n* = 19	Patients without GI symptoms at onset, *n* = 11	*P* value
Median age in yr, mean (range)	65 (41-90)	60 (44-90)	78 (41-89)	NS
Sex as women:men (%)	7:23 (23.3:76.7)	4:15 (21.0:79)	3:8 (27.3:72.7)	NS
COVID-19 classification				
Mild, *n* (%)	0	0	0	NS
Moderate, *n* (%)	30 (100)	19 (100)	11 (100)	NS
Coexisting illness				
Hypertension	9 (30.0)	6 (31.6)	3 (27.3)	
Diabetes mellitus	4 (13.3)	3 (15.8)	1 (9.1)	
Cardio-cerebrovascular disease	5 (16.7)	3 (15.8)	2 (18.2)	
Previous malignant tumor	2 (6.7)	1 (5.3)	1 (9.1)	
Chronic obstructive pulmonary disease	3 (10.0)	2 (10.5)	1 (9.1)	
Chronic kidney disease	3 (10.0)	2 (10.5)	1 (9.1)	
Obesity	2 (6.7)	2 (10.5)	0	
Chest CT				
Negative, *n* (%)	4 (13.3)	3 (15.8)	1 (9.1)	NS
Bilateral distribution of GGO with or without consolidation, *n* (%)	16 (53.3)	9 (47.4)	7 (63.6)	NS
Unilateral distribution of GGO, *n* (%)	2 (6.7)	1 (5.2)	1 (9.1)	NS
Bilateral interlobular septal thickening, *n* (%)	8 (26.7)	6 (31.6)	2 (18.2)	NS
Median time from first positive NPS (range), d	10 (1-25)	8.0 (1-25)	12.0 (3-24)	NS
SARS-CoV-2 RNA in feces[1], *n* (%)	6/14 (42.9)	4/11 (36.4)	2/3 (66.7)	NS
SARS-CoV-2 RNA in RNA-preservation medium rectal mucosa biopsy[2], *n* (%)	0	0	0	-
SARS-CoV-2 RNA in FFPE rectal mucosa biopsy[3], *n* (%)	0	0	0	-
Histological examination				
Glandular architecture: Normal/altered, *n* (%)	30/0 (100/0)	19/0 (100/0)	11/0 (100/0)	NS
Edema of the lamina propria: Absent/slight, *n* (%)	4/26 (13.3/86.7)	3/16 (15.8/84.2)	1/10 (9.1/90.9)	NS
Inflammatory lymphoplasmacytic infiltration in the lamina propria: Mild/moderate, *n* (%)	28/2 (93.3/6.7)	18/1 (94.7/5.3)	10/1 (90.9/9.1)	NS
Eosinophilic granulocytes in the lamina propria: Absent/occasional/scattered, *n* (%)	10/18/2 (33.3/60.0/6.7)	7/10/2 (36.8/52.6/10.7)	3/8/0 (27.3/72.2/0)	NS
Neutrophil granulocytes in the lamina propria: Absent/rare, *n* (%)	28/2 (93.3/6.7)	18/1	10/1 (90.9/9.1)	NS
Enterocyte damage: Absent/present, *n* (%)	30/0 (100/0)	19/0	11/0 (100/0)	NS
Clinical outcome, *n* (%)				
Discharged	30 (100)	19 (100)	11 (100)	NS
Died	0	0	0	NS

[1]Available in 14 cases.
[2]Available in 23 cases.
[3]Available in 30 cases.
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; GI: Gastrointestinal; NPS: Nasopharyngeal swab; SD: Standard deviation; FFPE: Formalin-fixed paraffin-embedded; GGO: Ground glass opacities; COVID-19: Coronavirus disease 2019; CT: Computed tomography.

DISCUSSION

In the present study, SARS-CoV-2 RNA was identified in only two cases out of the 59 intestinal samples (3.4%). In both cases the viral load was very low; the intestinal samples consisted of colonic tissue of patients with active neoplastic disease (a patient with acute myeloid leukemia who was receiving immunosuppressive therapy and a patient with metastatic colon cancer), and there was a nosocomial transmission of SARS-CoV-2. Interestingly, in the biopsy group, two patients had previously undergone surgery for cancer (prostate and cervix) and in both cases, no viral RNA was identified in the rectal samples. Several studies have suggested that people with neoplastic disease are more likely to contract COVID-19 and to develop more severe disease or die from it than the general population. A recent systematic review on COVID-19 patients with active malignancy, defined as current malignant disease or treatment for malignancy within the last 12 mo, showed that cancer constitutes a co-morbidity in 2.6% [95% confidence interval (CI): 1.8%-3.5%, I^2: 92.0%] of hospitalized COVID-19 patients and that the pooled in-hospital mortality risk was 14.1% (95%CI: 9.1%-19.8%, I^2: 52.3%)[20]. Nahshon *et al*[21] suggested a severe clinical course of 50.6% and a mortality rate of 34.5% in COVID-19 patients with cancer. The worst COVID-19 outcomes include acute respiratory distress syndrome, septic shock, acute myocardial ischemia and death[22]. These severe events occurred more frequently in patients with stage IV cancer as compared to those with non-stage IV cancer (70.0% *vs* 44.4%, respectively) and if the last antitumor treatment was within 14 d (hazard ratio = 4.079, 95%CI: 1.086-15.322, P = 0.037)[22]. The pathogenesis of severe COVID-19 in cancer patients may be due to the aggravation of inflammatory cytokine storms, the imbalance of immune responses, and multiple organ damage[23]. In a multicenter retrospective cohort study on 232 patients with cancer and 519 statistically matched patients without cancer, Tian *et al*[23] identified elevated levels of interleukin (IL)-6, tumor necrosis factor α (TNF-α) and N-terminal pro-B-Type natriuretic peptide (NT-proBNP) and a reduced level of CD4+T cells and albumin-globulin ratio as risk factors of COVID-19 severity in patients with cancer. Similarly, in a retrospective cohort study which included 2052 patients hospitalized with COVID-19 (cancer, n = 93; non-cancer, n = 1959), Cai *et al*[24] reported that immune dysregulation was an important feature in cancer patients with COVID-19, which might account for their poorer prognosis; they found that COVID-19 patients with cancer had ongoing and significantly elevated inflammatory factors and cytokines (C-reactive protein, procalcitonin, IL-2 receptor, IL-6, IL-8) as well as a decreased number of immune cells (CD8 + T cells, CD4 + T cells, B cells, nature killer cells, T-helper and T- suppressor cells) than those without cancer. In patients with weakened immune systems, SARS-CoV-2 could infect vascular epithelial cells and organs, such as the lungs, heart, kidneys, liver, and intestine, expressing high levels of ACE2[25]. Autopsy data have reported viral infection in several organs, indicating hematogenic spread of the virus[26,27]. Moreover, serum SARS-CoV-2 nucleic acid (RNAemia) was associated with COVID-19 severity [odds ratio (OR) = 5.43, 95%CI: 3.46-8.53], increased risk of multiorgan failure (OR = 7.33, 95%CI: 2.46-21.88) and mortality (OR = 11.07, 95%CI: 5.60-22.88)[28]. Notably, viral RNA was undetectable in any of 53 rectal biopsies performed on the 30 patients hospitalized for moderate COVID-19 (biopsy group). The inability of the authors to detect viral RNA in the rectal samples contrasted with some data which have identified SARS-CoV-2 in intestinal samples[8,9]. Xiao *et al*[8] evaluated the viral nucleocapsid protein in the GI tissues of a COVID-19 patient who developed severe respiratory distress and an upper GI bleed. At endoscopy, they observed mucosal damage in the esophagus and found viral nucleocapsid protein in the cytoplasm of gastric, duodenal, and rectal glandular epithelial cells with immunofluorescent staining[10]. Lin *et al*[9] detected SARS-CoV-2 RNA at endoscopy in esophageal, gastric, duodenal, and rectal specimens in 2 out of 6 COVID-19 patients having GI symptoms. In this study, the presence of viral RNA in the GI tissue was also associated with severe disease; in fact, SARS-CoV-2 RNA was found in the samples of 2 patients with severe disease but not in those of 4 patients with non-severe disease[9]. Therefore, although the authors' failure to detect SARS-CoV-2 in the rectal samples could have been due to the mild disease course of the cases selected, the possibility that the viral load was below the detection limit of their RT-PCR assay cannot be excluded. This could justify the presence of a mild to moderate inflammatory infiltrate in the lamina propria in all the rectal samples at histological examination. In fact, plasma cells, lymphocytes, and granulocytes can migrate to the extravascular space to reach the possibly infected tissues. These findings are in line with the low number of endoscopic and histological examinations of intestinal samples of COVID-19 patients which showed inflammatory infiltration in the lamina propria. Endoscopy and biopsy samples of the esophagus, stomach, duodenum and rectum were taken from a 78-year-old patient with COVID-19 who showed symptoms of upper GI bleeding. Numerous infiltrating plasma cells and lymphocytes with interstitial edema were found in the lamina propria of the stomach, duodenum and rectum of this patient[8]. In a surgical rectal specimen obtained during the incubation period in a COVID-19 patient with rectal adenocarcinoma, histological examination showed prominent lymphocytes and macrophages infiltrating the lamina propria without significant mucosal damage. T lymphocytes and macrophages were found to be more numerous than B lymphocytes in the lamina propria[29].

Six patients out of the 14 cases examined (42.9%) tested positive for SARS-CoV-2 RNA in the stool. Multiple studies have reported the positive detection of viral nucleic acids in the fecal samples of COVID-19 patients, finding rates varying from 15.3% to 66.7%[6,14]. In a meta-analysis, the authors

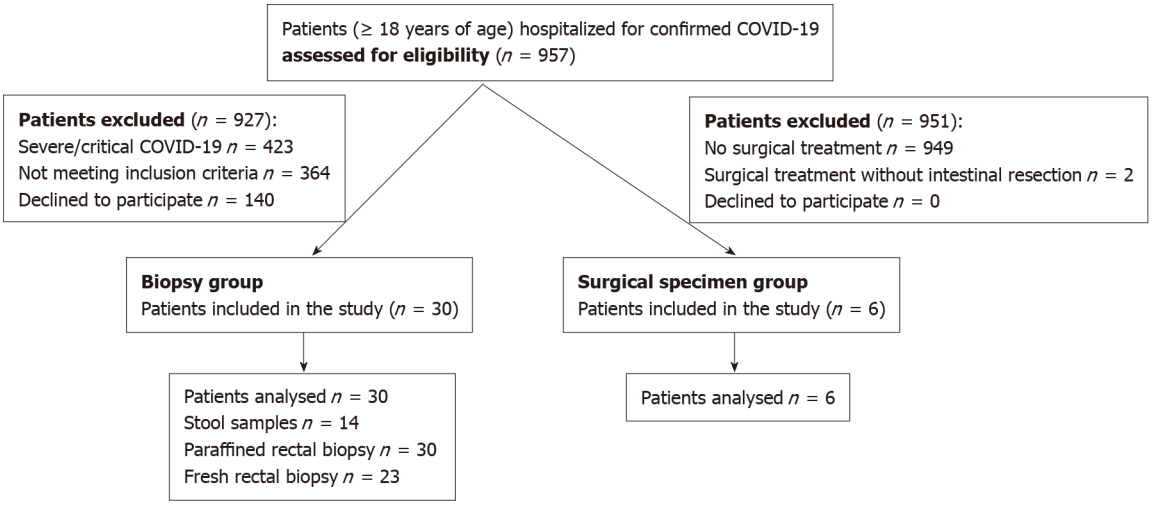
Table 2 Baseline characteristics of the surgical specimen group

Characteristic	Patient no. 1	Patient no. 2	Patient no. 3	Patient no. 4	Patient no. 5	Patient no. 6
Age in yr	93	76	78	96	56	62
Coexisting illness	COPD, CVD	COPD, metastatic colon cancer	COPD, CVD, chronic kidney disease	COPD, hypertension	Psychiatric disease, obesity	Acute myeloid leukemia
COVID-19 classification	Moderate	Moderate	Mild	Mild	Moderate	Moderate
Thorax CT imaging	Bilateral distribution of GGO with consolidation	Unilateral distribution of GGO with consolidation	Bilateral distribution of GGO with consolidation	Unilateral distribution of GGO with consolidation	Bilateral distribution of GGO with consolidation	Bilateral distribution of GGO with consolidation
Time from first positive NPS	10	1	8	1	20	7
Reason for admission	COVID-19	Spontaneous pneumothorax	COVID-19	Intestinal occlusion	COVID-19	Chemotherapy for acute myeloid leukemia
Reason for surgical treatment	Rectal perforation after enema	Fecal peritonitis from perforated colon cancer	Fecal peritonitis from perforated diverticulitis	Small bowel obstruction secondary to bridle	Ischemic colitis	Acute abdomen
Surgical treatment	Colorectal resection and end-colostomy	Right colectomy and end-ileostomy	Sigmoid resection and end-colostomy	Segmental intestinal resection	Total colectomy and ileostomy	Appendectomy and sigmoid resection
GI symptoms at onset	No	No	No	No	No	Diarrhea
SARS-CoV-2 RNA in FFPE tissue/viral load (*n*° copies/microg)	No	29	No	No	No	< 25
Histological examination						
Glandular architecture	Normal	Normal	Normal	Normal	Normal	Normal
Edema of the lamina propria	Slight	Absent	Slight	Slight	Slight	Absent
Inflammatory lymphoplasmacytic infiltration in the lamina propria	Mild	Mild	Mild	Mild	Mild	Moderate
Eosinophilic granulocytes in the lamina propria	Occasional	Occasional	Occasional	Occasional	Occasional	Occasional
Enterocyte damage	Absent	Absent	Absent	Absent	Absent	Absent
Vasculitis	Absent	Focal	Absent	Absent	Absent	Focal
Granulocyte, macrophage and plasma cell infiltrate in the muscle wall and adipose tissue	Absent	Moderate	Absent	Absent	Absent	Severe
Clinical outcome	Died	Died	Died	Died	Died	Died
Time from surgical treatment in d	6	4	4	35	17	20

GI: Gastrointestinal symptoms; COPD: Chronic obstructive pulmonary disease; CVD: Cardiovascular disease; NPS: Nasopharyngeal swab; FFPE: Formalin-fixed paraffin-embedded; GGO: Ground glass opacities; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; CT: Computed tomography.

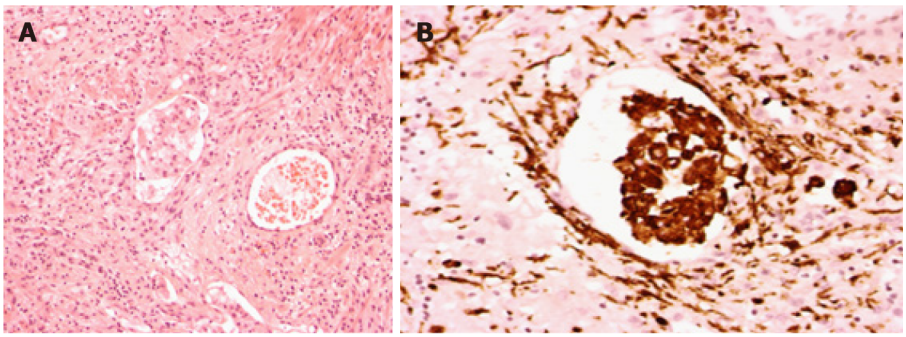
showed that viral RNA may be present in the feces in 48.1% of patients[30]. The mechanism of diarrhea in patients with COVID-19 is still largely unknown. Various etiopathogenetic hypotheses have been advanced to explain the occurrence of diarrhea in COVID-19 patients including alterations in gut microbiota, osmotic diarrhea due to malabsorption or inflammation, release of virulent proteins or toxins, and viral-induced intestinal fluid and electrolyte secretion by activation of the enteric nervous system[31,32].

Currently, the exact mechanism of intestinal involvement in COVID-19 is not yet well understood. Intestinal epithelial cells could be primarily infected by SARS-CoV-2 *via* the oral-fecal route or SARS-CoV-2 may invade the enteric cells after respiratory infection *via* lympho hematogenic spread. As COVID-19 is also associated with the involvement of different organs and systems, such as the liver,



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Figure 1 Flow diagram of the participants in the trial. COVID-19: Coronavirus disease 2019.



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Figure 2 Histological examination of intestinal samples. A: Histological examination showed many macrophages in the vascular lumen (hematoxylin and eosin, magnification 10 ×); B: CD68 antibody highlighted the macrophages (magnification 20 ×).

kidneys, heart, blood, and nervous system; it has been hypothesized that in severe COVID-19 patients and in those with compromised immunity, SARS-CoV-2 has not been successfully eradicated and can spread from the lungs to target organs, such as the intestine[33]. Although the present data are unable to support the observations suggesting that enteric infection can occur in COVID-19 patients, in the two cases positive for viral RNA, histological examination showed an inflammatory infiltrate characterized by the presence of macrophages, granulocytes, plasma cells, and focal vasculitis. Thus, it could be hypothesized that in these cases, there were both a direct viral infection and immune hyperactivation. Hyperactivation of the immune system in response to infection can cause severe complications and organ damage. The host immune response is thought to play a vital role in the pathogenesis of COVID-19[34-36]. The present study has several limitations. The biopsies were performed only in the rectum of patients with moderate COVID-19. On the other hand, due to the high risk of viral spreading during endoscopic procedures, it is difficult to obtain samples from the gastric, intestinal and colic mucosa in patients who do not complain of GI symptoms, especially in cases of severe illness. Moreover, it is not possible to exclude that the two positive colon samples could be contaminated by SARS-CoV-2 positive stool or blood. Another limitation of the present study was its observational nature which made it difficult to identify the causes of the observed phenomena. Nonetheless, the detection of the viral RNA observed and the inflammatory cell infiltration to the colonic tissue of patients with active cancer could serve as hypothesis generators, leading to the analyzing of more comprehensive autopsy or surgical specimens in order to assess the potential link between SARS-CoV-2 and enteric infections in this population. The strengths of this study include the use of RT-PCR which is the gold standard for detecting SARS-CoV-2 infection. Moreover, the rectal biopsies were performed in two different sites and stored in both RNA preservation medium and in 10% buffered formalin to reduce the risk of false negatives.

CONCLUSION

SARS-CoV-2 RNA was found in only a small percentage of the intestinal samples analyzed (3.4%). Nevertheless, more comprehensive autopsy or surgical specimens are needed to provide histological evidence of intestinal infection.

ARTICLE HIGHLIGHTS

Research background

Although some observations provide evidence for intestinal infection of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the mechanisms leading to this infection are not known.

Research motivation

The detection of viral RNA in gastrointestinal (GI) tissue samples has not been adequately investigated and results are conflicting. More GI tissue samples, comprehensive autopsy and surgical specimens are needed to provide histological evidence of intestinal infection.

Research objectives

Intestinal mucosal samples from mild-moderate coronavirus disease 2019 (COVID-19) patients were analyzed with the primary objective of detecting SARS-CoV-2 RNA and evaluating histological features.

Research methods

This is a monocentric trial in which real time reverse transcriptase-polymerase chain reaction and histological features were used to detect SARS-CoV-2 RNA in intestinal mucosal samples. The study population was composed of two groups of patients hospitalized for COVID-19. In the first group (biopsy group), the patients were eligible for inclusion if they had mild to moderate disease and if they agreed to have a rectal biopsy regardless of the presence or absence of GI symptoms; in the second group (surgical specimen group), patients were eligible for inclusion if they underwent intestinal resection during index hospitalization. The data obtained in this study are valuable because rectal biopsies were carried out on 30 patients who did not need the procedure to frame their disease status. The study therefore provides data that are not only more numerous but also qualitatively different from those available up to now.

Research results

Overall, we analyzed 53 rectal biopsies and 6 surgical specimens. Viral RNA was not detectable in any of the rectal biopsies performed (0/53). Histological examination showed no enterocyte damage, but slight edema of the lamina propria with mild inflammatory lymphoplasmacytic infiltration. Viral RNA was detected in 2 surgical specimens of the 6 examined, both of which were from patients with active neoplastic disease. Histological examination also pointed out abundant macrophages, granulocytes and plasma cells infiltrating the muscular layer and adipose tissue, and focal vasculitis.

Research conclusions

Mild-moderate COVID-19 may not be associated with rectal infection by the virus. Although the present data are unable to support the observations suggesting that enteric infection can occur in COVID-19 patients, the detection of the viral RNA observed and the inflammatory cell infiltration to the colonic tissue of patients with active cancer could serve as hypothesis generators, leading to the analyzing more comprehensive autopsy or surgical specimens in order to assess the potential link between SARS-CoV-2 and enteric infections in this population.

Research perspectives

Does intestinal infection lead to increased expression of inflammatory cytokines in the intestine and/or serum? Since the two positive samples were both from patients with active cancer, could a weakened immune system, induced by the neoplastic disease, increase the risk of the intestinal infection of SARS-CoV-2?

FOOTNOTES

Author contributions: Lazzarotto T and Poggioli G contributed equally to this work. Cuicchi D, Lazzarotto T, D'Errico A and Poggioli G participated in the conceptualization and design of the study; Cuicchi D collected data and carried out the initial analyses; Cuicchi D, Gabrielli L and Tardio ML drafted the initial manuscript; Gabrielli L and Rossini G carried out the virological evaluation; Tardio ML, D'Errico A and Poggioli G carried out the histological evaluation;

D'Errico A, Poggioli G and Lazzarotto T made substantial contributions to all aspects of the writing of the manuscript, which included contribution to conception, design, analysis and interpretation of the article; Lazzarotto T contributed to the review and revision of the manuscript; Poggioli G interpreted data, reviewed and revised the manuscript, and supervised and provided mentorship throughout all stages of the project and writing of the manuscript; and all authors approved the final version to be submitted.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Randomized controlled trial to evaluate the efficacy and safety of fexuprazan compared with esomeprazole in erosive esophagitis

Kang Nyeong Lee, Oh Young Lee, Hoon Jai Chun, Jin Il Kim, Sung Kook Kim, Sang Woo Lee, Kyung Sik Park, Kook Lae Lee, Suck Chei Choi, Jae-Young Jang, Gwang Ha Kim, In-kyung Sung, Moo In Park, Joong Goo Kwon, Nayoung Kim, Jae Jun Kim, Soo Teik Lee, Hyun Soo Kim, Ki Bae Kim, Yong Chan Lee, Myung-Gyu Choi, Joon Seong Lee, Hwoon-Yong Jung, Kwang Jae Lee, Jie-Hyun Kim, Hyunsoo Chung

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Abstract

BACKGROUND

Fexuprazan, a novel potassium-competitive acid blocker, reversibly suppresses the K^+/H^+-ATPase enzyme in proton pumps within gastric parietal cells. Fexuprazan's suppression of gastric acid was maintained in healthy individuals for 24 h in a dose-dependent manner.

AIM

To compare fexuprazan to esomeprazole and establish its efficacy and safety in patients with erosive esophagitis (EE).

METHODS

Korean adult patients with endoscopically confirmed EE were randomized 1:1 to receive fexuprazan 40 mg or esomeprazole 40 mg once daily for eight weeks. The primary endpoint was the proportion of patients with healed EE confirmed by endoscopy at week 8. The secondary endpoints included the healing rate of EE at week 4, symptom response, and quality of life assessment. Safety profiles and serum gastrin levels were compared between the groups.

RESULTS

Of the 263 randomized, 218 completed the study per protocol (fexuprazan 40 mg, n = 107; esomeprazole 40 mg, n = 111). Fexuprazan was non-inferior to esomeprazole regarding the healing rate at week 8 [99.1% (106/107) *vs* 99.1% (110/111)]. There were no between-group differences in the EE healing rate at week 4 [90.3% (93/103) *vs* 88.5% (92/104)], symptom responses, and quality of life assessments. Additionally, serum gastrin levels at weeks 4 and 8 and drug-related side effects did not significantly differ between the groups.

CONCLUSION

Fexuprazan 40 mg is non-inferior to esomeprazole 40 mg in EE healing at week 8. We suggest that fexuprazan is an alternative promising treatment option to PPIs for patients with EE.

Key Words: Gastroesophageal reflux; Esophagitis; Proton pump inhibitors; Heartburn; Quality of life

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Core Tip: A mainstay therapy of erosive esophagitis (EE) is acid suppression using proton pump inhibitors (PPIs), which have shortcomings such as their slow absorption and variability in metabolism. Acid suppression can be competitively and reversibly achieved by a novel potassium-competitive acid blocker, fexuprazan. We compared the efficacy and safety of fexuprazan and esomeprazole (each 40 mg once daily) in patients with EE for 8 wk. We conclude that fexuprazan is a new alternative to PPIs, by showing that fexuprazan is not inferior to esomeprazole in endoscopic healing rate of EE at week 8 and in safety profiles.

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INTRODUCTION

Gastro-esophageal reflux disease (GERD) is characterized by heartburn and acid regurgitation symptoms resulting from abnormal gastric reflux into the esophagus[1]. GERD prevalence is increasing in Asian and Western countries[2]. A recent report documented the worldwide prevalence of GERD as 13.3% (10.0% in Asia, 15.4% in North America, and 17.1% in Europe)[3]. The percentage change in age-standardized GERD prevalence in South Korea was 7.6% between 1990 and 2017[2]. GERD is classified as erosive esophagitis (EE) or non-erosive reflux disease (NERD) based on the presence of esophageal mucosal breaks *via* endoscopic examination[4]. Approximately one-third to half of the patients with EE complain of the typical symptoms of GERD[5]. In addition to typical symptoms, atypical and extraesophageal symptoms in patients with EE may impair health-related quality of life (HRQL)[6,7]. However, a poor HRQL is more likely associated with symptom frequency and severity rather than the presence or absence of EE[8].

A main treatment of GERD has been the use of proton pump inhibitors (PPIs). Current practical guidelines recommend PPIs as the first-line therapy for patients with EE[9]. PPIs irreversibly inhibit (H^+/K^+)-ATPase within the parietal cells of the gastric mucosa[10]. Studies have demonstrated that PPIs are 40%-50% more effective than placebo in healing of EE and resolving GERD symptoms[11,12]. Furthermore, complete healing of EE has been reported in 80% to 90% of patients after four and eight weeks of PPI treatment, respectively[11]. However, there are shortcomings of PPIs in GERD treatment, including unsatisfactory efficacy in atypical and extraesophageal symptoms and typical symptoms[13]. This might be due to the pitfalls of PPIs: The variability in PPI metabolism based on cytochrome P450 (CYP) 2C19 polymorphisms and the delayed onset of PPIs owing to their slow absorption associated with enteric coating to prevent degradation by acid.

As an alternative to PPI in GERD treatment, a novel potassium-competitive acid blocker (P-CAB), fexuprazan (Daewoong Pharmaceutical Co., Ltd., Seoul, South Korea), was developed[14]. In contrast to PPIs, metabolism of fexuprazan is independent of CYP 2C19; enteric coating is not needed because of acid stability. While PPIs bind irreversibly to only the active forms of the proton pump, fexuprazan can bind to both the active and inactive forms of the proton pump competitively and reversibly. A previous study on healthy individuals demonstrated the effect of fexuprazan's acid suppression and tolerability, observing that gastric pH > 4 was reached within 2 h and maintained for 24 h in a dose-dependent manner[14].

Nevertheless, the effectiveness and safety of fexuprazan compared to esomeprazole, one of the most widely used PPIs in GERD, have not been confirmed among patients with EE. Therefore, this phase III, double-blind, randomized, active-controlled, multi-center study was conducted to compare the efficacy and safety between fexuprazan and esomeprazole in patients with EE.

MATERIALS AND METHODS

Study design and treatments

This randomized, double-blind, parallel-group, multicenter, and phase III trial was performed in 25 institutions in South Korea between December 2018 and August 2019. Adult patients provided written informed consent prior to enrolment, and then screening test including the endoscopy was performed. Eligible participants were randomized 1:1 to receive either fexuprazan 40 mg or esomeprazole 40 mg following the screening test. At this point, participants were stratified according to Los-Angeles (LA) Classification Grade classified by the result of upper gastrointestinal endoscopy.

To ensure the double-blinded nature of the study, patients were administered once daily with two tablets of the study medication (fexuprazan 40 mg or esomeprazole 40 mg with its matching placebo in the study and control groups, respectively) for eight weeks.

Compliance of the study medication was ascertained at each visit by participants returning the unused portion and empty packaging, and was calculated using the total numbers of tablets to be taken, of tablets actually taken, and of returned and unreturned tablets in each participant.

This study was approved by the institutional review boards of each institution, conducted in compliance with the relevant ethics guidelines, and registered at ClinicalTrials.gov (NCT03736369). All the study medications and procedures performed were in accordance with the 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards.

Participants

Eligible participants were male or female patients (20-75 years old) with EE (LA Classification Grades A to D) confirmed by endoscopy at the same institution within 14 d of study treatment initiation. The major exclusion criteria were Barrett's esophagus (> 3 cm); esophageal stricture; active peptic ulcers; ulcer-related stenosis; gastrointestinal bleeding; eosinophilic esophagitis; Zollinger-Ellison syndrome; inflammatory bowel diseases; irritable bowel syndrome; pancreatitis; psychiatric disorders; acquired immune deficiency syndrome (AIDS); viral hepatitis; history of gastric acid suppression surgery; significant morbidities in the cardiovascular, respiratory, hepatic, renal, neurologic, endocrine, hematologic, and urologic systems; history of malignancies within five years; drug or alcohol abuse; and hypersensitivity to drugs containing active constituents of esomeprazole or other similar drugs (benzimidazoles and antibiotics). Also excluded were those who had abnormal laboratory values, including alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), total bilirubin, creatinine, and blood urine nitrogen > 2´ upper limits of the normal range, and women with child-bearing potential who did not consent to appropriate contraceptive methods use during the study.

Protocol

Endoscopy was performed at the start of the screening period and at weeks 4 and 8. EE healing was defined as the complete absence of mucosal breaks. If mucosal breaks did not heal at week 4, the patients continued to receive the study drug until week 8, when endoscopy was performed again. Two weeks after the confirmation of EE healing from the centralized endoscopic evaluation, the patients were evaluated for safety *via* telephone interviews, and where applicable, they underwent additional tests and procedures (Figure 1).

The primary efficacy endpoint was the proportion of patients with endoscopically confirmed EE healing at week 8. The secondary efficacy endpoints were EE healing rate at week 4; the patients' reported symptom outcomes, symptom assessment by reflux disease questionnaire (RDQ), and GERD-health related quality life (GERD-HRQL). Symptoms were evaluated based on patients' symptom diaries. Symptom severity in the daytime and at night were measured according to the five-point scale (0: none, 1: mild, 2: moderate, 3: severe, 4: very severe).

The parameters for assessing symptom responses were the first day of the complete resolution of symptoms (heartburn, acid regurgitation, and heartburn/acid regurgitation) after treatment, the proportion of patients without symptoms in the first 7 d and through the 8 wk of treatment, and the proportion of symptom-free days in the first 7 d and through the 8 wk of treatment. Changes in symptoms and GERD-HRQL from baseline at weeks 4 and 8 were evaluated using the RDQ and GERD-HRQL scales, respectively. The RDQ is a self-administered questionnaires comprising of 12 items to assess the frequency and severity of heartburn, acid regurgitation, and dyspepsia. Each item for frequency and severity was scored from 0 to 5; the higher score, the more severe or frequent symptoms [15]. The RDQ demonstrated the validity and reliability for diagnosis of GERD in primary care and community settings[16]. The GERD-HRQL scale comprises 11 items for the symptoms of heartburn and dysphagia, medication effects, and the patients' health conditions[17]. Each item was scored from 0 to 5; the higher the score, the lower the quality of life. The GERD-HRQL was validated and considered as an appropriate instrument to evaluate typical GERD symptoms[18]. Therefore, previous clinical studies performed in South Korea have used the RDQ and GERD-HRQL to evaluate the therapeutic effect in patients with GERD[19,20].

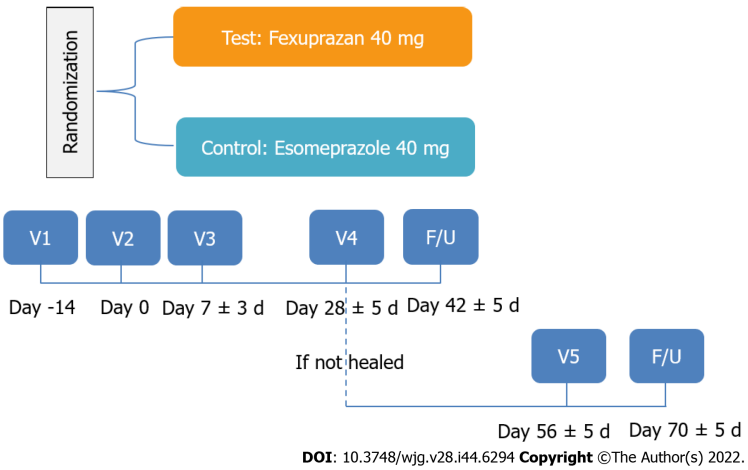


Figure 1 Study schema. V: Visit; F/U: Follow-up.

Additional analyses included heartburn and extraesophageal symptoms of GERD (chronic cough and throat irritation) in terms of the proportion of patients without symptoms and the proportion of symptom-free days in the first 3 d, 7 d and through the 8 wk of treatment. Patients with moderate/severe heartburn (RDQ ≥ 3) were also compared between the groups in terms of the proportion of patients without symptoms and the proportion of symptom-free days in the first 3 d, 7 d and through the 8 wk of treatment.

The patients' baseline characteristics included age, sex, body mass index (BMI), smoking history, drinking history, LA grade, *Helicobacter pylori* (*H. pylori*) infections, and CYP2C19 extensive metabolizer (EM)/poor metabolizer (PM) status. Serum gastrin levels were measured at weeks 4 and 8. Safety outcomes were measured by the analysis of adverse events (AEs), vital signs, physical examination, electrocardiogram (ECG) findings and laboratory tests. Adverse events (frequency, severity and seriousness) and concomitant medications were monitored throughout the study. Treatment-emergent adverse event (TEAE) was defined as an AEs newly occurred after the randomization and the first administration of study medication, and adverse drug reaction (ADR) was defined as any untoward and unintended response to the study medication of which causal relationship cannot be excluded.

Sample size

Based on previous studies, we estimated the sample size, assuming that the complete healing rate of mucosal breaks was 94.8% at week 8 after treatment with fexuprazan 40 mg and esomeprazole 40 mg [21,22]. Based on this threshold parameter, the sample size was 104 patients per treatment group, using the following conditions of the PASS program: non-inferiority margin of 10%[23], a one-sided significance level of 2.5%, 90% statistical power, and 1:1 randomization.

Randomization

This study was used stratified block randomization method base on LA grades (A/B/C/D) by the result of upper gastrointestinal endoscopy. An independent statistician generated a randomization list based on stratification factor (LA grades) using the PLAN (Proc Plan) procedure of SAS (ver. 9.4, SAS Institute, Cary, NC, United States). Eligible participants were randomly assigned by the investigators in a ratio of 1:1 *via* an interactive web-response system (IWRS). Neither participants nor relevant investigators were aware of these assignments.

Statistical analysis

Efficacy was evaluated by both the full analysis set (FAS) and per-protocol set (PPS), and PPS findings were interpreted as the main results. For the safety assessment, statistical analysis was performed on the safety set (SS). The FAS, based on the intention-to treat principle, included patients who received at least one dose of the study drug after randomization and had at least one primary efficacy assessment. The PPS included patients in the FAS who completed the study without any major protocol deviation. The SS group included all patients who received the study drug at least once after randomization.

For symptoms responses daily (day-time and night-time) assessment in the efficacy analysis, missing symptom in day-time or night-time was imputed using the last observation carried forward. Except for this, missing value was set to missing without imputation, and the results of patients who were completed the study early as mucosal breaks were completely healed up to week 4 were used as the results of the week 8.

Summaries of baseline characteristics of patients were presented in FAS. To assess the difference between the treatment group, the two sample t-test or Wilcoxon rank-sum test were used after normality evaluation in continuous baseline characteristics variables, and the chi-square test or Fisher's exact test were used in categorical baseline characteristics variables.

The primary objective of the study was to demonstrate the non-inferiority of fexuprazan 40 mg compared with esomeprazole 40 mg. The cumulative healing rate of EE and corresponding two-sided 95% confidence interval (CI) were presented for visit (up to week 4 or week 8) by treatment group. The common risk difference of the healing rate of EE up to week 8 between the treatment groups (fexuprazan 40 mg group - esomeprazole 40 mg group) and corresponding two-sided 95% CI using the Cochran-Mantel-Haenszel method adjusted by a stratification factor (baseline LA grade) were presented in the PPS. The non-inferiority of fexuprazan 40 mg to esomeprazole 40 mg was determined the lower limit of its two-sided 95%CI is larger than the non-inferiority margin of -10%. The same analyses were performed for the non-inferiority of healing rate of EE up to week 4. Furthermore, continuous data were analyzed using an analysis of covariance (ANCOVA) model, including treatment group as treatment effect, and baseline score (included if evaluation data were changed from baseline) and baseline LA grade as covariates. The changes from baseline within-treatment group were used the paired t-test or Wilcoxon signed rank test after normality evaluation as a post hoc analysis. Categorical data were analyzed using the Cochran-Mantel-Haenszel method adjusted by baseline LA grade. For the safety analysis, the chi-square test or Fisher's exact test was used to compare the difference in the incidence of AEs between the treatment groups. All statistical analyses were performed using SAS (ver. 9.4, SAS Institute, Cary, NC, United States) with a two-sided significance level of 5% for all tests.

RESULTS

Baseline characteristics of the participants

Of the total of 470 patients screened, 263 patients with EE were randomized to receive either fexuprazan 40 mg or esomeprazole 40 mg (Table 1). In total, 231 patients [152 men (65.8%) and 79 women (34.2%); 54.4 ± 12.7 mean age] were included in the FAS and completed the study (n = 116 and 115 in the fexuprazan and esomeprazole groups, respectively). Thirteen patients with study medication-related deviation, visit window deviation and consent withdrawal were excluded from the FAS (9 and 4 in the fexuprazan and esomeprazole groups, respectively), and 218 patients completed the study on the PPS (n = 107 and 111 in the fexuprazan and esomeprazole groups, respectively). The SS included 131 patients each in the fexuprazan and esomeprazole groups (Figure 2).

There were no significant differences in baseline characteristics between both groups, except CYP2C19 genotypes (EM or PM). A statistically significant difference was seen in the classification of CYP2C19 genotype (P = 0.007), but the result was obtained from only some of the participants who agreed to genotyping (n = 51 and 56 in the fexuprazan and esomeprazole groups, respectively).

The mean compliance rates were 98.6% ± 8.1% and 99.0% ± 2.6% at weeks 4 and 8, and the overall compliance rate with study medication exceeded 95% in all treatment groups without between-group differences.

Efficacy

Healing rate of EE: In the PPS, the proportions of patients with complete absence of mucosal breaks at week 8 were 99.1% (106/107) and 99.1% (110/111) in the fexuprazan and esomeprazole groups, respectively. The difference in proportions of patients with complete absence of mucosal breaks adjusted by baseline LA grade [fexuprazan 40 mg group – esomeprazole 40 mg group] was 0.9% (95%CI, -0.9 to 2.6) (Figure 3). The lower limit of two-sided 95%CI at week 8, -0.9%, was greater than the non-inferiority margin of -10%, indicating the non-inferiority of 8-week treatment of fexuprazan 40 mg to esomeprazole 40 mg in EE healing in GERD. At week 4, the healing rates of EE were not different between the two groups [90.3% (93/103) in the fexuprazan group and 88.5% (92/104) in the esomeprazole group, respectively] with a difference of 2.6% (95%CI: -5.7 to 10.9). The lower limit of 95%CI, -5.7%, was also greater than the non-inferiority margin of -10%. These results demonstrate that fexuprazan 40 mg was non-inferior to esomeprazole 40 mg in EE healing in GERD at weeks 4 and 8.

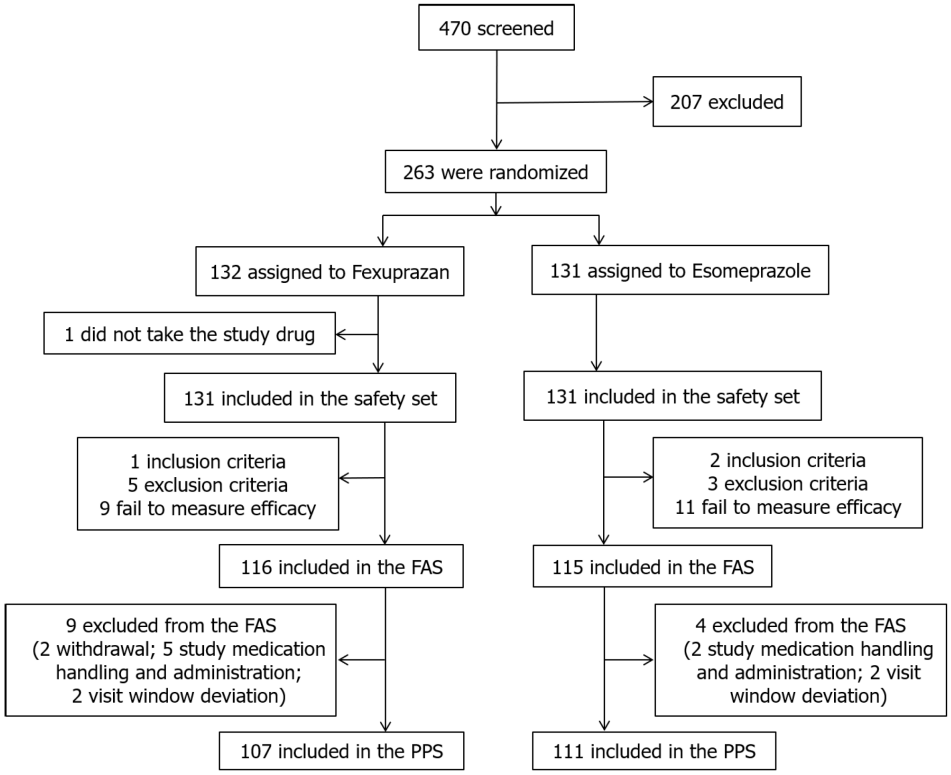
As the results in the exploratory analysis, healing rates of EE were not statistically significantly different according to CYP2C19 genotype (EM or PM) and *H. pylori* infection (positive or negative). Healing rates of EE at weeks 4 and 8 in EM participants (n = 88) were not different between fexuprazn and esomeprazole groups [91.7% (33/36) *vs* 89.4% (42/47) at week 4; 100.0% (36/36) *vs* 98.1% (51/52) at week 8]. EE healing rates at weeks 4 and 8 in PM participants (n = 14) were not different between the treatment groups [70.0% (7/10) *vs* 100.0% (3/3) at week 4; 90.9% (10/11) *vs* 100.0% (3/3) at week 8]. Healing rates of EE in *H. pylori*-positive participants (n = 47) were 100.0% (17/17) and 88.46% (23/26) in the fexuprazan and esomeprazole groups at week 4, and all of *H. pylori*-positive participants were completely healed at week 8. Healing rates of EE in *H. pylori*-negative participants (n = 169) were 88.2% (75/85) *vs* 88.3% (68/77) at week 4, and 98.9% (87/88) *vs* 98.8 (80/81) at week 8 in the fexuprazan and esomeprazole groups.

Table 1 Baseline characteristics of the patients (full analysis set)

Variables		Fexuprazan 40 mg (*n* = 116)	Esomeprazole 40 mg (*n* = 115)	*P* value
Age, yr (mean ± SD)[5]		53.70 ± 12.44	55.05 ± 12.89	0.343[w]
Sex, *n* (%)[6]				
	Men	78 (67.2)	74 (64.3)	0.643[c]
	Women	38 (32.8)	41 (35.7)	
BMI, kg/m^2 (SD)[5]		24.42 ± 3.08	24.81 ± 3.25	0.529[w]
Smoking history, *n* (%)[6]				
	Non-smokers	67 (57.8)	66 (57.4)	0.978[c]
	Current smokers	25 (21.6)	26 (22.6)	
	Past smokers	24 (20.7)	23 (20.0)	
Drinking history, *n* (%)[6]				
	Non-drinkers	15 (12.9)	15 (13.0)	0.992[c]
	Current drinkers	77 (66.4)	77 (67.0)	
	Past drinkers	24 (20.7)	23 (20.0)	
LA classification[1], *n* (%)[6]				
	Grade A	75 (64.7)	76 (66.1)	0.630[f]
	Grade B	33 (28.4)	31 (27.0)	
	Grade C	6 (5.2)	8 (7.0)	
	Grade D	2 (1.7)	0 (0.0)	
Helicobacter pylori[2], *n* (%)[6]				
	Positive	20 (17.4)	31 (27.2)	0.075[c]
	Negative	95 (82.6)	83 (72.8)	
CYP2C19[3], *n* (%)[6]				
	EM	39 (76.5)	53 (94.6)	0.007[c]
	PM	12 (23.5)	3 (5.4)	
Severity for heartburn[4], *n* (%)[6]				
	Mild	53 (45.7)	50 (43.5)	0.735[c]
	Moderate/severe	63 (54.3)	65 (56.5)	

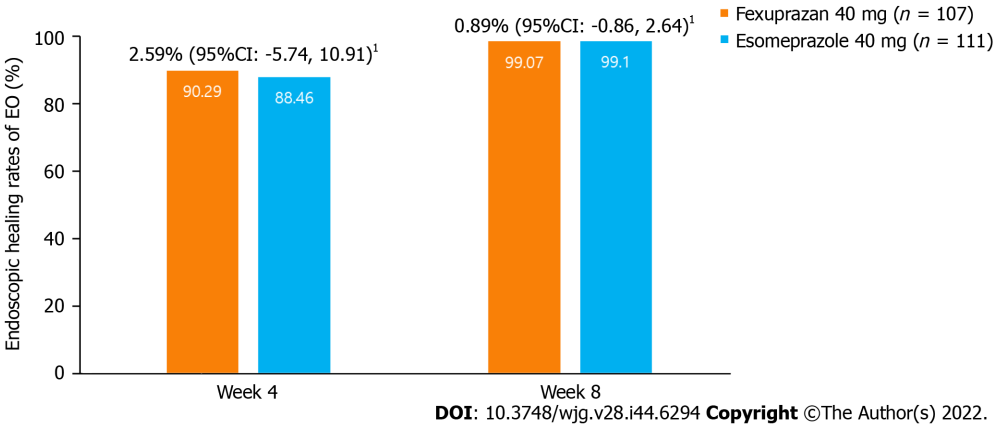
Values are mean SD or the number of the patients with percentage, where appropriate.
[1]LA Classification (Grade A: One (or more) mucosal break(s) no longer than 5 mm, that does not extend between the tops of two mucosal folds, Grade B: One (or more) mucosal break(s) more than 5 mm long, that does not extend between the tops of two mucosal folds, Grade C: One (or more) mucosal break(s) that is continuous between the tops of two or more mucosal folds, but which involve(s) less than 75% of the oesophageal circumference, Grade D: One (or more) mucosal break(s) which involve(s) at least 75% of the oesophageal circumference).
[2]Two subjects (Fexuprazan 40 mg: 1 subject, Esomeprazole 40 mg: 1 subject) did not have *H. pylori* results at baseline.
[3]CYP2C19 genotype results were collected only from the subjects who agreed to the informed consent for genetic testing.
[4]Severity for heartburn was defined based on the baseline Reflux disease questionnaire (RDQ). Mild: RDQ ≤ 2 (Less than or equal to 1 d out of 7 d), Moderate/Severe: RDQ ≥ 3 (Greater than or equal to 2 d out of 7 d).
[5]Testing for difference between treatment groups after normality evaluation [Wilcoxon rank-sum test (w)].
[6]Testing for difference among treatment groups [Chi-square test (c) or Fisher's exact test (f)].
BMI: Body mass index; LA: Los-Angeles; CYP2C19: Cytochrome P 2C19; EM: Extensive metabolizer; PM: Poor metabolizer.

Symptom response: Fexuprazan exhibited an overall symptom relief comparable to esomeprazole. The differences between the groups were not significant with respect to the first day of the complete resolution of symptoms (resolution of typical symptoms for 7 d) after treatment: the median values of days to complete resolution for heartburn, acid regurgitation, and heartburn/acid regurgitation were 13, 8, and 18 d *vs* 10, 6, and 16 d in the fexuprazan and esomeprazole groups, respectively. There were no statistically significant differences in the proportions of patients without symptoms in the first 7 d (26.2%, 25.2%, and 15.0%, *vs* 21.6%, 27.9%, and 11.7%, for heartburn, acid regurgitation, and



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Figure 2 Flowchart of the study patients. SS: Safety set; FAS: Full analysis set; PPS: Per-protocol set.



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Figure 3 Erosive esophagitis healing rate at weeks 4 and 8 (per protocol set). Erosive esophagitis (EE) healing was defined as the complete absence of mucosal breaks confirmed by the endoscopy. [1]Common risk difference of the healing rate of EE between the treatment groups (two-sided 95%CI) using the Cochran-Mantel-Haenszel method adjusted by baseline Los Angeles grade. EE: Erosive esophagitis; CI: Confidence interval.

heartburn/acid regurgitation, in the fexuprazan and esomeprazole groups, respectively) and through the 8 wk (20.6%, 21.5%, and 10.3%, *vs* 17.1%, 27.0%, and 9.9%). Similarly, there were no statistically significant differences in the proportion of symptom-free day/night-time in the first 7 d and through the 8 wk between both groups. (Supplementary Tables 1-4).

In the RDQ and GERD-HRQL, the frequency and severity of heartburn and acid regurgitation improved in both groups, with no significant difference in changes from baseline at weeks 4 and 8 (Tables 2 and 3).

In the results of subgroup analyses, fexuprazan demonstrated better heartburn relief in patients with moderate-to-severe symptoms who had experienced heartburn for 2 or more days in the week before treatment: the proportions of those without heartburn on the first day 3 were significantly greater in the fexuprazan group than in the esomeprazole group (22.4% *vs* 7.9%, P = 0.026 at the day/night-time; 29.3% *vs* 12.7%, P = 0.037 at the day-time; 34.5% *vs* 17.5%, P = 0.035 at the night-time) (Supplementary Table 5). The extraesophageal symptom of chronic cough improved better with fexuprazan:

Table 2 Change in reflux disease questionnaires symptom scores from baseline at weeks 4 and 8 (per protocol set)

	Fexuprazan 40 mg			Esomeprazole 40 mg		
	Baseline (*n* = 107)	Week 4 (*n* = 103)	Week 8 (*n* = 107)	Baseline (*n* = 111)	Week 4 (*n* = 104)	Week 8 (*n* = 111)
Frequency						
Heartburn						
mean ± SD	1.92 ± 1.23	0.91 ± 1.37	0.86 ± 1.33	2.12 ± 1.42	0.80 ± 1.29	0.70 ± 1.28
Change from baseline (mean ± SD)	-	-0.96 ± 1.50	-1.06 ± 1.49	-	-1.33 ± 1.69	-1.42 ± 1.64
P value[1]	-	< 0.001[w]	< 0.001[w]	-	< 0.001[w]	< 0.001[w]
LS mean difference from esomeprazole	-	0.19	0.22	-	-	-
P value[2]	-	0.280	0.184	-	-	-
Reflux						
mean ± SD	2.14 ± 1.29	0.93 ± 1.48	0.92 ± 1.49	1.95 ± 1.29	0.61 ± 1.07	0.59 ± 1.12
Change from baseline (mean ± SD)	-	-1.19 ± 1.58	-1.22 ± 1.53	-	-1.32 ± 1.44	-1.36 ± 1.40
P value[1]	-	< 0.001[w]	< 0.001[w]	-	< 0.001[w]	< 0.001[w]
LS mean difference from esomeprazole	-	0.28	0.28	-	-	-
P value[2]	-	0.112	0.101	-	-	-
Severity						
Heartburn						
mean ± SD	1.81 ± 1.18	0.57 ± 0.78	0.53 ± 0.77	2.10 ± 1.24	0.45 ± 0.75	0.42 ± 0.79
Change from baseline (mean ± SD)	-	-1.23 ± 1.26	-1.28 ± 1.26	-	-1.63 ± 1.29	-1.68 ± 1.27
P value[1]	-	< 0.001[w]	< 0.001[w]	-	< 0.001[w]	< 0.001[w]
LS mean difference from esomeprazole	-	0.16	0.16	-	-	-
P value[2]	-	0.116	0.121	-	-	-
Reflux						
mean ± SD	2.06 ± 1.22	0.61 ± 0.92	0.58 ± 0.90	1.97 ± 1.21	0.40 ± 0.69	0.39 ± 0.73
Change from baseline (mean ± SD)	-	-1.43 ± 1.23	-1.48 ± 1.20	-	-1.55 ± 1.23	-1.58 ± 1.19
P value[1]	-	< 0.001[w]	< 0.001[w]	-	< 0.001[w]	< 0.001[w]
LS mean difference from esomeprazole	-	0.20	0.18	-	-	-
P value[2]	-	0.066	0.089	-	-	-

[1]Testing for change within-treatment groups [paired *t*-test (t) or Wilcoxon signed rank test (w)].
[2]Testing for difference between treatment groups (ANCOVA model with treatment group as a factor, baseline score and stratification factor (baseline LA classification) as covariates).
Note: If subjects did not have any RDQ assessment data by week 4, were treated as missing at week 4. RDQ: Reflux disease questionnaires; LS mean: Least square mean.

the least squares (LS) means of days without chronic cough were significantly greater in the fexuprazan group than in the esomeprazole group on the days 3, 7, and week 8 (P = 0.006, P = 0.003, and P = 0.002, respectively). The extraesophageal symptom of throat irritation improved in both groups on days 3, 7, and week 8 without significant differences between the treatment groups (Supplementary Table 6).

Table 3 Change in gastroesophageal reflux disease-health related quality of life score from baseline at weeks 4 and 8 (per protocol set)

GERD-HRQL	Fexuprazan 40 mg			Esomeprazole 40 mg		
	Baseline (*n* = 107)	Week 4 (*n* = 102)	Week 8 (*n* = 106)	Baseline (*n* = 111)	Week 4 (*n* = 104)	Week 8 (*n* = 111)
mean ± SD	11.88 ± 8.11	4.21 ± 6.17	4.01 ± 6.20	12.98 ± 9.62	3.42 ± 5.04	3.32 ± 5.54
Change from baseline (mean ± SD)	-	-7.71 ± 8.37	-7.90 ± 8.56	-	-9.84 ± 8.70	-9.67 ± 8.56
P value[1]	-	< 0.001[w]	< 0.001[t]	-	< 0.001[w]	< 0.001[w]
LS mean difference from esomeprazole	-	1.06	1.05	-	-	-
P value[2]	-	0.137	0.151	-	-	-

[1]Testing for change within-treatment groups [paired *t*-test (t) or Wilcoxon signed rank test (w)].
[2]Testing for difference between treatment groups (ANCOVA model with treatment group as a factor, baseline score and stratification factor (baseline LA classification) as covariates).
Note: If subjects did not have any Gastroesophageal reflux disease-health related quality of life (GERD-HRQL) assessment data by week 4, were treated as missing at week 4. One subject in fexuprazan 40 mg did not have GERD-HRQL assessment data post baseline. GERD-HRQL: Gastroesophageal reflux disease-health related quality of life; LS mean: Least square mean.

Safety

Safety analyses were performed for 262 patients who received at least one dose of the study meditation. The overall incidences of TEAEs and ADRs were not significantly different between the treatment groups; TEAEs were reported by 22 patients (16.8%) and 25 (19.1%), and ADRs were reported by 9 patients (6.9%) and 7 patients (5.3%) in the fexuprazan and esomeprazole groups, respectively (Table 4). The severity of TEARs was mostly mild (61 events), with six moderate events (diarrhea, nausea, dysgeusia, pruritus, pain, and cystitis) and only one severe event (influenza). All ADRs were either mild (21 events) or moderate (3 events). There were 2 patients (1.5%) with ADRs (diarrhea and pruritus) leading to discontinuation of the study medication in the fexuprazan group, not esomeprazole group. However, there was no statistically significant difference in the incidence of ADRs leading to discontinuation between both groups. No serious TEARs or ADRs were reported in either group of patients (Supplementary Table 7). The most frequently occurring (≥ 2%) TEAEs were shown in Table 4.

The serum gastrin levels intended to increase, and their differences between the treatment groups were not significant at weeks 4 and 8 (Figure 4). There were no clinically significant changes in the laboratory test, vital signs, physical examination and ECG findings, and no liver enzyme elevations were reported.

DISCUSSION

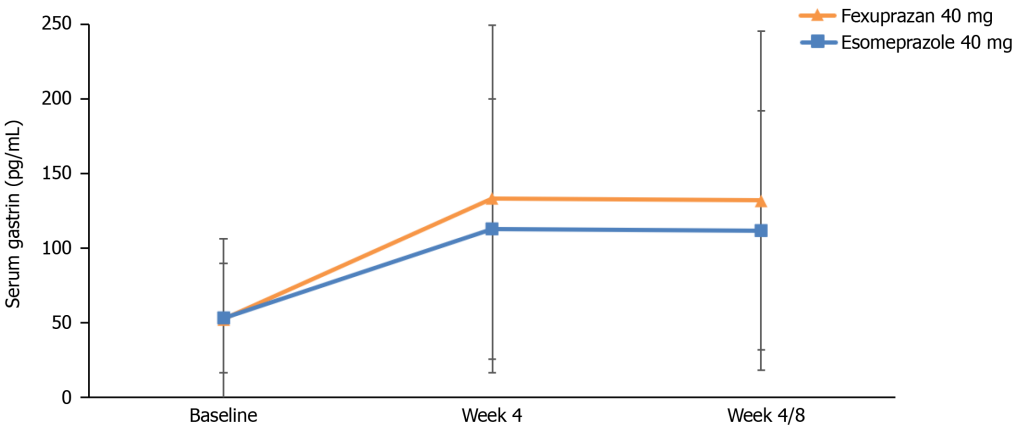
This study demonstrated the non-inferior efficacy and safety of fexuprazan 40 mg once daily to esomeprazole 40 mg once daily in the healing of EE at week 8 in patients with EE. The rates of healing EE were not different between the two groups at week 4. No differences between the groups were found in the secondary endpoints regarding symptom responses, including the first day of the complete resolution of symptoms (heartburn and acid regurgitation) and the proportions of patients without symptoms along with the proportions of symptom-free days in the first 7 d and throughout 8 wk of the treatment period. Furthermore, the two groups did not differ in the changes in RDQ and in GERD-HRQL from baseline at weeks 4 and 8. Serum gastrin levels and safety-related TEAEs and ADRs did not differ.

Our results were consistent with those of other P-CABs (tegoprazan and vonoprazan) in comparison with PPIs. Studies in patients with GERD and healthy volunteers have revealed the efficacy and safety of tegoprazan and vonoprazan, to be similar to those of PPIs. In a phase I study of tegoprazan, which has been used since 2018 after approval in South Korea, it safely inhibited acid secretion compared to esomeprazole[24]. In a multicenter, randomized, double-blind, and parallel-group non-inferiority study on 302 patients with endoscopically confirmed EE[19], tegoprazan 50 mg and 100 mg indicated cumulative healing rates of 98.9% and 98.9% at week 8, respectively, compared to the 98.9% healing rate of esomeprazole 40 mg. Regarding vonoprazan, its efficacy has been identified in clinical and pharmacological factors, including healing EE, symptom responses, maintenance treatment effect after healing EE, efficacy in refractory GERD, the effect of intermittent therapy, and the pH 4 holding time ratio[25-28]. A study of short-term symptom response at week 4 was similar: 88.0% and 81.8% in the esomeprazole 20 mg and vonoprazan 20 mg groups, respectively[29]. In a dose-ranging study,

Table 4 Overall Summary of treatment-emergent adverse events (safety set)

	Fexuprazan 40 mg (*n* = 131)	Esomeprazole 40 mg (*n* = 131)	Total (*n* = 262)
	n (%) [number of event]		
Subjects with TEAEs	22 (16.8) [34]	25 (19.1) [34]	47 (17.9) [68]
95%CI	[10.4, 23.2]	[12.4, 25.8]	[13.3, 22.6]
P value[1]			0.629[c]
Subjects with ADRs	9 (6.9) [13]	7 (5.3) [11]	16 (6.1) [24]
95%CI	[2.5, 11.2]	[1.5, 9.2]	[3.2, 9.0]
P value[1]			0.606[c]
Subjects with serious TEAEs	0	0	0
Subjects with serious ADRs	0	0	0
Most frequently occurring (≥ 2%) TEAEs by system organ class and preferred term			
System organ class preferred term			
Gastrointestinal disorders			
Diarrhoea	4 (3.1) [4]	2 (1.5) [2]	6 (2.3) [6]
Nervous system disorders			
Dizziness	1 (0.8) [1]	3 (2.3) [3]	4 (1.5) [4]

[1]Testing for difference among treatment groups [Chi-square test (c)].
Note: Denominator of percentage is the number of subjects in each treatment group. TEAEs: Treatment-emergent adverse events; ADRs: Adverse drug reactions.



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Figure 4 Changes from baseline in serum gastrin levels at weeks 4 and 8 (per-protocol set).

vonoprazan 5, 10, 20, and 40 mg exhibited non-inferior efficacy to lansoprazole 30 mg in the healing rates of EE at week 8[30]. In those with severe grades of EE and extensive metabolizers, treatments with vonoprazan 20 mg and lansoprazole 30 mg for 8 wk did not differ in the rates of EE healing[23]. Additionally, the recurrence rates of EE were significantly lower after a 24-wk treatment using 10 mg and 20 mg vonoprazan than with lansoprazole 15 mg[31]. Regarding the effect of vonoprazan on gastric acidity, the pH 4 holding time ratio significantly increased from 73.21% to 96.46% and from 69.97% to 100.00% in the 20 mg and 40 mg groups, respectively[26].

In this study, fexuprazan led to rapid treatment response in patients with moderate-to-severe heartburn. The proportion of patients without heartburn on day 3 who had moderate-to-severe symptoms was significantly higher with fexuprazan than with esomeprazole, in both day-time and night-time, and also at night-time only. Nocturnal heartburn was reportedly presented in approximately 80% of patients with frequent heartburn and impaired sleep quality and daytime HRQL[6,32]. Moreover, the continuous use of PPIs was not effective for nocturnal heartburn in 30% of patients with

reflux esophagitis[33], and in over 50% of patients with symptomatic EE[34]. Thus, this study suggests that fexuprazan may provide rapid symptom resolution in patients with nocturnal heartburn and refractory response to PPI treatment. The rapid response of symptoms to P-CABs was identified in another study revealing that vonoprazan 20 mg relieved heartburn symptoms on day 1 in more patients than lansoprazole 30 mg[35]. Although the present study did not demonstrate faster healing of EE, there have been studies showing rapid healing of EE after 2-week treatment of vonoprazan than PPIs[23,36]. Accordingly, in conjunction of this faster healing in EE with our finding of rapid symptom response by fexuprazan, it is cautiously suggested that patients with EE may need a relatively short-term treatment period by fexuprazan than PPIs. Further studies on shorter treatment in EE by fexuprazan are needed.

The pharmacodynamic and pharmacokinetic profiles of fexuprazan explain the rapid onset and sustained inhibition of acid secretion in GERD. Studies of fexuprazan in healthy individuals revealed that the mean percentage of time of gastric pH > 4 was achieved in 80% of 24 h and even at night. However, esomeprazole achieved a lower mean percentage time of gastric pH > 4, which was also lower at night[14]. With regard to the pharmacokinetic parameters, C_{max} was reached within 1-4 h after dosing, and the mean elimination half-life was approximately 9 h. Fexuprazan also exhibited dose-response relationships. Plasma concentrations increased proportionately with the doses ranging from 10-320 mg, whereas multiple doses did not cause significant accumulation. The elimination pathway of fexuprazan was not a renal route but probably *via* the liver or gut. Furthermore, in contrast to PPIs, food intake was not necessary for optimal action, as the parameters of gastric pH and plasma concentrations of fexuprazan did not change with a high-fat diet. Adverse drug effects on the liver were not higher with fexuprazan than with placebo, in contrast to the 0.2% potential liver toxicity in the pre-clinical experiment of vonoprazan[37]. Moreover, the gastrin-increasing effect of fexuprazan was similar to that of other PPIs, and was less frequent than that of vonoprazan[38]. Furthermore, the effects on gastric acid suppression, serum gastrin elevation, and dose response relationship were also consistent in different populations including Korean, Caucasian, and Japanese ethnicities[39].

In our study, fexuprazan improved one of the extraesophageal symptoms of GERD better than esomeprazole. Despite its unknown pathophysiology, patients with GERD-related chronic cough have been treated with PPIs with unsatisfactory symptom control. The superior efficacy of PPIs over placebo has not been confirmed in patients with GERD-related chronic cough in recent randomized controlled trials (RCTs)[40,41]. In addition, a meta-analysis of 5 RCTs did not suggest any evidence in favor of PPI therapy[42]. Taken the effect of fexuprazan in this study with the overall inadequate efficacy of PPIs in chronic cough, we suggest that fexuprazan could provide a better solution than PPIs for GERD-related chronic cough.

This study revealed elevated serum gastrin levels, but these were not significantly different between both groups. Previous reports have revealed higher serum gastrin levels in the P-CAB group than in the PPI group[37,43]. In the study of 212 outpatients, the serum gastrin in the P-CAB group had 2-3 fold and 1-2 fold increases than the normal and PPI groups, respectively[43]. However, increased serum gastrin levels were limited, particularly in patients with normal mucosa or mild atrophic gastritis. Additional limitations were the treatment periods of less than one year and the sampling time at pre-meal rather than at the peak level of 30 min after meals.

This study had some limitations. First, the number of patients classified as LA grade C/D was small. Actually, those with LA grades C/D accounted for only 6.2% of the fexuprazan and 7.0% of the esomeprazole groups. Therefore, it was difficult to confirm the advantage of fexuprazan, better clinical performances due to unique pharmacokinetics and pharmacodynamics of fexuprazan in severe EE than PPIs, as in other P-CAB studies[30]. Future fexuprazan studies need to be focused on significantly larger number of patients with severe EE (LA grades C/D). Second, the treatment period was only eight weeks, and studies on the long-term safety or recurrence rates after EE healing are required in the future, considering the insufficient data regarding the long-term safety of P-CABs. Third, when evaluating symptom severity, the possible effects of comorbidities such as chronic obstructive pulmonary disease were not considered[44].

CONCLUSION

We concluded that fexuprazan 40 mg once daily has non-inferior efficacy and safety to esomeprazole 40 mg once daily in healing EE at weeks 4 and 8. From the symptom evaluation through the symptom diary, RDQ and GERD-HRQL, it was confirmed that fexuprazan improved symptoms of heartburn and acid regurgitation and quality of life similarly to esomeprazole. The increase in serum gastrin levels by fexuprazan was not different from that of esomeprazole. Future research on fexuprazan is needed to evaluate the long-term efficacy and safety of fexuprazan in GERD including EE, PPI-refractory GERD, and other acid-related diseases along with the long-term maintenance therapy including on demand or intermittent treatment.

ARTICLE HIGHLIGHTS

Research background

Currently, a mainstay therapy of erosive esophagitis (EE) is proton pump inhibitors (PPIs), which have disadvantages like their delayed absorption and variable efficacy due to differences in drug metabolism A novel potassium-competitive acid blocker, fexuprazan, suppresses the K^+/H^+-ATPase enzyme reversibly and competetively in proton pumps within gastric parietal cells.

Research motivation

A previous study of fexuprazan on healthy individuals demonstrated the effect of its acid suppression and tolerability, by showing that gastric pH > 4 was reached within 2 h and maintained for 24 h in a dose-dependent manner. However, the efficacy and safety of fexuprazan in EE have not been compared to esomeprazole, one of the most widely used PPIs in gastro-esophageal reflux disease (GERD) including EE.

Research objectives

The aim of this phase III, double-blind, randomized, active-controlled, multi-center study was to compare the efficacy and safety between fexuprazan and esomeprazole in patients with EE.

Research methods

Adult patients who have EE confirmed by endoscopy were randomized 1:1 to receive fexuprazan 40 mg or esomeprazole 40 mg once daily for eight weeks in South Korea between December 2018 and August 2019. The primary endpoint was healing rates confirmed by endoscopy at week 8. The secondary endpoints included the proportion of patients with healed EE at week 4, symptom response, and GERD-related quality of life assessed from the evaluation through the symptom diary, reflux disease questionnaire (RDQ) and GERD-health related quality life (GERD-HRQL) questionnares. We also compared safety profiles and serum gastrin levels between the groups.

Research results

This study shows that fexuprazan 40 mg once daily is non-inferior to esomeprazole 40 mg once daily in healing rates of at weeks 4 and 8 and in symptom improvement of heartburn and acid regurgitation and RDQ and GERD-HRQL. In 218 participants who completed the study per protocol (fexuprazan 40 mg, n = 107; esomeprazole 40 mg, n = 111), fexuprazan was non-inferior to esomeprazole regarding the healing rate at week 8 [99.1% (106/107) *vs* 99.1% (110/111)], and at week 4 [90.3% (93/103) *vs* 88.5% (92/104)], symptom responses, and quality of life assessments. Also, serum gastrin levels at weeks 4 and 8 and drug-related side effects were not significantly different between the groups.

Research conclusions

This study results indicate that fexuprazan 40 mg once daily can be an alternative of esomeprazole 40 mg once daily for patients with erosive esophagitis in terms of efficacy and safety.

Research perspectives

Further research on fexuprazan should be directed to evaluate the long-term efficacy and safety of fexuprazan in various acid-related gastrointestinal diseases including NERD, PPI-refractory GERD, H. pylori infection, peptic ulcer diseases, and so on.

FOOTNOTES

Author contributions: Lee OY contributed to study design, acquisition and interpretation of data, and critically reviewed and edited the manuscript; Lee KN contributed to the data interpretation, and drafting and editing the manuscript; All authors contributed to enrolment of patients, agreed to be responsible for every aspect of this work, reviewed and finally approved the manuscript.

Institutional review board statement: This study was approved by the institutional review boards (IRBs) of each institution, conducted in compliance with the relevant ethics guidelines. All the study medications and procedures performed were in accordance with the 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards.

Clinical trial registration statement: This study is registered at ClinicalTrials.gov. The registration identification number is NCT03736369.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The corresponding author, Lee OY, had been a member of outside directors at the Daewoong Pharmaceutical Co., Ltd, form the period March 24 2018 to Nov 2 2021. All other authors declare no conflict of interests regarding this study.

Data sharing statement: Data are available upon reasonable request.

CONSORT 2010 statement: The authors have read the CONSORT 2010 Checklist, and the manuscript was prepared and revised according to the CONSORT 2010 Checklist.

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LETTER TO THE EDITOR

Comment on "Prognostic value of preoperative enhanced computed tomography as a quantitative imaging biomarker in pancreatic cancer"

Jian Yang, Ying Liu, Shi Liu

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies because of its high invasiveness and metastatic potential. Computed tomography (CT) is often used as a preliminary diagnostic tool for pancreatic cancer, and it is increasingly used to predict treatment response and disease stage. Recently, a study published in *World Journal of Gastroenterology* reported that quantitative analysis of preoperative enhanced CT data can be used to predict postoperative overall survival in patients with PDAC. A tumor relative enhancement ratio of ≤ 0.7 indicates a higher tumor stage and poor prognosis.

Key Words: Pancreatic ductal adenocarcinoma; Computed tomography; Tumor relative enhancement ratio; Diagnostic imaging; Quantitative analysis; Prognosis

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Core Tip: Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal malignancies because of its high invasiveness and metastatic potential. The purpose of this letter is to highlight that a quantitative parameter based on enhanced computed tomography, namely the tumor relative enhancement ratio, can reveal the correlation between high malignant potential because of hypervascularity and poor prognosis in PDAC.

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TO THE EDITOR

The stroma of pancreatic ductal adenocarcinoma (PDAC) is a fibroproliferative microenvironment mainly composed of fibroblasts, and its low vascular supply severely limits the tumor utilization of oxygen and nutrients[1,2]. In such a situation, invasion into fertile tissue becomes an acquired behavior of the tumor in response to severe metabolic stress[3,4]. We were extremely interested in a retrospective study by Gao *et al*[5] published in the June 2022 issue of *World Journal of Gastroenterology*. This was a moderate-quality observational study with a Newcastle-Ottawa Quality Assessment Scale score of 6 (3, 1, 2) that was assessed independently by two of our authors[6]. The importance of this study was that it revealed the ability to predict the overall survival of patients with resectable pancreatic cancer (PC) from an imaging perspective, providing assistance in developing early treatment plans and improving patient prognosis. Gao *et al*[5] initially found that enhanced computed tomography (CT) characterizing vascular perfusion could be used as a quantitative imaging biomarker (QIB) of the malignant potential of PC. Based on this innovative idea and combined with data analysis, the authors demonstrated the value of QIB for predicting the prognosis of patients with PC. In addition, the authors proposed some new concepts to calculate the difference between the region of the overall tumor of the portal venous (PV) phase and that of the non-enhancement phase as the tumor enhancement amplitude (TEA), and the difference between the pancreatic tissue outside the tumor of the PV phase and that of the non-enhancement phase was used as the pancreatic enhancement amplitude (PEA) outside the tumor[5]. The tumor relative enhancement ratio (TRER) was then derived as TEA/PEA. Based on a retrospective analysis of 67 patients with resectable PC, the conclusions drawn by the authors properly summarize the data in the study. Furthermore, this study provided the unique insight that preoperative enhanced CT is a simple and effective predictive tool for overall survival in patients with PDAC and highlighted the need for close monitoring of patients with a TRER ≤ 0.7 because their prognosis is likely to be poor. We would like to thank Gao *et al*[5] for this study, which helped to advance clinical diagnosis and treatment.

In recent years, QIB has become more widely used in clinical practice because the objective features obtained from *in vivo* images measured on a scale of proportions or intervals can serve as indicators of normal biological processes, pathogenic processes, or responses to therapeutic interventions[7]. We therefore use an open multidisciplinary citation analysis database based on artificial intelligence techniques termed *Reference Citation Analysis*. We used "quantitative imaging biomarker" and "pancreatic cancer" as search terms to find the most recent (last 5 years) and relevant cutting-edge research. Overall, the application of QIB is mainly combined with a clinical perspective, and it plays an important role in characterizing tissue, detecting disease, identifying phenotypes, defining longitudinal changes, or predicting outcomes[7]. As previously mentioned, the highly invasive and metastatic nature of PC makes the search for prognostic biomarkers with high accuracy challenging. Numerous studies developed different QIB models that, in addition to characterizing microvascular density[8], significantly compensate for the survival prediction rate of clinical models[9] and contribute to clinical decision making. Next, we provide a brief analysis of PC survival prediction based on the study by Gao *et al*[5] and in the context of the current state of research.

At present, radiomics research concerning the prediction of the prognosis of resectable PC mainly focuses on the analysis of tumor texture features based on CT images[10,11]. Low-attenuation radiomic features of tumors are associated with poorer survival[12,13]. In addition, current radiomics data suggest that first-order entropy is associated with overall survival in PDAC patients and can significantly improve prediction accuracy[14]. Gao *et al*[5] revealed that PDAC hypervascularity was positively associated with poorer survival based on a quantitative analysis of vascular perfusion imaging, which is consistent with the aforementioned low blood supply of highly invasive PDAC[1,2]. In addition, TRER is calculated using CT, which is simple and more easily accepted by clinicians and supports its strong practicability.

We are extremely concerned about the study of PDAC invasion and metastasis because high invasion and metastasis are the characteristics of PDAC itself[15]. Several current radiomics studies identified several predictors of survival following treatment in patients with unresectable or advanced PDAC, including the mean value of positive pixels and kurtosis[16], age and homogeneity on unenhanced CT [17], skewness[18], and cluster tendency with a square root filter[19]. Gao *et al*[5] cited several limitations, including the absence of patients with metastasis. We anticipate future research by Gao *et al* [5] on the use of TRER based on enhanced CT to predict the treatment response and survival of patients with metastatic PDAC after treatment, which will bring great benefits concerning the diagnosis and treatment of patients. In conclusion, quantitative analysis based on enhanced CT imaging (TRER) has

good acceptability and utility for predicting the prognosis and survival of patients with PDAC.

FOOTNOTES

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EVIDENCE REVIEW

Liver injury induced by COVID 19 treatment – what do we know?

Lekha Saha, Soumya Vij, Kajal Rawat

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Abstract

The severity of coronavirus disease 2019 (COVID-19) may be correlated with the risk of liver injury development. An increasing number of studies indicate that degrees of hepatotoxicity have been associated with using some medications in the management of COVID-19 patients. However, limited studies have systematically investigated the evidence of drug-induced liver injury (DILI) in COVID-19 patients. An increasing number of studies indicate that degrees of hepatotoxicity have been associated with using some of these medications in the management of COVID-19 patients. Significantly, it was relieved after the cessation of these agents. However, to our knowledge, no studies have systematically investigated the evidence of DILI in COVID-19 patients. In this review, we discussed the association between hepatotoxicity in COVID-19 patients and the drugs used in these patients and possible mechanisms of hepatotoxicity. The currently available evidence on the association of different therapeutic agents with hepatotoxicity in COVID-19 patient was systematically reviewed.

Key Words: Liver injury; COVID-19; Anti-COVID drugs; Mechanisms; Clinical evidences

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Core Tip: Liver damage is cited as the most frequent clinical extrapulmonary manifestation in a number of coronavirus disease 2019 (COVID-19) clinical trials. Herein, we summarized the available clinical data concerning liver injury during COVID-19. Transaminases and bilirubin levels were high in a significant majority of patients; however, the exact cause of liver damage is not entirely clear. A worse clinical course of COVID-19 may be associated with severe liver enzyme abnormalities. However, it is impossible to rule out a number of additional cofactors (drug-induced liver damage, an inflammatory response to an infection, hypoxic hepatitis, or underlying liver disease).

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INTRODUCTION

A novel coronavirus known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was initially discovered in Wuhan, China in early December 2019. This discovery was followed by an outbreak that spread across the globe. SARS-CoV-2 is a new pathogen in the coronavirus family, and the condition it causes is called coronavirus disease 2019 (COVID-19), a severe acute respiratory condition [1]. As of August 28, 2022, over 598 million confirmed cases and over 6.4 million deaths had been reported globally by the World Health Organization[1]. Patients commonly present with symptoms of fever, dry cough, and fatigue; however, symptom severity varies. Severe symptoms include respiratory distress, high-grade fever, chest discomfort, and loss of appetite. The disease is usually self-limiting, with recovery in nearly 80% of patients with no significant interventions required. Up to 15% may need respiratory support, and 5% of patients need intensive care due to severe disease[2,3].

COVID-19 consists of a systemic disease that can present numerous complications. Complications from the severe illness include acute respiratory distress syndrome, septic shock, and thrombolytic and multiorgan failure affecting the liver, heart, and kidneys[4]. The virus can affect not only the lungs of the patient but also other tissues that express the angiotensin-converting enzyme (ACE)-2 receptor, such as the vascular endothelium, gastrointestinal tract, and squamous epithelium of the nasal, oral mucous, and pharynx. Therefore, COVID-19 is a systemic illness that may cause arrhythmia, thrombolytic episodes, prolongation of the QT interval, acute coronary syndrome, myocardial dysfunction, ketoacidosis, hepatocellular damage, kidney injury, neurological symptoms, hyperglycemia, sepsis, and, in more severe cases, multiorgan failure.

Patients with COVID-19 experience various degrees of liver function abnormalities. Numerous studies have demonstrated that liver damage is frequent in people with COVID-19 and may contribute to the severity of the disease[5-7]. Liver damage can be complicated and varied, requiring a thorough investigation and continuous monitoring. In the context of COVID-19, clinicians will have to determine whether the liver injury is related to underlying liver disease, drugs used to treat COVID-19, the direct effect of the virus, or a complicated disease course. Recent studies have suggested a number of potential mechanisms of probable liver injury in COVID-19 patients. However, the exact cause and specific means of COVID-associated liver injury need to be elucidated further. Drug-induced liver injury (DILI) should be closely monitored in COVID-19 patients, especially considering the widespread off-label usage of medications in preventive and therapeutic regimens.

Liver disorders in COVID-19 patients using several concomitant off-label drugs (potentially causing further liver damage) should be a warning sign for rapid identification and early intervention, thus preventing severe impairment in patients. The inherent liver damage associated with COVID-19 patients and the use of numerous concurrent off-label medications leading to increased severity of liver damage should be a warning indicator for early diagnosis and treatment, averting severe impairment in COVID-19 patients. This review summarizes current evidence related to hepatobiliary complications in COVID-19, provides an overview of the available evidence and critically elucidates the proposed mechanisms. We anticipate that this review will assist medical professionals in developing more effective methods for managing such patients.

COVID-19 AND ITS IMPACT ON THE LIVER

The effect of COVID-19 on liver functions can be observed by abnormalities in the levels of liver enzymes. There may be a decrease in the albumin levels as well as increasing bilirubin levels. Laboratory findings of liver injury include an increase in aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase, lactate dehydrogenase, hyperbilirubinemia, prolonged prothrombin time. and hypoalbuminemia (Table 1)[8-11].

Any alterations in liver function can be either due to parenchymal injury, cholestasis, or mixed type of etiology[12,13]. The incidence of a parenchymal, cholestatic, and mixed form of liver injury was observed to be 75.0%, 29.2%, and 43.4%, respectively, in patients with COVID-19 infections. The elevation in liver enzymes (AST and ALT) was found to be within five times the upper limit of normal. The rates of ALT and AST increases were observed to be in a range of 2.5%-50.0% and 2.6%-61.1%, respectively. It was observed that 56% elevation in AST and 28% in ALT was observed in those patients who had severe COVID-19 infection[14]. Elevation of gamma-glutamyl transferase may occur in up to 28% of patients, whereas ALT elevation was observed in nearly 23% of patients as are the AST levels.

Increases in gamma-glutamyl transferase have been observed to increase mostly in severe forms of infection and could be possibly linked to drug toxicity[15]. AST level increases can be linked to hepatocyte injuries and mitochondrial damage by the virus. It was observed that an AST/ALT ratio > 1 helps predict the occurrence of severe pneumonia, need for intensive care, and risk of mortality. Elevations in ALP were rare but occurred in patients who had either multiorgan failure, liver failure or died due to COVID-19 infection[16].

Liver damage in patients with COVID-19 is currently limited to moderate to severe cases and the damage may be transient, with liver tests returning to normal without needing specific treatment[4,17]. The acute or chronic liver failure occurrence is yet to be investigated. However, the severity of the condition, the likelihood that a patient would require admission to the intensive care unit or a lengthy hospital stay[18], and the risk of death, all increase with higher serum levels of AST/ALT and total bilirubin[19]. In patients with pre-existing conditions, the occurrence of liver injury may be aggravated or accelerated. Some of these conditions include fatty liver disease with co-existing metabolic disorders, reactivation of viral hepatitis, as well as other causes of chronic liver diseases.

Some liver biopsy and histopathology specimens that were analyzed from patients with COVID-19 showed moderate microvascular steatosis and mild lobular and portal vein activity. There was evidence of infiltration of lymphocytes, sinusoidal expansion of the central lobule, and patchy necrosis[4]. There were no signs of severe liver injury such as extracellular fibrosis, coagulation necrosis, or severe cholestasis in the biopsy specimens examined. The difficulty to perform autopsy and liver biopsies of COVID-19 patients due to the virus' high transmissibility and the lack of guidelines for defending medical practitioners during the start of the pandemic lead to the deficit of histological information in the literature[17].

Several studies have reported that severe cases of COVID-19 were more likely to have severe liver injury than mild cases[20-22]. Male patients were more likely to have liver function injuries than females ($P < 0.05$)[23]. The association of multiorgan abnormalities has also been observed to correlate with the severity of the disease and the worsening of clinical outcomes. Liver involvement is not necessarily associated with a picture of multiorgan failure but may be seen in early disease stages. However, in these patients, there is no need for additional therapy for recovery of hepatic functions and is generally reversible. The frequency of liver damage in COVID-19 patients ranges from 14.8% to 53.0%[24].

POTENTIAL MECHANISMS OF LIVER INJURY IN PATIENTS WITH COVID-19

Direct effect of viral infection on the liver

The etiopathogenesis of liver damage related to direct COVID-19 infections has not been completely determined yet. However, it is hypothesized to be direct hepatocellular damage, as is observed by the elevation in the cytonecrosis enzymes levels (Figure 1). ACE2 is a key receptor of the SARS-CoV-2 virus [25]. The ACE2 receptor is expressed in the lungs and the bile duct cells and acts as a gateway for the entry of the virus. The cell-specific expression of the ACE2 receptor in healthy liver tissues was evaluated by Chai *et al*[6] and found that ACE2 was expressed in 2.6% of hepatocytes and 59.7% of cholangiocytes, suggesting that liver injury may be caused by direct viral invasion. The hypothesis of direct viral damage can also be explained by liver biopsies that show signs of hepatocyte apoptosis, along with the presence of viral RNA in the tissue[26]. A recent study by Wu *et al*[27] found that half of the COVID-19-infected patients who had completely cleared respiratory tract infections had virus shedding in their fecal specimens up to 11 d after viral detection in the respiratory tract samples became negative. This suggests that there may be viral replication in extrapulmonary sites (digestive tract and liver).

Role of endotheliitis in hepatic injury

Another suggested mechanism of liver injury is secondary to the hypercoagulability leading to thrombosis of the portahepatic system. Occurrence of hypoxia as a result of pulmonary involvement may lead to a state of hypoxia in liver tissue followed by reperfusions, thereby causing an influx of reactive oxygen species and worsening the proinflammatory state[28]. The inflammation of the vascular endothelium caused by SARS-CoV-2 infection leads to microvascular dysfunction, which leads to a hypercoagulable state, tissue edema, and organ ischemia[29,30]. It was previously reported that a variety of viruses, such as dengue fever virus, human immunodeficiency virus (HIV), and Ebola virus, have been affecting the coagulation system[29]. The underlying mechanism of the hepatic injury associated with COVID-19 may be the liver ischemia-reperfusion injury, a pathophysiological process that frequently occurs following a rapid recovery of blood circulation to the liver tissue. When there is vascular endothelial damage, either directly by virus or through immune-mediated inflammation, the vessels have a vasoconstriction and procoagulant state (Figure 1). Studies in the literature demonstrated significantly higher plasma levels of fibrinogen and D-dimer in severe cases of COVID-19 than those in healthy controls[31-33].

Table 1 Clinical evidence of coronavirus disease 2019-mediated impact on various biochemical indexes

Biochemical parameters	Observed range (minimum to maximum)	Inference	Ref.
Hepatobiliary function markers			
Alanine aminotransferase	15-107 U/L (7590 U/L for one patient with severe hepatitis)	Elevated serum levels	[8-11]
Aspartate transaminase	15-132 U/L (1445 U/L for one patient with severe hepatitis)	Elevated serum levels	[8-11]
Alkaline phosphate	48-118 U/L	Elevated serum levels	[8,10,11]
Total bilirubin	5.0-33.7 μmol/L	Elevated serum levels	[8-11]
Gamma-glutamyl transferase	15-161 U/L	Elevated in severe cases	[8-11]
Albumin	4-45 g/L	Reduced serum levels	[8-11]
Lactate dehydrogenase	124.8-716 U/L	Elevated serum levels	[8,10,11]
Inflammatory markers			
Lymphocytes count	(0.64×10^9)-$(1.10 \times 0\ 10^9)$/L	Reduced serum levels	[10]
C-Reactive protein	6.9-88.6 mg/L	Elevated serum levels	[10]
Interleukin-6	28.72 ng/L	Elevated serum levels	[10,33]
CD8+ T cell count	(220×10^7)-(220×10^9) cells/L	Reduced blood levels	[10]
Erythrocyte sedimentation rate	Maximum 58 mm/h	Elevated	[10]
Other markers			
D-dimer levels	0.20-4.55 μg/mL	Elevated plasma levels	[32,33]
Fibrinogen levels	220.0-528.0 mg/dL	Elevated plasma levels	[31,33]

Inflammatory storm in COVID-19-associated hepatic injury

The excessive immune response induced by COVID-19 infection causes an inflammatory cytokine storm that might also be one of the critical factors in hepatic injury (Figure 1)[34,35]. Lower lymphocyte counts (both helper T cells and suppressor T cells) and higher plasma levels of various inflammatory cytokines [monocyte chemoattractant protein 1, interleukin (IL)-2, IL-10, IL-7, GCSF, MIP1A, IP10, and tumor necrosis factor-alpha] were commonly observed in patients with COVID-19, especially in the critically ill patients[3,36]. Increases in IL-6 and IL-10 as well as a decrease in CD4+ T cells were found to be independent risk factors for severe liver damage in a cohort study of 192 patients[37]. Another study discovered an independent relationship between lymphopenia and C-reactive protein levels and hepatic damage[38].

Systemic inflammatory response syndrome in patients presenting with moderate to severe illness leads to uncontrolled immune-mediated inflammatory response secondary to the release of cytokines and other inflammatory markers such as interleukins and tumor necrosis factors. The state of cytokine storm leads to secondary liver injury by causing systemic inflammation, and the patients can often present with multiorgan failure[39].

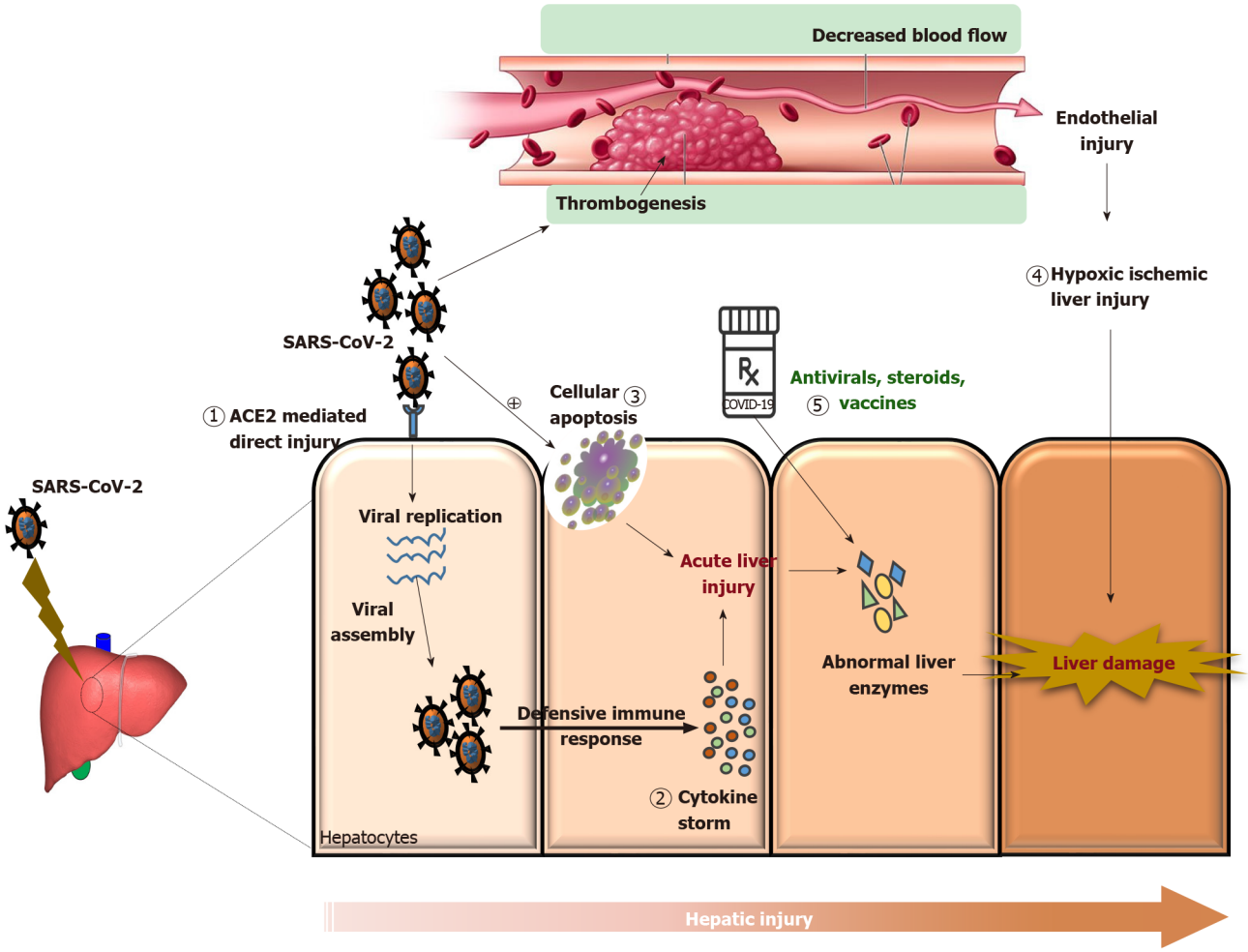
The liver injury associated with COVID-19 was determined to lead to a poorer patient outcome. This could be due to the involvement of other organ systems as well. In severe and critical patients, there was a rise in the biomarkers of inflammation, myocardial injury, liver and kidney functions, and coagulation functions[20]. Patients with liver injury usually had a longer hospital stay, and in those with a pre-existing liver disease, the risk of hospitalization and mortality was higher. Systematic reviews have shown that an elevation in liver biochemistry during the first visit was also found to be an indicator of the disease severity.

In a metanalysis of over 15000 patients with COVID-19, liver injury was reported in 23.1% patients in the early period and 24.4% incidence in the overall disease course. They found that 48.5% of the cases of liver injury were reported within the 1st 2 wk of admission, and 26.7% patients with severe pneumonia had liver injury as well[12]. The incidence of liver injury in patients who have died from COVID-19 was found to be as high as 58%-78%[40,41].

The poor prognosis of patients developing liver injury is possibly related to patients older than 60 years, severe COVID-19 infection, and underlying metabolic diseases such as hypertension and diabetes. Therefore, it was concluded that for patients with other comorbidities, there is a greater need for in-depth monitoring and individualizing the treatment given[42].

COVID-19 DILI: An overview

DILI is a rare diagnosis and can occur *via* many drugs, leading to liver damage. DILI may be severe but rarely fatal; recovery after drug discontinuation generally occurs. DILI is defined by increased liver



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Figure 1 Molecular mechanisms of severe acute respiratory syndrome coronavirus 2 and coronavirus disease 2019 drug-induced liver injury. 1: Direct action of the virus, *i.e.*, angiotensin-converting enzyme 2 receptor mediated action; 2: Immuno-inflammation caused by cytokine storm leading to liver damage; 3: Hepatocellular apoptosis induced by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection; 4: SARS-CoV-2-induced thrombosis leading to vascular endothelial damage and causing hypoxic-ischemic liver injury; 5: Several drugs and therapies used to counteract coronavirus disease 2019 including antivirals, immunomodulatory agents, and vaccines reportedly led to abnormal liver enzymes causing drug-induced liver injury. All these mechanisms aggravate liver injury by causing abnormal liver enzymes like increasing the levels of alanine aminotransferase, aspartate transaminase, alkaline phosphate, gamma-glutamyl transferase, lactate dehydrogenase, and total bilirubin and reducing the albumin levels. ACE2: Angiotensin converting enzyme-2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019.

enzymes either due to hepatocellular necrosis, cholestasis, or both[43]. The hepatocellular injury pattern resembles acute viral hepatitis-induced injury, hallmarked by hepatocyte necrosis and inflammation. Agents that typically cause this injury pattern include isoniazid, nitrofurantoin, and methyldopa. Cholestatic injury resembles bile duct obstruction, hallmarked by bile stasis, portal inflammation, and proliferation or injury of bile ducts and ductules. Drugs that exhibit this type of injury include amoxicillin/clavulanate, ciprofloxacin, and sulfonylureas. Mixed type liver injury is most characteristic of DILI, with the liver biopsy showing necrosis and inflammation along with bile stasis[44].

DILI may be caused by either an intrinsic or a distinctive mechanism. The clinical pattern associated with DILI is usually varied and can mimic other forms of liver disease. The patient's presentation can resemble acute hepatitis, acute liver failure, chronic hepatitis, biliary obstruction, or fatty liver disease. As the evidence of DILI concerning various drugs is often large and scattered across multiple disciplines, it becomes difficult for clinicians and researchers to search for evidence related to each drug. Therefore, the reference for the most established evidence related to a drug and its ability to cause liver injury is *via* databases such as LiverTox[44].

Intrinsic DILI is usually dose-dependent and predictable and occurs after a brief latency period. The most common DILI is idiosyncratic, and it is primarily unpredictable, with a variable latency period [45]. The severity of DILI may vary, and up to 10% of severe cases may need a liver transplant for the same. Mortality is three times higher in patients with pre-existing liver disease, along with a DILI. Monitoring is vital in a patient exhibiting signs of DILI. Any signs of jaundice, hyperbilirubinemia, hepatomegaly, or new onset right upper quadrant pain warrant further investigations and discontinuation of the suspected drugs[4]. Mostly, liver injury is reversible, with recovery after drug discon-

tinuation. One possible treatment option is ursodeoxycholic acid, which protects elevated transaminase and serum total bilirubin levels[46].

The Roussel Uclaf Causality Assessment Method scale is a seven-domain scale, which considers the temporal association of liver injury, various risk factors such as age, alcohol intake, and concomitant hepatotoxic drugs, and occurrence of repeated liver damage on the administration of the suspect drug. It also helps in the exclusion of other causes of transaminase elevations, such as ischemia, myositis, cytokine storm, and previous chronic liver disease[4].

The societal pressures of COVID-19 infections, including hospital bed shortages and added stress to the health system, are further compounded by liver injury secondary to drug use. Although in mild to moderate cases, the risk of mortality remains low, it still leads to the added burden on healthcare systems due to prolongation of hospital stays and an increased risk of contracting a nosocomial infection[47].

The treatment of COVID-19 saw the use of many therapeutic options, from the repurposing of older drugs to new drug trials and the administration of new vaccines to prevent infection. Assessing the hepatotoxicity profile of drugs used in COVID-19 is complicated as many drugs have been used off-label and with dosages not routinely used. Thereby, reports of DILI may be rarely encountered in routine practice with some drugs but may be more often when used in COVID-19[48].

LITERATURE REVIEW

The general information on SARS-CoV-2-associated hepatic injury was gathered by using a systematic search strategy. The eligible systematic reviews, meta-analysis, and review articles on COVID-19-induced and COVID-19 DILI were searched in four databases: EMBASE, Scopus, MEDLINE, and Web of Science. The literature search was performed through September 5, 2022 and terms used for search across all the databases included: ((COVID-19) AND (Drug-induced) AND (Hepatotoxicity)) OR ((SARS-CoV-2) AND (Drug-induced) AND (Hepatotoxicity)) OR ((COVID-19) AND (Drug-induced) AND (Hepatic injury)) OR ((SARS-CoV-2) AND (Drug-induced) AND (Hepatic injury)) OR ((COVID-19) AND (Drug-induced) AND (Liver injury)) OR ((SARS-CoV-2) AND (Drug-induced) AND (Liver injury)). The search returned a total of 113 articles, which were further screened for duplicates. A total of 56 articles were included, all fitting the eligible criteria, *i.e.,* reviews and meta-analyses on COVID-19-induced and COVID-19 DILI.

Evidence of hepatotoxicity risk associated with COVID-19 therapy

One meta-analysis reported the incidence of drug toxicity with COVID-19 therapy as high as 25.4%[49]. Some of the most commonly reported cases of hepatotoxicity have been associated with the prescription of antipyretics such as acetaminophen, anti-inflammatories such as tocilizumab, and antivirals such as favipravir, remdesivir, lopinavir/ritonavir (LPV/r), chloroquine, oseltamivir, and ribavirin (Table 2). In patients with pre-existing chronic liver disease or with severe infection, the risk of developing hepatotoxicity was reported to be higher[50]. The liver is the leading site for metabolism and elimination for many drugs. Some of the drugs that were repurposed for COVID-19 treatments such as nucleoside analogs and protease inhibitors are primarily metabolized in the liver[10] (Table 2).

ANTIVIRAL DRUGS AND LIVER INJURY

One of the antiviral drugs explored for therapy in COVID-19 included lopinavir-ritonavir, a combination widely prescribed in the treatment of HIV/AIDS. As a combination, it has been previously reported to be associated with moderate to severe elevations in serum aminotransferases. The mechanism of liver injury is probably due to metabolism through the CYP3A4 system[51]. Various studies reported liver function abnormalities when patients were treated with LPV/r[52]. The incidence of reported adverse events with the use of this combination was as high as 63.8% in one study, with the second most common adverse event being liver injury[53] (Table 2).

A pooled meta-analysis reported the incidence of DILI with LPV/r as 37.2%[49]. Once hepatotoxicity develops with LPV/r or ribavirin, restarting the drug is not recommended[54]. LPV/r prescription is associated with moderate to severe elevations in serum transaminase levels. Ritonavir, given as a boosting drug in this combination, acts as an enzyme inhibitor and may also lead to compounded hepatotoxicity of co-administered medicines[55] (Table 2).

Favipiravir is an inhibitor of RNA-dependent RNA polymerase enzyme. It has shown efficacy against Ebola, West Nile virus, yellow fever virus, and influenza. It acts by embedding in the viral RNA, causing mutations and leading to viral death[56]. It has been demonstrated to have activity against the SARS-CoV-2 virus by interfering with the viral replication by competing with the purine nucleosides. There have been some reports of elevation in the hepatic transaminases with favipiravir therapy, but it was generally mild and reversible[57] (Table 2).

Table 2 Clinical evidence of coronavirus disease 2019 drug-induced liver injury

Therapeutic agent	Molecular mechanism against SARS-CoV-2	Impact on liver	Ref.
Antiviral agents			
Remdesivir	It inhibits RNA-dependent RNA polymerase of SARS-CoV-2 and hence hinders its replication post infection	Mild-to-moderate increase in serum ALT, AST, and total bilirubin levels	[10,11, 13,52, 60,83, 84]
Lopinavir-Ritonavir	It is a protease inhibitor that disrupts viral replication and its release from host cells	Elevated serum enzyme levels varying from hepato-cellular to cholestatic, increased Golgi fragmentation, and organelle stress response leading to hepatic injury	[10,11, 13,52, 60]
Favipiravir	It inhibits RNA-dependent RNA polymerase of SARS-CoV-2 and hence hinders its replication post infection	Abnormal liver function test (elevated serum ALT, AST, and total bilirubin levels)	[10,11, 52,57, 60,85]
Oseltamivir	It selectively inhibits viral neuraminidase and alters its release from host cells	Elevated serum ALT levels and total bilirubin levels in some patients	[10,11, 52,58, 60,83]
Nevirapine	It is a non-nucleoside reverse transcriptase inhibitor. It inhibits the replication of the viral genome and helps in reducing viral load	Significant elevations in serum ALT levels leading to symptomatic impact on patient care	[10,11, 13,52]
Umifenovir	It is a direct acting virucidal agent; it inhibits SARS-CoV-2 spike protein/ACE2 membrane fusion and hence inhibits viral entry into host cells	Elevated serum ALT levels	[10,11, 52,59, 60]
Antibiotics and antiparasitic agents			
Ivermectin (anthelmintic drug)	Several mechanisms have been proven using *in silico* and *in vitro* studies like inhibition of viral entry, viral replication, and host importin α/β-1 of nuclear transport protein	Elevations in serum AST levels and clinically apparent liver damage	[10,11, 13,52]
Chloroquine and hydroxy-chloroquine (antimalarial drug)	It has been proposed to inhibit the viral fusion with the ACE1 receptors and hence prevents viral entry into the host cells	Increased serum enzyme levels at high doses and trigger acute liver injury with sudden onset of fever	[10,11, 13,52, 60]
Azithromycin (macrolide antibiotic)	It inhibits viral entry into the host cells and also inhibits several cytokines	Elevated serum ALT levels and cholestatic hepatitis persisting even after discontinuing medication	[10,11, 13,52]
Voriconazole	It is an antifungal agent and was used in the management of COVID-19-associated pulmonary aspergillosis	Elevated serum AST and ALT levels, and a case of acute liver failure had also been reported	[10,11, 13,52]
Anidulafungin	It has been proposed that it acts by inhibiting viral protease and hence disrupts viral replication process in host cells	Transient elevation in serum ALT levels	[10,11, 13,52]
Immunomodulatory agents			
Tocilizumab (IL-6 receptor blocker)	It is an IL-6 receptor blocker and is approved for treatment of cytokine storm associated with COVID-19	Mild-to-moderate increase in serum ALT, AST levels and clinically apparent liver injury with jaundice, with the odds of at least one affected patient dying of liver injury	[10,11, 13,52, 60,84]
Dexamethasone (corticosteroid)	Reduces aggressiveness Inflammatory response	Corticosteroids are associated with severe liver injury, which can cause hepatomegaly and steatosis and can trigger nonalcoholic steatohepatitis and sometime leads to liver failure and death	[10,11, 13,52, 60]
Baricitinib Tofacitinib Imatinib	It is a Janus kinase inhibitor; it reduces inflam-matory responses associated with COVID-19 infection	It is associated with idiosyncratic hepatotoxicity (possibly cholestatic) in patients with COVID-19. However, the liver damage has not been reported in COVID-19 patients so far	[67,68]
Colchicine	It has several potential anti-inflammatory mechanisms like inhibiting inflammasome signaling, reducing neutrophil chemotaxis, and reduced production of cytokines	Overdose of colchicine was associated with self-limiting liver injury	[10,11, 13,52]
Miscellaneous therapies			
Paracetamol	It has an antipyretic and analgesic effect and is widely used as an adjunct to reduce fever during COVID-19	Overdose of acetaminophen is associated with acute liver injury and when taken for several days at the therapeutic dose, there can be a slight increase in aminotransferases levels	[13,72, 73]

Enoxaparin	It is low molecular weight heparin and was indicated in thromboembolic venous complications of COVID-19	Elevated serum AST levels and mild elevation in serum alkaline phosphate and total bilirubin levels	[10,11, 13,52]
LMWH	It is an anticoagulant used in COVID-19 to reduce the risk of thromboembolism	LMWH are associated with acute hepatotoxicity, and elevations in the liver enzymes are usually observed after 5-8 d of initiation of therapy	[74,85]
SARS-CoV-2 vaccines (Pfizer-BioNTech, Moderna, and Oxford-AstraZeneca)	mRNA and viral vector encoding SARS-CoV-2 spike protein were used for vaccinations in healthy subjects	The clinical phenotype is mostly hepatocellular and showed features of immune-mediated hepatitis	[77,78]

ALT: Alanine aminotransferase; AST: Aspartate transaminase; ACE: Angiotensin converting enzyme; IL-6: Interleukin 6; LMWH: Low molecular weight heparin; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019.

Oseltamivir has also been initially explored for therapy in patients with COVID-19. The efficacy of oseltamivir could not be established in various trials and is now no longer prescribed. Some case reports have reported a transient rise in liver enzymes associated with its use; however evidence in COVID-19 is lacking[58]. Umifenovir is used for influenza treatment and was prescribed initially in COVID-19 in China. It is metabolized *via* CYP3A4, and this mechanism may be associated with the occurrence of DILI. Its use in cirrhotic patients is cautioned due to risk of liver injury[59,60] (Table 2).

Remdesivir is a nucleotide analogue that was explored for the treatment of hepatitis C virus and Ebola. One case series reported 25% and 33% increases AST and ALT, respectively[61]. The risk of abnormalities in the AST and ALT levels were higher with the use of remdesivir as compared to any other therapy for COVID-19. Elevations were noted immediately after starting remdesivir, but the gradual improvement was noted afterwards[62]. Prior to starting remdesivir, a baseline liver function test should be obtained. If the transaminase level is increased more than 10 times, then the use of remdesivir should be discontinued[63]. However, hepatotoxicity is generally asymptomatic, fully reversible, and not associated with any jaundice[64] (Table 2).

IMMUNOMODULATORS AND LIVER INJURY

Hydroxychloroquine and chloroquine are immunomodulator drugs, mainly used in autoimmune disorders. During the early days of the pandemic, these drugs were widely explored for the treatment of COVID-19 infections due to their proposed role in decreasing the hyperinflammatory states associated with COVID-19. Hydroxychloroquine is metabolized in the liver, and its metabolites may also accumulate in the liver leading to hepatotoxicity, although rarely reported[44]. One case of a 10-fold increase in transaminases was reported with administration of hydroxychloroquine, which was reversible and recovery occurred after withdrawal of the drug[65] (Table 2).

Tocilizumab is a monoclonal antibody against the IL-6 receptor and is indicated for use in cytokine storm associated with severe disease. It has received Food and Drug Administration approval for use in COVID-19 infections. It has been demonstrated to have a transient elevation in the hepatic enzymes, but rare cases of severe liver injury have occurred[55]. Patients with a pre-existing hepatitis B viral infection have experienced reactivation of the virus after treatment with tocilizumab[66]. The recommendation suggests patients treated with tocilizumab should receive regular liver function test monitoring, and hepatitis B virus-DNA levels should be rechecked in patients with pre-existing infections after discharge from the hospital[39] (Table 2).

Baricitinib, tofacitinib, and imatinib belong to a class of Janus kinase inhibitors. Of these, baricitinib has been used the most for treating COVID-19 because of its effect on decreasing the inflammatory response to COVID-19 infection[67]. There is evidence of a nearly 1% incidence of liver enzyme and bilirubin increases with patients, but no data has been published so far for patients with COVID-19. In the randomized controlled trial conducted for baricitinib in COVID-19, there were no reports of liver damage[68] (Table 2).

Azithromycin was widely used in the beginning of the pandemic, as it had some demonstrated antiviral and anti-inflammatory activity[69]. Combined use of hydroxychloroquine and azithromycin was explored by a French study. However, the findings were disputed, and the study was withdrawn [70]. Azithromycin has a concern over causing cardiotoxicity, but there have been instances of hepatotoxicity reported with azithromycin use as well[44]. The risk of liver injury is higher in patients with pre-existing liver disease, and they are at a higher risk of developing more severe complications[71] (Table 2).

MISCELLANEOUS THERAPIES AND LIVER INJURY

Paracetamol has both antipyretic and analgesic effects and is a well-known cause of dose-dependent DILI; it is usually difficult to manage and can be fatal to the patients. The underlying mechanism for toxicity is direct damage through a metabolite generated from liver metabolism[72]. Paracetamol is used as an adjunct therapy, along with many other medications, but the actual incidence of hepatotoxicity associated with acetaminophen is still undetermined[73] (Table 2).

Low molecular weight heparin is one of the most commonly prescribed anticoagulants used in COVID-19 to reduce the risk of thromboembolism and its sequelae. Low molecular weight heparin is known to cause hepatotoxicity in the range of 4.3%-13.0%[74]. Liver enzyme elevation usually occurs 5-8 d after initiation of therapy and are reported to normalize within 2 wk of stopping the drugs[75] (Table 2).

One of the cornerstones of treatments is the systemic administration of corticosteroids. Corticosteroids have been shown to reduce mortality in mechanically ventilated patients and in patients who require supplemental oxygen support at the time of admission. Although, corticosteroid use is associated with many adverse events, ranging from deranged glycemic control to thromboembolism, secondary infections and reactivation of hepatitis B infection[39]. The prolonged use of corticosteroids may be associated with hepatic steatosis. However, this effect is less likely to be seen with the relatively short duration of corticosteroid therapy in COVID-19 infections[76] (Table 2).

Vaccinations in healthy subjects for prophylaxis against COVID-19 had been reported to be associated with acute liver injury in some patients. A world-wide case series involving 87 patients administered with COVID-19 vaccines, including 59% of cases with the Pfizer-BioNTech (BNT162b2) vaccine, 23% of cases with the Oxford-AstraZeneca (ChAdOX1 nCoV-19) vaccine, and 18% of cases with the Moderna (mRNA-1273) vaccine, had reported clinical features of acute liver injury associated with SARS-CoV-2 vaccination[77,78]. However, the benefits of COVID-19 vaccines during the pandemic cannot be neglected because of the rare events of liver injury in the small proportion of the overall vaccinated population (Table 2).

DIAGNOSIS AND MANAGEMENT OF DILI

DILI is a diagnosis by exclusion. Patient workup includes thorough patient history, including a history of chronic liver disease, alcohol or drug abuse, and complete information on past and concomitant drug history[79]. A complete history of herbal and dietary supplement use should also be taken[80]. Patients developing liver injury require regular monitoring of ALT, AST, total and direct bilirubin, albumin, prothrombin time, and international normalized ratio[24].

According to the updated Roussel Uclaf Causality Assessment Method, the cutoffs for the diagnosis of DILI is as follows: ALT > 5 times the upper limit of normal and/or ALP > 2 times upper limit of normal. Based on the ratio of serum ALT to ALP values (R-value), DILI pattern may be hepatocellular (≥ 5), cholestatic (≤ 2), or mixed (> 2 and < 5)[43]; 50% of the patients with DILI exhibit a hepatocellular pattern[64].

Acute liver injury in patients with COVID-19 is usually mild and is transient, with recovery without requiring any specific therapy[79]. However, supportive therapy is often needed, such as albumin infusion or administration of fresh frozen plasma. In patients with pre-existing liver dysfunction and severe infection, the drugs prescribed should be carefully monitored, taking care that no more than two drugs with the potential to cause DILI should be administered at the same time. Also, regular monitoring of liver functions should be done for such patients. If patients develop DILI, early discontinuation should be initiated, or dosage should be altered if complete discontinuation is not possible[81].

There are no defined guidelines for managing DILI in patients with COVID-19. The most effective option available is to discontinue the offending agent. This leads to recovery in 90% of patients. In cases where required, therapy can be initiated with medications such as ursodeoxycholic acid[82]. Some other therapeutic options available include drugs such as polyene phosphatidylcholine, glycyrrhizic acid, and adenosylmethionine. Glycyrrhizic acid has been demonstrated to have a strong affinity for liver enzymes and has anti-inflammatory, anti-allergic, and hepatoprotective effects[52]. Patients with COVID-19 are at a higher risk of hepatic injury, and therefore drugs such as nonsteroidal anti-inflammatory drugs, acetaminophen, and antibiotics, which have the potential of causing liver injury, should be given to these patients cautiously. A small number of healthy subjects presented SARS-CoV-2 vaccination-associated liver injury, predominantly immune-mediated hepatitis, were managed using corticosteroid therapy[78].

CONCLUSION

There are variable degrees of impaired liver function in patients with COVID-19. Liver damage can be complicated and varied, requiring significant investigations and continuous monitoring. Clinicians

treating patients with COVID-19 must first evaluate if the liver damage is a result of underlying liver illness, therapies used to treat COVID-19, a direct result of the virus, or a complex disease course. Recent studies proposed a number of mechanisms regarding possible causes of liver damage in COVID-19 patients. This review gave an overview of the case series that are currently available, highlighted the most recent research on hepatobiliary consequences in COVID-19, and critically explained the putative mechanisms underlying liver damage. We anticipate that this review will assist medical professionals in creating more effective plans for managing such patients.

FOOTNOTES

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REVIEW

Gastrointestinal microbiota: A predictor of COVID-19 severity?

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Abstract

Coronavirus disease 2019 (COVID-19), caused by a severe acute respiratory syndrome coronavirus 2 infection, has raised serious concerns worldwide over the past 3 years. The severity and clinical course of COVID-19 depends on many factors (*e.g.*, associated comorbidities, age, *etc*) and may have various clinical and imaging findings, which raises management concerns. Gut microbiota composition is known to influence respiratory disease, and respiratory viral infection can also influence gut microbiota. Gut and lung microbiota and their relationship (gut-lung axis) can act as modulators of inflammation. Modulating the intestinal microbiota, by improving its composition and diversity through nutraceutical agents, can have a positive impact in the prophylaxis/treatment of COVID-19.

Key Words: Gut microbiota; COVID-19; Prognostic biomarkers; Gut-lung axis; Probiotics; Nutraceuticals

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Core Tip: In the last 10 years, the intestinal microbiota has been intensively studied in relation to various diseases from gastrointestinal to cardiovascular, respiratory, and even neurological or psychiatric diseases. Coronavirus disease 2019 (COVID-19) has been a challenge in this regard. Thus, in this review we highlighted the link between microbiota and COVID-19, aspects of the clinical and imaging manifestation and the potential role of some nutraceuticals in this widespread respiratory viral disease.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), first reported as a new infectious disease in December 2019 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, can result in acute respiratory syndrome and has raised serious global concerns[1]. The clinical course of COVID-19 ranges from asymptomatic and mild to life-threatening forms[2]. Interindividual variability influences clinical symptoms and disease outcomes and is related to varying genetic profiles of the host immune response and angiotensin converting enzyme 2 (ACE2) binding affinity of SARS-CoV-2[3,4].

Disease severity and clinical course of COVID-19 depends on the patient's associated comorbidities, including cardiovascular disease, hypertension, diabetes, chronic pulmonary disease, age, and smoking status[5]. The hyperreactivity of the host immune responses caused by SARS-CoV-2 infection, known as "cytokine storm", leads to a massive and uncontrolled activation of proinflammatory pathways, which ultimately results in multiorgan failure and mortality[6,7]. Evolving research data suggests several conventional serum biomarkers are correlated with disease onset and disease severity, including white blood cells, D-dimers, fibrinogen, C-reactive protein (CRP), procalcitonin, lactate dehydrogenase, serum ferritin, interleukin 6 (IL-6), alanine aminotransferase, aspartate aminotransferase (AST) and total bilirubin[8].

The human microbiota is a complex microecosystem composed of bacteria, viruses, fungi and archaea within the oral cavity, gut, lung, skin and vagina, with the highest abundance and diversity in the gut [9]. The role of microbiota in health and disease conditions has been recently shown in experimental and clinical setting[10]. The involvement of gut microbiota and its associated metabolites in maintaining body homeostasis includes regulation of host immunity, influencing physiological functions, such as digestion and nutrition and biosynthesis of vitamins and numerous active compounds[10-12]. Gut bacterial compounds play a critical role in regulating disease pathogenesis and recovery and providing promising therapeutic targets for stroke[13], neurodegenerative diseases[14], cardiovascular dysfunctions[15] and cancer-related diseases[16].

Targeting inflammatory responses triggered by SARS-CoV-2 infection by modulating the gut-lung axis represents a promising therapeutic target[17]. A clinical perspective of gut bacterial changes associated with disease severity and outcomes might provide predictive fecal and serum biomarkers with prognostic and diagnostic value[18]. Metabolomic and microbiome profiling research studies depicting bacterial changes during and after COVID-19 may provide a better understanding of the role of gut microbiota in COVID-19 pathogenesis[19,20].

This narrative review aimed to provide new insight into the involvement of gut microbiota in COVID-19 patients by modulating inflammatory responses and disease severity. For a better understanding of the translational relevance of gut microbiota as disease modifying therapy in COVID-19 disease, we summarized the most common changes in gut composition abundance of commensal and pathogenic species in relation to disease onset and severity.

MATERIALS AND METHODS

This narrative review aimed to provide an overview of the current knowledge of the involvement of gut microbiota in COVID-19 patients. We performed an electronic search in the databases of Medline (PubMed, PubMed Central) by using different term combinations of "COVID-19" or "Sars-Cov-2" and "microbiota", "airway microbiota", "lung microbiota", "gut microbiota", "dysbiosis" and "leaky gut".

SHORT OVERVIEW OF SARS-COV-2 INFECTION: FROM ORIGINS TO RECENT DATA

It is known that in the last two decades three coronaviruses have been described to cause life-threatening severe infection in humans: SARS-CoV, Middle East respiratory syndrome-CoV and SARS-CoV-2[21-23]. SARS-CoV, originated in China generated a global pandemic in 2002, having a mortality rate of 10%[21]. Middle East respiratory syndrome-CoV-2, was first reported in Saudi Arabia in 2012 and caused another transmissible disease impacting the public health sector[22]. The most recent pandemic declared was the COVID-19 pandemic, first reported in Wuhan China with a quick spread around the world[23].

The newly acquired infection is continuing to spread because of the mutations that occur in the genome and leads to an intensive viral replication with a high risk of reinfection, reducing the antibodies produced by vaccination or previous infections[24]. Being an RNA virus, SARS-CoV-2 has an important potential to adapt to new hosts, developing mutations and leading to different variants with different characteristics. To identify the new variants genomic sequencing is used. At the beginning of the pandemic, the mutation, D614G, was very contagious but not very dangerous with severe manifestations[24-26].

After this mutation, another large variety of variants have been found and named variants of concern. A variant of SARS-CoV-2 infection is a variant of concern when it impacts the public health sector. Variants of concern are linked to high transmissibility, virulence, decreased effectiveness to vaccines or medical treatment and with the capacity to evade detection.

These mutations with a high transmissibility have an increased hospital admission rate and mortality rate[27]. The five variants found to be variants of concern since the beginning of the pandemic, according to World Health Organization, are illustrated in Table 1[28]. The initial step of the infection is the recognition of the receptor, which is the key to tissue tropism[29]. The affinity of the spike glycoprotein to bind to the ACE2 receptor influences the replication and the severity of the disease[29-31].

The spike protein is formed by two subunits: The S1 subunit, which contains the receptor-binding domain and recognizes ACE2 on the host cells; and the S2 subunit, which mediates the fusion of the viral and cellular membrane. Mutations that appear in the receptor-binding domain lead to a higher viral replication and contagion. They allow the virus to not respond to vaccine-elicited antibodies[32,33]. The viral protein is cleaved by transmembrane serine protease 2, a host cell molecule involved in viral entry[34]. It has been shown that the expression of ACE2 and transmembrane serine protease 2 is increased in the nasal and oral mucosa, airways, lungs and intestine[35,36].

GUT MICROBIOTA IN HEALTH AND DISEASE

The human gut microbiota is a complex ecosystem composed of all microorganisms (10^{13} to 10^{14}) at this level, including bacteria, viruses, fungi and archaea[9]. The human microbiota, known as the "hidden organ" is composed of all the microorganisms in the oral cavity, gut, lung, skin, vagina, *etc*, but the greatest diversity and abundance is in the gut[37]. At this level, there are two dominant phyla, Firmicutes and Bacteroidetes, in healthy adults. For each of the two phyla, there are several dominant genera: *Lactobacillus, Faecalibacterium, Clostridium, Enterococcus* for Firmicutes; and *Bacteroides* and *Prevotella* for Bacteroidetes[38].

The microbiota has an important role in maintaining body homeostasis, can modulate host immunity, and has the ability to influence physiological functions[11]. Communication between the gut microbiota and the immune system is mainly carried out through mediators. Short chain fatty acids (SCFAs) are included in this category[39]. These mediators (SCFAs), represented by acetate, propionate and butyrate, play important roles through interactions with host immune cells and represent an important carbon source for colonocytes[40].

THE INVOLVEMENT OF GUT MICROBIOTA IN COVID-19 PATHOGENESIS

Intestinal microbiota and intestinal permeability have an important role both in regulating the transition of beneficial elements (*e.g.*, nutrients) and in stopping the penetration and transfer of harmful particles from the intestinal lumen into the circulation[41]. It has been shown that probiotics can regulate the composition of the microbiota and thus contribute to maintaining the body's homeostasis[42]. Management of COVID-19 by administration of probiotics or other nutraceutical agents that can modulate the microbiota has not been a mainstay in the pandemic. However, the influence of COVID-19 by modulating the microbiota was not completely neglected during that period[43].

Table 1 Variants of concern reported for coronavirus disease 2019

VOCs	First time reported	Country of origin
Alpha (B.1.1.7)	December 2020	United Kingdom
Beta (B.1.351)	December 2020	South Africa
Gamma (P.1)	January 2021	Brazil
Delta (B.1.617.2)	December 2020	India
Omicron (B.1.1.529)	November 2021	South Africa

VOC: Variants of concern.

THE GUT-LUNG AXIS IN COVID-19

Gastrointestinal symptoms account for frequent complaints of patients with SARS-CoV-2 infection[44,45]. Mounting preclinical and clinical evidence pointed out the relationship between pulmonary injury and intestinal dysfunction within viral lung infection[46], influenza A virus infection[47], bronchial asthma[48], chronic obstructive pulmonary disease[49] and cystic fibrosis[50].

Though distinguished by their functional and compositional microflora, *i.e.*, species and density, both the gut and lung microbiota systems contribute to host homeostasis by mediating local and systemic inflammatory responses, forming the so-called "gut-lung axis"[51]. Immunomodulatory effects of the gut-lung axis are mediated by mucosal-related immune systems, consisting of gut-associated lymphoid tissue and bronchial-associated lymphoid tissue[52-54].

In healthy conditions, a similar signature of microbial phyla is shared between gut and lung microbiota being provided by Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria, with Fusobacteria and Verrucomicrobia only found in the intestinal microbiota[55,56]. However, there is a distinctive pattern in the compositional bacterial genera of gut and lung microbiota, with *Lactobacillus, Clostridium, Bacillus, Enterococcus* and *Prevotella* dominant in gut microbiota and *Streptococcus, Veillonella, Fusobacterium* and *Haemophilus* dominant in lung microbiota[55,56].

The crosstalk between the gut and lung microbiota is bidirectional, with dysbiosis of either tract influencing each other[57]. Therefore, once gut microbiota are dysregulated an enrichment of blood flow with microbiota-derived products will result in a systemic inflammatory state, affecting multiple organ systems, including the lungs[58]. Several pulmonary diseases have been associated with altered samples of gut microbiota, including asthma, chronic respiratory dysfunction and pulmonary allergic responses [59,60]. However, pulmonary dysfunction during acute and chronic inflammatory lung diseases trigger intestinal changes by altering intestinal permeability and promoting bacterial translocation[61-63].

Differences in gut microbiota diversity have been reported in multiple pulmonary diseases[64]. Among 43 patients with chronic pulmonary dysfunction, an overgrowth of Proteobacteria, *i.e.*, *Haemophilus spp.*, and Firmicutes with a decreased proportion of Bacteroidetes, *i.e.*, *Prevotella spp.*, have been shown[64]. Environmental factors, such as dietary fibers, antibiotics and pre/probiotics, impacted gut microbiota, providing therapeutic insights into the microbiota-associated gut and lung dysfunction to re-establish the homeostasis in the gut-lung axis[65]. A high-fiber diet has been associated with bacterial changes in the gut-lung axis by increasing the abundance of Bacteroidaceae and decreasing the ratio of Firmicutes/Bacteroidetes in both the feces and lungs[17]. Moreover, in an experimental model of allergic lung inflammation, mice treated with a high-fiber diet or propionate showed changes in the bacterial composition of the gut and lung microbiota and an enhanced capacity of bone marrow to generate macrophage and dendritic cell precursors[17]. Moreover, in an asthma mouse model, a SCFA and fiber diet increased the phagocytic capacity of dendritic cells in the lungs and regulated T helper 2 cell-promoting inflammatory responses[17]. This experimental finding suggested that dietary fermentable fibers and SCFAs might regulate immunological tolerance in atopic asthma patients[17].

Microbiota-derived metabolites mediate the immune cross-talk between gut and lung microbiota[66,67]. SCFAs, the most dominant microbiota-derived metabolites, derived from dietary fermentable fibers in anaerobic conditions, are represented by fatty acid molecules, which are formed by chains of up to six carbon atoms, consisting of acetate, propionate or butyrate[68-70]. SCFAs play an essential role in maintaining the integrity of the intestinal epithelial barrier and mitigating inflammatory events within the gut and respiratory tract by regulating the expression of G-protein coupled receptors or histone deacetylases[68,69,71]. Circulatory acetate or propionate stimulate bone marrow hematopoiesis and enhance airway immunity by activating the differentiation of T helper cells and monocytes and increasing the expression of various chemoattractant molecules on immune cells, thereby promoting immunomodulatory mechanisms against respiratory tract infections[72,73].

The development of asthma has been shown to be influenced by the synthesis and secretion of bacterial-derived metabolites[74]. The correlation between SCFAs, specifically acetate concentration in feces, and the risk of asthma in 319 pediatric subjects demonstrated the link between dysregulation of

bacterial metabolites and pulmonary dysfunction[74]. The unstable state of gut microbiota associated with an increased risk of asthma in a cohort of pediatric patients was driven by a decrease in *Faecalibacterium, Veillonella, Lachnospira* and *Rothia*[74].

Increasing experimental models ascertained the role of microbiota metabolites in immune cell differentiation. In an *in vivo* model of experimental colitis, butyrate promoted the differentiation of regulatory T cells and alleviated the development of colitis[67]. Butyrate enhanced IL-10 synthesis and decreased the production of IL-6 by binding GPR109a on dendritic cells and macrophages. Several expression targets were activated *via* interaction with SCFAs. For instance, in healthy conditions, butyrate could activate peroxisome proliferator-activated receptor gamma[75].

In gut microbiota analysis, Giron *et al*[76] showed that a systemic inflammatory response was linked to elevated serum markers of tight junction permeability markers and microbial translocation. Regarding the lung microbiome, Rueca *et al*[18] found the absence of *Bifidobacterium* and *Clostridium* species in the nasal/oropharyngeal samples of COVID-19 patients. An outgrowth of *Proteobacteria* and *Firmicutes* has been reported during respiratory diseases[77]. Growing clinical evidence showed an altered profile of the gut microbiome in stool samples of COVID-19 patients. A recent research study reported that changes in bacterial microbiota in COVID-19 patients could be driven by an active replication of SARS-CoV-2 within the gut[78]. In a functional analysis study of gut microbiota, a study demonstrated an increase in bacterial proliferation of *Collinsella tanakaei*, *Collinsella aerofaciens*, *Morganella morganii* and *Streptococcus infantis*, with a high metabolic rate for de novo synthesis of amino acid and nucleotides[78].

The differences in gut microbial species between diseased and healthy control patients have been correlated with disease severity and complications[20,79]. We provided a synopsis of the most common gut bacterial species changes during COVID-19 in Table 2. More studies to analyze the metabolomic and microbiome profiling data on large cohorts of COVID-19 patients to further depict the role of gut-lung axis in COVID-19 pathogenesis are needed.

CLINICAL COURSE OF SARS-COV-2 INFECTION

Several comorbidities associated with a higher risk of infection, such as cardiovascular disease, hypertension, diabetes, chronic pulmonary disease, age and smoking, have been reported. These factors can modulate the expression of ACE2[5]. The association between ACE2 expression and advanced age and being male are controversial[90]. Smoking led to an upregulation of ACE2 with an increased risk of severe disease[91]. Another condition that predicts severe outcomes is obesity[92]; it increased intensive care unit (ICU) admission and the requirement of invasive mechanical ventilation[93]. Also, attention should be taken in psychiatric patients (with anxiety and depression) because some possible associations between these comorbidities and sleep problems were reported[94]. Regarding the complications developed in the context of the infection, one meta-analysis showed that acute respiratory distress syndrome, shock and acute kidney injury are conditions associated with a worse prognosis and with a higher rate of ICU admission[95]. There are other complications associated with high severity like disseminated intravascular coagulation, superinfections, arrhythmias and cardiac trauma.

Several feasible circulation biomarkers used to assess disease severity included lymphocyte count, thrombocytes, serum ferritin, lactate dehydrogenase, CRP and D-dimer levels[96]. It has been shown that lymphopenia is an important and useful predictor for the severity as it was reported in those with a bad prognosis[97]. In a study of 52 critically ill COVID-19 patients, 80% reported lymphopenia[98], whereas another study of 99 patients reported a rate of only 25% in those with mild COVID-19 infection [99]. These results suggest that lymphopenia can be used as an important marker in the diagnosis of the new coronavirus infection in the evaluation of disease severity. It shows that a high number of immune cells, especially T lymphocytes, are consumed, and the immune function is inhibited[100].

In the context of COVID-19 pneumonia, a cytokine storm can be released, and the cytokines (IL-6, tumor necrosis factor-α) stimulate hepatocytes to produce CRP. It has been demonstrated that CRP is correlated with COVID-19 progression and severity[101-103]. In addition, the chronic conditions associated with hyperinflammation such as metabolic syndrome, atherosclerosis and hypertension can predict worse outcomes[104]. A study by Alamdari *et al*[105] on 459 patients with high body mass index demonstrated that high levels of CRP, lymphopenia, hypomagnesemia and creatinine at admission were associated with a higher mortality.

High levels of serum ferritin: High levels of serum ferritin are observed in many inflammatory diseases and is considered a biomarker in different conditions such as rheumatologic disorders or different cancers[106]. In the context of SARS-CoV-2 infection, due to the inflammatory process, the cytokines, and in particular IL-6, stimulate hepcidin production, which is involved in the regulation of ferritin[107-109].

The studies showed that high levels of ferritin were observed in COVID-19 patients *vs* controls, and those with severe or critical disease had increased levels of ferritin than those with mild or moderate disease. Moreover, it was shown that non-survivors had increased levels of serum ferritin than survivors. One meta-analysis showed that the sensitivity of serum levels of ferritin in predicting the

Table 2 Changes in gut and airway microbiota bacterial species during coronavirus disease 2019

Changes	COVID-19 *vs* non-COVID-19 patients	Number of patients COVID-19 *vs* non-COVID-19	Ref.
Gut microbiota	Increase: *Ruminococcus gnavus*, *Ruminococcus torque*, *Bacteroides dorei*	100 *vs* 78	[20]
	Decrease: *Bifidobacterium adolescentis*, *Faecalibacterium prausnitzii*, *Eubacterium rectale*		
	Increase: *Streptococcus*, *Rothia*, *Veillonella*, *Actinomyces*	30 *vs* 30	[80]
	Increase: *Blautia*, *Coprococcus*, *Collinsella*	53 *vs* 76	[81]
	Decrease: *Streptococcus*, *Weissella*, *Enterococcus*, *Rothia*, *Lactobacillus*, *Actinomyces*		
	Increase: *Bifidobacterium*, *Bacteroides*, *Parabacteroides*, *Escherichia-Shigella*	22 *vs* 40	[82]
	Decrease: *Faecalibacterium*, *Dorea*, *Enterobacter*		
	Increase: *Corynebacterium*, *Campylobacter*, *Klebsiella*	50 *vs* 34	[83]
	Increase: *Streptococcus*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*	64 *vs* 40	[84]
	Decrease: *Bacteroidetes*, *Roseburia*, *Faecalibacterium*, *Coprococcus*, *Parabacteroides*		
Airway microbiota	Increase: *Veillonella*, *Staphylococcus*, *Corynebacterium*, *Neisseria*, *Actinobacillus*, *Selenomonas*	192 *vs* 95	[85]
	Decrease: *Haemophilus*, *Alloiococcus*		
	Increase: *Lactobacillus fermentum*, *Lactobacillus reuteri*, *Lactobacillus delbrueckii*, *Lactobacillus salivarius*	19 *vs* 23	[86]
	Increase: *Corynebacterium_1*, *Staphylococcus*, *Dolosigranulum*, *Peptoniphilus*, *Lawsonella*	38 *vs* 21	[87]
	Decrease: *Leptotrichia*, *Fusobacterium* (especially *Fusobacterium periodonticum*), *Haemophilus*	18 *vs* 12	[88]
	Increase: *Propionibacteriaceae*	31 *vs* 9	[89]
	Decrease: *Corynebacterium accolens*		

COVID-19: Coronavirus disease 2019.

severity of the disease is about 91% with a cutoff level of > 548.5 ng/mL[96].

D-dimers: D-dimers are one of the fragments produced when plasmin cleaves fibrin to break down clots. They are assessed as an algorithm in the thrombosis exclusion, but any pathologic or non-pathologic process that increases the production or disruption of fibrin can lead to high D-dimer levels [110]. Infections, venous thromboembolism and deep vein thrombosis are the most common causes of increased D-dimer levels[111].

A study by Yao *et al*[112] on 248 patients revealed that increased D-dimers at hospital admission for SARS-CoV-2 infection, after excluding pulmonary embolism and deep vein thrombosis, were associated with increased severity and with in-hospital mortality. Also, they showed a significant correlation between D-dimer levels and COVID-19 severity classified by lung involvement on computed tomography (CT) scan, oxygenation index and clinical staging according to the Novel Coronavirus Pneumonia Diagnosis and Treatment Guideline (6th edition) by the National Health Commission of China. It was highlighted that D-dimers are a useful marker to assess the severity even before the thoracic CT scan.

The hepatic injury with increased liver enzymes was reported. There are some potential mechanisms through which the liver is affected: Direct liver injury; associated inflammatory responses; congestive hepatopathy; hepatic ischemia; drug-induced liver injury; and muscle breakdown. The levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and total bilirubin were elevated and increased in a disease progression manner. The AST level was correlated with disease severity. According to Moon *et al*[113], AST levels had the highest correlation with mortality rate compared with other circulating markers, reflecting the involvement of liver injury in disease progression. An overview of circulating biomarkers that were correlated with COVID-19 infection is provided in Table 3.

Table 3 Biomarkers associated with coronavirus disease 2019

Decreased levels	Increased levels
Lymphocytes	White blood cells
Platelets	D-dimers
Eosinophils	Fibrinogen
	C-reactive protein
	Procalcitonin
	Lactate dehydrogenase
	Ferritin
	IL-6
	ALT, AST
	Alkaline phosphatase
	Total bilirubin

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IL-6: Interleukin 6.

DIAGNOSTIC TOOLS FOR THE ASSESSMENT OF SARS-COV-2 INFECTION AND DISEASE SEVERITY

Chest imaging in the diagnosis and monitoring of COVID-19 pneumonia plays a significant role, and all available methods should be used. Chest radiography may show no abnormalities at symptom onset, with considerable findings visible only 10-12 d later[114]. Similarly, thoracic CT scans performed in the first 5 d after symptom onset may reveal isolated ground-glass opacities or consolidations in limited distribution. The full extent of the acute pulmonary manifestations increases over the 1st wk, with a peak on day 10[115]. The available imaging tools are chest radiography, chest CT and lung ultrasound (LUS).

Chest radiography

Chest X-ray (CXR) is commonly used as the first imaging examination when pneumonia is suspected [116]. Despite not being a substitute for real-time polymerase chain reaction test or chest CT, CXR can provide a prompt and cost-effective diagnosis in a small percentage of patients (approximately 15%) [117]. Chest radiography shows low sensitivity, as low as 25%, and high specificity, estimated at 90%, in detecting COVID-19 abnormalities, thus it should not be used as a screening method[118]. Frequent chest radiographic findings are airspace opacification, pulmonary consolidation and ground-glass opacification. Pneumonia in COVID-19 tends to be bilateral, with a pattern involving predominantly peripheral lung regions and lower lobes[1]. A proposed radiological scoring of pneumonia severity describes four disease degrees based on the percentage of lung involvement as it follows: Mild if < 25%; moderate if 25%-50%; severe 50%-75%; and critical if > 75%[119].

Chest CT

CT is the most sensible imaging examination and is best correlated with the severity of the disease. To reduce the patient's exposure time to radiation, imaging tools should be performed in patients with moderate to severe symptomatology and in those with progressive alteration of respiratory parameters [120]. Frequent findings in COVID-19-positive patients are ground-glass opacities, consolidations, interlobular septal thickening and crazy paving. Additional findings consist of the reverse halo sign, air bronchogram sign, tree in bud, pleural or pericardial effusions and mediastinal lymphadenopathies. The ground-glass opacities reflect the parenchymal involvement, and they represent the most consistent feature, being found in almost all affected patients, symptomatic or asymptomatic[6]. Compared to other types of pneumonia, COVID-19 pneumonia presents with multifocal and multilobar involvement of both lungs, with a subpleural and basal distribution[121].

Disease severity can be appreciated by different scoring systems. The percentage of the overall parenchymal involvement may predict mild (< 25% lung involvement), moderate (25%-50%), severe (50%-75%) or critical (> 75%) forms of disease. Another score uses the visual estimation of the surface affected of each of the five lung lobes, with each lobe being given a score from 0 to 5, 0 meaning no involvement, 1 involving < 5%, 2 involving < 25%, 3 involving < 50%, 4 involving < 75% and 5 involving > 75% of the lobar surface. The total score obtained is the sum of the scores attributed to each lobe, and it varies from 0 to 25[122]. In a retrospective study, Bernheim *et al*[123] proposed a similar score by assessing the degree of involvement of each of the five lobes. The only difference from the previous

score is that involvement of 1%-25% was attributed 1 point resulting in a score from 0 to 4 for each lobe. The total CT severity score ranged from 0 to 20. In a study of 739 patients, the authors proposed a semi-quantitative scoring system in order to predict the outcome of infected patients. They visually appreciated the pulmonary involvement by assessing each of the five lobes separately for ground-glass opacities and consolidations. Each lobe had a score varying from 0 to 7, and the maximum total score was 35[124].

Despite its high accuracy, CT may be unsuitable for critical ICU patients who may not be able to undergo transfer to the radiology department in order to perform a CT scan[120,125,126]. Moreover, there is a potentially increased risk of disease transmission to CT technicians and other patients who require imaging investigations in the same department[127]. For monitoring ICU patients, portable CXR or LUS are preferable.

LUS

LUS is a widely accessible, non-invasive, non-irradiating and cost-effective tool that can be used in the initial assessment and monitoring of symptomatic patients[128,129]. The main advantages of this examination reside in the possibility of being performed in children and pregnant women and at the patient's bedside. It is portable and offers replicable examination for follow-up[130,131]. Portable ultrasound devices can be used in ICU departments, both by radiologists and clinicians, offering real-time information about a patient's evolution.

Numerous authors stated that LUS can offer similar diagnostic information to chest CT in the evaluation of COVID-19 pneumonia[126,132,133]. Gibbons *et al*[132] concluded that LUS compared to portable CXR had a higher sensitivity for detection of viral pneumonia. LUS findings in COVID-19 patients include multiple B lines below the pleural surface, subpleural consolidations, pleural thickening and irregularity[134]. The B lines depict the interstitial involvement and represent the most common ultrasonographic pattern found in patients with COVID-19[129,132,135]. Despite having a high sensitivity in detecting COVID-19 pneumonia in subpleural lung regions, the deep pulmonary parenchyma remains inaccessible to LUS due to air interposition leading to an underestimation of the disease extent[136]. LUS findings are not distinctive for viral cases of pneumonia, but just as it was previously discussed in the case of chest radiography and chest CT scans, the bilateral and predominantly basal distribution is a strong indicator for COVID-19 pneumonia rather than influenza or bacterial pneumonia[137]. Nonetheless, LUS has a low specificity, since it cannot distinguish from other pulmonary and cardiac conditions such as acute respiratory distress syndrome, heart failure and subpleural lung masses[125,136].

TRANSLATIONAL RELEVANCE OF GUT MICROBIOTA IN MODIFYING DISEASE SEVERITY AND OUTCOMES OF COVID-19 PATIENTS

The involvement of gut microbiota in modifying disease outcomes and therapeutic responses of COVID-19 patients might represent a promising therapeutic strategy. Emerging clinical studies suggested that dysfunctional immune response triggered by gut microbiota dysregulation upon SARS-CoV-2 infection might influence the severity and the course of COVID-19[20,138].

The proinflammatory state triggered by the host immune response against SARS-CoV-2 infection promotes changes in gut commensal microflora, leading to dysbiosis, which will further result in alteration of the intestinal epithelial barrier[51,138,139]. Once the integrity of the intestinal barrier is disrupted, the high permeable state of the intestine creates the most favorable conditions for entering into the circulation of bacterial products and toxins, activating a systemic inflammatory response[140].

Evolving data examined predictive biomarkers of disease severity from serum samples of COVID-19 patients, which were correlated with inflammatory response and disease severity[20,138]. Compared to controls, the serum samples of COVID-19 patients exhibited high levels of fatty acid-binding protein 2, peptidoglycan and lipopolysaccharide, markers of gut permeability, suggesting the unstable state of the intestinal barrier within these patients[138].

Significant dysbiosis in 146 COVID-19 patients has been reported by Prasad *et al*[138]. The phylogenetic changes in the serum microbiome of COVID-19 patients consisted of enrichment of *Actinobacteria spp.* and underrepresentation of *Bacteroides spp.*, with an increased ratio of Firmicutes to Bacteroidetes [138]. Therefore, the unstable state of gut microbiota in COVID-19 patients is reflected by a decrease in beneficial bacteria, *i.e.*, *Bifidobacterium*, and an increase in deleterious bacteria related to bacteremia or sepsis, *i.e.*, *Brevibacterium* and *Pantoea*[138].

By RNA and DNA sequencing of blood and stool samples of COVID-19 patients, Yeoh *et al*[20] depicted a distinct signature of gut microbiota composition in 100 positive subjects. A decreased abundance of gut commensals such as *Faecalibacterium prausnitzii*, *Eubacterium rectale* and *Bifidobacterial* have been reported up to 30 d after disease course in 87 hospitalized COVID-19 patients, suggesting the long-term dysregulation of gut microbiota[20].

At the species level, the authors identified a significant association between the compositional abundance of gut microbiota and disease severity. The microbial species, *Faecalibacterium prausnitzii*, *Bifidobacterium bifidum*, *Bifidobacterial adolescentis* and *Eubacterium rectale* negatively correlated with disease severity, the mean value of the gut microbial composition decreased compared to non-COVID-19, and mild COVID-19 samples correlated to the lowest value compared to the severe and critical patients[20].

Analysis of gut microbial samples might provide valuable prognostic serum markers, which could predict disease severity and outcomes[20]. Correlational analysis between different microbial taxa and cytokine and chemokine levels suggested the role of gut microbiota in regulating the magnitude of immune response and modifying disease severity of COVID-19 disease[20].

Thus, the decrease in the abundance of *Bifidobacterium adolescentis*, *Faecalibacterium prausnitzii* and *Eubacterium rectale* in COVID-19 patients was associated with elevated cytokine levels of tumor necrosis factor-α, IL-10, C-C motif chemokine ligand 2 and CXCL10[20]. In identifying the microbial species associated with disease severity, Schult *et al*[79] analyzed gut microbial profiles and observed a different bacterial composition in COVID-19 patients with a low risk of complications, with a predominance of *Faecalibacterium prausznitzii*, and high risk complications, in which *Parabacteroides spp.* Dominates. The changes in the abundance of microbial species were more pronounced in patients with severe associated conditions, such as acute kidney injury and acute respiratory distress syndrome, followed by a lesser microbiota change in acute cardiac events and venous thromboembolism. Moreover, the authors proposed 12 gut microbial species as cocktail biomarkers with an accuracy of 0.94 for predicting the progression of disease and the severity of COVID-19 patients[79]. Thus, the abundance of *Ruthenibacterium lactatiformans*, *Clostridium innocuum* and *Alistipes finegoldii* was correlated with inflammatory blood markers, such as white blood cells, CRP and procalcitonin, and disease progression. In severe and fatal cases, the microbial profile of the gut exhibited depleted levels of *Blautia luti*, *Faecalibacterium prausnitzii*, *Alistipes putredinis*, *Dorea longicatena* and *Gemmiger formicilis*[79].

Aging, diet and comorbidities, such as obesity, diabetes and cardiovascular diseases, have a significant impact on the microbial profile of the gut, leading to dysbiosis[141-144]. Age and comorbidity-related changes in the gut microbial profile of COVID-19 patients influence immune regulatory mechanisms, which might explain the severe forms of disease and the associated complications in older and comorbid patients[145].

In relation to the mechanistic data mentioned, patients with severe forms of COVID-19 faced more pronounced gastrointestinal symptoms, suggesting the association between clinical symptoms and disrupted gut microbiota during COVID-19 disease[146,147]. Another study revealed a depressed state of bacterial composition species, consisting of lower levels of beneficial symbionts and higher levels of opportunistic pathogens, such as *Streptococcus*, *Rothia*, *Actinomyces* and *Veillonella*[80]. The authors proposed five gut microflora biomarkers, including *Intestinibacter*, *Erysipelatoclostridium*, *Actinomyces*, *Fusicatenibacter* and *Romboutsia* with diagnostic value to distinguish between COVID-19 patients and healthy controls[80].

The long-term dysregulated effects of SARS-CoV-2 have been revealed in fecal samples of COVID-19 patients, with persisted gut dysbiosis after clearance of the virus[148]. Prognostic valuable bacterial-based markers include *Coprobacillus*, *Clostridium ramosum* and *Clostridium hathewayi*, which were associated with COVID-19 severity. In a murine gut model, some beneficial bacterial species, including *Bacteroides dorei*, *Bacteroides thetaiotaomicron*, *Bacteroides massiliensis* and *Bacteroides ovatus*, downregulate ACE2 expression level, negatively correlating with the viral load of SARS-CoV-2[148].

At the basis of immune interactions between lung and gut microbiota are microbiota-derived metabolites that modulate host immune cells in a direct or indirect manner[66,149]. Some bacteria species, such as *Anaerostipes butyraticus*, *Faecalibacterium prausnitzii* and *Roseburia intestinalis*, display enzymatic systems to digest the complex carbohydrates, which resulted in SCFA products[70,150]. By analyzing oral microbiota, Firmicutes, Actinobacteria and Bacteroidetes were enriched in the COVID-19 group compared with healthy controls. Moreover, the oral microbiota exhibited fewer levels of butyric acid-producing bacteria and more lipopolysaccharide-producing bacteria in the positive patients[77].

Changes in the metabolomic profile of fecal samples of COVID-19 patients have been correlated with different microbial composition profiles[19]. A better understanding of metabolic changes in serum or fecal COVID-19 samples will further provide new insight into the gut-lung axis and propose putative prognostic markers in COVID-19. Up to 20 metabolites were changed in the fecal sample of COVID-19 patients, including monosaccharides, *i.e.*, D-allose, D-glucose and D-arabinose, nucleotides, *i.e.*, hypoxanthine, pseudouridine and inosine, and amino acids, *i.e.*, l-tyrosine and l-tryptophan, and were associated with bacterial species modifications[19].

Targeting severe immune responses in COVID-19 represents the main therapeutic approach[151]. Recent studies pointed out the role of a high-fiber diet and probiotics as disease-modifying therapy in COVID-19[151,152]. The role of nutraceutical compounds, consisting of vitamins, dietary supplements and pro/prebiotics in COVID-19, have been reported to improve the clinical course and severity of COVID-19 disease (Table 4). Several clinical trials investigating the role of probiotics enriched with different types of beneficial species are in progress (NCT04854941, NCT05080244, NCT04390477).

Table 4 Nutraceuticals used to improve disease severity and outcomes of coronavirus disease 2019 patients

Nutraceuticals	Number of patients with *vs* without nutraceutical agent	Results	Ref.
Probiotic combined *Bifidobacterium*, *Lactobacillus* and *Enterococcus*	179 *vs* 196	Shorter time to clinical improvement (fever, hospital stay, viral shedding) in hospitalized COVID-19 subjects	[153]
Probiotic *Lactobacillus hamnosus* GG	566 *vs* 566	Extended time until the development of infection with COVID-19, reduced the severity of the disease, changed the composition of the intestinal microbiota in the household contact infected with COVID-19 (after 28 d)	[154]
Probiotic *Lacticaseibacillus rhamnosus*, *Bifidobacterium bifidum*, *Bifidobacterium longum subsp. infantis*, *Bifidobacterium longum subsp. longum*	99 *vs* 101	The duration of diarrhea was shorter in patients who received the probiotic than in those who did not. No significant effect on mortality, no change in most biomarkers in patients with COVID-19 in hospitalized patients (at 14 d)	[155]
Vitamin D3 (single oral bolus of 80000 IU)	57 *vs* 9	The severity of COVID-19 decreased. Improved survival rate	[156]
25-hydroxyvitamin D3	50 *vs* 26	Reduced the need for treatment in the ICU in patients hospitalized due to proven COVID-19	[157]
Quercetin	21 *vs* 21	Decreased virus clearance, frequency of symptoms and level of LDH and ferritin parameters	[158]

ICU: Intensive care unit; LDH: Lactate dehydrogenase; COVID-19: Coronavirus disease 2019.

CONCLUSION

There is evidence that changes in gut microbiota are an important factor in the pathogenesis of COVID-19. An important role in this disease is also played by the relationship between the gut and the lungs, known as the "gut-lung axis". Modulating gut microbiota to increase diversity and abundance can positively influence the severity of COVID-19. Further studies are needed to explore the microbiota in COVID-19 patients with varying degrees of severity, in post-COVID-19 patients and their medical history with nutraceutical agents.

FOOTNOTES

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MINIREVIEWS

The mononuclear phagocyte system in hepatocellular carcinoma

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Abstract

The mononuclear phagocyte system (MPS) consists of monocytes, dendritic cells and macrophages, which play vital roles in innate immune defense against cancer. Hepatocellular carcinoma (HCC) is a complex disease that is affected or initiated by many factors, including chronic hepatitis B virus infection, hepatitis C virus infection, metabolic disorders or alcohol consumption. Liver function, tumor stage and the performance status of patients affect HCC clinical outcomes. Studies have shown that targeted treatment of tumor microenvironment disorders may improve the efficacy of HCC treatments. Cytokines derived from the innate immune response can regulate T-cell differentiation, thereby shaping adaptive immunity, which is associated with the prognosis of HCC. Therefore, it is important to elucidate the function of the MPS in the progression of HCC. In this review, we outline the impact of HCC on the MPS. We illustrate how HCC reshapes MPS cell phenotype remodeling and the production of associated cytokines and characterize the function and impairment of the MPS in HCC.

Key Words: Mononuclear phagocyte system; Hepatocellular carcinoma; Monocyte; Dendritic cell; Macrophage

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Core Tip: The hepatocellular carcinoma (HCC) is a complex disease affected or initiated by many factors, including chronic hepatitis B virus infection, hepatitis C virus infection, metabolic disorders or alcohol consumption. Innate immune system can shape the acquired immune response, which can surveillance HCC directly. As the main component of innate immunity, the mononuclear phagocyte system (MPS) plays a vital role in HCC. In this review, we outline the impact of HCC on MPS. We illustrate how HCC reshapes MPS cell phenotype remodeling and producing the associated cytokines, and characterize the function and impairment of MPS in HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and is predicted to be the sixth most-diagnosed cancer and the fourth-leading cause of death among all types of cancers[1-3]. HCC is influenced or initiated by many factors, including chronic hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, metabolic disorder or chronic alcohol consumption[4-7]. More than 85% of HCC cases are accompanied by HBV infection in China[8-9]. Evidence has shown that HCC is a type of tumor with low or moderate immunogenicity[10]. Chronic inflammation creates an immunosuppressive microenvironment in the liver, facilitating HCC tumorigenesis and progression[11]. Although great progress has been made in the treatment of HCC in recent decades, the long-term survival rate of HCC is still poor[12]. Many factors, such as liver function, tumor stage and the performance status of patients, affect HCC clinical outcomes. According to previous studies, targeted treatment of dysregulated tumor microenvironments may improve the efficacy of HCC treatments[11,12].

The mononuclear phagocyte system (MPS) includes monocytes, dendritic cells (DCs) and macrophages and was proposed by Van Furth in the late 1960s and early 1970s[13,14]. Monocytes, DCs and macrophages are important members of the innate immune system and are capable of modulating adaptive immune responses. The immune system is precisely balanced between immune activation and tolerance. Within this system, antigen presenting cells (APCs) play a critical role in orchestrating the immune response[15]. Although stellate cells, endothelial cells and hepatocytes in the liver have the potential to present antigens to T cells, the MPS plays a major role in determining the nature of the immune response and keeps the liver as an immune tolerance organ. The MPS appears to be superior at sampling the environment through phagocytosis and presenting antigens to T cells *via* MHC class II molecules[16]. APCs of the MPS appear to have a commensurately increased expression of antigen presentation and costimulatory molecules and are potent secretors of modulatory cytokines[17].

In addition to its role in tissue development, homeostasis, inflammation and innate immune defense against pathogens, the MPS also plays a vital role in cancer[17,18]. Innate immune cell-derived cytokines can regulate T-cell differentiation, thereby shaping adaptive immunity, which is associated with the prognosis of HCC. It is important to illustrate the critical role of the MPS in the progression of HCC. In this review, we discuss how the HCC microenvironment remodels MPS cell phenotypes and cytokine production and describe the function and impairment of MPS components in HCC.

IMPACT OF THE MPS ON HCC

Monocytes and HCC

Monocytes originate in the bone marrow and spleen and account for approximately 5%-10% of human peripheral blood mononuclear cells[13]. Monocytes transform into macrophages and DCs during inflammation[19-23]. It has been reported that the increase in activated monocytes [human leukocyte antigen (HLA)-DR^{high}CD68^{+} cells] in the liver is related to disease progression[24]. Chemokine (C-C motif) ligand 15 (CCL15) recruits CCR1^{+}CD14^{+} monocytes to the edge of HCC tissue. High expression of CCL15 is associated with poor clinical prognosis. CCR1^{+}CD14^{+} monocytes suppress antitumor immunity, facilitate tumor metastasis and promote tumor cell proliferation and invasion[25]. Blocking CCL2/CCR2-mediated macrophage accumulation has been proposed as a treatment strategy for HCC [26-29].

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid progenitors and immature myeloid cells[30]. MDSCs in the tumor microenvironment, which are essential for tumor progression and are effective inhibitors of natural killer (NK) cells in HCC patients, play an important

role in immunosuppression[31-33]. In humans, MDSCs usually express CD11b and CD33 and have low or no expression of HLA-DR. Monocyte-derived MDSCs (mMDSCs) express CD14, while granulocyte-derived MDSCs named gMDSCs express CD15 and CD66b[34]. MDSCs inhibit T and NK-cell proliferation and have an inhibitory effect on conventional NK cells in a TIGIT-dependent manner. Compared with conventional NK cells, adaptive NK cells express lower levels of TIGIT and resist the inhibitory effect of MDSCs[35]. MDSCs express CD155, which is induced by reactive oxygen species, and inhibit NK-cell function *via* the CD155-TIGIT interaction[35]. Compared with normal liver tissues, hepatoma tissues are rich in granulocytes and mononuclear cells. Mononuclear cells and MDSCs suppress the $CD8^+$ T-cell response[36].

S100A9 is a marker that can be used to distinguish MDSCs from monocytes[37]. Studies have identified $CD14^+HLA\text{-}DR^{low/neg}$ monocytes as MDSCs in HCC patients. The frequency of $CD14^+HLA\text{-}DR^{low/neg}$ MDSCs in the peripheral blood and ascites fluid of these patients is increased. $CD14^+HLA\text{-}DR^{low/neg}$ MDSCs cannot induce T-cell proliferation. MDSCs induce $FoxP3^+$ regulatory T (Treg) cell proliferation, while $CD14^+HLA\text{-}DR^+$ monocytes induce Th17-cell proliferation and IL-17 secretion[33]. MDSCs induce Treg cell generation *via* all-trans retinoic acid[38]. In human HCC, the fibrotic tissue around the tumor is rich in mMDSCs, which are significantly associated with a low survival rate[26].

DCs and HCC

DCs are a class of bone marrow-derived cells in the blood, epithelia and lymphoid tissues and are the most powerful professional APCs[39]. DCs participate in the regulation of innate and adaptive immunity[40]. DCs have antigen delivery capabilities, which make them attractive carriers for therapeutic tumor vaccines and platforms for vaccine development[41]. Human DCs are usually induced from monocytes isolated from peripheral blood mononuclear cells by M-CSF and IL-4 stimulation and develop into mature DCs (mDCs) after being loaded with antigens[42].

Human DCs express CD45 and HLA-DR and are divided into different populations according to CD11c, CD123 and IL-3Rα expression. Myeloid DCs (mDCs) are important antigen-presenting cells and express high levels of CD11c but low levels of CD123. Plasmacytoid DCs (pDCs) express high levels of CD11c, CD123, Toll-like receptor (TLR)-7 and TLR-9[43,44]. In normal liver tissues, $CD141^+$ hepatic DCs express CLEC9A, ILT3 and ILT4, but cell percentages and functions are damaged in hepatocellular carcinoma[45-47]. Evidence indicates that DCs have high antitumor and cytotoxic activity against HCC cells *in vitro* and *in vivo*[48]. DCs inhibit the growth of HCC cells by loading LCSC antigens[49].

Peptide-pulsed DCs present antigens to naive T cells, thereby activating and inducing naive T cells to become antigen-specific cytotoxic T lymphocytes (CTLs) to kill tumor cells[50]. DCs incubated with epithelial adhesion molecule peptides have a significant inhibitory effect on tumor growth[51]. DC precursors that are sensitized by dexamethasone can effectively trigger the major histocompatibility complex class I (MHC I)-restricted CTL response, allowing DCs to make full use of secondary antigen peptides, thereby maximizing the specificity of the HCC immune response[52].

Macrophages and HCC

Macrophages originating in the liver are generally described as Kupffer cells (KCs)[53]. KCs express MHC class II and have varying levels of costimulatory markers, such as CD40, CD80, and CD86, and express the inhibitory marker Z39Ig[20,54]. The activation of STAT3 is involved in the differentiation of monocytes into macrophages[55]. Although tumor-infiltrating macrophages are generally believed to be derived from circulating monocytes, emerging evidence suggests that tissue macrophages can be maintained through self-renewal[56]. Liver resident macrophages are established by progenitor cells derived from the fetal liver and are maintained by self-proliferation and monocyte migration[57,58]. KCs in the liver are negatively correlated with patient prognosis by inhibiting T-cell antitumor functions [59].

Tumor-associated macrophages (TAMs) are the most abundant immune cells in the tumor microenvironment and play a key role in immunosuppression[60]. TAMs are important in tumor-related inflammation and can be polarized into M1 or M2 phenotypes[61]. M1 macrophages are induced by LPS and IFN-γ; M2 macrophages are induced by IL-4 and IL-13[62]. It is generally believed that M1 TAMs have antitumor functions, while M2 TAMs promote tumor proliferation[63]. Studies have shown that IL-10 is involved in the polarization of TAMs to the M2 phenotype[64]. CD68 and CD163 are two popular TAM cell surface markers[65,66]. It has been reported that $CD68^+HLA\text{-}DR^+$ TAMs enhance HCC proliferation through the NFκB/FAK pathway[67].

TAMs induce $CD8^+$ T-cell exhaustion *via* the PD-1/PD-L1 and Tim^3 signaling pathways in the tumor microenvironment[68,69]. $CCR2^+$ TAMs accumulate and express the inflammatory marker S100A9 at the border of highly vascularized HCC, while $CD163^+$ TAMs accumulate in the center area of HCC tissue [70]. Silencing SIRT4 in TAMs regulates macrophage activation and significantly promotes HCC cell growth[71]. Downregulation of SIRT4 expression is related to an increase in macrophage infiltration in HCC tissues and a high ratio of M2/M1 macrophages[72]. The miR-148B/CSF1 pathway regulates the entry of TAMs into HCC tissue. A lack of miR-148B induces CSF1 expression and macrophage infiltration, thereby promoting liver cancer metastasis and poor prognosis[73].

THE REGULATORY FUNCTION OF HCC ON THE MPS

Studies have shown that the tumor environment can trigger early activation of monocytes in the surrounding tumor tissues[74,75]. Tumor environmental factors induce monocytes to express PD-L1 transiently in the early stage, activate monocytes, and induce PD-L1 expression in an autocrine cytokine-dependent manner[75]. HCC cells promote monocyte-to-macrophage differentiation *via* the PKM2 pathway[76]. In addition, monocytes in human peripheral blood differentiate into immature dendritic cells[77]. Monocyte-derived TNF-α works synergistically with tumor-derived soluble factors to induce neutrophils to produce the metastasis-promoting factor OSM. Tumor-infiltrating monocytes mediate the production of CXCL2 and CXCL8 through the PFKFB3/NFκB signaling pathway. The level of PFKFB3 and the production of CXCL2/CXCL8 in monocytes are positively correlated with the infiltration of OSM-producing neutrophils in human HCC tissues[78].

The tumor microenvironment inhibits DC maturation and activation, leading to an immunosuppressive phenotype and function[79]. In addition, hepatoma cells recruit immunosuppressive DCs and MDSCs to suppress $CD8^+$ T cells, leading to tumor escape from immune surveillance[80]. Tumor-derived exosomes impair the differentiation and maturation of DCs through the IL-6/STAT3 signaling pathway, which prevents myeloid precursor cells from differentiating into $CD11c^+$ DCs and induces apoptosis, thereby reducing T-cell activation and mediating immunosuppression[81,82].

The proliferation of tumor-infiltrated macrophages is more powerful than that of macrophages in nontumor tissues. Increased levels of macrophage proliferation are positively correlated with the density of macrophages in the tumor and the poor prognosis of HCC patients. Proliferating macrophages can be induced by small soluble adenosine, which is derived from tumor cells. GM-CSF released by tumors stimulates macrophages to express the A2A receptor, which then coordinates with adenosine to cause HCC-infiltrating macrophage proliferation[83]. Mitochondrial nucleoid structural changes lead to the release of mitochondrial DNA (mtDNA) into the cytoplasm, which is considered to be mtDNA stress, thereby regulating innate immunity[84].

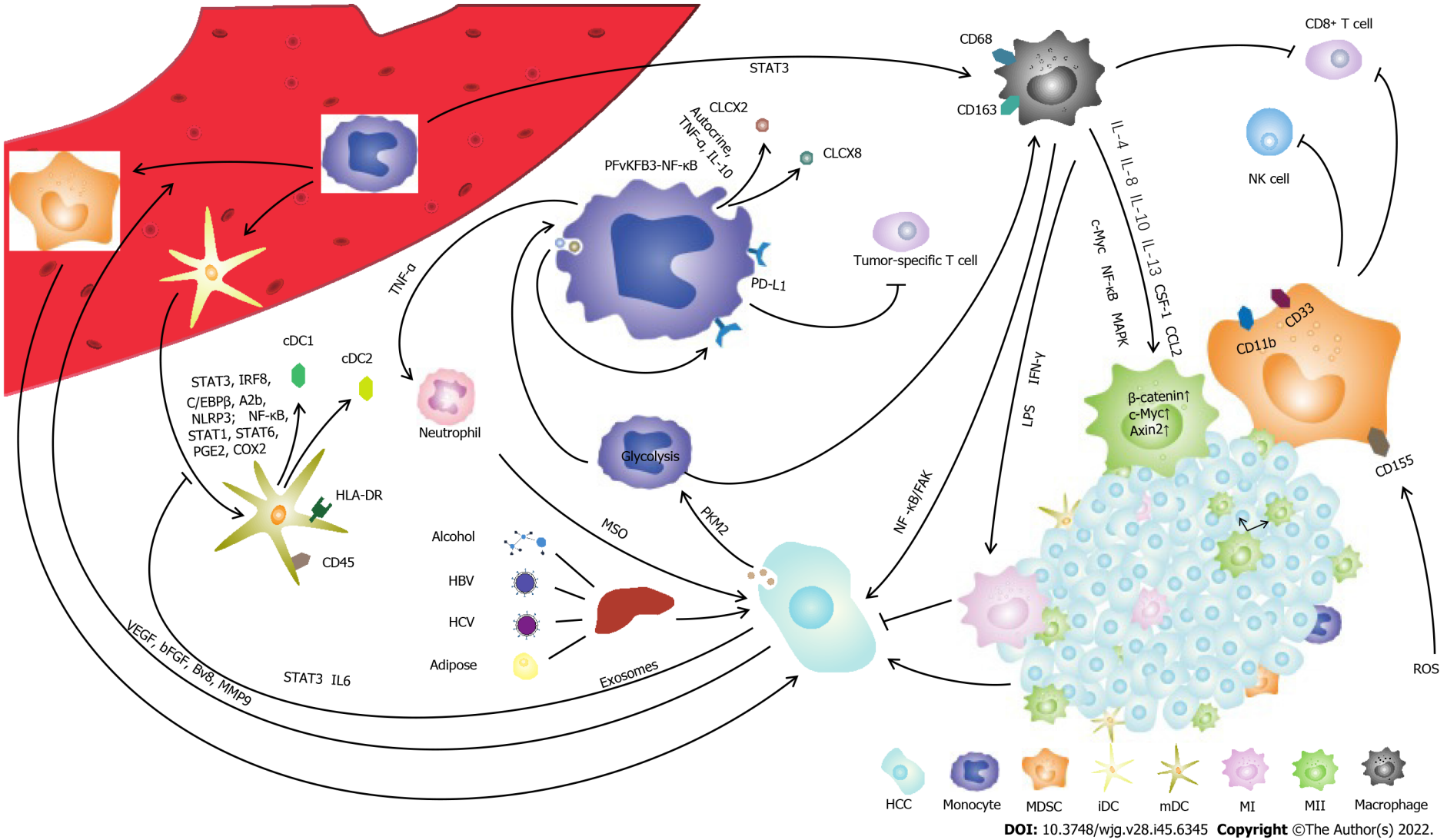
CSF-1 and CCL2 in the tumor microenvironment polarize macrophages to the M2 phenotype[85,86]. Crosstalk between macrophages and liver tumor cells can also affect the polarization of TAMs through the Wnt/β-catenin signaling pathway[87]. Compared to those in M1 cells, β-catenin, c-Myc and Axin2 are more enriched in M2 TAMs. It is believed that the c-Myc signaling pathway promotes the polarization of TAMs toward the M2 phenotype[88]. Neurotensin-induced IL-8 polarizes TAMs to the M2 phenotype and promotes epithelial-mesenchymal (EMT) in HCC through the MAPK and NFκB pathways[89]. In addition, hypoxia-inducible factors 1α (HIF-1α) and HIF-2α cause TAM polarization through hypoxia in the tumor microenvironment, leading to HCC malignancy[90,91]. Hypoxia-induced high mobility group protein box 1 may affect tumor progression by regulating TAMs, leading to poor clinical prognosis[92].

CONCLUSION

Components of the MPS have important but distinct roles in HCC. The MPS is associated with specific cell phenotypes and functions in the HCC microenvironment. Monocytes are progenitors of DCs and macrophages, which play an important role in inhibiting the immune response. In addition, MDSCs are a heterogeneous population composed of immature myeloid cells and myeloid progenitor cells. Increasing evidence suggests that tumor progression is associated with the accumulation of MDSCs, which cause local and systemic immunosuppression[93]. Furthermore, increased accumulation of MDSCs is associated with early tumor recurrence and is considered to be one of the predictors of poor prognosis in HCC patients who undergo clinical treatment[94]. Cytokine-induced killer (CIK) cells include a wide variety of T-cell receptor-specific effector cells, which are a mixed cell population. CIK cells have cytotoxic activity against tumor cells, which is not MHC-restricted. Studies have shown that adjuvant immunotherapy for HCC using CIK cells can reduce tumor recurrence and improve overall survival[95]. MDSCs inhibit CIK cell lysis *via* ARG1 and iNOS.

Human DCs are usually induced from monocytes isolated from peripheral blood. DCs have antigen delivery abilities, which make them attractive carriers for therapeutic tumor vaccines and platforms for vaccine development. In normal liver tissues, hepatic DCs have powerful antigen presentation functions and immunomodulatory abilities, but their percentages and functions are damaged in hepatocellular carcinoma[45]. Monocytes act as precursor cells that differentiate into DCs and macrophages under normal physiological conditions. The following are questions that remain to be investigated. Will monocyte impairments be inherited by DCs and macrophages? Will DCs and macrophages acquire immunosuppressive phenotypes and functions in HCC after differentiation?

Although the majority of previous studies have focused on individual MPS components, interactions and cooperation among various MPS components are common[96-98]. For instance, KCs are the main immune cells in liver tissues and play a key role in DC recruitment to the liver[97]. pDC survival and IFN-α production are affected by IL-10 and TNF-α secretion by monocytes. MDSCs produce increased levels of IL-10, which leads to pDC apoptosis. How do MDSCs interact with pDCs in the HCC microen-



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Figure 1 The interaction of mononuclear phagocyte system and hepatocellular carcinoma in the tumor microenvironment. (1) Activated monocytes secrete TNF-α and IL-10 to increases the expression of PD-L1 on the surface of monocytes, inhibiting the function of Tumor-specific T cell. TNF-α, CXCL2 and CXCL8 produced by monocytes stimulate neutrophils to produce MSO which promote tumor development; (2) Tumor-derived growth factors stimulate myeloid-derived suppressor cell differentiation through STAT3, IRF8, A2B, NLRP3 pathways and PEG2, COX2 produced by tumor stroma stimulate myeloid-derived suppressor cell differentiation through NK-κB, STAT1, STAT6 pathways. Myeloid-derived suppressor cell promotes tumor progression by producing VEGF, BV8 and MMP9. Myeloid-derived suppressor cells inhibits the function of NK cells and CD8+ T cells. Reactive oxygen species induce the expression of CD155 on myeloid-derived suppressor cells

inhibiting natural Killer cell function; (3) The exosomes PKM2 produced by hepatocellular carcinoma in tumor microenvironment mediate the production of CXCL2 and CXCL8 through the PFKFB3-NF-κB signaling pathway; (4) Monocytes in peripheral blood mononuclear cells differentiate into macrophages through STAT3 pathway. LPS and IFN-γ stimulated tumor-associated macrophages polarizing into M1 phenotype, but IL-4, IL-8, IL-10, IL-13, CSF-1 and CCL2 stimulated tumor-associated macrophages polarizing into M2 phenotype through c-Mcy, NFκB and MAPK signaling pathway; and (5) Monocytes in peripheral blood mononuclear cells can be differentiated into immature dendritic cell, loaded with antigen to produce mature dendritic cell. Tumor-derived exosomes impair the activation and maturation of dendritic cells through IL-6 STAT3 signaling pathway. HCC: Hepatocellular carcinoma; MDSC: Myeloid-derived suppressor cells; iDC: Immature dendritic cell; mDC: Mature dendritic cell; M1: M1-type macrophages; M2: M2-type macrophages; ROS: Reactive oxygen species.

vironment? Additionally, monocyte- or macrophage-derived IL-15 and pDC-derived IFN-α synergistically stimulate IFN-γ production in NK cells during HCV infection[96]. Does this interaction occur in HCC? Other forms of cooperation between different MPS components remain to be analyzed.

In this review, we described the impact of HCC on each component of the MPS and the immune function of the MPS in HCC development (Figure 1). Monocytes are precursors of DCs and macrophages, which play an important role in the regulation of immune function in HCC. Through crosstalk with hepatoma cells or regulation of the tumor microenvironment, monocytes are capable of producing high levels of IL-10, resulting in an inhibitory microenvironment. DCs can suppress the stimulation of T cells and inhibit efficient antitumor T-cell functions. TAMs are the most abundant immune cells in the tumor microenvironment and play a key role in immunosuppression. The tumor microenvironment inhibits DC maturation and activation, leading to an immunosuppressive phenotype and function. The HCC microenvironment remodels MPS cell phenotypes and cytokine production and impairs MPS components in HCC.

FOOTNOTES

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MINIREVIEWS

Rifabutin as salvage therapy for *Helicobacter pylori* eradication: Cornerstones and novelties

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Abstract

When several *Helicobacter pylori* eradication treatments fail, guidelines recommend a cultured guided approach; however, culture is not widely available. Therefore, a rifabutin based regimen could be the best solution. Rifabutin indeed shows a low rate of antibiotic resistance. Rifabutin is generally used in combination with amoxicillin in a triple therapy, with eradication rates about 80% in third-line regimens. The ideal duration of this therapy should range between 10 and 12 d. Combinations with antibiotics other than amoxicillin have demonstrated even better results, such as vonoprazan, which is a type of novel acid suppressor drug. Finally, a new formulation of triple therapy in a single capsule is under investigation, which is a field that deserves further investigation. Some notes of caution about rifabutin should be mentioned. This drug is used to treat tuberculosis or atypical mycobacteria; therefore, before starting a rifabutin-based eradication regimen, *Mycobacterium tuberculosis* infection should be thoroughly tested, since its use could promote the development of antibiotic resistance, thus affecting its effectiveness against Koch's bacillus. Additionally, some serious side effects must be evaluated before starting any rifabutin-based therapy. Adverse effects include fever, nausea, vomiting and bone marrow suppression. For this reason, full blood count surveillance is required.

Key Words: *Helicobacter pylori*; Eradication; Rifabutin; Antibiotic resistance; Rescue therapy; Treatment

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Core Tip: Rifabutin is an antibiotic that is commonly used to treat tuberculosis or atypical mycobacteria. However, it shows antimicrobial effect against *Helicobacter pylori* as well. It is indicated when multiple eradication treatments have failed. In this review, we summarized current evidence about traditional triple therapy containing amoxicillin and rifabutin as salvage therapy, based on the most recent meta-analyses. Furthermore, other novelties regarding rifabutin based regimens have been mentioned.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a widespread cause of infectious disease, mainly causing chronic gastritis, peptic ulcer disease, but also causing gastric cancer or mucosa-associated lymphoid tissue lymphoma [1]. In Italy, it is estimated that more than one-third of the adult population is infected by this[2]. The Kyoto consensus report on gastritis appointed *H. pylori* gastritis as a nosologically distinct entity in the new International Classification of Disease 11th Revision; this entails that all *H. pylori*-infected patients must be treated, regardless of clinical manifestations[3].

The diagnosis of *H. pylori* infection is understood by performing different tests; however, the most appropriate one to achieve an accurate diagnosis is still being debated. On the other hand, an increase of *H. pylori* resistance to previously efficacious antimicrobics has been observed, thus making eradication of the bacterium more and more complex[4]. The eradication of this bacterium requires the combination of multiple antibiotics, which in return reduces patients adherence to the treatment and increases rates of adverse events secondary to therapy. Molecular methods, such as real-time polymerase chain reaction may allow an understanding of if the isolated strain carries genes that confer resistance to antibiotics (mostly against levofloxacin or clarithromycin)[5]. The Maastricht VI/Florence consensus report suggests the microbiological culture is a gold standard for antibiotic susceptibility; however, culture cannot be considered a routine diagnostic test as it is complex, expensive, and requires dedicated personnel[6].

The first-line eradication therapy should be chosen according to the local prevalence of antimicrobial resistance; however, in several areas of Italy it is unknown, but in some areas of Central and Southern Italy there is proof regarding the high prevalence of clarithromycin resistance, close to the 30%[7]. International guidelines recommend a 10-14-d regimen based on quadruple therapy as a first-line choice in countries with high (> 15%) resistance to clarithromycin: (1) Bismuth-based quadruple therapy: Proton pump inhibitor (PPI) + bismuth + tetracycline + metronidazole, also known as Pylera®, with an eradication rate of 90%[8,9]; and (2) Non-bismuth concomitant quadruple therapy: PPI + clarithromycin + amoxicillin + metronidazole/tinidazole, which raises the eradication rate to 75%. If the first-line bismuth quadruple therapy regimen fails, levofloxacin containing regimen is recommended as the second line. However, after multiple treatment failures, empirical rescue regimens have been suggested and rifabutin has proven to be effective in this scenario[10,11].

RIFABUTIN MECHANISM OF ACTION, PHARMACODYNAMICS, AND PHARMACOKINETICS

Rifabutin is a rifampicin derivative compound; it has a high lipid-solubility, an elevated oral absorption (with high tissue-to-plasma ratio), and chemical stability at a wide pH range (*i.e.*, in the gastric environment): In an *in vivo* study in rats, the concentration of rifabutin in gastric secretion was 10-17 times superior to that in plasma, suggesting considerable gastric secretion[12]. Rifabutin is extensively metabolized, which means dosage adjustments are necessary in patients with severe renal or hepatic dysfunction. This drug shows a broad spectrum of antimicrobial activity; it is mostly used against mycobacteria (*Mycobacterium leprae*, *M. tuberculosis*, and atypicals[13]), some gram-positive and gram-negative bacteria, *Toxoplasma gondii*, and *Chlamydia trachomatis*.

The *in vitro* sensitivity of *H. pylori* to this antibiotic is high (with minimum inhibitory concentration (MICs) lower than that found for amoxicillin, clarithromycin, and metronidazole[14,15]), and it does not share resistance to clarithromycin, metronidazole or levofloxacin[16,17], making rifabutin-based rescue regimen a potential treatment after multiple failures[18-20]. Rifabutin acts by inhibiting the β-subunit of bacterial DNA-dependent RNA polymerase encoded by the beta subunit of RNA polymerase (rpoB)

gene, thus having bactericidal action.

H. PYLORI RESISTANCE TO RIFABUTIN

H. pylori antibiotic resistance is the main worldwide problem affecting current eradication regimens; *H. pylori* shows great *in vitro* susceptibility *in vitro* to rifabutin[21,22], and resistance to this antimicrobic is lower than that found for amoxicillin, clarithromycin, and metronidazole[23]. The reference methodology to identify resistance is microbiological testing, which is often hard to perform because the culture of this germ may be difficult, and requires expert hands.

This antibiotic is used for tuberculosis (TB) treatment, especially in subjects with human immunodeficiency virus co-infection. For this reason, before starting a rifabutin-based eradication regimen, *M. tuberculosis* infection should be tested, as its use could promote the development of antibiotic resistance, thus affecting its effectiveness against Koch's bacillus[24]. Some laboratory mutants of *H. pylori*, obtained *in vitro*, with amino acid alterations in codons from 524 to 545 or in codon 585 of rpoB, showed resistance to rifabutin[25]. In a Japanese study, a negligible resistance rate (0.24%) to rifabutin was observed in cultures of strains isolated from more than 400 patients. Only one rifabutin-resistant strain was found in a subject with previous rifampin therapy for lung tuberculosis[26]. It was observed that previous rifampicin exposure may be related to high MICs to rifabutin, with point mutations in the rpoB gene, hinting at possible cross-resistance between rifabutin and rifampicin[27].

It has been postulated that multiple strains of *H. pylori*, either resistant and/or susceptible to different antibiotics, can be present in the same patient, thus suggesting the combined use of rifabutin with other antibiotics. In fact, several studies have shown that the risk of antimicrobial resistance onset is lower when it is used in combination with other antibiotics such as amoxicillin[28].

EFFICACY OF RIFABUTIN REGIMENS IN *H. PYLORI* ERADICATION

Recent studies have revealed that the prevalence of *H. pylori* resistance to rifabutin and amoxicillin is minimal, so therapy with the association of rifabutin and amoxicillin could achieve satisfactory eradication rates. This regimen is recommended for rescue therapy in some consensus reports[29-31].

A systematic review by Malfertheiner *et al*[32] showed that rifabutin containing rescue therapy is a powerful therapy after several (usually three) previous eradication failures. The prevalence rate of rifabutin resistance of only about 1% was found, and furthermore, when studies included patients naïve to *H. pylori* eradication treatment, the data were even lower (0.6%). In general, mean-weighted *H. pylori* eradication rate (at intention-to-treat analysis) was 73%; eradication rates of second-, third-, and fourth-/fifth-line regimens were 79%, 66%, and 70%, respectively. All studies examined in the review used rifabutin at the dose of 300 mg/d, which seemed to be more successful than 150 mg/d. The optimal treatment duration for rifabutin was 10 to 12 d.

A systematic review and meta-analysis by Liu *et al*[33] analyzed 537 articles from medical journals (PubMed, the Cochrane Central Register of Controlled Trials, Embase, and SCI) of randomized clinical trials evaluating *H. pylori* therapy, recruiting a treatment group with a PPI, rifabutin, and amoxicillin. Twenty-one articles were selected, and the overall eradication rate was 70.4% at intent-to-treat (ITT) and 72.0% by per-protocol (PP) analyses. The eradication effectiveness obtained with rifabutin and amoxicillin was lower than the other triple therapies (68.4% *vs* 81.9% success rate). The effectiveness of the combination was not greater than the association of amoxicillin and levofloxacin. The effectiveness of the association of amoxicillin and rifabutin was comparable to the quadruple therapy, which included a PPI and amoxicillin. The cure rate of rifabutin plus amoxicillin was lower than bismuth-containing quadruple therapy. This review established that a regimen with PPI, rifabutin, and amoxicillin for *H. pylori* infection could not be the optimal choice for rescue therapy after several eradication failures.

Gingold-Belfer *et al*[34] conducted another meta-analysis of 33 randomized controlled trials, which used triple therapy with rifabutin and amoxicillin and found a pooled success of 71.8%. Lee *et al*[35] analyzed 84 patients' overall resistance rates to amoxicillin, clarithromycin, metronidazole, and moxifloxacin and found that they were 13.1%, 83.3%, 47.6%, and 71.4%, respectively. A susceptibility-guided therapy was proposed, based on culture, and it was shown that it was both effective and devoid of complications, even for patients reporting high antimicrobial resistance; in particular, in the arm receiving rifabutin due to multiple resistances, the eradication rate was 100%.

In 2022, Nyssen *et al*[36] data analyses based on the European multicenter prospective observational registry about *H. pylori* management was performed, analyzing 18 different rifabutin-containing treatments including two or three other antibiotics and recruiting 500 patients. Rifabutin was mostly used in second-line (32%), third-line (25%), and fourth-line (27%) regimens, with a success rate of 78%, 80% and 66%, respectively, according to modified intention-to-treat analysis.

In 2022, Inokuchi *et al*[37] enrolled patients who did not respond to second-line therapy to assess the efficacy and safety of 7-d rifabutin, amoxicillin, and vonoprazan triple therapy [20 mg vonoprazan twice daily (b.i.d.), 500 mg amoxicillin four times daily (q.i.d.), and 150 mg rifabutin twice daily (q.d.)] lasting 7 d as third- or later-line treatment for *H. pylori* infection. Intention-to-treat and PP analyses showed a high eradication rate [91.2%, 95% confidence interval (CI): 84%-99% and 92.7%, 95%CI: 86%-100%, respectively]. The results indicate that this regimen is efficient and safe as a third-line treatment or in successive efforts of *H. pylori* eradication.

New drugs, combining rifabutin all-in-one with other drugs, are in course of study: A phase three, double-blind study (ERADICATE Hp) driven by Kalfus *et al*[38], randomized (2:1) treatment-naïve dyspeptic patients with *H. pylori* infection to RHB-105 (Talicia®), a new all-in-one association of omeprazole 40 mg, amoxicillin 1000 mg, and rifabutin 50 mg, randomized *vs* placebo, both given every 8 h for 2 wk. The study showed an *H. pylori* eradication rate ITT of 89.4%.

An association of rifabutin with other antibiotics has been tried: An intervention study in Southern Italy considered rifabutin and tetracycline association after three or more eradication therapy attempt failures[39]. Only rifabutin and tetracycline were tested in a relevant number of patients, reporting an eradication rate of 80.4% (per protocol) and 77.4% (intention-to-treatment).

Italian guidelines suggest the 12-d rifabutin-amoxicillin triple therapy (*i.e.,* PPI at standard dose b.i.d., amoxicillin 1 g b.i.d., and rifabutin 150 mg b.i.d.) as a rescue regimen[40], which has been demonstrated to be useful after several previous therapeutic failures[19]. In other studies, such as the one performed by Malfertheiner *et al*[32], an ideal length of treatment from 10 to 12 d was suggested, whereas the latest publication of Inokuchi *et al*[37] suggested a 7-d regimen.

SIDE EFFECTS

Despite the effectiveness of rifabutin-based regimens, serious side effects must be evaluated before starting any rifabutin-based therapy. Adverse effects include fever, nausea, vomiting, with a "not common" and reversible effect, *i.e.,* bone marrow suppression. For this reason, full blood count surveillance is required. Uveitis has recently been described in patients under an association of rifabutin and other antimycobacterial drugs[41,42].

In the study by Inokuchi *et al*[37], adverse events occurred in 31.6% of the patients; also in the article by Nyssen *et al*[36] one or more side effects were recorded in the 26% of the patients (nausea was the most common), and only one severe bone marrow adverse event (0.2%) was described. Furthermore, it is possible that rifabutin may induce changes in the intestinal microbiota even if there are no studies in the literature on this topic, to the best of our knowledge. Presumably, this can be explained by the limited use of this antibiotic in *H. pylori* infection therapy.

CONCLUSION

H. pylori eradication is currently a worldwide challenge for clinicians. In 2017, the World Health Organization classified resistance to clarithromycin as a "high-priority" issue for *H. pylori*[43]. Microbiological cultures are advised[7], but they are difficult to perform for the slow bacterial growth and particular nutritional requirements, making it very expensive as they require specialized staff with a specialized laboratory. Furthermore, in latest studies, Pylera® therapy eradication rates are comparable to culture-tailored therapies[44].

Facing treatment failures, rifabutin has an interesting role against *H. pylori,* since such drug shows excellent *in vitro* effectiveness, and the diffusion of its resistance is very low (< 1%). Side effects should be weighed, even though severe adverse events are exceptional. In all the studies analyzed, rifabutin has a great effectiveness, safety and tolerability when used as a "rescue regimen", *i.e.,* third or fourth-line therapy; in conclusion, the use of rifabutin as a new first-line treatment alternative for *H. pylori* gastritis should be thoroughly pondered, by evaluating the risk of microbial resistance, the high cost of treatment and the wide availability and effectiveness of alternative drugs. This could be precociously evaluated in the eradication algorithm in high resistance areas.

FOOTNOTES

Author contributions: Losurdo G and Di Leo A planned the investigation; Borraccino AV and Celiberto F carried out the research; Borraccino AV, Losurdo G, and Ierardi E wrote the paper; Di Leo A supervised the study; and all authors read and validated the final version.

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MINIREVIEWS

Deep learning based radiomics for gastrointestinal cancer diagnosis and treatment: A minireview

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Abstract

Gastrointestinal (GI) cancers are the major cause of cancer-related mortality globally. Medical imaging is an important auxiliary means for the diagnosis, assessment and prognostic prediction of GI cancers. Radiomics is an emerging and effective technology to decipher the encoded information within medical images, and traditional machine learning is the most commonly used tool. Recent advances in deep learning technology have further promoted the development of radiomics. In the field of GI cancer, although there are several surveys on radiomics, there is no specific review on the application of deep-learning-based radiomics (DLR). In this review, a search was conducted on Web of Science, PubMed, and Google Scholar with an emphasis on the application of DLR for GI cancers, including esophageal, gastric, liver, pancreatic, and colorectal cancers. Besides, the challenges and recommendations based on the findings of the review are comprehensively analyzed to advance DLR.

Key Words: Radiomics; Deep learning; Gastrointestinal cancer; Medical imaging

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Core Tip: Radiomics, especially deep-learning-based radiomics (DLR), has revolutionized the diagnosis, assessment and prognosis of gastrointestinal (GI) cancer. This review provides an analysis and status of DLR in GI cancer and identifies future challenges and recommendations.

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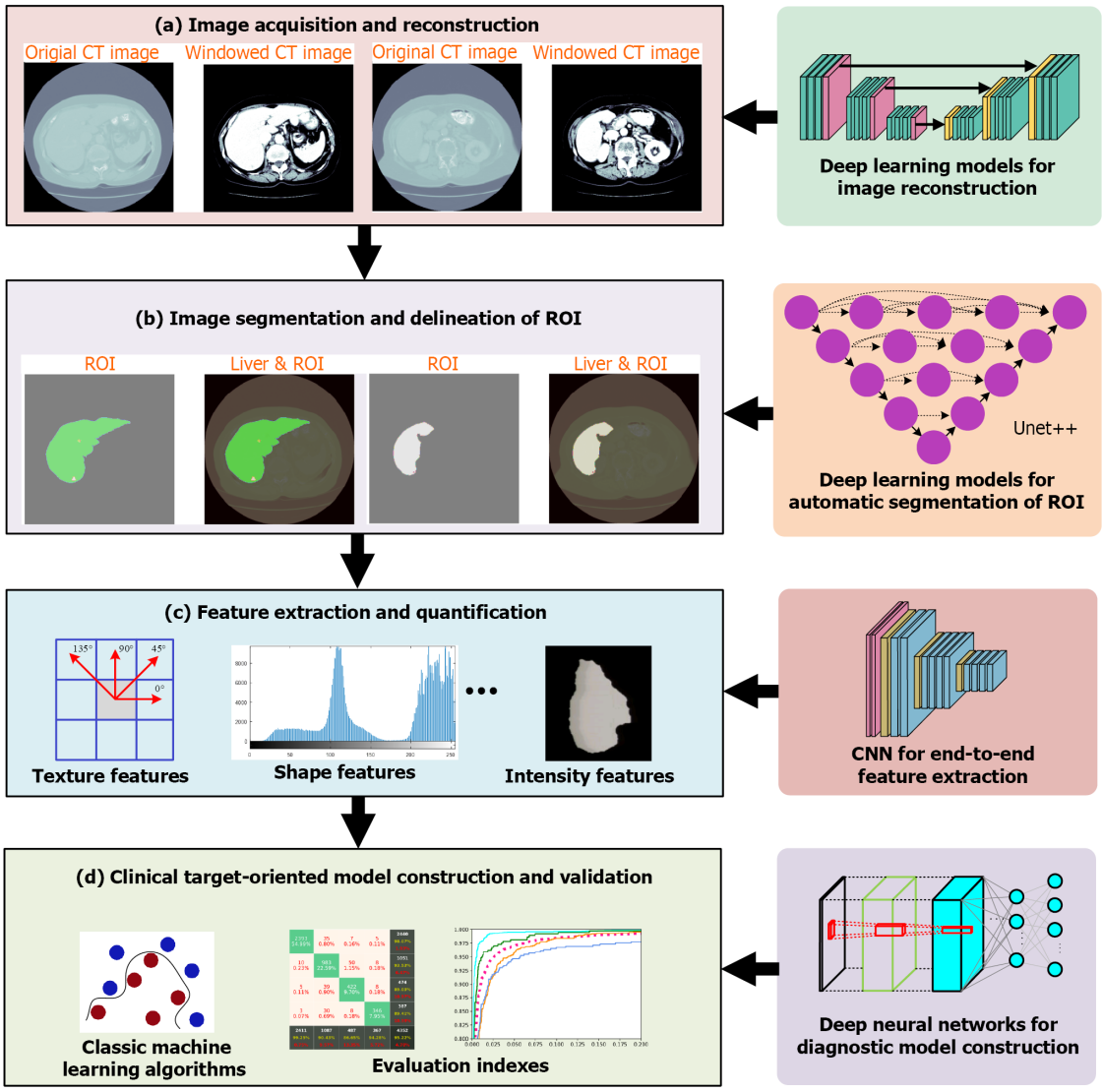
INTRODUCTION

Gastrointestinal (GI) cancers, mainly include colorectal, gastric, liver, esophageal, and pancreatic cancers, and are the leading cause of cancer-related mortality globally[1]. According to CANCER TOMORROW[2], a forecast of the global burden of cancer mortality and incidence, by 2040, new cases of GI cancer and deaths will increase significantly. In recent years, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), ultrasound (US) and other medical imaging techniques have been widely used in GI cancer diagnosis and treatment[3,4]. It is foreseeable that with the increase in GI cancer, the amount of medical imaging data will continue to grow. However, manual reading cannot cope with this growth, and the disparity in expertise among radiologists causes a high rate of missed diagnosis and misdiagnosis. In addition, traditional CT, MRI, PET, US, and other imaging examinations cannot observe changes in tumor heterogeneity, which can provide a better understanding of the causes and progression of cancer[5]. The development of radiomics technology provides new opportunities and methods to solve these dilemmas.

Radiomics is an emerging method for quantitative analysis and prediction of tumor phenotypes using machine learning or statistical models, and was proposed by Lambin *et al*[6] in 2012. In recent years, radiomics has been widely used in GI cancer and showed notable outcomes in tumor characterization, therapy response assessment, and prediction of survival rate after surgery[7-11]. Compared with the conventional method of using only manual inspection, radiomics can extract high-dimensional features that are difficult to be quantitatively described by the doctors from massive radiological images, and to correlate them with clinical and pathological data of patients in order to improve diagnosis and prognostication[12]. The fundamental premise of radiomics is that the developed descriptive models may produce useful prognostic, predictive and diagnostic information. Radiomics can be divided into two main categories: conventional radiomics, also referred to handcrafted radiomics (HCR) and deep-learning-based radiomics (DLR), also referred to as discovery radiomics[13]. Given the benefits of these two approaches, hybrid solutions that mix HCR and DLR also exist.

The HCR workflow is divided into multiple steps: (1) Image acquisition and reconstruction; (2) image segmentation and delineation of region of interest (automatic, semi-automatic, or manual delineation); (3) feature extraction and quantification. This is the core step of HCRs. The extracted features are mainly handcrafted features (also referred as pre-designed features), including shape, texture and intensity features. Some features may be highly correlated or redundant, so feature dimensionality reduction is an important step in feature analysis; and (4) Clinical target-oriented model building and validation. At this step, classic machine learning algorithms are usually used to develop high-precision and high-efficiency prediction models, and the models are trained and validated with sufficient data. The workflow of HCR is depicted in Figure 1.

Although HCR has been widely adopted in GI cancer and has achieved significant results, it has some deficiencies, such as low degree of automation and standardization, cumbersome and time-consuming feature extraction steps, and insufficient robustness and accuracy. Recently, deep learning, a promising technique in characterization of medical images, has gained much attention[14-17]. Many researchers have adopted DLR to overcome the limitations of conventional radiomics[18-22]. DL refers to a broad class of algorithms rather than a specific model. As long as a deep neural network structure is used to represent features at a deeper level, it can be called DL model. One of the most popular DL models used in medical imaging is convolutional neural networks (CNNs), which can automatically learn representative features from medical images. The use of CNNs in radiomics makes it easy to build an end-to-end feature extraction process, thereby avoiding the tedious and handcrafted feature extraction process. CNNs can also be used in image reconstruction and segmentation to improve the automation level of



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Figure 1 Overview of steps in handcrafted radiomics workflow and steps that can be done with deep learning models. ROI: Region of interest.

HCR, and the accuracy and reliability of diagnosis and prediction (Figure 1).

DL techniques are revolutionizing radiomics. In the field of GI cancer diagnosis and treatment, while there are several surveys on HCR[9-11], there is no specific review on the application of DLR. To provide a comprehensive overview of DLR in GI cancer, the performance of DLR in gastroenterology is summarized in this review, with an emphasis on the diagnosis and treatment of GI cancers, including esophageal, gastric, liver, pancreatic, and colorectal cancers. The original contributions to knowledge of this review are: (1) A unique interdisciplinary viewpoint on radiomics by discussing state-of-the-art DLR solutions; and (2) the challenges and recommendations based on the findings of the review are thoroughly analyzed to advance the field.

DLR FOR ESOPHAGEAL CANCER

Esophageal cancer is the seventh most prevalent form of cancer and the sixth most lethal cancer globally [1], and it is classified into esophageal squamous cell carcinoma (ESCC) or esophageal adenocarcinoma according to the type of cells. In consideration of the low overall 5-year survival rate of patients and the variation in responsiveness of patients to the current treatments such as neoadjuvant chemotherapy (NAC) and neoadjuvant chemoradiotherapy (NCRT) due to tumor heterogeneity, it is vital to have accurate diagnosis, pretreatment evaluation and survival rate prediction. The number of DLR studies regarding esophageal cancer has been growing, with most of the studies exploring treatment response, and the others investigating disease classification and survival rate prediction.

An important preoperative topic of esophageal cancer is diagnosis, yet the number of relevant DLR studies for diagnosis is minimal. Takeuchi *et al*[23] fine-tuned VGG16 to develop a DLR model for the diagnosis of esophageal cancer from CT scans, and its performance was comparable to that of the radiologists during testing, with a higher accuracy of 84.2% and specificity of 90.0%.

Response to treatment, especially NAC and NCRT, is one of the most popular research interests in the field of esophageal cancer. Hu *et al*[24] designed a CT-based model to predict the pathological complete response to NCRT of patients with ESCC using DL features, in which the support vector machine (SVM) classifier executed the classification action. The DL features were extracted using pretrained models, and the optimal one used ResNet50 that achieved an area under the receiver operating characteristic curve (AUC) of 0.805 and accuracy of 77.1% for the testing cohort, which achieved better results than using handcrafted features. Ypsilantis *et al*[25] designed a 3S-CNN model that extracted DL features from PET scans and predicted whether the patient with esophageal cancer was non-responsive to NAC. This model was also compared with other competitive machine learning algorithms and results showed that it surpassed the other models with an average specificity, sensitivity and accuracy of 80.7%, 81.6%, and 73.4% respectively. Amyar *et al*[26] presented a novel 3D CNN model named 3D RPET-NET that predicted the response to CRT using esophageal cancer images of FDG-PET scans, and a comparative analysis with other approaches in the literature was also carried out. Three-dimensional RPET-NET obtained the best results with an accuracy of around 72% and even reached 75% when using tumor volume with an isotropic margin of 2 cm. Li *et al*[27] proposed a CT-based 3D DLR model (3D-DLRM), which was modified from ResNet34. Its aim was to predict whether patients with locally advanced thoracic ESCC had an objective or nonobjective response to concurrent CRT, achieving a validation AUC and positive predictive value of 0.833 and 100%, respectively. They also evaluated a model integrating the 3D-DLRM with clinical selected factors that even outperformed the individual 3D-DLRM, reaching a validation AUC of 0.861.

Other research interests of esophageal cancer include patient survival rate prediction. Wang *et al*[28] compared the use of an HCR model, DLR model and DLR nomogram for the prediction of the survival rate of esophageal cancer patients after 3 years of CRT, in which DL features were extracted and selected by DenseNet-169 to build the DLR model. This DLR nomogram attained the highest validation AUC of 0.942 and Harrell's concordance index (C-index) of 0.784, surpassing the results produced by the sole use of HCR and DLR models. Yang *et al*[29] proposed a 3D-CNN model based on ResNet18 to predict esophageal cancer patient survival rate using PET scans. The model was initially pretrained to classify abnormal and healthy esophagus, and then trained to classify whether patients survived or expired within a year after diagnosis in the second stage, and the model obtained an AUC of 0.738. Gong *et al*[30] developed a hybrid radiomics nomogram to predict local recurrence-free survival (LRFS) of locally advanced ESCC patients who received definitive CRT from contrast-enhanced CT (CECT) scans, and it was combined with radiomic features, features extracted by 3D-DenseNet and prognostic clinical risk factors. The final model achieved a C-index of 0.76 for its external validation set, indicating the effectiveness of the addition of DL features for better prediction performances.

Some studies also discuss the application of DLR to prediction of lymph node (LN) metastasis, which is an effective prognosis factor of ESCC. Wu *et al*[31] built a model involving HCR, computer vision and DLR to predict the LN status of ESCC patients, and they also constructed two simpler models for efficacy comparison, and they exploited Convolution Neural Network-Fast (CNN-F) to extract DL features from CT images. The model with all signatures involved performed the best with C-statistic of 0.875, 0.874, and 0.840 for training, internal validation, and external validation cohorts, and those demonstrate its satisfactory discriminative ability.

The studies about the application of DLR for esophageal cancer are summarized in Table 1.

DLR FOR GASTRIC CANCER

Gastric cancer (GC) is the fifth most prevalent form of cancer and the fourth most lethal cancer globally [1]. To ameliorate the low survival rate of patients, early diagnosis of disease and systematic treatment methods are necessary. The application of DLR in GC has been a promising area for research with a rising number of relevant studies published every year, that aim to tackle or refine the existing concerns regarding GC.

Many studies focused on prediction of treatment response of patients. Cui *et al*[32] constructed a pretreatment venous-phase CT-based DLR nomogram that combined handcrafted features, DL features and remarkable clinicopathological factors to identify locally advanced GC patients with good response to NAC. The nomogram achieved better than the clinical model and the separate use of two features that were built for comparison, attaining C-index values of 0.829, 0.804, and 0.827 in its internal validation cohort and two external validation cohorts, respectively. Li *et al*[33] developed a combined artificial intelligence (AI) model that incorporated feature outputs from HCR and DLR models, which aimed to determine whether the patients had signet ring cell carcinoma (SRCC) of GC and predict survival and treatment response to postoperative chemotherapy from CECT images. They also compared its efficacy with the clinical, HCR and DLR models, and the AI model obtained the best

Table 1 Summary of studies using deep-learning-based radiomics for esophageal cancer

Ref.	Imaging	Study design	Study aim	DL model	Dataset	Outcomes
Takeuchi *et al* [23], 2021	CT	Retrospective	Detection of esophageal cancer	VGG16	1646 CT images (1500 images for training and validation, 146 for testing)	Accuracy: 84.2%; F value: 74.2%; Sensitivity: 71.1%; Specificity: 90%) in test set
Hu *et al*[24], 2021	CT	Retrospective	Evaluation of response to NCRT to ESCC	ResNet50	231 patients (161 in training cohort, 70 in testing cohort)	AUC: 0.805; C-index: 0.805; Accuracy: 77.1%; Sensitivity: 83.9%; Specificity: 71.8%) for the testing cohort
Ypsilantis *et al* [25], 2015	PET	Retrospective	Prediction of response to NAC in patients with esophageal cancer	3S-CNN	107 patients	Sensitivity: 80.7%; Specificity: 81.6%; Accuracy: 73.4%
Amyar *et al* [26], 2019	PET	Retrospective	Prediction of response to radio-chemotherapy in patients with esophageal cancer	3D RPET-NET	97 patients	Accuracy: 75.0%; Sensitivity: 76.0%; Specificity: 74.0%; AUC: 0.74
Li *et al*[27], 2021	CT	Retrospective	Prediction of treatment response to CCRT among patients with locally advanced TESCC	ResNet34	306 patients (203 in training cohort, 103 in validation cohort)	AUC: 0.833; PPV: 100%
Wang *et al*[28], 2022	CT	Retrospective	Prediction of survival rates for patients with esophageal cancer after 3 yr with chemoradio-therapy	DenseNet- 169	154 patients (116 in training cohort, 38 in validation cohort)	AUC: 0.942; C-index: 0.784
Yang *et al*[29], 2019	PET	Retrospective	Identification of esophageal cancer patients with poor prognosis	3D-CNN based on ResNet18	1107 scans	AUC: 0.738
Gong *et al*[30], 2022	CECT	Retrospective	Prediction of LRFS in esophageal cancer patients after 1 yr of definitive chemoradio-therapy	3D-Densenet	397 patients	C-index: 0.76
Wu *et al*[31], 2019	CT	Retrospective	Prediction of LN status of patients with ESCC	CNN-F	411 patients	C-index: 0.840

CT: Computed tomography; PET: Positron emission tomography; CECT: Contrast-enhanced computed tomography; NCRT: Neoadjuvant chemoradio-therapy; NAC: Neoadjuvant chemotherapy; ESCC: Esophageal squamous cell carcinoma; CCRT: Concurrent chemo-radiation therapy; TESCC: Thoracic esophageal squamous cell carcinoma; LRFS: Local recurrence-free survival; LN: Lymph node; CNN-F: Convolutional neural network-fast; AUC: Area under the receiver operating characteristic curve; PPV: Positive predictive value; DLR: Deep-learning-based radiomics.

results with an AUC of 0.786 and accuracy of 71.6% for diagnosing SRCC for the test cohort. The AI model also evaluated that SRCC patients with higher risks had shorter median overall survival (OS) and insignificant improvements in median OS after receiving adjuvant chemotherapy than those of lower risk, indicating its good capability to predict survival and response to treatment. Tan *et al*[34] built a dual-energy CT delta radiomics model to predict the treatment response to chemotherapy of patients with far-advanced GC. They developed a V-Net segmentation model, and the application of this semi-automatic segmentation model to the delta radiomics model shortened the diagnostic time and achieved better results in terms of mean AUC (0.728 *vs* 0.687 in the testing cohort, 0.828 *vs* 0.749 in the independent validation cohort) than using manual segmentation.

Survival rate prediction is also a popular topic for DLR of GC. Hao *et al*[35] combined clinical variables, radiomic features and DL features to build a CT-based prediction Cox proportional-hazard model, which served to predict the OS and progression-free survival (PFS) of patients with GC. The model acquired the highest C-index of 0.783 and 0.770 for OS and PFS when using postoperative clinical variables, and the most dominant variables for survival prediction were identified as important prognostic factors in the subsequent survival analysis. Some studies only made use of DL techniques to build predictive models for similar purposes. Zhang *et al*[36] proposed a multi-focus and multi-level fusion feature pyramid network (MMF-FPN) to predict OS risks of GC patients from CT images, and other models using existing methods in the literature were used for comparison. The experimental results showed that MMF-FPN was the finest model that attained the highest C-indexes (validation: 0.74, testing: 0.76) and hazard ratios (validation: 3.50, testing: 9.46).

To do a preoperative prediction of early recurrence of patients with advanced GC from CT images, Zhang *et al*[37] designed a radiomics nomogram that utilized clinical characteristics and radiomics signature containing handcrafted and DL features as input. The radiomics nomogram reached an AUC and accuracy of 0.806 and 0.723, respectively, while having considerable k values of 0.932 for both intra-

and inter-reader agreement, exceeding the results obtained by the radiomics signature and clinical modal built for comparison.

Accurate prediction of LN status of GC, which is a remarkable prognostic factor, is of importance to determine the appropriate treatment. Guan *et al*[38] explored the efficacy of using different DL models to extract features and machine learning classifiers (*i.e.*, SVM and random forest) to build a CT-based predictive model for the evaluation of LN status. Other models using radiomic features and integrated features were built for comparison, and the best model was ResNet50-RF with an AUC and accuracy of 0.9803 and 98.10%, respectively. A nomogram based on DL feature scores and clinical risk factors was also developed and a higher AUC of 0.9914 was achieved in the testing cohort. Dong *et al*[39] proposed a similar DLR nomogram to evaluate the number of LN metastases of locally advanced GC patients before surgery, in which radiomics signatures that contained handcrafted and DL features and clinical characteristics were used. The performance of the model was evaluated with four validations sets; three of which were collected from China and one from Italy. The model showed its good discriminative capability to identify N-staging of GC with higher C-indexes of 0.797 in the validation sets from China and 0.822 in the set from Italy, and it outperformed other predictors such as clinical models and single signatures. To predict the LN status and prognosis of patients, the dual-energy CT-based DLR nomogram created by Li *et al*[40] incorporated CT-reported LN and two radiomics signatures for arterial-phase and venous-phase CT images, in which DL features were extracted *via* CNN. The nomogram performed better and gained a higher AUC of 0.82 than the clinical model built alongside for comparative analysis, and the associated prognosis prediction was satisfactory in terms of PFS (C-index: 0.64) and OS (C-index: 0.67). Jin *et al*[41] developed a DLR model that adopted ResNet-18 to evaluate the LN status in nodal stations using CECT, and the high value of the median AUC of the 11 stations (0.876) proved the excellent prediction ability of the model. The authors attempted to build a nomogram combining the DL features with clinical features, but no significant improvements in the results were observed.

Other prognostic factors of GC have also been investigated in previous studies. Sun *et al*[42] exploited DL techniques to build a CT-based radiomics nomogram for evaluating the status of serosal invasion of advanced GC patients. Three radiomics signatures were generated based on the three phases of CT images with their DL features extracted using CNNs, and they were integrated with clinical characteristics to form the nomogram. The final model outperformed other models, such as clinical and phenotypic models, and its AUC for test sets I and II was 0.87 and 0.90, respectively. Li *et al*[43] compared the use of DL features and radiomic features to create a CECT-based GC risk (GRISK) model using similar procedures for the prediction of the status of lymphovascular invasion in patients with localized GC. The team explored the use of deep transfer learning models to build a gastric imaging marker, in which five pretrained models and an auto-encoder were utilized for feature extraction and reduction, respectively. Then, it was integrated with patient clinical and radiological characteristics to construct its own GRISK model. The GRISK model with deep transfer learning gastric imaging marker obtained comparable AUC (0.722 *vs* 0.725) and accuracy (0.671 *vs* 0.710) with the other model with the radiomics gastric imaging marker but did not surpass the latter model.

The studies investigating the usage of DLR for GC are summarized in Table 2.

DLRs FOR LIVER CANCER

Primary liver cancer is the sixth most prevalent form of cancer and the third most lethal cancer globally, and some of its common phenotypes are hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma[1]. Taking the high mortality caused by this disease into account, the clinical application of early diagnosis, individualized evaluation and prognosis prediction are valued. The exploitation of DLR technology in liver cancer has been rapidly developing, and various solutions for the issues in different phases of diagnosis and treatment are emerging.

Computer-aided diagnosis does not only aid radiologists such as shortening the diagnosis time, but also allow them to evaluate appropriate treatments at earlier stages of liver cancer. Ding *et al*[44] constructed a CT-based DLR model that fused a radiomics signature and a DL model, to differentiate HCC into low or high grade. The DL model was an alteration of VGG19 and it performed better than the radiomics signature, with better AUC (0.7513 *vs* 0.7475) and accuracy (66.31% *vs* 65.78%). The fused DLR model was the optimal model with observable improvements in the results, achieving an AUC of 0.8042 and accuracy of 72.73%.

Accurate prediction of patient response to different therapies is critical to realize personalized treatment at different stages of HCC. Peng *et al*[45] developed a multi-class DL model from ResNet50 to predict four treatment responses to transarterial chemoembolization (TACE) therapy of HCC patients using CECT scans. Its performance was assessed using confusion matrices and receiver operating characteristic curves, and the model attained an AUC over 0.90 for all four classes in both validation sets, and accuracies of 85.1% and 82.8% for validation sets 1 and 2, respectively. In the next year, they combined conventional radiomics and DL to build a new CECT-based DLR model that served to predict the initial treatment response to TACE of HCC patients preoperatively[46]. Different from their prior

Table 2 Summary of studies using deep-learning-based radiomics for gastric cancer

Ref.	Imaging	Study design	Study aim	DL model	Dataset	Outcomes
Cui *et al* [32], 2022	CT	Retrospective	Prediction of response to NAC in patients with LAGC	DenseNet-121	719 patients	C-index: 0.829
Li *et al* [33], 2022	CT	Retrospective	Diagnosis and prediction of chemotherapy response to SRCC patients	Modified U-Net	855 patients (598 in training cohort; 257 in testing cohort)	For diagnosis, AUC: 0.786; accuracy: 71.6%; sensitivity: 77.3%; specificity: 69.2% for testing cohort
Tan *et al* [34], 2020	CT	Retrospective	Prediction of response to chemotherapy in patients with gastric cancer	V-Net	116 patients	Mean AUC: 0.728 (testing cohort); 0.828 (validation cohort) when using semi-segmentation
Hao *et al* [35], 2021	CT	Retrospective	Prediction of OS and PFS after gastrectomy; evaluation of effects of variables on survival prediction	Attention-guided VAE	1061 patients (743 for training; 318 for testing)	C-index of OS: 0.783; C-index of PFS: 0.770 when only using postoperative variables
Zhang *et al*[36], 2021	CT	Retrospective	Prediction of OS risks of patients with gastric cancer	MMF-FPN	640 patients (337 in training set; 181 in validation set; 122 in test set)	C-index: 0.76; hazard ratio: 9.46 in test set
Zhang *et al*[37], 2020	CT	Retrospective	Prediction of early recurrence of patients with AGC	DCNNs	669 patients	AUC: 0.806; accuracy: 0.723; sensitivity: 0.827; specificity: 0.667
Guan *et al* [38], 2022	CT	Retrospective	Prediction of preoperative status of LNM of gastric cancer patients	ResNet50-RF	347 patients (242 for training; 105 for testing)	AUC: 0.9803; accuracy: 98.10%; sensitivity: 98.39%; specificity: 0.9767% for testing of ResNet50-RF
Dong *et al* [39], 2020	CT	Retrospective	Prediction of the number of LNM in LAGC	DenseNet-201	730 patients	C-index: 0.822 in validation set
Li *et al* [40], 2020	CT	Retrospective	Prediction of LNM and prognosis in gastric cancer patients	DCNNs	204 patients (136 in training set, 68 in test set)	AUC: 0.82 in test set; C-index of OS: 0.67; C-index of PFS: 0.64
Jin *et al* [41], 2021	CT	Retrospective	Prediction of LNM status in LN stations of gastric cancer patients	ResNet-18	1699 patients	Median AUC: 0.876; median Sensitivity: 0.743; median Specificity: 0.936 in validation cohort
Sun *et al* [42], 2020	CT	Stage I: Retrospective; stage II: Validation	Prediction of serosa invasion of AGC patients	DCNNs	572 patients (252 in training set, 176 in test set I, 144 in test set II)	AUC: 0.87; accuracy: 80%; sensitivity: 0.73; specificity: 0.85 in test set I. AUC: 0.90; accuracy: 85%; sensitivity: 0.75; specificity: 0.93 in test set II
Li *et al* [43], 2022	CT	Retrospective	Evaluation of lymphovascular invasion of localized gastric cancer patients	SqueezeNet, ResNet50, Inception V3, VGG19, DeepLoc	1062 patients (728 for training, 334 for testing)	AUC: 0.725; sensitivity: 73.2%; specificity: 60.3%; accuracy: 71.0% for radiomics GRISK model (final model) in testing cohort

CT: Computed tomography; NAC: Neoadjuvant chemotherapy; LAGC: Locally advanced gastric cancer; SRCC: Signet ring cell carcinoma; OS: Overall survival; PFS: Progression-free survival; AGC: Advanced gastric cancer; LNM: Lymph node metastasis; LN: Lymph node; DCNNs: Deep convolutional neural networks; MMN-FPN: Multi-focus and multi-level fusion feature pyramid network; UC: Area under the receiver operating characteristic curve; C-index: Harrell's concordance index; GRISK: Gastric Risk; RF: Random forest; VAE: Variational auto-encoder; DLR: Deep-learning-based radiomics.

work, they designed their own CNN for feature extraction and prediction, and the DL model was integrated with five radiomics models built with different classic machine learning algorithms or tumor size feature to build integrated models for efficacy comparison. The DL model outperformed all individual radiomics models with an AUC of 0.972, while all integrated models yielded higher values of AUC than merely using DL model. The combination of DL with random forest classifier obtained the highest AUC of 0.994.

Survival prediction is also an important research area to facilitate individualized HCC treatment. To predict the OS of HCC patients who were treated with stereotactic body radiation therapy, Wei *et al*[47] established a CECT-based DL network model that comprised two variational-autoencoder-based survival models and one CNN-based model for extracting radiomic features, clinical features and CT features. The performance of the separate models and the integrated radiomics model using either DL network or Cox hazard model was compared by C-index, in which the integrated model produced the

highest C-index of 0.650 in repeated cross-validation among all models. Liu *et al*[48] developed two separate DLR models to differentiate HCC patients who received radiofrequency ablation (RFA) or surgical resection into high or low risks using CEUS images, and the corresponding radiomics signatures were built. Afterwards, two radiomics nomograms were constructed by combining the signatures with clinical variables to predict the 2-year PFS of patients and both models. Both DLR models achieved satisfactory values of C-index (0.726 for RFA, 0.741 for surgical resection). The good agreement of the survival predictions of the nomograms was demonstrated from the calibration curves.

Postoperative recurrence of cancer is one of the primary causes of death, which extends to the increase in recurrence risk assessment using DLR. To predict the early recurrence of HCC patients using multi-phase CECT scans, Wang *et al*[49] explored the predictive ability of various kinds of models, and they included a DLR model based on ResNet, a clinical model extracting features from clinical data and three combined CNN-based models of different structures. Experimental results demonstrated that the integration of DL features and clinical features improved the prediction accuracy, and one combined model obtained the highest AUC of 0.825. The team improved their study by comparing the DL model with a conventional radiomics model, and one more combined model of another structure was added to the comparative analysis of their previous work[50]. The DL model performed better than the radiomics model with an average AUC of 0.7233 and accuracy of 69.52%, while one of the combined models surpassed the rest in the comparative analysis and reached 0.8248 and 78.66% in its average AUC and accuracy, respectively. They also investigated the effect of attaching a joint loss function to the best model on the average AUC and accuracy, and the two metrics were improved to 0.8331 and 80.49%. He *et al*[51] presented an intelligent-augmented DL model for Risk Assessment of Post LIver Transplantation (i-RAPIT) model in their study, which was a multi-network model that estimated the recurrence risk of HCC patients after liver transplantation. The i-RAPIT model was composed of two deep CapsNet networks for feature extraction from MR and pathological images, and a natural-language-processing-based radial basis function (NLP-based RBF) for extracting clinical features. Before the MR images were entered into the model, U-Net was also exploited for tumor and liver detection in the images. The model achieved a total accuracy of 82%, and AUC of 0.87 and F-1 score of 84% when comparing with other network combinations.

Early detection of microvascular and macrovascular invasion is another practical approach to select the proper therapy for HCC patients and reduce mortality. Jiang *et al*[52] adopted 3D-CNN to build a CT-based DL model for predicting the status of microvascular invasion of HCC patients, and three models based on radiomics features, radiologic features, and integration of the two kinds of features and clinical characteristics was also used for comparison. The results produced by the four models were excellent, with the DL model achieving better results for a few metrics such as AUC (0.906), and sensitivity (93.2%) in the validation set. Wang *et al*[53] devised a new DL model named MVI-Mind that consisted of a light-weight transformer for segmentation and a CNN for prediction of microvascular invasion, and several DL techniques were used to compare the proposed methods. The MVI-Mind attained highest mean intersection over union of 0.9006 and accuracy of 99.47% as compared with other DL segmentation algorithms, and it maintained its superiority in prediction and obtained AUC values of 0.9223, 0.8962, and 0.9100 for arterial phase, portal venous phase and delayed period CT images, respectively. For estimating the status of macrovascular invasion using CT scans, Fu *et al*[54] utilized the concept of multi-task DL neural network (MTnet) to build predictive models. Radiomic features from CT images, clinical and radiological factors were fused to construct the proposed model, and it was modified from U-Net that contained modules engaged in tumor segmentation, feature extraction and prediction. It exhibited the most outstanding performance with an AUC of 0.836 among all models built for comparison.

The studies investigating the implementation of DLR for liver cancer are summarized in Table 3.

DLR FOR PANCREATIC CANCER

Pancreatic cancer is the seventh most deadly cancer worldwide, in which pancreatic adenocarcinoma or pancreatic ductal adenocarcinoma (PDAC) are the most prevalent, accounting for the high mortality rate [1]. The number of deaths caused by this disease is almost equivalent to the number of cases due to the overall poor prognosis, so the introduction of advanced AI technologies is essential and urgent to rectify the situation. In these few years, the field of DLR in pancreatic cancer has flourished and more critical issues such as disease differentiation and survival prediction have been discussed.

Achieving an accurate diagnosis of PDAC gives a great contribution to avoiding false predictions and improving the survival outcomes of patients. For distinguishing between PDAC and autoimmune pancreatitis using CT scans, Ziegelmayer *et al*[55] developed a DLR model that utilized VGG19 to extract DL features, and its efficacy was compared with a model trained on handcrafted radiomic features. The former model performed better with higher mean values in AUC, sensitivity, and specificity (0.90, 89% and 83%) over the cross-validation procedure. Liao *et al*[56] used a DL model based on the coarse-to-fine network architecture search (C2FNAS) to perform segmentation of CECT images for radiomic feature extraction, and they were used for training the machine learning model for

Table 3 Summary of studies using deep-learning-based radiomics for liver cancer

Ref.	Imaging	Study design	Study aim	DL model	Dataset	Outcomes
Ding *et al* [44], 2021	CT	Retrospective	Evaluation of HCC differentiation	VGG19	1234 patients (799 in training cohort, 248 in validation cohort; 187 in independent testing cohort)	AUC: 0.8042; accuracy: 72.73%; sensitivity: 70.75%; specificity: 75.31% in testing cohort for the fused DLRs model
Peng *et al* [45], 2020	CT	Retrospective	Prediction of different treatment responses to TACE in HCC patients	ResNet50	789 patients (562 in training cohort; 89 and 138 in validation cohorts 1 and 2)	Accuracy: 85.1% in validation cohort 1; accuracy: 82.8% in validation cohort 2
Peng *et al* [46], 2021	CT	Retrospective	Prediction of initial response to TACE in HCC patients	CNN	310 patients (139 in training cohort; 171 in validation cohort)	AUC: 0.994
Wei *et al* [47], 2021	CT	Retrospective	Prediction of OS of HCC patients treated with SBRT	CNN	167 patients	C-index: 0.650 in cross validation
Liu *et al* [48], 2020	US	Retrospective	Prediction of PFS of HCC patients treated with RFA or surgical resection	CNN	214 RFA patients (149 for training; 65 for validation), 205 SR patients (144 for training; 61 for validation)	C-index of RFA: 0.726; C-index of surgical resection: 0.726
Wang *et al*[49], 2019	CT	Retrospective	Prediction of early recurrence of HCC patients	ResNet	167 patients	AUC of best model: 0.825
Wang *et al*[50], 2020	CT	Retrospective	Prediction of early recurrence of HCC patients	ResNet	167 patients	For the best model with joint loss function, AUC: 0.8331; accuracy: 80.49%
He *et al* [51], 2021	MRI and pathological data	Retrospective	Evaluation of HCC recurrence risk of liver transplantation recipients	U-net, CapsNet	109 patients (87 for training; 22 for testing)	Total accuracy: 82%; recall: 80%; precision: 89%; AUC: 0.87; F-1 score: 84%
Jiang *et al* [52], 2021	CT	Retrospective	Prediction of microvascular invasion status of HCC patients	3D-CNN	405 patients (324 in training set, 81 in validation set)	AUC: 0.906; sensitivity: 75.7%; specificity: 93.2%; accuracy: 85.2%; F-1 score: 87.2% in validation set
Wang *et al*[53], 2022	CT	Retrospective	Prediction of microvascular invasion status of HCC patients	Transformer, CNN	138 patients	For arterial phase images in validation set, AUC: 0.9223; Average accuracy: 86.78%
Fu *et al* [54], 2021	CT	Retrospective	Prediction of macrovascular invasion status in HCC patients	Modified U-Net	366 patients (281 in training cohort, 85 in validation cohort)	AUC: 0.836 in validation cohort

CT: Computed tomography; MRI: Magnetic resonance imaging; US: Ultrasound; HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; PFS: Progression-free survival; RFA: Radiofrequency ablation; SBRT: Stereotactic body radiation therapy; CNN: Convolutional neural network; AUC: Area under the receiver operating characteristic curve; C-index: Harrell's concordance index; DLR: Deep-learning-based radiomics.

prediction. The DL segmentation model obtained a mean Dice score of 0.773 for segmentation while the prediction model yielded an AUC of 0.960 when distinguishing between PDAC and the control group (non-cancerous diseases and normal pancreas). Tong *et al*[57] constructed a ResNet-50-based DLRs model to classify PDAC and chronic pancreatitis patients from CEUS images, and the outputs were the probability of being PDAC or chronic pancreatitis, and heatmaps with highlighted regions that displayed the detected lesions. A two-round reader study was conducted to test the effectiveness of the model. The model achieved an AUC of 0.967 and 0.953 in two validation sets and outperformed the radiologists in the first round, while radiologists could obtain higher accuracies in determining the disease with the aid of the model in the second round.

Prediction of treatment response is also a critical aspect in the field of DLR in pancreatic cancer. Watson *et al*[58] built a CNN model based on LeNet to classify, using CT scans, whether PDAC patients had a pathological response or no response to NAC. It was compared with two models: a hybrid DL model that had the same architecture as the pure DL model but captured both CT image features and one clinical feature [≥ to 10% decrease in carbohydrate antigen (CA)-19], and a CA-19 model only taking in the feature regarding CA-19 decrease. Both DL models could produce superior results than the CA-19 model, and the hybrid DL model obtained a slightly higher AUC than the pure DL model (0.784 *vs* 0.738).

Survival prediction is another vital feature of PDAC that occupies a substantial portion of the existing DLR studies. Muhammad *et al*[59] designed a CNN architecture modified from AlexNet to evaluate the survival risk of PDAC patients that received radiomic features extracted from CECT images, and the

model reached a C-index of 0.85, indicating itself as a good survival model. Zhang *et al*[60] also made use of a CNN that was pretrained with non-small cell lung cancer images to construct their CT-based survival model for patients with resectable PDAC, in which a modified loss function was used. The proposed model accomplished finer prognostic predictions than the conventional radiomic model with an index of prediction accuracy of 11.81% and C-index of 0.651. They released another paper in the same year and compared the efficacy of DL and radiomic features from CECT images by feeding them separately to a random forest classifier to build a DLR model for predicting OS[61]. Similar to their prior work, the DL features were extracted by a pretrained CNN model but with a different structure. The model that used DL features attained an AUC of 0.81, which was higher than the other model based on radiomic features and gained a hazard ratio of 1.38 when the respective risk scores (predicted probabilities of deaths) were tested in survival analyses. Later, they further modified their previous DLR model to a risk score-based feature fusion model to predict 2-year OS[62]. Two small models based on DL and radiomic features separately were embodied in the framework to generate their corresponding risk scores, and these risk scores were used to train the main prediction model. The performance of the proposed model was later assessed with other models using different feature reduction techniques, and the risk score model achieved the highest AUC of 0.84. Yao *et al*[63] devised a new multi-task network model to perform both survival and tumor surgical margin prediction of resectable PDAC patients simultaneously using multi-phase CECT scans. Inside the model, a 3D-CNN model incorporated with a nnUNet for pancreas segmentation was exploited for the margin prediction part, while the combination of 3D-ResNet18 and Contrast-Enhanced 3D Convolutional Long Short-Term Memory (CE-ConvLSTM) network was responsible for survival prediction. The model achieved the results exceeding all other deep models in the comparative analysis, which yielded a C-index of 0.705 in predicting survival outcome and a balanced accuracy of 73.6% in determining the resection margin. They revised their preliminary work by incorporating pancreatic anatomical features into the model and switching to implement an automatically self-learning segmentation method that used 3D UNet as the network architecture and nnUNet as the backbone model for training[63]. The new model attained the highest survival C-index of 0.667 and balanced accuracy of 67.1% for resection margin prediction among all the models including their previous model and other DL and radiomics models.

LN metastasis also possesses a high prognostic value in pancreatic cancer and it is noteworthy to have an early and accurate prediction of its status. An *et al*[64] developed a DLR model with different radiomics signatures extracted from dual-energy CT scans for the prediction of LN metastasis by a pretrained ResNet-18 model. Experiments of adding key clinical features were conducted to compare the effectiveness of using different approaches. The combined model integrated DL features and key clinical features yielded the highest AUC of 0.92 and accuracy of 86%.

The expression of various genes is an influential factor for patient prognosis and preoperative prediction of these prognostic factors can assist the diagnosis and treatment evaluation process. To predict the status of *HMGA2* and *C-MYC* gene expression of PDAC patients, Li *et al*[65] compared the use of radiomic features, DL features (extracted by pretrained CNN) and integration of both features in a CT-based model using an SVM classifier. Region of interest segmentation was conducted by two experienced radiologists individually, and the model was tested with different segmented images for improving the validity of the study. A model using DL features and all features achieved similar values in all evaluation metrics for both *C-MYC* and *HMGA2* tests, while DL features selected by Doctor B obtained outstanding average AUC scores (*C-MYC*: 0.90, *HMGA2*: 0.91) and accuracies (*C-MYC*: 95%, *HMGA2*: 88%) in the two gene tests.

The studies investigating the application of DLR for pancreatic cancer are summarized in Table 4.

DLR FOR COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common kind of cancer and the second leading cause of cancer-related fatalities worldwide[1]. It is crucial to carry out research on the diagnosis, treatment response prediction, and survival prediction of CRC, which can improve the prognosis of patients and significantly reduce the social and medical burden. In recent years, promising research results have emerged in the preoperative, intraoperative and postoperative stages of CRC using DLRs technology, covering the entire process of CRC diagnosis and treatment.

DLR is revolutionizing the treatment options for CRC. When making treatment decisions for CRC patients, identifying *KRAS* mutations, which may contribute to the continued proliferation of tumors, can help personalize treatment and care for CRC patients[66]. For preoperative prediction of *KRAS* mutations in patients with CRC, HCR and DLR were merged into a noninvasive model created by Wu *et al*[67]. The model, which mixed the handcrafted and DLR radiomics features, produced a C-index for the original cohort of 0.815 and the validation cohort of 0.832, which was higher than using HCR or DLR alone. For the individualized treatment decision-making in colorectal liver metastases (CRLM) management, the prediction of chemotherapeutic response is crucial. To predict the response to chemotherapy in CRLM, Wei *et al*[68] developed a ResNet10-based DLR model that used contrast-enhanced multidetector CT images as inputs. They also developed an HCR model for comparison. The

Table 4 Summary of studies using deep-learning-based radiomics for pancreatic cancer

Ref.	Imaging	Study design	Study aim	DL model	Dataset	Outcomes
Ziegelmayer *et al*[55], 2020	CT	Retrospective	Identification of PDAC and AIP	VGG19	86 patients (44 AIP patients and 42 PDAC patients)	Sensitivity: 89%; specificity: 83%; AUC: 0.90
Liao *et al*[56], 2022	CT	Retrospective	Identification of PDAC, non-cancerous pancreatic diseases and normal pancreas	CNN	3120 images (1872 for training, 624 for validation, 624 for testing)	Sensitivity: 89.9%; specificity: 91.3%; AUC: 0.960 when distinguishing between PDAC and control group
Tong *et al*[57], 2022	US	Retrospective	Identification of PDAC and CP	ResNet-50	558 patients	AUC: 0.967; sensitivity: 87.2%; specificity: 100%
Watson *et al*[58]	CT	Retrospective	Prediction of pathologic response of PDAC patients to NAC	LeNet	81 patients (65 for training and validation; 16 for testing)	AUC: 0.785; brier score: 0.174; sensitivity: 81.4%; specificity: 60.4% in test set of hybrid deep learning model
Muhammad *et al*[59], 2018	CT	Retrospective	Evaluation of survival hazard of PDAC patients	AlexNet	159 patients	C-index: 0.76; hazard ratio: 9.46 in test set
Zhang *et al*[60], 2020	CT	Retrospective	Evaluation of survival probability of PDAC patients	CNN	520 patients	IPA: 11.81%, C-index: 0.651 in testing cohort
Zhang *et al*[61], 2020	CT	Retrospective	Prediction of OS of PDAC patients; Evaluation of risk scores to distinguish patients with high or low risk	CNN	98 patients (68 in training cohort; 30 in testing cohort)	AUC: 0.81; hazard ratio: 1.86
Zhang *et al*[62], 2021	CT	Retrospective	Prediction of 2-yr OS of resectable PDAC patients	CNN	98 patients (68 in training cohort; 30 in testing cohort)	AUC: 0.84; specificity: 68%; sensitivity: 91%
Yao *et al*[63], 2021	CT	Retrospective	Prediction of survival risk and tumor resection margin of resectable PDAC patients	CNN	205 patients	C-index: 0.705 for survival prediction; balanced accuracy: 73.6%, sensitivity: 81.3%, specificity: 65.9% for resection margin prediction
Yao *et al*[63], 2021	CT	Retrospective	Prediction of survival risk and tumor resection margin of resectable PDAC patients	CNN	1209 patients	C-index: 0.667 for survival prediction; balanced accuracy: 67.1%; sensitivity: 59.8%; specificity: 74.3% for resection margin prediction
An *et al*[64], 2022	CT	Retrospective	Prediction of LNM status and OS in PDAC patients	ResNet-18	148 patients (88 in training cohort, 25 in validation cohort, 35 in testing cohort)	For combined model, AUC: 0.92; accuracy: 86%; sensitivity: 94%; specificity: 78% in testing cohort
Li *et al*[65], 2019	CT	Retrospective	Prediction of *HMGA2* and *C-MYC* gene expression status of PDAC patients;Prediction of survival time of patients	CNN	111 patients	Average AUC score: 0.90; accuracy: 95%; sensitivity: 92%; specificity: 98% in *C-MYC* test with deep features selected by Doctor B; average AUC score: 0.91; accuracy: 88%; sensitivity: 89%; specificity: 88% in *HMGA2* test with deep features selected by Doctor B

CT: Computed tomography; US: Ultrasound; PDAC: Pancreatic ductal adenocarcinoma; AIP: Autoimmune pancreatitis; CP: Chronic pancreatitis; NAC: Neoadjuvant chemotherapy; LNM: Lymph node metastasis; OS: Overall survival; AUC: Area under the receiver operating characteristic curve; C-index: Harrell's concordance index; CNN: Convolutional neural network; IPA: Index of prediction accuracy; DLR: Deep-learning-based radiomics.

DLR model achieved a higher AUC than the HCR model when predicting the response to chemotherapy in CRLM (training: 0.903 *vs* 0.745; validation: 0.820 *vs* 0.598). Microsatellite instability (MSI) function is a predictive biomarker for clinical outcomes and predicts responses to adjuvant 5-fluorouracil and immunotherapy in CRC. A DL model that was created using the MobileNetV2 architecture by Zhang *et al*[69] was adopted to predict the MSI status of CRC based on MR images. With AUC values of 0.868, the best model successfully identified 85.4% of the MSI status, indicating that the suggested model may aid in locating individuals who might benefit from chemotherapy or immunotherapy.

DLR in CRC also emphasizes the need to predict treatment response. For improving NCRT response prediction in locally advanced rectal cancer, Fu *et al*[70] compared the handcrafted and DL features extracted from pre-treatment diffusion-weighted MR images. The DLR approach produced a mean AUC of 0.73, while the HCR method yielded a mean AUC of 0.64, which demonstrated that DLR may achieve higher classification performance compared with HCRs. To predict the distant metastasis in

locally advanced rectal cancer patients receiving NCRT, Liu *et al*[71] exploited the use of a DLR model based on MR images. DLR achieved a C-index of 0.747 and AUC of 0.894 at 3 years. In order to define tumor morphological change for response evaluation in patients with metastatic CRC, Lu *et al*[72] offered a DLR study using CNN and recurrent neural network. They discovered that the DL network performed better than the size-based equivalent with C-index (0.649 *vs* 0.627), and was capable of predicting the early on-treatment response in metastatic CRC. The predictive performance could be improved by the integration of DL network with size-based methodology.

LN metastasis, which is a key prognostic factor for CRC, is among the other study topics of CRC. Ding *et al*[73] adopted a DLR nomogram based on faster region-based CNN (Faster R-CNN) to predict LN metastasis in patients with CRC. Patient age, Faster R-CNN-detected LN metastasis, and tumor differentiation were predictors in the Faster R-CNN nomogram for predicting LN metastasis, with AUCs in the training and validation sets of 0.862 and 0.920, respectively. Zhao *et al*[74] applied a DLR model related with genomics phenotypes for predicting LN metastasis in CRC and showed good performance with AUCs of 0.81, 0.77, and 0.73 in the training, testing and validation sets, respectively. Li *et al*[75] examined the performance of the three most popular classification techniques-DL, conventional machine learning, and deep transfer learning-to determine the most efficient way for automatic classification of CRC LN metastases. Deep transfer learning was the most successful, with an accuracy of 0.7583 and AUC of 0.7941. All of these studies have shown that DLR technology has good performance in the prediction and classification of LN metastasis.

The studies exploring the creation of DLR for CRC are summarized in Table 5.

CHALLENGES AND RECOMMENDATIONS

In the past several years, with the development of DL technology, the research and application of DLR in tumor diagnosis, treatment and prognosis have been increasing. To perform a systematic evaluation of the status of DLRs for GI cancer, we conducted an extensive review on all original publications between January 1, 2015, and August 30, 2022. Even though several published articles have confirmed the exceptional performance of DLR, there are still many issues that algorithm designers and doctors must address. Below is a list of the challenges and recommendations for DLR in future research summarized by our team.

Prospective and multi-center studies

According to the most recent research, most studies on DLR were retrospective and single center. Retrospective studies may have sample selection bias and cannot truly reflect the distribution of clinical cases, which could jeopardize the precision of DLR models. As different centers have different machine parameters, scanning settings, and diagnostic rules, single-center studies limit the generalization of the DLR models. Prospective and multi-center studies can evaluate the reliability and accuracy of the DLR models, enhance their generalization, and bridge the gap between academic studies and clinical applications. Thus, carrying out prospective and multi-center studies is the key to accelerating the clinical application of DLRs models.

Development of user-friendly DL models

We found that many physicians do not really want to use DLR methods for related research because the models usually have complex structures, large parameters, poor interpretability, non-existence of gradients, overfitting, and other problems, which limit the promotion and use of DLR technology. Therefore, it is necessary to develop simple and user-friendly models and training schemes for non-professional users. Publication of more source codes and pre-training weights are ways to reduce the development and training difficulty of DL models. For overfitting problems, development of automatic data augmentation schemes and image synthesis schemes can increase the amount of training data. For the black box nature of DL models, attention maps and network dissection schemes can be integrated into the model to improve interpretability.

Establishment of accessible datasets

For DLR, the dataset is the new oil. DLR analysis requires a large amount of data to train and validate models; however, most studies are based on private datasets and do not use uniform construction standards, which will hinder the reproducibility of the studies and deployment of DLR models. Thus, a professional data development organization that combines multi-center data should be established. The organization should also standardize the development process of multiple kinds of datasets and make the datasets publicly accessible. Additionally, to reward data contributors, researchers who use these datasets could charge appropriate fees.

Efficient fusion of multiple features

DLR is a new technology in the field of AI for medical image analysis. Although its performance is satisfactory, it is not a panacea, especially in the case of extreme shortage of data. Numerous studies

Table 5 Summary of studies using deep-learning-based radiomics for colorectal cancer

Ref.	Imaging	Study design	Study aim	DL model	Dataset	Outcomes
Wu *et al*[67], 2020	CT	Retrospective	Predicting *KRAS* status in patients with CRC	CNN	Primary cohort: 279 patients; validation cohort: 119 patients	C-index of 0.815 for the primary cohort and 0.832 for the validation cohort
Wei *et al*[68], 2021	CT	Retrospective	Predicting the response to chemotherapy in CRLM	ResNet10	192 patients	AUC of DLR: 0.820; AUC of HCR: 0.598
Zhang *et al* [69], 2021	MRI	Retrospective	Predicting the MSI status of CRC	MobileNetV2	491 patients	Accuracy: 85.4%; AUC: 0.868
Fu *et al*[70], 2020	MRI	Retrospective	Predicting NCRT response in patients with LARC	VGG19	43 patients	AUC of DLR: 0.73; AUC of HCR: 0.64
Liu *et al*[71], 2021	MRI	Retrospective	Predicting the distant metastasis of LARC patients receiving NCRT	ResNet18	235 patients	C-index of 0.747 and AUC of 0.894 in the validation cohort
Lu *et al*[72], 2021	CT	Retrospective	Prediction of early on-treatment response in mCRC	CNN + RNN	1028 patients	C-index: 0.649
Ding *et al* [73], 2020	MRI	Retrospective	Prediction of metastatic LN in CRC	Faster RCNN	545 patients	AUC for training: 0.862; AUC for validation: 0.920
Zhao *et al* [74], 2022	CT	Retrospective	Prediction of metastatic LN in CRC	Autoencoder	423 patients	AUC for training: 0.81; AUC for validation: 0.73; AUC for testing: 0.77
Li *et al*[75], 2020	MRI	Retrospective	Classification of CRC LN Metastasis images	AlexNet	3364 samples (1646 positive; 1718 negative)	Accuracy: 75.83%; AUC: 0.7941

CRC: Colorectal cancer; CT: Computed tomography; MRI: Magnetic resonance imaging; CRLM: Colorectal liver metastases; MSI: Microsatellite instability; NCRT: Neoadjuvant chemoradiotherapy; LARC: Locally advanced rectal cancer; mCRC: Metastatic colorectal cancer; LN: Lymph node; CNN: Convolutional neural network; RNN: Recurrent neural network; AUC: Area under the receiver operating characteristic curve; C-index: Harrell's concordance index; DLR: Deep-learning-based radiomics; HCR: Handcrafted radiomics; Faster R-CNN: Faster region-based convolutional neural network.

have demonstrated that combining HCR and DLR, can result in better performance. Thus, we suggest integrating other clinical features, genomics, handcrafted features, and DL features to build an optimal solution. Moreover, a suitable feature dimensionality reduction scheme should also be adopted to reduce the redundancy of the integrated features. In addition to imaging features, features extracted from clinical data sources, such as gene expression, clinical characteristics, and blood biomarkers, can also be combined to enhance radiomic features.

CONCLUSION

Globally, GI cancers account for a large portion of cancer-related fatalities. For the diagnosis and treatment of GI cancer, DLR can offer a simpler, quicker and more reliable approach. This article is the first comprehensive review on DLR in the GI tract. The status, difficulties, and suggestions discussed in this review can help engineers create optimal radiomics products to support clinical decision-making and offer guidance for diagnosis and treatment of other tumors. Despite the success of DLR in GI cancer, prospective and multi-center studies are still needed. Development of user-friendly DL models, the creation of large public databases, and the fusion of multiple features are also necessary to encourage the clinical application of radiomics.

FOOTNOTES

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ORIGINAL ARTICLE

Observational Study

Best therapy for the easiest to treat hepatitis C virus genotype 1b-infected patients

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Abstract

BACKGROUND

The revolution in treatment of patients with chronic hepatitis C virus (HCV) infection dates back to the introduction of direct-acting antivirals (DAAs). The increase in efficacy was most pronounced in patients infected with genotype (GT) 1b, as this was the most poorly responsive population to treatment during the interferon era.

AIM

To identify the most effective interferon-free therapy for GT1b-infected patients and to determine positive and negative predictors of virological response.

METHODS

This real-world retrospective analysis included patients chronically infected with GT1b HCV whose data were obtained from the multicenter observational EpiTer-2 database. Treatment effectiveness was evaluated for each therapeutic regimen as the percentage of sustained virological responses (SVR). Assessment of the safety was based on the evaluation of the course of therapy, the occurrence of adverse events including serious ones, deaths during treatment and in the post 12-wk follow-up period.

RESULTS

The studied population consisted of 11385 patients with a mean age of 53 ± 14.8 years and a female predominance (53.4%). The majority of them were treatment-naïve (74.6%) and patients with cirrhosis accounted for 24.3%. Of the DAA regimens used, 76.9% were GT-specific with ombitasvir/paritaprevir/ritonavir + dasabuvir ± ribavirin being the most used option (32.4%). A total of 10903 patients responded to treatment resulting in a 98.1% in the per-protocol analysis after excluding 273 patients without SVR data. The effectiveness of all regimens exceeded 90% and the highest SVR of 98.9% was achieved in patients treated with a combination of glecaprevir/pibrentasvir. Logistic regression analyses showed that the virologic response was independently associated with female sex [odds ratio (OR) = 1.67], absence of decompensated cirrhosis at baseline (OR = 2.42) and higher baseline platelets (OR = 1.004 per 1000/μL increase), while the presence of human immunodeficiency virus (HIV) coinfection significantly decreased the odds of response (OR = 0.39). About 95%-100% of patients completed therapy irrespective of the drug regimen. At least one adverse effect occurred in 10.9%-36.3% and most of them were mild. No treatment related deaths have been reported.

CONCLUSION

We documented very high effectiveness and a good safety profile across all DAA regimens. Positive predictors of SVR were female sex, absence of decompensated cirrhosis at baseline and higher platelet count while HIV coinfection reduced the effectiveness.

Key Words: Hepatitis C; Genotype 1b; Direct-acting antivirals; Pangenotypic; Genotype-specific; Sustained virologic response

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Core Tip: Our real-world analysis focused on a population of patients chronically infected with the genotype 1b of hepatitis C virus. In the interferon era, this was considered to be difficult to treat due to low cure rates. Although we documented the very good effectiveness of all interferon-free regimens, we have shown that in what is now described as the easiest to treat population, the chance of response to antiviral treatment is reduced by coinfection with human immunodeficiency virus, while female sex, absence of decompensated cirrhosis and higher platelets count at baseline, increase the chances of a cure.

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INTRODUCTION

The revolution in antiviral treatment of chronic infection with hepatitis C virus (HCV) dates to 2011 when direct-acting antivirals (DAA) were introduced into therapeutic regimens. The first registered drugs in this group were used in combination with an existing regimen containing pegylated interferon (pegIFN) and ribavirin (RBV) and their activity was limited to HCV genotype (GT) 1 infections[1]. Evaluated in the retrospect, it seems that it is patients infected with HCV GT1 who have become the beneficiaries of this therapeutic revolution.

GT1 infections are nearly equally divided into subtypes 1a and 1b, are the most common worldwide accounting for about 49% of all cases of chronic hepatitis C (CHC), which, according to the latest data, is estimated at 58 million people[2-4]. With the highest global prevalence, GT1 infection is the leading cause of the most severe complications of CHC, such as cirrhosis and hepatocellular carcinoma (HCC) in the chronically HCV-infected population. It was therefore of great public health importance that GT1-infected patients were also the first to gain access to highly effective and safe IFN-free regimens, termed GT-specific due to their activity against GT1 and GT4 only[5]. As a result, the treatment efficacy of GT1-infected patients increased to 95% and above compared to the approximately 50% efficacy of pegIFN + RBV therapy[6]. It is additionally worth noting that pegIFN + RBV therapy was less effective against HCV subtype 1b than subtype 1a, and this difference was independent of other factors that may favor viral clearance[7]. At the same time, for patients with GT3 infection, the second most common HCV-infected population with the share of 18%, the only IFN-free regimen available was a combination of sofosbuvir (SOF) + RBV with a suboptimal efficacy of 75% comparable to that achieved with pegIFN + RBV therapy[5,6]. In this way the population of GT1-infected individuals defined as "difficult to treat" in the IFN era became "easy to treat" in the time of DAA therapies. And conversely to what happened in the IFN era among patients with GT1 infection, the best treatment results with DAA were obtained in those infected with subtype 1b, who can therefore be called the "easiest to treat". This is due to resistance associated substitutions with the potential to reduce efficacy which are more common in the subtype 1a[8].

The differences in the treatment efficacy between GT1 and GT3-infected patients have balanced out with the registration of pangenotypic regimens with documented activity against all HCV GTs[9,10]. The purpose of this analysis was to evaluate the effectiveness and safety of antiviral treatment in patients with GT1b infection from real-world experience (RWE) settings since the beginning of the IFN-free era with the identification of the most effective type of therapy and to identify positive and negative predictors that affect the likelihood of virological response in this "easiest to treat" but most numerous populations among HCV-infected patients.

MATERIALS AND METHODS

Study population

Patients chronically infected with GT1b HCV, whose data were obtained from the EpiTer-2 database, were included in this analysis. EpiTer-2 is an ongoing real-world multicenter observational project evaluating antiviral treatment of CHC since the beginning of the availability of IFN-free therapy in Poland. From mid-2015 to the end of 2021, 15156 patients in 22 Polish hepatology centers started DAA therapy and their data were retrospectively entered into an online questionnaire administered by TIBA. The study is supported by the Polish Association of Epidemiologists and Infectiologists.

The choice of the IFN-free regimen by the treating physician, as well as the monitoring of treatment, were guided by the recommendations of the Polish Group of Experts of HCV and the regulations on drug reimbursement under the drug program of the National Health Fund. Patients provided informed consent for treatment and the processing of personal data as required by the therapeutic program and national regulations[11,12]. Due to the observational, non-interventional nature of the study using approved drugs, ethics committee approval was waived in accordance with local law (Pharmaceutical Law of 6th September 2001, art. 37al).

The database includes patients' data, such as sex, age, body mass index (BMI), HCV GT, liver disease severity, current DAA regimen, history of previous therapy, human immunodeficiency virus (HIV) and HBV co-infection, comorbidities and comedications, and baseline laboratory test results, including

aminotransferases activity, international normalized ratio, complete blood count, bilirubin, albumin and creatinine concentration.

Liver disease severity

Assessment of liver disease advancement included measurement of liver stiffness using non-invasive elastography with Fibroscan or Aixplorer with correlating METAVIR fibrosis values[13]. Patients with a fibrosis value of F4 were evaluated for the presence of esophageal varices, decompensation of liver function in the past and at the start of antiviral therapy and were assessed on the Child-Pugh scale. Data were also collected on the diagnosis of HCC and past liver transplantation.

Antiviral treatment

GT-specific regimens include asunaprevir (ASV) + daclatasvir (DCV), ledipasvir (LDV)/SOF ± RBV, ombitasvir (OBV)/paritaprevir (PTV)/ritonavir + dasabuvir (r + DSV) ± RBV, grazoprevir (GZR)/elbasvir (EBR) ± RBV, and others which included SOF, DCV, simeprevir used in various combination with or without RBV. Pangenotypic regimens include the combination of SOF and velpatasvir (VEL), voxilaprevir (VOX) with SOF/VEL, glecaprevir (GLE) and pibrentasvir (PIB), and GLE/PIB plus SOF and RBV.

Treatment effectiveness

Data on HCV RNA measured by reverse transcriptase-polymerase chain reaction with a lower limit of detection 10 IU/mL assessed at baseline, at the end of therapy and 12 wk after the treatment completion were collected. Based on these, the effectiveness of the treatment which is a sustained virological response (SVR), is determined and defined as undetectable HCV RNA 12 wk after the end of therapy. Patients lost to follow-up without HCV RNA evaluation 12 wk after the end of therapy were considered non-virological failures, while those with detectable viral load at this time point were considered virological failures.

Treatment safety

During therapy and up to 12 wk after its completion, data were collected on the treatment course, incidence of adverse events (AEs) with an assessment of their severity, and deaths with an assessment of their relation to antiviral therapy.

Statistical analysis

The results are expressed as mean ± SD or *n* (%). *P* values of < 0.05 were considered to be statistically significant. The significance of difference was calculated by Fisher's exact or chi square tests for nominal variables and by Mann-Whitney *U* test for continuous and ordinal variables. Forward stepwise logistic regression models with Bayesian Information Criterion as a model selection criterion were performed with virological response as the dependent variable. Baseline independent variables were selected by significance in univariate analyses and included sex, fibrosis advancement, coinfection with HIV, liver cirrhosis decompensation at baseline, the history of cirrhosis decompensation, HCC, therapy, BMI, serum albumins, bilirubin, alanine aminotransferase (ALT), and platelets. Logistic regression models were calculated by use of Statistica 13.0 (TIBCO Software Inc., Palo Alto, CA, United States).

RESULTS

Patients' characteristics

The population consisted of 11385 GT1b-infected patients with a mean age of 53 ± 14.8 years and a female predominance (53.4%). Detailed characteristics of patients are presented in Table 1. The majority of them had comorbidities with the most prevalent arterial hypertension (35.2%) followed by diabetes (12.7%), 60.1% were under pharmacological treatment for coexisting diseases. The largest group in terms of fibrosis was F1 patients who accounted for 39% of the analyzed population, patients with F4 corresponding to cirrhosis accounted for nearly a quarter of the cohort. The majority of them were compensated, 13.1% were scored as B and 0.1% as C in CP scale (Table 2). Hepatic decompensation at baseline in the form of ascites and encephalopathy was reported in 5.9% and 0.3% of them, respectively. Eight hundred and sixty (31%) patients had documented esophageal varices.

Antiviral treatment

Three-quarters of the studied patient population was treatment-naive; among those who had been previously treated, nonresponse was the most common type of treatment failure (Table 3). The most frequently used prior regimen was the combination of pegIFN + RBV, 155 patients (1.4%) were unsuccessfully treated with IFN-free options. Of the currently used regimens, 76.9% were GT-specific, with OBV/PTV/r + DSV ± RBV being the most commonly used option (32.4%). The GLE/PIB combination was the most frequently administered (14.8%) among the pangenotypic therapies. Twelve

Table 1 Baseline characteristics of 11385 genotype 1b-infected patients treated with interferon-free regimens

Parameter	GT1b patients, *n* = 11385
Sex, female/male, *n* (%)	6085 (53.4)/5300 (46.6)
Age in yr, mean ± SD; min-max	53 ± 14.8; 17-97
Females	54.8 ± 15.1; 18-92
Males	51 ± 14.3; 17-97
BMI, kg/m^2 mean ± SD; min-max	26.3 ± 4.5; 13.2-57.4
Comorbidities, *n* (%)	
Any comorbidity	7280 (63.9)
Hypertension	4013 (35.2)
Diabetes	1447 (12.7)
Renal disease	470 (4.1)
Autoimmune diseases	251 (2.2)
Non-HCC tumors	237 (2.1)
Other	5330 (46.8)
Concomitant medications, *n* (%)	6842 (60.1)
Liver fibrosis *n*(%)	
F0	237 (2.1)
F1	4440 (39)
F2	2135 (18.8)
F3	1587 (13.9)
F4	2768 (24.3)
No data	218 (1.9)
HCC history, *n* (%)	175 (1.5)
OLTx history, *n* (%)	122 (1.1)
HBV coinfection (HBsAg+), *n* (%)	106 (0.9)
HIV coinfection, *n* (%)	359 (3.1)
ALT IU/L, mean ± SD	75.8 ± 65.5
Bilirubin mg/dL, mean ± SD	0.8 ± 0.9
Albumin g/dL, mean ± SD	4.3 ± 3.2
Creatinine mg/dL, mean ± SD	0.9 ± 1.8
Hemoglobin g/dL, mean ± SD	14.4 ± 1.8
Platelets × 1000/μL, mean ± SD	195.3 ± 77.8
HCV RNA × 10^6 IU/mL, mean ± SD	2.1 ± 4.7

ALT: Alanine transaminase; BMI: Body mass index; F: Fibrosis; GT: Genotype; HBsAg+: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; IFN: Interferon; OLTx: Orthotopic liver transplantation; SD: Standard deviation.

patients were treated with triple pangenotypic regimens, three with GLE/PIB + SOF + RBV and nine with VOX/VEL/SOF, and all of them responded to treatment. In all cases, it was a rescue therapy used after prior DAA failure.

Treatment effectiveness

A total of 10903 patients responded to antiviral treatment, resulting in a 98.1% cure rate in the per protocol (PP) analysis after excluding 273 patients without SVR data. The effectiveness of different treatment regimens varied; in the PP analysis, the lowest 90.2% was reported for the GT-specific ASV +

Table 2 Genotype 1b-infected patients with liver cirrhosis treated with direct-acting antivirals

Parameter	GT1b patients, *n* = 2768
History of hepatic decompensation, *n* (%)	
Ascites	322 (11.6)
Encephalopathy	87 (3.1)
Documented esophageal varices, *n* (%)	860 (31)
Hepatic decompensation at baseline, *n* (%)	
Ascites - moderate responded to diuretics and tense	164 (5.9)
Encephalopathy	72 (0.3)
Child-Pugh, *n* (%)	
B	362 (13.1)
C	20 (0.1)
No data	223 (8.1)

GT: Genotype.

DCV regimen, while the highest effectiveness of 98.9% was achieved in the group of patients treated with GLE/PIB pangenotypic option (Table 4, Figure 1). The significantly higher percentage of men, patients with higher BMI, HIV co-infection and those taking concomitant medications were reported in virologic non- responders compared to patients with SVR (Table 5).

This population also documented a lower proportion of patients with F1 fibrosis and a higher proportion of those with F4 fibrosis, patients with a history of decompensated liver function, as well as those with the presence of esophageal varices and decompensation at baseline. Statistically significant differences were also documented for laboratory test results consistent with advanced liver disease such as platelet count, bilirubin and albumin levels.

Virologic responders were more likely to be treatment-naive and significantly more frequently received the GLE/PIB regimen, while the proportion of ASV + DCV and SOF/VEL ± RBV were lower. There was no statistically significant difference in SVR by type of therapy, GT-specific *vs* pangenotypic. Logistic regression analyses showed that virologic response in GT1b-infected patients was independently associated with female sex [odds ratio (OR) = 1.67], absence of cirrhosis decompensation at baseline (OR = 2.42) and higher baseline platelets (OR = 1.004 per 1000/μL increase), while the presence of HIV coinfection significantly decreased the odds of response (OR = 0.39) (Table 6). Importantly, there was no association with liver fibrosis, history of prior therapy, BMI or serum ALT, albumin and bilirubin levels.

Treatment safety

Regardless of the drug regimen used, the vast majority of patients (95.3%-100%) completed therapy as planned (Table 7). At least one AE occurred in 10.9%-36.3% of patients, with the highest percentage in those treated with ASV + DCV. This population also experienced the highest rate of serious AEs (SAEs) and discontinuation of therapy due to AEs, 5.2% and 1.5%, respectively. Among 7 SAEs reported during ASV + DCV therapy, two events were described as a transient elevation of aminotransferases activity; the remaining SAEs were not related to liver disease and antiviral therapy. In one person with elevated aminotransferases activity, the ASV + DCV was discontinued for this reason at week 20th but finally this patient achieved an SVR. There were 64 deaths in the entire study population, none of which were judged by the treating physician to be related to antiviral treatment.

DISCUSSION

In our work, we analyzed a sizeable real-world population of patients infected with GT1b which is the most common HCV GT in Europe and in Poland accounts for about 82% of all cases of CHC[2,14]. Dominating among HCV-infected patients, GT1b is also mainly responsible for the most severe complications of the disease, thus posing a public health burden which is why this population came under our attention.

We followed a cohort of 11385 patients from the beginning of the IFN-free era to the final stage which was the introduction of pangenotypic therapies using the retrospective national multicenter EpiTer-2 database. DAA therapy was effective in 98% of the analyzed population demonstrating what a huge

Table 3 Treatment characteristics of genotype 1b-infected patients treated with direct-acting antiviral

Parameter	GT1b patients, *n* = 11385
History of previous therapy	
Treatment-naïve	8497 (74.6)
Non-responder	949 (8.3)
Relapser	831 (7.3)
Discontinuation due to safety reason	338 (3)
Unknown type of response	728 (6.4)
No data	42 (0.4)
Previous treatment regimen in treatment-experienced, *n* = 2846	
IFN ± RBV	149 (5.2)
PegIFN ± RBV	2080 (73.1)
IFN + RBV + DAA	438 (15.4)
DAA	155 (5.4)
No data	24 (0.9)
Genotype-specific treatment regimens	
ASV + DCV	135 (1.2)
LDV/SOF ± RBV	2652 (23.3)
OBV/PTV/r + DSV ± RBV	3685 (32.4)
GZR/EBR ± RBV	2270 (19.9)
Other (SOF ± SMV ± DCV ± RBV, SMV ± DCV ± RBV)	16 (0.1)
Pangenotypic regimens	
GLE/PIB	1684 (14.8)
GLE/PIB + SOF + RBV	3 (< 0.1)
SOF/VEL ± RBV	931 (8.2)
VOX/VEL/SOF	9 (0.1)

ASV: Asunaprevir; DAA: Direct-acting antiviral; DCV: Daclatasvir; DSV: Dasabuvir; EBR: Elbasvir; GLE: Glecaprevir; GT: Genotype; GZR: Grazoprevir; IFN: Interferon; LDV: Ledipasvir; OBV: Ombitasvir; PegIFN: Pegylated interferon; PIB: Pibrentasvir; PTV/r: Paritaprevir; RBV: Ribavirin; SMV: Simeprevir; SOF: Sofosbuvir; VEL: Velpatasvir; VOX: Voxilaprevir.

leap in treatment effectiveness has made in these patients compared to the IFN era when SVR was around 50%. Such a result supports our thesis that this is currently the easiest way to treat the HCV-infected population and is in line with the results of many RWE analyses on populations with GT1 infection[15,16]. However, it should be noted, that there are several papers that concentrate exclusively on GT1b-infected patients, and furthermore, some of them analyze only a specific DAA regimen[17-21]. Focusing on patients with GT1b infection with the inclusion of all registered treatment regimens since the beginning of the DAA era in the analysis makes our study unique.

Regardless of which therapeutic options patients were treated with, effectiveness exceeded 90% and was higher than 98% for most regimens. The lowest SVR rate was documented for the GT-specific ASV + DCV regimen which was the DAA option registered only for patients with GT1b infection and available in Poland until 2018. However, it should be noted that the effectiveness of 90.2% we achieved in Polish GT1b-infected patients using this therapeutic option was higher than the SVR of 85% documented in a meta-analysis in nine clinical trials[22].

The highest success rate among widely used regimens of 98.9% was achieved in patients treated with the pangenotypic option, GLE/PIB. Univariate analysis confirmed a significantly higher proportion of patients treated with this regimen among responders compared to virologic failures (P = 0.01). Our findings regarding the very high effectiveness of GLE/PIB are consistent with the results of many RWE analyses and clinical trials documenting efficacy of this regimen at 99%-100%[23-25]. Despite the disparity between the two regimens discussed above, we did not show a statistically significant difference in SVR overall between GT-specific and pangenotypic options. This finding is consistent with

Table 4 The overall treatment effectiveness and according to treatment regimens in relation to different risk factors among the studied patients (per protocol analysis)

Parameter	Whole population	Females	Males	F4	T-ex	History of hepatic decompensation	≥ 70-year-old	eGFR ≤ 60 mL/min	Obese[1]	HIV+
All regimens	10903/11112 (98.1)	5891/5969 (98.7)	5012/5143 (97.5)	2590/2678 (96.7)	2726/2795 (97.5)	319/336 (94.9)	1490/1509 (98.7)	281/284 (98.9)	1880/1923 (97.8)	327/341 (95.9)
ASV + DCV	119/132 (90.2)	72/79 (91.1)	47/53 (88.7)	47/55 (85.5)	40/47 (85.1)	0/1 (0)	22/24 (91.7)	5/5 (100)	26/28 (92.9)	1/1 (100)
LDV/SOF ± RBV	2534/2582 (98.1)	1381/1398 (98.8)	1153/1184 (97.4)	679/707 (96)	680/696 (97.7)	173/181 (95.6)	386/389 (99.2)	67/67 (100)	446/459 (97.2)	103/107 (96.3)
OBV/PTV/r + DSV ± RBV	3563/3627 (98.2)	1949/1973 (98.8)	1614/1654 (97.6)	969/992 (97.7)	1490/1516 (98.3)	72/74 (97.3)	397/403 (98.5)	80/82 (97.6)	602/611 (98.5)	26/28 (92.9)
GZR/EBR ± RBV	2179/2216 (98.3)	1201/1215 (98.8)	978/1001 (97.7)	362/372 (97.3)	291/297 (98)	19/20 (95)	397/401 (99)	87/88 (98.9)	380/389 (97.7)	48/48 (100)
GLE/PIB	1625/1643 (98.9)	869/874 (99.4)	756/769 (98.3)	264/268 (98.5)	112/114 (98.2)	12/12 (100)	147/150 (98)	24/24 (100)	267/267 (100)	72/76 (94.7)
GLE/PIB + SOF + RBV	3/3 (100)	1/1 (100)	2/2 (100)	1/1 (100)	3/3 (100)	-	-	-	1/1 (100)	-
SOF/VEL ± RBV	857/884 (96.9)	409/419 (97.6)	448/465 (96.3)	260/275 (94.5)	91/101 (90.1)	40/44 (90.9)	138/139 (99.3)	16/16 (100)	158/167 (94.6)	74/78 (94.9)
VOX/VEL/SOF	9/9 (100)	2/2 (100)	7/7 (100)	3/3 (100)	7/7 (100)	-	3/3 (100)	-	-	3/3 (100)

[1]Body mass index > 30 kg/m^2.

Data are presented as number/total number of patients (percentage). ASV: Asunaprevir; DCV: Daclatasvir; DSV: Dasabuvir; EBR: Elbasvir; eGFR: Estimated glomerular filtration rate; F: Fibrosis; GLE: Glecaprevir; GZR: Grazoprevir; HIV: Human immunodeficiency virus; LDV: Ledipasvir; OBV: Ombitasvir; PIB: Pibrentasvir; PP: Per protocol; PTV/r: Paritaprevir; RBV: Ribavirin; SOF: Sofosbuvir; SVR: Sustained virological response; T-ex: Treatment-experience; VEL: Velpatasvir; VOX: Voxilaprevir.

the results of another Polish RWE analysis evaluating GT-specific *vs* pangenotypic regimens in GT1b-infected patients and according to our best knowledge there are no other analyses of a comparative nature[18]. We are aware that our study does not compare the two types of regimens head-to-head, it is a retrospective analysis and the results of comparison in this area should be approached with caution. However, considering the clinical trials and RWE studies evaluating the effects of therapy in GT1b-infected patients with particular therapeutic regimens, belonging to both GT-specific and pangenotypic options, it should be concluded that our results are comparable to them. The largest group in our analysis were patients treated with the OBV/PTV/r + DSV ± RBV regimen, the first available DAA option in our country[26]. This means that it was used in patients at the beginning of the IFN-free era. Based on our previous study on the change in profile of patients in EpiTer-2 database, we can assume that they were older, more likely to have been diagnosed with cirrhosis, treatment-experienced, and had a greater burden of comorbidities, that is, more difficult to treat, compared to patients further down the DAA availability timeline[27]. The excellent 98.2% effectiveness of this regimen demonstrated by us supports the findings of numerous clinical trials and RWE analyses carried out in GT1b-infected patients[15-17,28,29]. We demonstrated in patients treated with LDV/SOF ± RBV, almost identical effectiveness. Based on the clinical trial results, this combination could be used even in patients with decompensated liver cirrhosis[30]. Other RWE studies reported lower effectiveness of LDV/SOF ± RBV at the level of 93%-96% compared to 98.1% achieved in the current analysis but this difference may be related to baseline patient characteristics[15,31,32]. The list of GT-specific options in our study is completed by the GZR/EBR combination, the last regimen from this group in terms of timeline availability, with the excellent SVR of 98.3%. This is almost the same effectiveness as documented in a meta-analysis of eight randomized clinical trials with GZR/EBR[33]. Patients infected with GT1b achieved an SVR of 98.4 while those with GT1a infection responded 95.7% supporting the statement of the easiest to treat HCV population. A slightly lower success rate of 97.3% was reported in the cohort of United States veterans treated with GZR/EBR, while Korean patients responded at a rate of 98.6%, comparable to our analysis[21,34].

Among the currently available regimens for GT1b-infected patients, we documented the lowest SVR of 96.9% for the pangenotypic option SOF/VEL ± RBV. This combination is registered for use in any stage of liver disease including patients with decompensated cirrhosis which may have influenced our results[35]. In addition, we did not separately analyze the regimen without and with RBV which could also have affected the total result obtained[35,36].

Table 5 The comparison of virological responders and non-responders to direct-acting antiviral therapy

Parameter	Responders, *n* = 10903	Non-responders, *n* = 209	*P* value
Sex, females/males, *n* (%)	5891 (54)/5012 (46)	78 (37.3)/131 (62.7)	< 0.001
Age in yr, mean ± SD; min-max	52.9 ± 14.8; 17-97	53.4 ± 13; 25-84	0.69
BMI, kg/m^2 mean ± SD; min-max	26.3 ± 4.5; 13.2-57.4	27 ± 4.6; 15.4-48	0.005
Genotype-specific treatment regimens, *n* (%)			
ASV + DCV	119 (1.1)	13 (6.2)	< 0.001
LDV/SOF ± RBV	2534 (23.2)	48 (22.9)	0.99
OBV/PTV/r + DSV ± RBV	3563 (32.6)	64 (30.7)	0.58
GZR/EBR ± RBV	2179 (20)	37 (17.7)	0.46
Other (SOF ± SMV ± DCV ± RBV, SMV ± DCV ± RBV)	14 (0.1)	2 (1)	0.03
Pangenotypic regimens, *n* (%)			
GLE/PIB	1625 (14.9)	18 (8.6)	0.01
GLE/PIB + SOF + RBV	3 (< 0.1)	0	0.06
SOF/VEL ± RBV	857 (7.8)	27 (12.9)	0.01
VOX/VEL/SOF	9 (0.2)	0	0.42
Regimen, *n* (%)			0.65
Genotype-specific	8409 (77.1)	164 (80.5)	
Pangenotypic	2494 (22.9)	45 (21.5)	
Comorbidities, *n* (%)			
Any comorbidity	6958 (63.8)	138 (65.7)	0.55
Hypertension	3842 (35.2)	75 (35.7)	0.90
Diabetes	1369 (12.6)	24 (11.4)	0.72
Renal disease	446 (4.1)	7 (3.3)	0.71
Autoimmune diseases	240 (2.2)	4 (1.9)	0.96
Non-HCC tumors	214 (2)	6 (2.9)	0.49
Other	5085 (46.6)	106 (50.5)	0.27
Concomitant medications, *n* (%)	6517 (59.8)	141 (67.5)	0.03
Liver fibrosis, *n* (%)			
F0	229 (2.1)	2 (1)	0.37
F1	4308 (39.5)	50 (23.9)	< 0.001
F2	2054 (18.8)	38 (18.2)	0.88
F3	1522 (14)	25 (12)	0.47
F4	2590 (23.8)	88 (42.1)	< 0.001
No data	200 (1.8)	6 (2.9)	0.40
History of previous therapy, *n* (%)			
Treatment-naïve	8138 (74.6)	140 (67)	0.01
Non-responder	908 (8.3)	24 (11.5)	0.13
Relapser	803 (7.4)	21 (10)	0.18
Discontinuation due to safety reason	320 (2.9)	9 (4.3)	0.34
Unknown type of response	695 (6.4)	15 (7.2)	0.74
No data	39 (0.4)	0	0.78

Previous DAA treatment	120 (1.2)	18 (9)	< 0.001
History of hepatic decompensation, *n* (%)			
Ascites	286 (2.6)	14 (6.7)	< 0.001
Encephalopathy	75 (0.7)	7 (3.3)	< 0.001
Documented esophageal varices, *n* (%)	778 (7.1)	38 (18.2)	< 0.001
Hepatic decompensation at baseline, *n* (%)			
Ascites	136 (1.3)	10 (4.8)	< 0.001
Encephalopathy	58 (0.5)	6 (2.9)	< 0.001
HCC history, *n* (%)	150 (1.4)	8 (3.8)	0.01
OLTx history, *n* (%)	117 (1.1)	2 (0.9)	0.86
Child-Pugh, *n* (%)			
B	306 (2.8)	23 (11)	< 0.001
C	15 (0.1)	0	0.67
HBV coinfection (HBsAg+), *n* (%)	102 (0.9)	2 (0.9)	0.74
HIV coinfection, *n* (%)	327 (3)	14 (6.7)	0.004
ALT IU/L, mean ± SD	75.6 ± 65.3	85.5 ± 71.1	0.005
Bilirubin mg/dL, mean ± SD	0.8 ± 0.8	1 ± 0.7	< 0.001
Albumin g/dL, mean ± SD	4.3 ± 3.3	4.1 ± 2.8	< 0.001
Creatinine mg/dL, mean ± SD	0.9 ± 1.9	0.8 ± 0.2	0.93
Hemoglobin g/dL, mean ± SD	14.4 ± 1.8	14.3 ± 1.9	0.71
Platelets, × 1000/μL, mean ± SD	196.5 ± 77.4	160.6 ± 73.3	< 0.001
HCV RNA × 10^6 IU/mL, mean ± SD	2.1 ± 4.8	2.2 ± 2.8	0.02

ALT: Alanine transaminase; ASV: Asunaprevir; BMI: Body mass index; DAA: Direct-acting antiviral; DSV: Dasabuvir; DCV: Daclatasvir; EBR: Elbasvir; F: Fibrosis; GLE: Glecaprevir; GZR: Grazoprevir; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; LDV: Ledipasvir; OBV: Ombitasvir; OLTx: Orthotopic liver transplantation; PIB: Pibrentasvir; PTV/r: Paritaprevir; RBV: Ribavirin; SD: Standard deviation; SOF: Sofosbuvir; SMV: Simeprevir; VEL: Velpatasvir; VOX: Voxilaprevir.

Twelve patients in our analysis were treated with triple pangenotypic regimens, GLE/PIB + SOF + RBV (*n* = 3) and VOX/VEL/SOF (*n* = 9) with 100% effectiveness. In each case, it was a rescue therapy used after prior DAA failure according to the guidelines[11,12,37,38]. Recommendations for the use of both regimens in DAA non- responders are based on the results of clinical trials documenting efficacy of 96%-100% of GT1b-infected patients[39,40]. It should be noted, however, that the clinical studies involved a small group of DAA-experienced patients with GT1b infection treated with VOX/VEL/SOF, only 69 individuals participated in the POLARIS-1 and POLARIS-4 studies, and in the case of GLE/PIB + SOF + RBV therapy it was only one patient participating in the MAGELLAN-3 study. While RWE publications with a much larger number of patients and such characteristics confirming the high effectiveness of the VOX/VEL/SOF regimen are numerous, for the GLE/PIB + SOF + RBV option, data from routine clinical practice concern only two patients who responded to treatment[41-43].

Along with the very high effectiveness of DAA regimens in GT1b-infected patients, we documented a good safety profile supporting literature data[44]. Regardless of the option used, the vast majority of patients completed therapy as planned and discontinuation of treatment course due to AEs was experienced by a maximum of 1.5% of patients in the ASV + DCV group. In this population, we also noted the highest rate of serious AEs, 5.2%, in two patients in the form of transient elevation of aminotransferases, a phenomenon of unknown pathogenesis previously reported by researchers[45]. None of the 64 deaths reported during therapy course and 12 wk of follow-up were considered by the treating physician to be related to antiviral treatment. The second objective of our analysis was to identify positive and negative predictors affecting the likelihood of virological response in GT1b-infected patients.

Multivariate analysis showed that virologic response in GT1b-infected patients was independently associated with female sex, absence of cirrhosis decompensation at baseline and a higher platelet count, while the presence of HIV co-infection significantly reduced the odds of response. Moreover, no association with liver fibrosis, history of prior therapy, BMI or serum ALT levels, albumin and bilirubin concentration was documented.

Table 6 Factors associated with virologic response in logistic regression model in studied hepatitis C virus-genotype 1b population

Effect	Effect measure	Wald stat	OR	-95%CI of OR	+95%CI of OR	*P*value
Intercept		10.742	24.208	3.600	162.775	0.001
Sex	Female	10.075	1.668	1.216	2.289	0.002
HIV-coinfection	Yes	9.278	0.393	0.216	0.717	0.002
Platelets × 1000/μL	Per 1 U increase	7.619	1.004	1.001	1.006	0.006
Decompensation at baseline	No	4.359	2.423	1.056	5.559	0.037
HCC	Yes	3.733	0.459	0.208	1.011	0.053
BMI kg/m^2	Per 1 U increase	3.154	0.971	0.940	1.003	0.076
Treatment-experience	No	0.972	1.180	0.849	1.639	0.324
Albumins g/dL	Per 1 U increase	0.822	1.027	0.970	1.088	0.365
Bilirubin mg/dL	Per 1 U increase	0.294	0.963	0.839	1.104	0.588
Fibrosis	1	0.020	0.902	0.217	3.749	0.888
Fibrosis	2	0.255	0.690	0.164	2.911	0.614
Fibrosis	3	0.054	0.840	0.194	3.639	0.816
Fibrosis	4	0.547	0.580	0.137	2.460	0.460
ALT IU/mL	Per 1 U increase	0.053	1.000	0.998	1.002	0.818
History of decompensation	No	0.010	1.039	0.500	2.160	0.918

ALT: Alanine transaminase; BMI: Body mass index; CI: Confidence interval; GT: Genotype; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; M: Male; OR: Odds ratio.

It is difficult to directly compare our results to other studies because to the best of our knowledge, apart from one single-center study from Poland, there is no analysis evaluating different DAA therapies all together in a GT1b-infected population with identifying negative predictors of SVR[18]. Therefore, we can refer to the prognostic factors of virologic response in the GT1b-infected patients established for a specific DAA regimen, but the findings are inconsistent. Male sex was the only negative prognostic factor for SVR in Polish patients with GT1b infection treated with different DAA options, as well as in Romanian patients receiving OBV/PTV/r + DSV ± RBV[17,18]. However, a study of Chinese GT1b-infected patients treated with another GT-specific regimen GZR/EBR showed that sex did not affect virological response[46]. A multivariate analysis involving a cohort of GT1-infected United States veterans treated with LDV/SOF documented cirrhosis and thrombocytopenia as negative predictors of virologic response, but GT1a-infected patients were the overwhelming majority[32]. In contrast to our study, an integrated analysis of the use of LDV/SOF in phase 2 and 3 trials showed prior treatment failure as a negative factor but once again, the analysis involved patients infected with both subtypes of GT1 with an advantage of 1a[47]. Both GT1 subtypes, also with predominance of 1a, were analyzed by the RWE study, Backus *et al*[16] established that the negative predictor of virological response in both LDV/SOF ± RBV and OBV/PTV/r + DSV ± RBV regimens is BMI > 30 kg/m^2. The same treatment regimens were evaluated in a study of Spanish GT1-infected patients but this time with a predominance of subtype 1b (83.7%) and the only negative predictor for SVR was low albumin level, while the degree of fibrosis did not affect the virologic response, as did in our analysis[15]. An interesting finding in our analysis is a documented HIV co-infection as an independent negative SVR predictor. In the era of IFN, this group of patients was considered difficult to treat, however, data from the DAA era indicate no negative impact of HIV co-infection on the SVR rate[34,48]. We do not have an answer to explain our results because there is no study directly assessing GT1b-infected patients with HCV monoinfection and HIV co-infection treated with DAA. It should be noted, however, that we did not collect data on adherence to treatment in our analysis which is a factor that may be important in the HIV-coinfected population.

This is one of the limitations of our study that we are aware of. Other limitations of the analysis arise from its retrospective nature with possible entry errors, bias and underreporting AEs. The strongest point of our study was the analysis of a large real-world population from an entire country which is homogeneous in terms of HCV GT and subtype, but very diverse in terms of other important factors, including liver fibrosis, history of previous therapy, comorbidities, coinfections and therapeutic regimens used. This means that the obtained results can be generalized to the entire population infected with GT1b HCV.

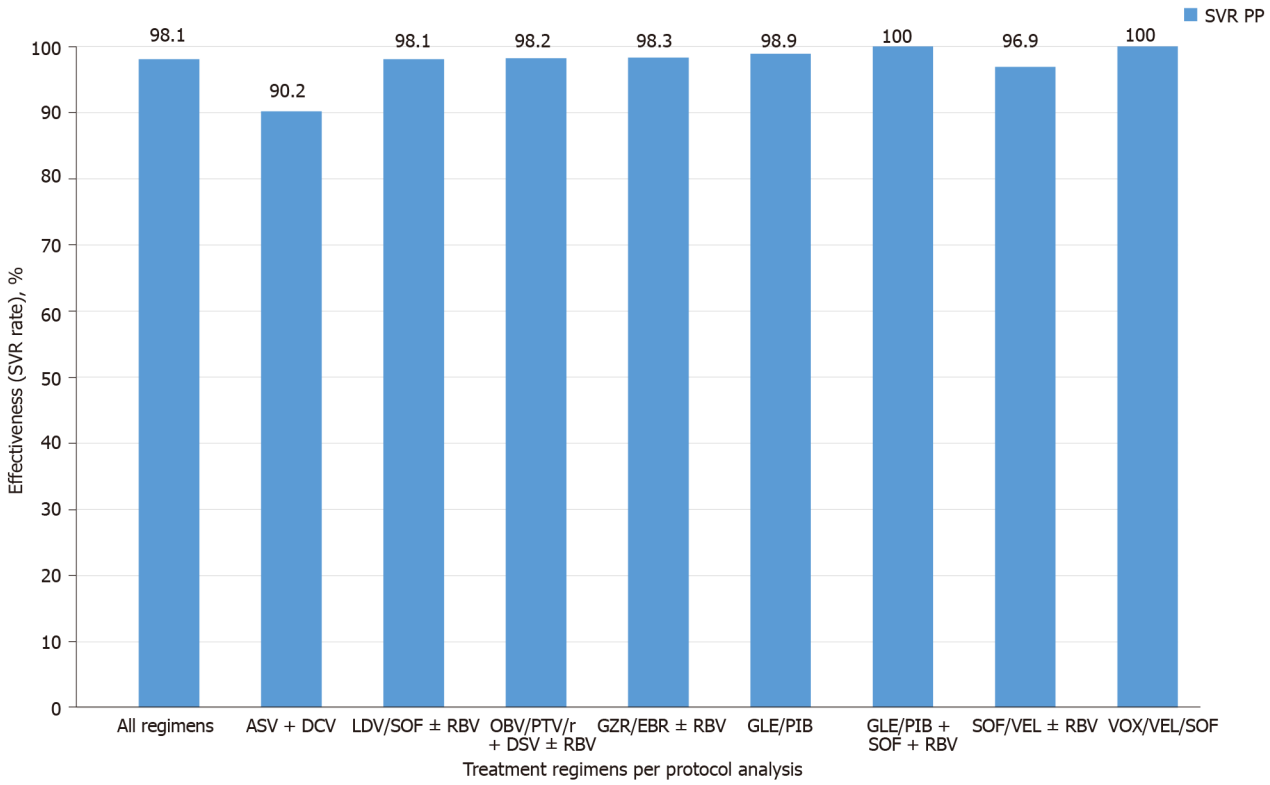
Table 7 Safety of antiviral therapy in genotype 1b-infected patients according to treatment regimens

Parameter	GT1b patients, *n* = 11385	ASV + DCV, *n* = 135	LDV/SOF ± RBV, *n* = 2652	OBV/PTV/r + DSV ± RBV, *n* = 3685	GZR/EBR ± RBV, *n* = 2270	GLE/PIB, *n* = 1684	GLE/PIB + SOF + RBV, *n* = 3	SOF/VEL ± RBV, *n* = 931	VOX/VEL/SOF, *n* = 9
Therapy course, *n* (%)									
According to schedule	11031 (96.9)	130 (96.3)	2549 (96.1)	3513 (95.3)	2245 (98.9)	1666 (98.9)	3 (100)	900 (96.7)	9 (100)
Discontinuation	134 (1.2)	5 (3.7)	27 (1)	60 (1.6)	18 (0.8)	8 (0.5)	0	16 (1.7)	0
Modification	151 (1.3)	0	62 (2.4)	76 (2.1)	1 (< 0.1)	3 (0.2)	0	9 (1)	0
No data	69 (0.6)	0	14 (0.5)	36 (1)	6 (0.3)	7 (0.4)	0	6 (0.6)	0
Patients with at least one AE, *n* (%)	2167 (19)	49 (36.3)	518 (19.5)	911 (24.7)	362 (15.9)	184 (10.9)	1 (33.3)	133 (14.3)	0
Serious adverse events, *n* (%)	123 (1.1)	7 (5.2)	27 (1)	59 (1.6)	11 (0.5)	5 (0.3)	0	14 (1.5)	0
AEs leading to treatment discontinuation, *n* (%)	82 (0.7)	2 (1.5)	20 (0.8)	47 (1.3)	7 (0.3)	4 (0.2)	0	2 (0.2)	0
Most common AEs (≥ 1%), *n* (%)									
Weakness/fatigue	1073 (9.4)	20 (14.8)	409 (15.4)	395 (10.7)	141 (6.2)	57 (3.4)	0	46 (4.9)	0
Anemia	142 (1.2)	1 (0.7)	65 (2.5)	55 (1.5)	9 (0.4)	1 (0.1)	1 (33.3)	5 (0.5)	0
Sleep disorders	214 (1.9)	4 (3)	49 (1.8)	95 (2.6)	29 (1.3)	25 (1.5)	0	12 (1.3)	0
Skin lesions	106 (0.9)	6 (4.4)	24 (0.9)	41 (1.1)	10 (0.4)	13 (0.8)	0	12 (1.3)	0
Itchy skin	205 (1.8)	0	31 (1.2)	135 (3.7)	6 (0.3)	27 (1.6)	0	5 (0.5)	0
Diarrhea	83 (0.7)	4 (3)	20 (0.8)	32 (0.9)	17 (0.7)	4 (0.2)	0	6 (0.6)	0
Nausea	136 (1.2)	4 (3)	29 (1.1)	50 (1.4)	25 (1.1)	20 (1.2)	0	8 (0.9)	0
Headaches	306 (2.7)	5 (3.7)	87 (3.3)	95 (2.6)	60 (2.6)	45 (2.7)	0	13 (1.4)	0
Myalgia/arthralgia	166 (1.5)	5 (3.7)	27 (1)	65 (1.8)	45 (2)	13 (0.8)	0	11 (1.2)	0
Abdominal pain	159 (1.4)	10 (7.4)	27 (1)	45 (1.2)	41 (1.8)	22 (1.3)	0	14 (1.5)	0
Loss of appetite	52 (0.5)	3 (2.2)	6 (0.2)	24 (0.7)	9 (0.4)	4 (0.2)	0	6 (0.6)	0
AEs of particular interest (cirrhotics), *n* (%)	*n* = 2768	*n* = 55	*n* = 737	*n* = 1019	*n* = 380	*n* = 278	*n* = 1	*n* = 290	*n* = 3
Ascites	60 (2.2)	0	31 (4.2)	21 (2.1)	2 (0.5)	0	0	6 (2.1)	0
Encephalopathy	44 (1.6)	0	22 (3)	16 (1.6)	1 (0.3)	2 (0.7)	0	3 (1)	0
Gastrointestinal bleeding	16 (0.6)	0	10 (1.4)	4 (0.4)	0	0	0	2 (0.7)	0
Death, *n* (%)	64 (0.6)	0	19 (0.7)	18 (0.5)	9 (0.4)	5 (0.3)	0	13 (1.4)	0

AE: Adverse event; ASV: Asunaprevir; DCV: Daclatasvir; DSV: Dasabuvir; EBR: Elbasvir; GLE: Glecaprevir; GT: Genotype; GZR: Grazoprevir; LDV: Ledipasvir; OBV: Ombitasvir; PIB: Pibrentasvir; PTV/r: Paritaprevir; RBV: Ribavirin; SOF: Sofosbuvir; VEL: Velpatasvir; VOX: Voxilaprevir.

CONCLUSION

In a large real-world population of patients infected with GT1b, we have documented a very high effectiveness and good safety profile of DAA therapy, regardless of the therapeutic regimen used. Positive predictors of SVR were female sex, absence of decompensated cirrhosis at baseline and a higher platelet count, while HIV coinfection reduced the chance of cure.



DOI: 10.3748/wjg.v28.i45.6380 **Copyright** ©The Author(s) 2022.

Figure 1 Treatment effectiveness (sustained virologic response rates) according to treatment regimens per protocol analysis. ASV: Asunaprevir; DCV: Daclatasvir; DSV: Dasabuvir; EBR: Elbasvir; GLE: Glecaprevir; GZR: Grazoprevir; LDV: Ledipasvir; OBV: Ombitasvir; PIB: Pibrentasvir; PP: Per protocol; PTV/r: Paritaprevir; RBV: Ribavirin; SOF: Sofosbuvir; SVR: Sustained virologic response; VEL: Velpatasvir; VOX: Voxilaprevir.

ARTICLE HIGHLIGHTS

Research background

The increase in the efficacy of antiviral therapy following the introduction of direct antiviral drugs [direct-acting antiviral (DAA)] was most pronounced in patients infected with hepatitis C virus genotype (GT) 1b described as difficult to treat in the interferon (IFN) era.

Research motivation

We aimed to identify the most effective IFN-free therapy for GT1b-infected patients and to determine positive and negative predictors of virological response in this patient population.

Research objectives

Data of 11385 GT1b-infected patients treated with DAA regimens derived from the Epiter-2 database were analyzed.

Research methods

The effectiveness of treatment for each regimen was assessed by the percentage of sustained virological response (SVR). The primary independent variables with SVR as the dependent variable for the logistic stepwise regression model were selected based on significance in univariate analysis.

Research results

A total of 10903 patients responded to antiviral treatment resulting in a 98.1% in the per-protocol analysis after excluding 273 patients without SVR data. The effectiveness of all regimens exceeded 90% and the highest SVR of 98.9% was achieved in the group of patients treated with a combination of glecaprevir/pibrentasvir. Logistic regression analyses showed that virologic response in GT1b-infected patients was independently associated with female sex, absence of cirrhosis decompensation at baseline, and higher baseline platelets, while the presence of human immunodeficiency virus (HIV) coinfection significantly decreased the odds of response.

Research conclusions

The very high effectiveness was documented across all DAA regimens. Positive predictors of SVR were

female sex, absence of decompensated cirrhosis at baseline and higher platelet count, while HIV coinfection reduced the chance of cure.

Research perspectives

The obtained data constitute the basis for selecting the most effective therapy in the case of GT1b-infected patients with no positive predictors and the presence of negative predictors of DAA therapy effectiveness.

FOOTNOTES

Author contributions: Zarębska-Michaluk D and Flisiak R conceived the study design, acquired the data; Zarębska-Michaluk D and Brzdęk M analyzed and interpreted the data; Zarębska-Michaluk D drafted the manuscript; Brzdęk M prepared tables and figures; Jaroszewicz J acquired the data, performed statistical analysis; Flisiak R prepared figures; Tudrujek-Zdunek M, Lorenc B, Klapaczyński J, Mazur W, Kazek A, Sitko M, Berak H, Janocha-Litwin J, Dybowska D, Supronowicz Ł, Krygier R, Citko J, Piekarska A acquired the data and approved the final version of the manuscript.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Endoscopic mucosal resection-precutting *vs* conventional endoscopic mucosal resection for sessile colorectal polyps sized 10-20 mm

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Abstract

BACKGROUND

The optimal method to remove sessile colorectal lesions sized 10-20 mm remains uncertain. Piecemeal and incomplete resection are major limitations in current practice, such as endoscopic mucosal resection (EMR) and cold or hot snare polypectomy. Recently, EMR with circumferential precutting (EMR-P) has emerged as an effective technique, but the quality of current evidence in comparative studies of conventional EMR (CEMR) and EMR-P is limited.

AIM

To investigate whether EMR-P is superior to CEMR in removing sessile colorectal polyps.

METHODS

This multicenter randomized controlled trial involved seven medical institutions in China. Patients with colorectal polyps sized 10-20 mm were enrolled and randomly assigned to undergo EMR-P or CEMR. EMR-P was performed following submucosal injection, and a circumferential mucosa incision (precutting) was conducted using a snare tip. Primary outcomes included a comparison of the rates of *en bloc* and R0 resection, defined as one-piece resection and one-piece resection with histologically assessed clear margins, respectively.

RESULTS

A total of 110 patients in the EMR-P group and 110 patients in the CEMR group were finally evaluated. In the per-protocol analysis, the proportion of *en bloc* resections was 94.3% [95% confidence interval (CI): 88.2%-97.4%] in the EMR-P group and 86% (95%CI: 78.2%-91.3%) in the CEMR group (P = 0.041), while subgroup analysis showed that for lesions > 15 mm, EMR-P also resulted in a higher *en bloc* resection rate (92.0% *vs* 58.8% P = 0.029). The proportion of R0 resections was 81.1% (95%CI: 72.6%-87.4%) in the EMR-P group and 76.6% (95%CI: 68.8%-84.4%) in the CEMR group (P = 0.521). The EMR-P group showed a longer median procedure time (6.4 *vs* 3.0 min; P < 0.001). No significant difference was found in the proportion of patients with adverse events (EMR-P: 9.1%; CEMR: 6.4%; P = 0.449).

CONCLUSION

In this study, EMR-P served as an alternative to CEMR for removing nonpedunculated colorectal polyps sized 10-20 mm, particularly polyps > 15 mm in diameter, with higher R0 and *en bloc* resection rates and without increasing adverse events. However, EMR-P required a relatively longer procedure time than CEMR. Considering its potential benefits for *en bloc* and R0 resection, EMR-P may be a promising technique in colorectal polyp resection.

Key Words: Colorectal polyps; Medium size; Polypectomy; Endoscopic mucosal resection with circumferential precutting; Conventional endoscopic mucosal resection

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Core Tip: The optimal method for removal of sessile colorectal lesions sized 10-20 mm remains uncertain. Piecemeal and incomplete resection are major limitations of conventional endoscopic mucosal resection (CEMR) in removing sessile colorectal polyps. In this study, we found that endoscopic mucosal resection with circumferential precutting achieved better *en bloc* resection (94.3% *vs* 86.0%; P = 0.041) with no increase in adverse events, and can serve as an alternative technique to CEMR in the removal of sessile colorectal lesions sized 10-20 mm.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men and second most common cancer in women[1]. In China, the incidence and mortality rates of CRC showed an increasing trend in 2000-2011; approximately 376.3 thousand new CRC cases and 191.0 thousand CRC deaths occurred in 2015[2], posing a severe threat to people's health. Endoscopic resection of colorectal polyps has proven effective in reducing CRC incidence and mortality[2,3]. Currently, all polyps must be resected except for diminutive (≤ 5 mm) rectal and rectosigmoid polyps as they are predicted with high confidence to be hyperplastic[4].

Endoscopic mucosal resection (EMR) is a well-established method for removing sessile colorectal polyps. However, as the polyp size increases, the piecemeal resection rate increases[5,6], which is a well-known risk factor for local recurrence after EMR[7-9]. Although endoscopic submucosal dissection (ESD) has been increasingly used to overcome the disadvantage of EMR, the technically challenging, time-consuming practice and longer hospital stay required limit its wider use[10]. Recently, EMR with circumferential precutting (EMR-P), an alternative to conventional EMR (CEMR), has emerged as an effective method[11,12]. However, most comparative studies were retrospective, with a small sample

size, involving large (≥ 20 mm) or difficult lesions (where *en bloc* resection could not be achieved by CEMR), and the quality of evidence was relatively poor.

To date, no high-quality evidence or specific recommendation is available in recent guidelines for the optimal resection of 10-20 mm sessile lesions. Therefore, a prospective comparative randomized study was conducted to compare the efficacy of EMR-P with that of CEMR in medium-sized (10-20 mm) colorectal polyps.

MATERIALS AND METHODS

Study design

This prospective multicenter randomized controlled trial involved seven Chinese institutions: the First Affiliated Hospital, School of Medicine, Zhejiang University (Institution A); Renmin Hospital of Yuyao City, Zhejiang (Institution B); Jinhua Municipal Central Hospital, Zhejiang (Institution C); Taizhou Hospital, Zhejiang (Institution D); the Central Hospital of Lishui City, Zhejiang (Institution E); Ningbo Medical Center, Lihuili Hospital, Zhejiang (Institution F); and Ningbo First Hospital, Zhejiang (Institution G). The trial complied with the Declaration of Helsinki, and the study protocol was approved by the Institutional Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University (No. 20191477); Ningbo First Hospital, Zhejiang (No. 2020-R013) and other participating institutions. This study was registered at ClinicalTrials.gov (NCT04191473).

Patients

Patients aged ≥ 18 years undergoing endoscopic resection for colorectal mucosal lesions (adenoma, intramucosal adenocarcinoma, or sessile serrated adenoma/polyps) 10-20 mm in size were included. Endoscopic diagnosis of mucosal lesions was based on macroscopic appearance[13], narrow-band imaging (NBI) findings, or classification of pit patterns on magnifying chromoendoscopy (MCE).

The exclusion criteria were as follows: (1) Pedunculated lesions; (2) residual lesions after endoscopic resection; (3) lesions with submucosal infiltration judged by advanced endoscopic imaging; (4) lesions in patients with inflammatory bowel disease; (5) familial polyposis; (6) electrolyte abnormality; (7) coagulation disorders; (8) severe organ failure; and (9) patients who were pregnant or nursing and who were taking NSAID drugs or anticoagulant medications. All the patients were informed of the research aims and endoscopy procedures and provided written informed consent for research participation.

Procedures

Colonoscopy was performed after bowel preparation with polyethylene glycol solution, magnesium sulfate, or sodium phosphate subject to the common practice of each center (the detailed information of used materials in each institution was listed in Supplementary Table 1). In both polypectomy procedures, a high-definition lower gastrointestinal endoscope was used (Olympus Medical Systems, Tokyo, Japan). In both groups, whether a cap was used or the bowel lumen was filled with air or carbon dioxide was left to the preference of the endoscopist. When the colonoscope reached the lesion location, NBI or MCE was used to identify the presence of submucosal invasion. If necessary, endoscopic ultrasound examinations were performed. When the lesion was confirmed to be noninvasive, EMR-P or CEMR was subsequently performed.

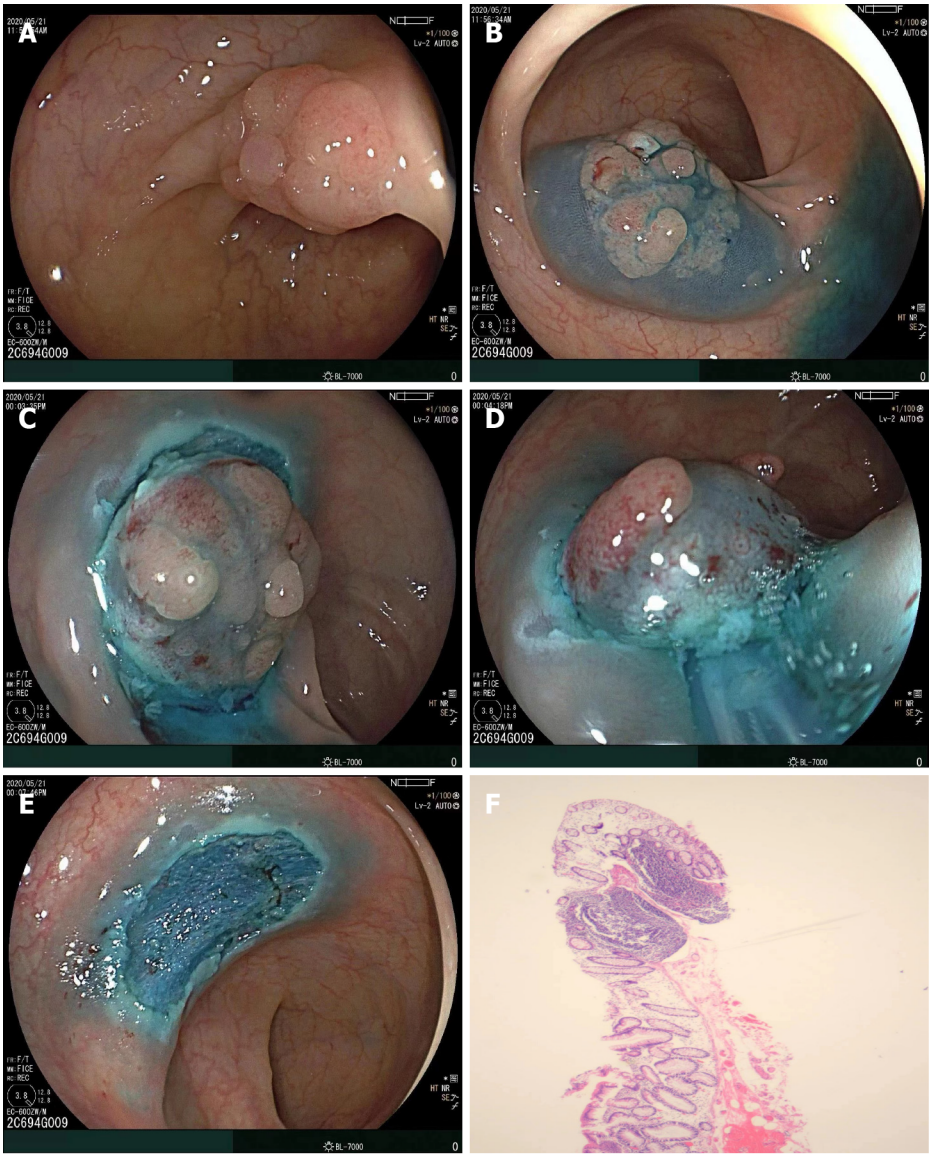
The EMR-P procedure included the following steps: (1) Submucosal solution injection; (2) a fully circumferential mucosa incision (precutting) using a snare tip to separate the lesion from the surrounding non-neoplastic mucosa (the polypectomy snares were chosen at the discretion of each institution); and (3) snaring the circumferential lesion and removing the tumor in the same manner as for CEMR. Following resection of the lesion, hemostatic forceps (with or without argon plasma coagulation) were used to prevent postoperative bleeding (Figure 1).

The Paris classification was used to classify the morphology of polyps with superficial appearance (category 0): Polypoid type [lesions 2.5 mm above the mucosal layer: Pedunculated (0-Ip), sessile (0-Is), or mixed (0-Isp)], nonpolypoid [lesions less than 2.5 mm (0-IIa), flat (0-IIb), or slightly depressed (0-IIc)], and mixed types[14].

Regarding patients with multiple lesions, only the lesion closest to the anus and sized 10-20 mm was registered to remove the subjective bias of researchers. All operations were performed by certain endoscopists in each center. Experienced endoscopists were defined as those who had performed > 1000 colonoscopies, > 300 EMRs, and > 10 ESDs. Compared with the size of open (approximately 7 mm) or closed (approximately 2 mm) biopsy forceps according to its endoscopic appearance, the size of the lesion was initially estimated and then was confirmed by comparison with an opened snare (20-30 mm) during treatment.

Randomization and concealment

The SPSS™ Statistics (version 21; Chicago, IL, United States) software was used to generate random numbers, and simple randomization was adopted to determine whether the EMR-P or CEMR grouping



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Figure 1 Following resection of the lesion. A: A sessile lesion (15 mm × 13 mm) located in the sigmoid colon; B: Submucosal injection of glycerol fructose was applied; C: A circumferential incision was made using a snare tip; D: The snare could grasp the submucosal tissues below the mucosal lesions; E: *En bloc* resection was achieved with no complications; F: Histological diagnosis was tubular adenoma with low-grade dysplasia (20×).

depended on a 1:1 ratio. The operation method corresponding to the random numbers was sent to each center within a closed envelope by the research coordinator. A randomized competitive enrollment mode was applied at each center until the envelopes were allocated completely. Every enrolled patient received an envelope, but the grouping information was blinded for them during the endoscopic procedures. Only immediately before starting the colonoscopy was the operator informed of the treatment allocated by a research assistant.

Outcome variables

The primary study outcomes were a comparison of the R0 and *en bloc* resection rates between the groups. The secondary outcomes included procedure time and adverse events (intraprocedural bleeding, postoperative bleeding, or perforation). *En bloc* resection was defined as one-piece resection, and R0 resection was defined as one-piece resection with histologically clear margins of lesions. Non-R0 resection included a positive resection margin (R1) or an unclear resection margin (RX). The procedure time of both polypectomy techniques was measured from the start of submucosal injection to complete removal of the polyp. Intraprocedural bleeding was defined as bleeding occurring during the procedure that persisted for more than 60 s and required any form of endoscopic hemostasis (*e.g.*, endoscopic coagulation or mechanical therapy, with and without adrenaline injection)[4]. Postoperative bleeding was defined as, within 14 d after EMR-P or CEMR, hematochezia, with a > 2 g/dL decrease in hemoglobin or requiring endoscopic hemostasis. All the patients were followed up within two weeks

after the operation to assess the presence of any adverse events.

Sample size

It was hypothesized that EMR-P would be superior to CEMR for endoscopic R0 resection of colorectal polyps sized 10-20 mm. As previous studies reported that the CEMR R0 resection rate for colorectal polyps 10-20 mm in size was 40%-60%[15], it was preliminarily estimated that the R0 resection rate of CEMR for intermediate-sized (10-20 mm) colorectal polyps in the present study would be 50%. Next, an assumption was made that EMR-P could increase the R0 resection rate to 70%. The enrollment of 100 patients in each group provided 80% statistical power ($\alpha = 0.05$) to detect a 20% difference between the groups. Considering a possibility of a dropout rate of 10%, it was planned to increase the sample size to 220 patients in total.

Statistical analysis

The primary endpoints were analyzed according to the principle of intention-to-treat (ITT). The chi-squared test and Fisher's exact test were used to compare categorical variables, while the Mann-Whitney U-test and the unpaired two-sample *t*-test were used for continuous variables. Additionally, logistic regression analysis was applied to calculate the odds ratios. All the analyses were two-sided, and *P* values < 0.05 were considered statistically significant. If the primary outcomes of a patient were missing or abnormal, the case data were discarded directly; if the secondary outcomes were missing or abnormal, the mean value of each group was used as a replacement. All statistical analyses were performed using SPSS™ Statistics (version 21; Chicago, IL, United States) and R (version 4.1.2) software.

Histological examination

After resection, the specimens were immersed in 10% formalin and sent to the pathology department of each institution for histological evaluation. All the retrieved specimens were evaluated in terms of histologic types and involvement of the resection margin. Histological diagnosis of the lesion and involvement of the resection margin were evaluated according to the 2019 World Health Organization Classification of Tumors of the Digestive System.

RESULTS

Participant flow

A total of 220 patients with 220 polyps were included in the study and were randomly assigned to two groups (the CEMR Group, $n = 110$; and the EMR-P Group, $n = 110$). These patients with 220 colorectal lesions were included in the ITT analysis for the primary endpoint. Three patients with pedunculated lesions in each group were excluded, while in the EMR-P group, one patient was excluded as the polyp was > 20 mm in size, leaving 213 patients (106 patients in the EMR-P group and 107 patients in the CEMR group) in the per-protocol analysis (PP analysis) (Figure 2).

Baseline data

No significant difference was observed in age, sex, location or morphology of lesions, polyp size or endoscopist experience between the groups. In this study, 97.0% of patients in the EMR-P group and 99.0% of patients in the CEMR group received prophylactic clipping of resected wounds. All 220 patients were hospitalized for endoscopic treatment (Table 1).

Procedure-related outcomes

The *en bloc* resection rate in the EMR-P group was higher than that in the CEMR group in both ITT and PP analyses (Table 1). However, only PP analysis showed a significant difference (94.3% *vs* 86.0%; $P = 0.041$). No significant difference was found in the R0, R1, and RX resection rates between the groups, though EMR-P showed a slightly but not significantly higher R0 resection rate. Histologically, most of the polyps in the two groups were adenomas (74.5% in the EMR-P group and 77.3% in the CEMR group), without a significant difference between the groups. The median operation times in the EMR-P and CEMR groups were 6.4 and 3.0 min ($P < 0.001$), respectively (Table 2).

Adverse events

In the EMR-P group, four cases had bleeding during precutting while six cases had bleeding during the snaring and removal procedure; however, the occurrence of intraprocedural bleeding was not significantly different between the groups during the whole operation. One case of intraprocedural perforation occurred in the EMR-P group but did not result in a significant difference compared with the CEMR group (Table 3). These adverse events were successfully addressed by hemostatic clip placement. During the two-week follow-up period, no postprocedural bleeding or perforation occurred in either group, except for one patient who was required to undergo additional surgery due to the pathological diagnosis of rectal cancer with submucosal infiltration above 3500 μm.

Table 1 Baseline characteristics of the study population

Characteristic	EMR-P (*n* = 110), %	CEMR (*n* = 110), %	*P* value
Male (%)	67 (60.9)	70 (63.6)	0.676
Mean age (yr, ± SD)	60.4 ± 10.4	59.9 ± 11.5	0.735
Height (cm)	164.4 ± 7.7	165.0 ± 7.5	0.566
Weight (kg)	61.4 ± 10.5	63.5 ± 10.7	0.151
Location, *n* (%)			0.725
Cecum	6 (5.5)	5 (4.5)	
Ascending	19 (17.3)	18 (16.4)	
Transverse	24 (21.8)	28 (25.5)	
Descending	7 (6.4)	9 (8.2)	
Sigmoid	26 (23.6)	31 (28.2)	
Rectum	28 (25.5)	19 (17.3)	
Morphology, *n* (%)			0.858
0-Ip	3 (2.7)	3 (2.7)	
0-Is	36 (32.7)	42 (38.2)	
0-IIa	68 (61.8)	62 (56.4)	
0-IIb	2 (1.8)	3 (2.7)	
0-IIc	1 (0.9)	0 (0.0)	
Institution, *n* (%)			0.570
A	16 (14.5)	9 (8.2)	
B	31 (28.2)	28 (25.5)	
C	6 (5.5)	5 (4.5)	
D	19 (17.3)	16 (14.5)	
E	9 (8.2)	14 (12.7)	
F	13 (11.8)	18 (16.4)	
G	16 (14.5)	20 (18.2)	
Tumor size (mm)	14.0 ± 3.7	13.1 ± 3.1	0.055
Operator experience, *n* (%)			0.072
Experienced endoscopist	109 (99.1)	103 (93.6)	
Non-experienced endoscopist	1 (0.9)	7 (6.4)	

Institution A: The First Affiliated Hospital, College of Medicine, Zhejiang University, China; Institution B: Renmin Hospital of Yuyao City, Zhejiang, China; Institution C: Jinhua Municipal Central Hospital, Zhejiang, China; Institution D: Taizhou Hospital, Zhejiang, China; Institution E: The Central Hospital of Lishui City, Zhejiang, China; Institution F: Ningbo Medical Center, Lihuili Hospital, Zhejiang, China; Institution G: Ningbo First Hospital, Zhejiang, China. EMR-P: Endoscopic mucosal resection with circumferential precutting; CEMR: Conventional endoscopic mucosal resection.

Subset analysis

An exploratory subgroup analysis was conducted and found that in lesions > 15 mm, EMR-P was better for *en bloc* resection (92.0% *vs* 58.8%; $P = 0.029$). However, in lesions ≤ 15 mm, no significant difference was detected in the *en bloc* resection rate (95.0% *vs* 91.1%; $P = 0.313$). Overall, EMR-P showed a trend toward a higher *en bloc* resection rate than CEMR, regardless of location, lesion size, institution, or operator experience (Figure 3).

DISCUSSION

The present multicenter randomized controlled trial demonstrated that for medium-sized (10-20 mm)

Table 2 Procedure-related outcomes in this study

Parameter	EMR-P group, %	CEMR group, %	*P* value
ITT analysis	*n* = 110	*n* = 110	
En bloc resection	103 (93.6) [87.5-96.9]	95 (86.4) [78.7-91.6]	0.072
R0 resection	89 (80.9) [72.6-87.2]	86 (78.2) [69.6-84.9]	0.616
R1 resection	9 (8.2) [4.4-14.8]	9 (8.2) [4.4-14.8]	> 0.999
RX resection	6 (5.5) [2.5-11.4]	6 (5.5) [2.5-11.4]	> 0.999
PP analysis	*n* = 106	*n* = 107	
En bloc resection	100 (94.3) [88.2-97.4]	92 (86.0) [78.2-91.3]	**0.041**
R0 resection	86 (81.1) [72.6-87.4]	83 (77.6) [68.8-84.4]	0.521
R1 resection	9 (8.4) [4.5-15.4]	9 (8.5) [4.5-15.2]	0.983
RX resection	6 (5.7) [2.6-11.8]	6 (5.6) [2.6-11.7]	0.987
Histological type, *n* (%)	*n* = 110	*n* = 110	0.354
Adenoma	82 (74.5)	85 (77.3)	
Tubular adenoma with low-grade dysplasia	62 (56.4)	63 (57.3)	
Tubular adenoma with high-grade dysplasia	6 (5.5)	7 (6.4)	
Villous adenoma with low-grade dysplasia	2 (1.8)	1 (0.9)	
Villous adenoma with high-grade dysplasia	0 (0.0)	0 (0.0)	
Tubular villous adenoma with low-grade dysplasia	10 (9.1)	12 (10.9)	
Tubular villous adenoma with high-grade dysplasia	2 (1.8)	2 (1.8)	
Hyperplastic	11 (10.0)	14 (12.7)	
Serrated lesions	9 (8.2)	7 (6.4)	
Cancer	4 (3.6)	4 (3.6)	
Intramucosal adenocarcinoma	3 (2.7)	4 (3.6)	
Submucosal invasive adenocarcinoma	1 (0.9)	0 (0.0)	
Other	4 (3.6)	0 (0.0)	
Procedure time (min)	6.4 (4.5, 10.3)	3.0 (1.9, 6.2)	**< 0.001**

EMR-P: Endoscopic mucosal resection with circumferential precutting; CEMR: Conventional endoscopic mucosal resection; ITT: Intention-to-treat analysis; PP: Per-protocol analysis; *En bloc* resection is defined as a once-piece resection; R0 resection is defined as *en bloc* resection with a histologically negative margin; R1 resection is defined as a positive resection margin; RX resection is defined as an unknown resection status.

sessile colorectal polyps, the *en bloc* resection rate of EMR-P (94.3%) was significantly higher than that of CEMR (86%), particularly in lesions > 15 mm. Additionally, compared with CEMR, EMR-P did not increase the incidence of adverse events.

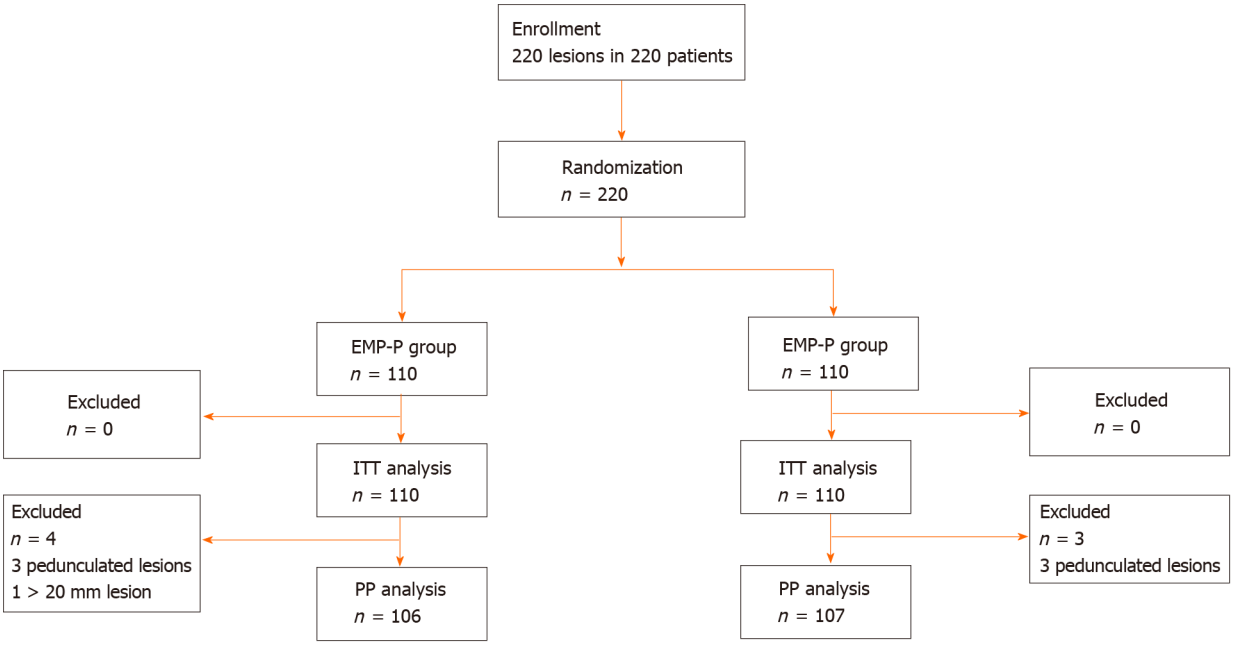
Although hot snare polypectomy (HSP) has been recommended by the European Society of Gastrointestinal Endoscopy Clinical Guidelines as a predominant technique to remove polyps sized 10-19 mm[4], incomplete resection remains an unavoidable issue. The Complete Adenoma Resection ("CARE") study reported a high incomplete resection rate of 17.3% after HSP in polyps sized 10-20 mm [16]. Additionally, incompletely resected lesions may lead to 10%-30% of post-colonoscopy CRC[17].

Currently, EMR was proven to be superior to HSP in terms of the complete resection rate (89% *vs* 73%; $P = 0.02$)[18], a finding also supported by another pooled study[19]. However, the increased lesion size was accompanied by an augmented piecemeal EMR resection rate[6], an important risk factor for local recurrence and metachronous neoplasia[20-23]. Notably, Oka *et al*[23] revealed that piecemeal resection and histologically positive margins were both risk factors for local recurrence in univariate analysis; however, only piecemeal resection was an independent risk factor in multivariate logistic regression analysis. Furthermore, as previously reported, piecemeal-resected lesions reduced the quality and reliability of histological evaluation[24], possibly leading to the inability to provide proper additional treatment and recommendations of appropriate surveillance intervals[4,25]. To improve the

Table 3 Adverse events in this study

Parameter	EMR-P group (*n* = 110)	CEMR group (*n* = 110)	*P* value
Intraprocedural bleeding	10 (9.1)	7 (6.3)	0.449
Postoperative bleeding	0	0	/
Intraprocedural perforation	1	0	/
Postoperative perforation	0	0	/

EMR-P: Endoscopic mucosal resection with circumferential precutting; CEMR: Conventional endoscopic mucosal resection.



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Figure 2 Flow diagram of the study. ITT: Intention-to-treat; PP: Per-protocol; EMR-P: Endoscopic mucosal resection-precutting; CEMR: Conventional endoscopic mucosal resection.

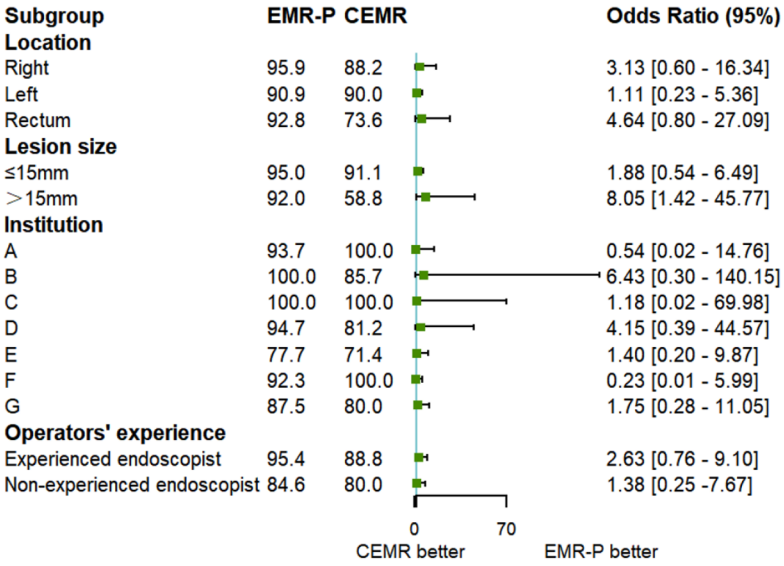
effectiveness and safety of endoscopic colorectal lesion resection, several improved EMR techniques have been developed, such as EMR-P, underwater EMR (UEMR), anchored EMR, and cap-shaped EMR [4,26-28].

Of these, EMR-P was first described by Hirao *et al*[29] in 1986 and has been reported as EMR with a small or circumferential incision or simplified ESD[12,30,31]. The application of this method for gastric neoplasms has also been reported[32-34]. In 2007, Repici *et al*[35] first performed a single-center nonrandomized trial to evaluate the safety and efficacy of EMR-P (an it-knife was used for precutting) in the *en bloc* resection of large colonic polyps. Although only 55.1% (16 of 29 patients) of the lesions (> 30 mm) achieved *en bloc* resection, the authors suggested EMR-P was a promising technique.

Although some studies have confirmed the superiority of EMR-P to CEMR for large polyps (> 20 mm)[12,36], even noninferior to ESD[37,38], only one other study has directly compared the efficiency of EMR-P with CEMR in medium-sized polyps[11]. That study reported that EMR-P had a higher complete resection rate (87.8% *vs* 67.3%; $P < 0.001$) and *en bloc* resection rate (98.0% *vs* 85.7%; $P < 0.004$) than CEMR. However, that study was retrospective with a small number of follow-up cases and an objective to analyze the efficacy of EMR-P for lesions that were challenging for standard EMR[11]. Thus, the guiding role of that study for clinical practice to tackle normal nonpedunculated lesions was limited.

In accordance with the results of PP analysis in the present study, EMR-P was superior to CEMR regarding the *en bloc* resection rate (94.3% *vs* 86%; $P = 0.041$), particularly in lesions > 15 mm (92.0% *vs* 58.8%; $P = 0.029$). However, these significant differences were not found in the ITT analysis.

Although EMR-P also showed a higher R0 resection rate, no significant difference was found (ITT analysis: EMR-P *vs* EMR, 80.9% *vs* 78.2%; PP analysis: EMR-P *vs* EMR, 81.1% *vs* 77.6%). Notably, the results in the present study showed an overall higher R0 removal rate than in previous studies, regardless of the method applied. In a trial by Yamashina *et al*[15], only a 50% R0 resection rate was achieved by CEMR for nonpedunculated colorectal polyps (median size: 13.5 mm); and UEMR, another



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Figure 3 Subset analysis for *en bloc* resection. EMR-P: Endoscopic mucosal resection-precutting; CEMR: Conventional endoscopic mucosal resection.

alternative technique for medium-sized colorectal polyps, only resulted in a 75% R0 resection rate. For polyps sized 15-20 mm, Imai *et al*[27] also indicated that only a 65.3% R0 resection rate was achieved by CEMR with added tip-in EMR in the whole group.

Despite a lack of direct comparisons, these data implied that the similar R0 resection rates between the groups might be due to the high R0 removal rate by CEMR in the present study, leaving less room for improvement by EMR-P. Theoretically, EMR-P does have some advantages over the above two alternative treatments of CEMR. As it does not need to fill the entire lumen only with fluid, compared with UEMR, EMR-P saves a lot of time in deflating the lumen completely; moreover, its visual field during operation is less affected by poor intestinal preparation or intraprocedural bleeding. In comparison with tip-in EMR, circumferential incision of EMR-P allows the snare to be closer to the vertical margins of the lesions when snaring the polyps, which may be conducive to a better R0 resection rate.

Owing to the additional precutting step, EMR-P required a slightly longer total procedure time (EMR-P *vs* EMR: 6.4 *vs* 3.0 min, respectively; $P < 0.001$). However, considering the potential cost of retreatment caused by the higher risk of recurrence, spending some three more minutes in clinical operation, with no additional devices needed, seems more cost-effective. Additionally, despite including circumferential incision, more intraprocedural or postoperative bleeding cases in the EMR-P group were not observed. In the present study, only one patient in the EMR-P group experienced intraprocedural perforation, which was resolved through defect closure with endoscopic clips and antibiotic use. However, as most of the endoscopists were experienced, confirming the safety of EMR-P was challenging, particularly for endoscopists with less experience. This finding requires further clarification with follow-up research.

Theoretically, EMR-P is more difficult than CEMR but simpler than ESD, and EMR-P could also become an intermediate link to ESD training programs in the future. In the present study, the better performance of EMR-P was because of the circumferential incision. This additional step could facilitate the snare to be placed along the incision and then grasp and remove the lesions more reliably. However, during the snaring process, the benefit for vertical resection margins of EMR-P seems limited, likely explaining why it is difficult for all improved EMR techniques to achieve a 100% R0 resection rate. Additionally, circumferential incision may lead to unfavorable injected submucosal fluid maintenance, resulting in poor uplifting of mucosa and affecting the visual field. Fortunately, this weakness could be resolved by using thicker submucosal injectates to provide a sustained lift, such as succinylated gelatin, hydroxyethyl starch, or glycerol[4,39].

The present study has several limitations. First, the recurrence rate was not evaluated because follow-up colonoscopies were not performed. Thus, in accordance with the European guideline, surveillance colonoscopy three years after complete endoscopic resection is recommended[25]. Second, the single-blind study design may cause better outcomes of the new operation method, an inevitable problem of randomized controlled trials concerning endoscopy. Third, biopsy of the resected site after EMR has been recommended as the gold standard for evaluating the completeness of resection[40,41]. In the present study, *en bloc* and histologically negative margins of the sample were used to define R0 resection, a strategy that might be prone to sampling error because marginal biopsies only represent part of the lesion margin.

CONCLUSION

The present randomized study revealed that EMR-P successfully achieved a > 90% *en bloc* resection rate in 10-20 mm nonpedunculated polyps without increasing the incidence of adverse events. Although EMR-P may take slightly longer and result in a similar R0 resection rate than CEMR, its potential benefits are promising in clinical practice, particularly in lesions > 15 mm.

ARTICLE HIGHLIGHTS

Research background

The optimal method for removing sessile colorectal lesions sized 10-20 mm remains uncertain. Piecemeal and incomplete resection are major limitations in current practice, including conventional endoscopic mucosal resection (CEMR). Recently, EMR with circumferential precutting (EMR-P) has emerged as an effective technique to overcome the deficiency of EMR. However, the current quality of evidence in comparative studies concerning about CEMR and EMR-P is limited.

Research motivation

To investigate a better method for removing sessile colorectal lesions sized 10-20 mm.

Research objectives

To investigate if EMR-P is superior to CEMR in colorectal lesions sized 10-20 mm.

Research methods

This is a single-blind multicenter randomized controlled trial which involved seven medical institutions in China. The EMR-P was performed following submucosal injection, and a circumferential mucosa incision (precutting) was conducted using a snare tip. The *en bloc* and R0 resection rate were the primary outcomes analyzed, defined as one-piece resection and one-piece resection with histologically assessed clear margins, respectively.

Research results

In the per-protocol analysis, the *en bloc* resection rate of EMR-P was significantly higher than CEMR (94.3% *vs* 86%, $P = 0.041$), while in subgroup analysis, EMR-P also resulted in a higher *en bloc* resection rate (92.0% *vs* 58.8% $P = 0.029$) for lesions > 15 mm. The R0 resection rate was 81.1% (95%CI: 72.6%-87.4%) in the EMR-P group, higher than in CEMR group (76.6%; 95%CI: 68.8%-84.4%), but without a significant difference between the groups. The EMR-P group had a longer median procedure time (6.4 *vs* 3.0 min; $P < 0.001$). No significant difference in the incidence of adverse events was found between the groups (EMR-P: 9.1%; CEMR: 6.4%; $P = 0.449$).

Research conclusions

EMR-P could serve as an alternative treatment for sessile colorectal polyps sized 10-20 mm.

Research perspectives

The factors affecting the *en bloc* or R0 resection rate of EMR-P should be further studied, such as the experience of endoscopists, the depth of circumferential incision, the size of the polyp and so on.

FOOTNOTES

Author contributions: Zhang XQ, Xu L, and Yu CH designed the research; Zhang XQ, Sang JZ, Xu L, Mao XL, Li B, Zhu WL, and Yang XY participated in the operation; Zhang XQ and Xu L analyzed the data; Zhang XQ wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of First Affiliated Hospital, School of Medicine, Zhejiang University (No. 20191477); Ningbo First Hospital, Zhejiang (No. 2020-R013) and other participating institutions.

Clinical trial registration statement: This study is registered at ClinicalTrials.gov. The registration identification number is NCT04191473.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All the authors have no conflicts of interest to declare.

Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at zhangxuequn@zju.edu.cn. Participants gave informed consent for data sharing.

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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META-ANALYSIS

Meta-analysis on the epidemiology of gastroesophageal reflux disease in China

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Abstract

BACKGROUND

No large-scale epidemiological survey on the prevalence of gastroesophageal reflux disease (GERD) in China has been conducted. China has a large population and a complex geographical environment. It is important to understand the prevalence and spatial distribution of GERD in China.

AIM

To explore the prevalence and the spatial, temporal, and population distributions of GERD in the natural Chinese population.

METHODS

We searched Chinese and English databases for literature on the prevalence of GERD in the natural Chinese population. The prevalence of GERD was pooled using a random-effects meta-analysis model. Subgroup analysis was performed according to time, region, and population. We used ArcGIS software to draw statistical maps and trend analysis charts. Spatial autocorrelation analysis was carried out using Geoda software. Spearman correlation analysis was used to assess the spatial distribution relationship between GERD and upper digestive tract tumours.

RESULTS

Altogether, 70 studies involving 276014 individuals from 24 provinces of China were included. The overall pooled prevalence of GERD was 8.7% (95%CI: 7.5%-9.9%) in mainland China. Over the past two decades, the prevalence of GERD in China has increased from 6.0% to 10.6%. GERD was more common in people aged 40-60, with body mass index ≥ 24, and of Uygur ethnicity. The prevalence was

higher in the west and east than in the centre, and there may be a local spatial autocorrelation between the Qinghai-Tibet Plateau and the southeast. GERD was correlated with gastric (r = 0.421, P = 0.041) and oesophageal tumours (r = 0.511, P = 0.011) in spatial distribution.

CONCLUSION
GERD is becoming common in China. The prevalence differs by region and population. The development of appropriate strategies for the prevention and treatment of GERD is needed.

Key Words: China; Meta-analysis; Gastroesophageal reflux disease; Prevalence; Epidemiology; Spatiotemporal trends

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Core Tip: The overall pooled prevalence of gastroesophageal reflux disease (GERD) was 8.7% in China. The prevalence was higher in the west and east than in the centre, and there may be a local spatial autocorrelation between the Qinghai-Tibet Plateau and the southeast. GERD was correlated with gastric and oesophageal tumours in spatial distribution.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) refers to the symptoms or complications caused by the reflux of gastric contents into the oesophagus or further into the oral cavity (including larynx) or lung field[1]. The main symptoms of GERD are heartburn, reflux, bulbar sensation, dysphagia, chest pain, hiccups, *etc*.[2]. It may also present extraesophageal symptoms, including chronic cough, laryngitis, asthma, and tooth erosion[3]. GERD is a common disease with a global prevalence of approximately 13%[4,5]. According to reports, the prevalence of GERD was highest in North America, ranging from 18.1% to 27.8%, followed by 8.8% to 25.9% in Europe, and the lowest from 2.5% to 7.8% in Asia[6,7]. As a chronic disease with a high prevalence rate, GERD has brought substantial economic and health burdens to patients and society[8,9] and may lead to tumours[10]. In addition to laboratory methods[11], several standard scales are used to evaluate GERD, including the gastroesophageal reflux disease questionnaire (GerdQ)[12] and the reflux disease questionnaire (RDQ)[13].

The prevalence of GERD in China is lower than the worldwide rates[4,5]. However, no ethnicity-wide large-scale epidemiological survey or meta-analysis on the majority of GERD in China has been conducted to date. Due to the differences in regions, periods, populations, sampling methods, and diagnostic criteria, there are significant differences in the survey results of existing studies. China has a vast area and many races, which will lead to significant differences in the prevalence of GERD. Therefore, we conducted this meta-analysis to explore the prevalence of GERD in the natural Chinese population, to analyze its spatial, temporal, and population distributions, and to explore its geographical connection with upper gastrointestinal cancer.

MATERIALS AND METHODS

Subject design

We searched the literature on GERD in adults in mainland China, conducted a meta-analysis to explore the prevalence of GERD, and assessed the disease's spatial, temporal, and population distributions through subgroup analysis. Then, using the pooled prevalence of different provinces as the dependent variable, we analyzed the spatial trend and spatial aggregation of GERD. Using the pooled prevalence in other cities, we analyzed the correlation between GERD and upper gastrointestinal tumours.

Literature search

The PubMed, EMBASE, WanFang, VIP, and CNKI databases were systematically and independently searched for studies reporting the prevalence of GERD in Chinese adults from database inception to January 1, 2022. The search terms used in this study were as follows: ('China' or 'Chinese') and

('Gastroesophageal reflux' or 'GERD') and ('prevalence' or 'epidemiology survey'). All included literature has been officially published. There were no language restrictions or publication date restrictions on this search. We also manually searched the references of the selected papers to avoid omission. In addition, the incidence rate of upper gastrointestinal tumours came from the "China Cancer Registration Annual Report 2019", which reported the tumour monitoring data of more than 200 cities. We supplement and improve the highlights of the latest cutting-edge research results using the Reference Citation Analysis (https://www.referencecitationanalysis.com/) in the process of revising the manuscript.

Study selection

Original studies that fulfilled the following criteria were included in the meta-analysis: the subjects were adults in mainland China, the study type was a cross-sectional epidemiological survey, the sample size and the number of patients could be obtained, and the sample size was more than 200. Articles were excluded if they met the following criteria: The same data were reported in multiple articles, the articles were case reports or reviews, and the articles included surveys for particular groups, such as hospital patients, students, soldiers, and miners.

Quality evaluation

Following the methods of a previous study[14], we developed a quality rating scale with eight items. For each item, 1 point was awarded for meeting the standard requirements, and 0 points were awarded for not mentioning or not conforming to the standard. The score of each article was between 0 and 8. Literature ≤ 3 points was considered "low quality" and was excluded.

Data extraction

LTL and ZJM independently searched for literature, evaluated the literature quality, and extracted the data. In case of disagreement, CCW participated in the discussion and helped to form an agreement. The extracted information included the survey period, the survey location (province and city), sampling method, diagnostic tools and standards, sample size, number of patients, and demographic data (including ethnicity, sex, gender, body mass index (BMI), residence, marital status, education, income, occupation, smoking, alcohol consumption, tea drinking, physical exercise, *Helicobacter pylori*, and life stress). In addition, the incidence rates of tumours in the oral cavity, nasopharynx, throat, oesophagus, and stomach of the cities involved were collected from the "2019 annual report on cancer registration in China".

Statistical analyses

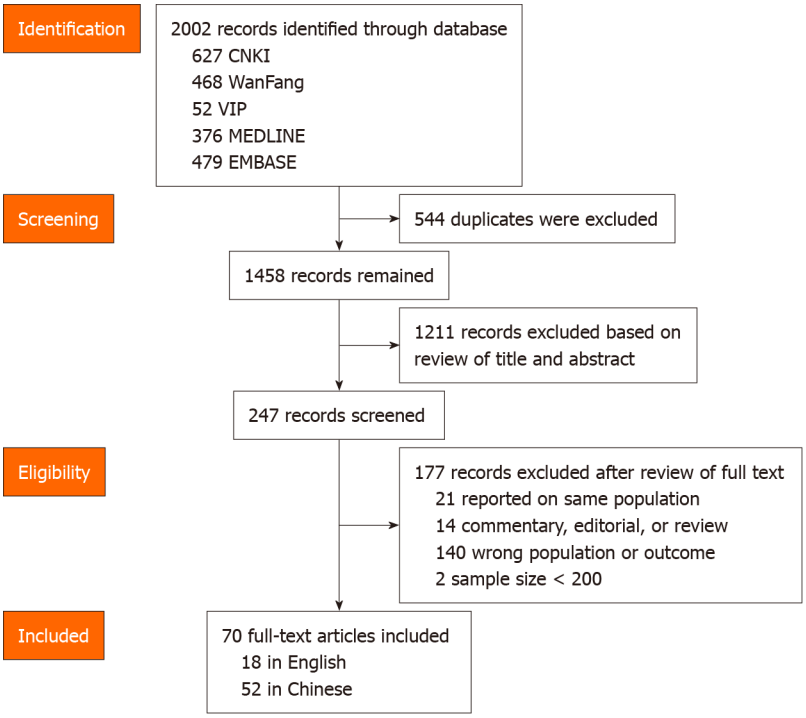
The statistical methods of this study have been published previously[15]. We used R software (version 4.0.1) for meta-analysis and subgroup analysis according to study design, survey period, survey area, and other information. The I^2 statistic test was used to evaluate the heterogeneity in the included literature. $I^2 > 50\%$ was considered heterogeneity, and a random effect model was adopted; If $I^2 \leq 50\%$, then a fixed effect model was used. We used Begg's test to assess publication bias and we used sensitivity analysis to assess the stability of the results. Meta-regression analysis was used to control the influence of interference factors.

We used ArcGIS software (version 10.6) to make a statistical map of the pooled prevalence rate in each province. ArcGIS was also used to create a trend analysis chart to evaluate the spatial changes in diseases[16]. The X-axis, Y-axis, and Z-axis of the chart represent longitude, latitude, and prevalence, respectively. Geoda software (version 1.12) was used for spatial autocorrelation analysis of diseases. Global Moran's I index was used to evaluate global spatial autocorrelation. The Lisa cluster map and Lisa significance map were used to describe local spatial autocorrelation[17,18]. Spearman correlation analysis was performed with R software to explore the effect of GERD on the incidence rate of upper gastrointestinal tumours. In addition, we used R software to draw a violin diagram, line charts and correlation heat diagram. A 2-sided $P < 0.05$ indicated a significant difference.

RESULTS

Literature screening results and the pooled prevalence

The literature screening process is shown in Figure 1. A total of 70 articles were included in our study, including 52 published in Chinese and 18 published in English. Limited by the article's length, the essential characteristics of the included literature are shown in Supplementary Table 1. A total of 276014 people from 24 provinces of mainland China were enrolled in the study, and 27386 people were diagnosed with GERD. The quality assessment ranged from 4 to 8, with an average of 6.01. In the 70 articles, the GERD prevalence ranged from 1.65% to 28.07%, and the pooled prevalence was 8.7% (95%CI: 7.5%-9.9%). The I^2 was 99.4%, so the random effect model was adopted. Begg's test indicated no publication bias in this study ($Z = 1.11$, $P = 0.269$). The results of the sensitivity analysis showed that the



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Figure 1 Flowchart of the selection of studies.

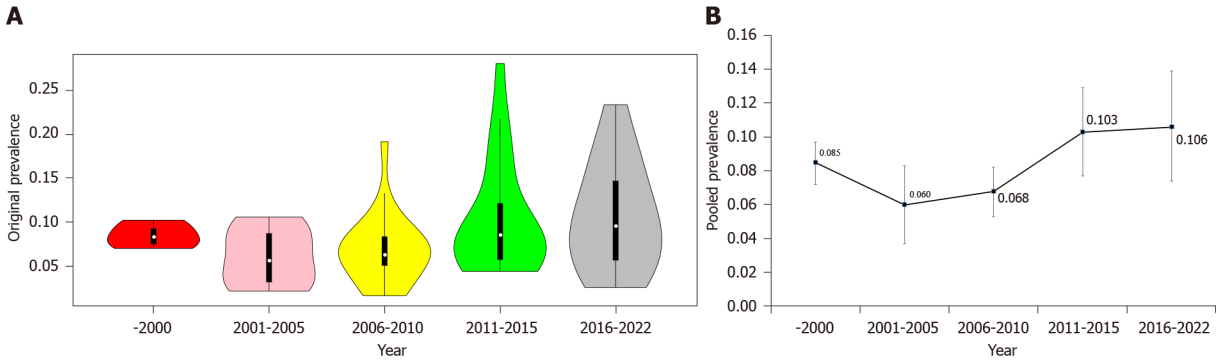
pooled prevalence remained stable. To exclude the influence of research design, we conducted subgroup analyses according to the questionnaire used, random sampling, quality score, and sample size. We found that the prevalence rates estimated by different questionnaires were inconsistent (Q = 15.88, P = 0.001). The GerdQ estimated the highest GERD prevalence rate, and the RDQ estimated the lowest. The other three indicators had no effect on the results (P > 0.05).

Spatial, temporal, and population distributions

Figure 2A is a violin chart of the actual prevalence, and Figure 2B is a line chart of the pooled prevalence in different periods. In the past 20 years, the prevalence of GERD in China showed an upwards trend (Q = 11.81, P = 0.019). After controlling for the influence of regions and questionnaires by meta-regression analysis, this trend still existed (t = 2.37, P = 0.020). Figure 3 shows the regional distribution of the prevalence of GERD. From the map, the prevalence of GERD was different in different provinces, with the highest prevalence in Xinjiang at 19.1% (95%CI: 13.5%-24.7%) and the lowest prevalence in Guangdong at 3.3% (95%CI: 2.2%-4.3%). Overall, the prevalence rate was high in the eastern and western regions and low in the central region (Q = 9.18, P = 0.010). Supplementary Figure 1 shows the geographical division. The difference was still significant after excluding the influence of times and questionnaires (t = 2.01, P = 0.049). Figure 4 shows a forest plot of GERD prevalence for subgroup analysis by demographic characteristics. We selected 15 indicators for demographic subgroup analysis. The results showed that age (Q = 48.86, P < 0.001), ethnicity (Q = 14.15, P = 0.027), and BMI (Q = 5.50, P = 0.049) were statistically significant.

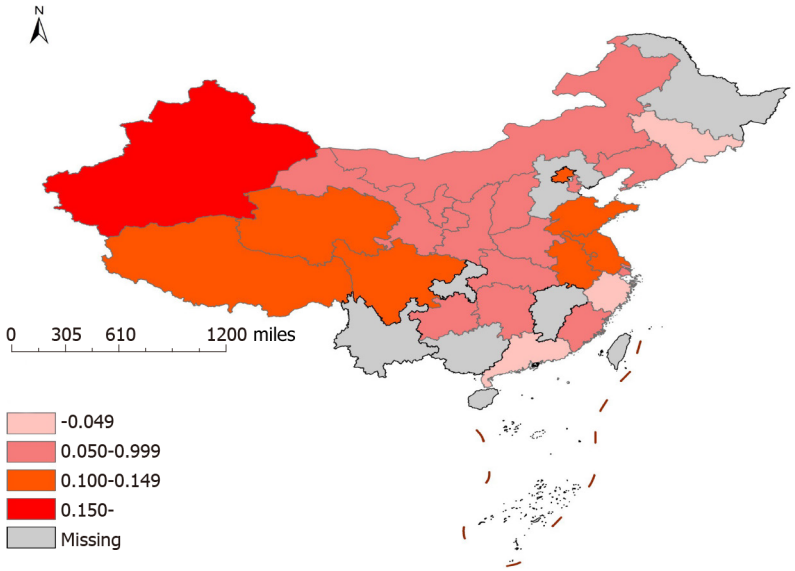
Spatial trend distribution and correlation analysis

The prevalence in the east-west distribution (X-axis) showed the change characteristics of falling and rising, and all fitted trend lines (green curve) showed a U-shape. In the north-south distribution (Y-axis), the fitting trend line (blue curve) was opposite, with low prevalence at both ends and high prevalence in the middle (see Figure 5A). In Figure 5B, the Moran's I value was 0.145, and the Monte Carlo simulation P value was 0.103, indicating no global spatial autocorrelation. However, as shown in Figures 5C and D, the results of local spatial autocorrelation analysis showed that the Qinghai-Tibet Plateau was a high-to-high aggregation area of GERD, and Hunan and Fujian were low-to-low aggregation areas. A total of 36 cities were involved in the meta-analysis, of which 24 cities were able to obtain tumour incidence rates and were included in the correlational analysis. The results showed that GERD was correlated with gastric (r = 0.511, P = 0.011) and oesophageal tumours (r = 0.421, P = 0.041) (see Figure 6).



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Figure 2 Prevalence of gastroesophageal reflux disease in different periods. A: Violin diagram of the original prevalence; B: Line chart of the pooled prevalence.



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Figure 3 Prevalence of gastroesophageal reflux disease in different provinces of China.

DISCUSSION

This is the first meta-analysis on the prevalence of GERD in the Chinese natural population. The majority of GERD cases in China were 8.7% (95%CI: 7.5%-9.9%), which was lower than that in European and American countries but higher than the estimated 2.5%-5.0% GERD prevalence in previous global reviews[4-7]. This meta-analysis included 70 studies, and the distribution of effect values showed nonnormality ($P < 0.001$). Therefore, we used the nonparametric Begg test to test publication bias and found that there was no bias in this study[19]. Sensitivity analysis showed that our results were stable.

To control the influencing factors, we also conducted a subgroup analysis according to the type of study design. Our results were not related to random sampling, literature quality or sample size but were influenced by the survey instrument used. In the meta-analysis stratified by prevalence survey, the prevalence was inconsistent among different survey tools, which has been previously reported[4,14]. This study applied the RDQ, GerdQ and other survey tools. Previous studies have shown that these questionnaires exhibit good psychometric characteristics and are suitable for application in primary health care[11-13]. The GerdQ and RDQ were considered to have the same screening effect[12]. However, in our study, the GerdQ estimate of the prevalence of GERD was higher than the estimate of RDQ. When we excluded the influence of survey time and area through meta-regression, there was no difference in the prevalence between survey tools.

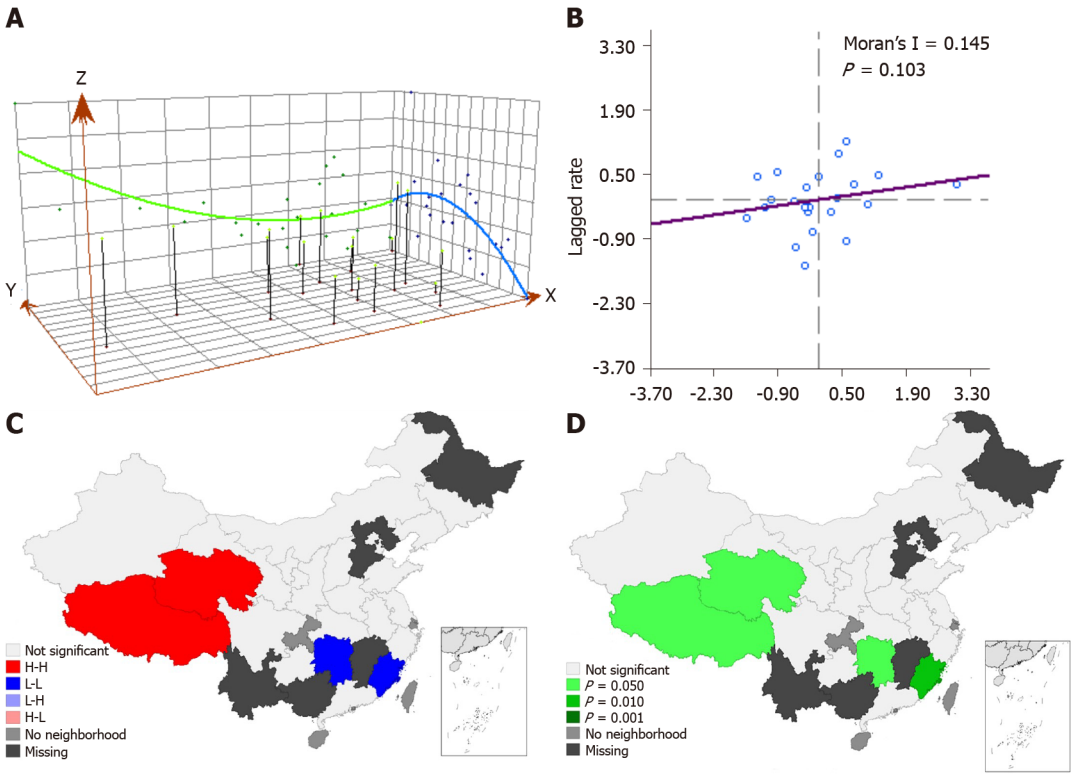
Our study revealed that the prevalence of GERD in China is rising. From 6.0% at the beginning of the 21st century to 10.6% at present, there was a significant difference in the prevalence among different periods ($P = 0.019$). After controlling for the survey tools and regions, differences still existed. Chen *et al* [20] reported that during the period 2000-2007, the indications for referral endoscopy secondary to GERD increased over time. A study found that in Hong Kong, the prevalence of weekly GERD rose by

Subgroup	Studies	Events	Total	Forest	Proportion [95%-CI]	Model	*P* (Begg)	*P* (Cochran Q)
Age								
<20	3	76	2095		0.028 [0.009; 0.046]	Random	0.602	<0.001
20~39	26	5111	48814		0.076 [0.054; 0.098]	Random	0.343	
40~59	29	8858	60915		0.119 [0.092; 0.146]	Random	0.099	
≥60	28	3423	25774		0.117 [0.094; 0.140]	Random	0.269	
Gender								
Male	37	11906	95582		0.092 [0.071; 0.113]	Random	0.583	0.967
Female	37	8040	78314		0.093 [0.076; 0.109]	Random	0.022	
Ethnicity								
Han	8	2097	18805		0.124 [0.074; 0.174]	Random	0.138	0.003
Uygur	5	2527	11822		0.216 [0.170; 0.262]	Random	0.624	
Tibetan	3	529	3106		0.136 [0.068; 0.203]	Random	0.117	
Other	7	471	4476		0.107 [0.082; 0.143]	Random	0.851	
Body mass index								
<24	15	4656	46234		0.083 [0.054; 0.111]	Random	0.458	0.019
≥24	15	8065	46524		0.156 [0.102; 0.209]	Random	0.083	
Residence								
City or town	9	1293	23732		0.054 [0.037; 0.072]	Random	0.06	0.578
Countryside	9	1035	18345		0.061 [0.044; 0.079]	Random	0.677	
Marital status								
Married	8	2122	28529		0.076 [0.049; 0.103]	Random	0.216	0.683
Single	8	397	6917		0.069 [0.043; 0.094]	Random	0.293	
Occupation								
Industry or agriculture	6	1274	18893		0.079 [0.055; 0.104]	Random	0.851	0.516
Other industries or no jobs	9	1085	15972		0.068 [0.042; 0.094]	Random	0.211	
Education								
Primary school or below	9	1472	13675		0.096 [0.056; 0.136]	Random	0.095	0.891
Secondary school	9	1959	22613		0.089 [0.055; 0.124]	Random	0.144	
College or above	8	732	9105		0.084 [0.053; 0.115]	Random	0.138	
Income								
High income	3	101	3316		0.036 [0.011; 0.062]	Random	0.602	0.571
low income	3	937	16759		0.048 [0.016; 0.080]	Random	0.117	
Life stress								
Small	5	1224	19509		0.079 [0.043; 0.116]	Random	0.624	0.522
Great	5	1009	9370		0.099 [0.050; 0.148]	Random	0.327	
Current drinking								
Yes	18	4971	30028		0.139 [0.098; 0.181]	Random	0.344	0.272
No	18	10164	77835		0.110 [0.087; 0.142]	Random	0.023	
Current smoking								
Yes	17	5633	35120		0.122 [0.082; 0.162]	Random	0.202	0.571
No	17	9298	71077		0.107 [0.075; 0.140]	Random	0.044	
Tea-drinking								
Yes	8	1342	9157		0.133 [0.085; 0.182]	Random	0.216	0.651
No	8	2196	17028		0.117 [0.068; 0.167]	Random	0.048	
Physical exercise								
Once a week or more	5	3203	31216		0.081 [0.026; 0.135]	Random	0.999	0.941
Less than once a week	5	5505	28980		0.085 [0.018; 0.188]	Random	0.327	
Helicobacter pylori infection								
Yes	3	248	2948		0.099 [0.014; 0.185]	Random	0.602	0.933
No	3	393	3661		0.106 [0.012; 0.224]	Random	0.602	



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Figure 4 Forest plot for subgroup analysis by demographic data.



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Figure 5 Spatial trend distribution of gastroesophageal reflux disease. A: Trend Analysis; B: Moran-scatterplot; C: Lisa concentration map; D: Lisa significance map.



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Figure 6 Correlation heat map of the relationship between gastroesophageal reflux disease and upper gastrointestinal tumors. [a]*P* < 0.05, [b]*P* < 0.01.

1.3% between 2002 and 2011, which represents at least a 50% relative increase[21]. Additionally, two other studies[7,22] provided evidence that the global GERD prevalence has increased over the past two decades. The increase in bulk may be due to changes in people's lifestyles caused by economic development[23].

A trend analysis showed that the prevalence of GERD in China was high in the eastern and western regions and low in the central areas. After controlling for the survey tools and period, the prevalence rates in different regions were still disparate. It has been reported that the prevalence of GERD in Central Asia is high[4]. Interestingly, this study found that the highest prevalence rate in China was in

Xinjiang, which is adjacent to Central Asia, and has an environment and diet that are similar to Central Asia. Dietary structure is an influencing factor of GERD[24,25]. For example, vegetarian diets were negatively related to GERD, while meat and fat consumption were positively related to GERD. Different dietary patterns, diet styles, and eating habits in other regions may be the reasons for regional differences in prevalence.

We divided the papers into 15 subgroups to investigate the prevalence of GERD in different populations, and significant differences among other ethnic groups, ages, and BMIs were found. The majority of GERD was the highest in populations over 40 years old, followed by those 20-39 years old; the lowest prevalence was in those under 20 years old, which is consistent with another meta-analysis[6] observing Asian populations. Leonardo found through meta-analysis that, compared with those aged < 50 years, the OR for GERD in those aged ≥ 50 years was 1.32 (95%CI: 1.12-1.54)[5]. A new global meta-analysis revealed that the prevalence rate was the highest in those aged 35-60 years old and decreased slightly in those over 60 years old[4]. The current consensus is that the prevalence of GERD is different among different ethnic groups[26]. Our study suggested that the prevalence of GERD in the Uygur population is significantly higher than that in other ethnic groups. This was also consistent with our regional distribution, as the Uygur people mainly live in Xinjiang Province, which is in western China. The prevalence, frequency and severity of GERD were reported to increase with BMI, which was consistent with our findings[26]. The results of a large-scale prospective study also suggested that a decrease in BMI can improve GERD-related symptoms, while an increase in BMI may aggravate GERD symptoms[27]. In addition, some researchers proposed that the decline in *Helicobacter pylori* infection will lead to a healthier stomach and decreased gastric acid secretion, leading to a reduction in reflux disease[28]. Our research did not support this statement. The other 11 factors evaluated had no effects on the prevalence of GERD, and their impact on GERD remains controversial.

We found that GERD did not have global spatial autocorrelation but had local spatial autocorrelation. In addition, the local spatial autocorrelation analysis results demonstrated that the area with the highest incidence of GERD was different from the highest spatial autocorrelation area of GERD. Tibet and Qinghai were high-to-high aggregation areas, and Hunan and Fujian were low-to-low aggregation areas. This unstable result may be related to the fact that we have many missing values. There were 7 provinces without data. More studies may be needed to explore the spatial autocorrelation of GERD in China. Our study found that GERD was positively correlated with oesophageal cancer and gastric cancer in spatial distribution. The association of GERD and oesophageal cancer has been reported[29], and we confirmed this conclusion from the perspective of the spatial distribution of the disease. The correlation between GERD and gastric cancer may be due to the increase in gastric acid secretion in some gastric cancers, which leads to the occurrence of GERD. A meta-analysis suggested that GERD may be a significant risk factor for laryngeal cancer[30]. However, future prospective controlled studies are needed. There was no correlation between GERD and oral cancer and oesophageal cancer, and no similar literature report has been found.

In our study, all respondents were healthy people (not patients or a specific group of people), and the study covered most provinces in China, both of which suggest that the research sample was satisfactory and representative. We conducted a number of subgroup analyses to explore the prevalence of different populations. In addition, we evaluated the temporal and spatial distributions of GERD in mainland China for the first time and analysed the spatial correlation between GERD and upper respiratory tract tumours. However, this meta-analysis has several limitations. First, there was considerable heterogeneity in the calculation of crude prevalence and subgroup analysis. Second, although we included many studies, the literature of some regions and subgroups was still insufficient. Finally, the lack of data in seven provinces may affect our spatial correlation analysis results.

CONCLUSION

In conclusion, this systematic review demonstrated that the summary estimate of the prevalence of GERD in Chinese people was 8.7%, indicating a trend of higher prevalence in the west and east than in the centre and an increasing prevalence over time. The prevalence rate of GERD was higher in people who were over 40 years old, obese and of the Uygur population. The prevalence of GERD was positively correlated with that of oesophageal cancer in terms of spatial distribution. Considering the vast and diverse population of China, further studies are needed to identify effective strategies to reduce the incidence of GERD in China.

ARTICLE HIGHLIGHTS

Research background

Gastroesophageal reflux disease (GERD) is a common digestive tract disease that is not easy to cure and has significant impact on the quality of life of patients. There is no large-scale epidemiological survey or

systematic review on GERD prevalence in China. China has the world's largest population, dozens of ethnic groups, vast land area and a complex geographical environment. Those factors have caused great challenges for the prevention and control of GERD in China.

Research motivation

It is important to understand the prevalence and spatial distribution of GERD in China because it will provide a reference for disease prevention and control.

Research objectives

The purpose of this meta-analysis was to understand the spatial, temporal and population distributions of GERD prevalence in China and to explore the correlation between the disease and upper gastrointestinal tumours in the spatial distribution.

Research methods

This article pooled the prevalence of GERD overall and in subgroups by using a random effect model. A statistical map of the spatial distribution of GERD prevalence was drawn. Spatial trends and spatial autocorrelations were analyzed. The interrelation between GERD and the prevalence of upper gastrointestinal tumours in different regions was also discussed.

Research results

The overall prevalence of GERD in mainland China was 8.7% (95%CI: 7.5%-9.9%). In the past 20 years, the prevalence of GERD in China has increased from 6.0% to 10.6%. GERD was more common in people aged 40-60, with BMI ≥ 24, and of Uygur ethnicity. The prevalence rate in the western region is the highest, followed by the eastern region, and the central region is the lowest. There may be a local spatial autocorrelation between the Qinghai-Tibet Plateau and the southeast. GERD was correlated with gastric tumours (r = 0.421, P = 0.041) and oesophageal tumours (r = 0.511, P = 0.011) in spatial distribution.

Research conclusions

The prevalence of GERD is on the rise in China. Prevalence varies in different regions and populations.

Research perspectives

In China, GERD should receive more attention. More efforts should be made to prevent and control GERD in key areas and populations.

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FOOTNOTES

Author contributions: Lu TL and Chen CW contributed to the study design and drafting of the manuscript; Lu TL, Zhang JM, Li SR and Chen CW contributed to the data collection; Lu TL and Li SR contributed to the analysis and interpretation of data; Zhang JM and Li SR contributed to the critical revision of the manuscript; All the authors did approval of the final version for publication.

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CASE REPORT

Germline BRCA2 variants in advanced pancreatic acinar cell carcinoma: A case report and review of literature

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Abstract

BACKGROUND

Pancreatic acinar cell carcinoma (PACC) is a rare tumor. Up to 45% of PACCs have alterations in the DNA damage repair pathway and 23% harbor rearrangements in the *BRAF* or *RAF1* genes. We present a PACC case with a germline *BRCA2* likely pathogenic variant (LPV) to highlight the impact of genomic testing on treatment decisions and patient outcomes. In our larger case series, we provide clinic-based information on additional 10 PACC patients treated in our center.

CASE SUMMARY

A 70-year-old male was diagnosed with advanced PACC. At presentation, he was cachectic with severe arthralgia despite prednisolone and a skin rash that was later confirmed to be panniculitis. He was treated with modified FOLFIRINOX (mFFX) with the knowledge of the germline *BRCA2* LPV. Following 11 cycles of mFFX, a computed tomography (CT) scan demonstrated significant tumor response in the pancreatic primary and hepatic metastases, totaling 70% from baseline as per Response Evaluation Criteria in Solid Tumors. Resolution of the skin panniculitis was also noted. We identified two additional PACCs with druggable targets in our case series. Our data contribute to practical evidence for

the value of germline and somatic profiling in the management of rare diseases like PACC.

CONCLUSION
This patient and others in our larger case series highlight the importance of genomic testing in PACC with potential utility in personalized treatment.

Key Words: Pancreatic acinar carcinoma; *BRCA*; Polyadenosine diphosphate-ribose polymerase inhibitor; Case report

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Core Tip: Pancreatic acinar cell carcinoma (PACC) is a rare tumor with distinct molecular features and a relatively high proportion of targetable mutations. In this article, we describe a case report of PACC with a germline *BRCA2* likely pathogenic variant, with a series of 10 additional cases, along with an in-depth look at the patients' therapeutic details. We aim to outline the advantages of genomic analysis and its outcome regarding treatment selection in this tumor type.

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INTRODUCTION

Pancreatic acinar cell carcinoma (PACC) is a rare subtype of pancreatic cancer, accounting for 1%-2% of exocrine pancreatic neoplasms[1]. While there is some clinical and genotype difference, patients with PACC and pancreatic ductal adenocarcinoma (PDAC) are often treated as one disease entity. Evolving data has increased our understanding of this rare tumor's biology, treatment, and prognosis. Due to the disease's rarity, most data about PACC are limited to reviews, case reports, and case series. The tumor biology of PACC is not well characterized due to the lack of tissue availability for large-scale molecular analysis. Intriguingly, data obtained in recent years have indicated that PACC has a distinctive mutational landscape[2-4]. There is increasing interest in this area, particularly regarding the homologous repair deficiency (HRD) signature in PACC. Chmielecki *et al*[2] reported up to 45% deficiency of the DNA damage repair (DDR) pathway genes in the study population. Oncogenic therapeutic targets including *RAF1* rearrangements and mismatch repair genes have proven elusive in a significant proportion of PACCs, and lack of tumor profiling probably contributes to low reporting[3,4].

Here, we present a PACC case to emphasize the clinical application of genomic profiling in the context of precision medicine for better patient outcomes. Although this patient was very unwell at the presentation, raising the question of suitability for modified FOLFIRINOX (mFFX), the knowledge of the *BRCA2* likely pathogenic variant (LPV) as predictive for mFFX sensitivity guided our decision to use this regime. In the case series section, we describe the clinical characteristics, therapeutic outcomes, and mutational signatures of additional 10 patients with PACC treated in our center. As proof of concept, we describe the immediate clinical impact for the patients with distinct genomic alterations that have been associated with sensitivity to specific chemotherapeutic or targeted agents.

CASE PRESENTATION

Chief complaints

The patient was a 70 male smoker with recurrent lower limb joint pain and was generally unwell for the previous year.

History of present illness

He presented to a rheumatology service with joint pain, which was diagnosed as gout and treated with short courses of prednisolone; however, during the steroid treatment, he also experienced central abdominal discomfort, reduced appetite, and 10 kg weight loss. He had progressive lower joint pain with tender, warm skin nodules, which restricted mobility.

History of past illness
He had encephalitis and asthma as a child.

Personal and family history
He had a history of transitional cell carcinoma of the renal pelvis at age 46 for which he underwent a left total nephrectomy and a non-small cell lung adenocarcinoma at age 51, which was treated by lung resection. His mother died of ovarian cancer at age 72.

Physical examination
Physical examination revealed a cachectic man with a palpable liver edge and ill-defined widespread erythematous subcutaneous nodules on bilateral lower limbs (Figure 1A). Eastern Cooperative Oncology Group performance status (PS) was 2.

Laboratory examinations
Initial blood tests demonstrated lipase > 6000 U/dL, elevated transaminases [alanine aminotransferase (ALT) 68 U/L and aspartate aminotransferase 58 U/L], total bilirubin 6 μmol/L, albumin 28 g/L and creatinine 120 μmol/L (estimated glomerular filtration rate 52 mL/min). Tumor markers were: Normal carbohydrate antigen 19-9 (CA19-9) 28 kU/L and raised alpha-fetoprotein 58 μg/L.

Imaging examinations
Initial computed tomography (CT) imaging showed a bulky pancreatic tumor measuring over 16 cm and multiple liver metastases. The largest liver lesion measured over 8 cm (Figure 2A). There was an ill-defined area of sclerosis in the right ischium which was suspicious of metastasis. Whole-body bone scintigraphy detected mild non-specific increased activity in the right ischium corresponding to the area of sclerosis but no significant bony abnormality. CT chest showed no evidence of thoracic metastases.

FURTHER DIAGNOSTIC WORK-UP
The patient underwent a liver biopsy that confirmed a poorly differentiated carcinoma staining positive for keratin 7, CAM5.2, claudin 4, glypican 3, and A1AT. Negative markers included keratin 20, arginase 1, hepPar1, synaptophysin, chromogranin, CD56, and TTF1. The tumor was mismatch repair proficient. An additional skin biopsy of one of the subcutaneous nodules confirmed pancreatic lobular panniculitis. Germline testing identified a *BRCA2* LPV (c.4356delinsCA, p. Gln1452Hisfs*8).

CASE SERIES
Population and clinical data
We treated 11 PACC patients between August 2014 and July 2021 at Princess Margaret Cancer Centre (PMCC), Toronto. These comprised 6 (55%) pure and 5 (45%) mixed PACC. Approximately 2000 pancreatic carcinoma patients were managed at PMCC during this period. The median age at diagnosis of the PACC patients was 65 years (range 57-74) and all were male. At diagnosis, 2 (18%) were resectable, 2 (18%) locally advanced, and 7 (64%) metastatic. The full demographic features of all patients are summarized in Table 1.

Four (36%) patients had curative-intent surgery. Three of them developed systemic relapse and received subsequent treatment with palliative chemotherapy. All seven metastatic patients had chemotherapy. Altogether, ten patients received palliative chemotherapy: mFFX (6), Gemcitabine plus Nab-paclitaxel (GnP) (3), and Gemcitabine (1).

The median time to progression from the date of surgery to the first systemic relapse for the resected patients was 10.5 mo (1.5-10.6). After a follow-up period of 20.4 mo, 6 (55%) patients had died of the disease while five are still alive. The median overall survival (OS) of the cohort was 20.4 mo (range 4.6-36.0) but this variable is temporally immature. The median OS of the four resected patients was 30.3 mo (28.2-36.0).

Genomic data
Eligibility for germline genetic testing in Ontario has evolved with the advent of next-generation sequencing, newly identified genes, and the association of established genes with different cancer types. In April 2021, Ontario Health expanded the availability of germline testing to all individuals with pancreatic cancer regardless of age or family history[5]. Before this, germline testing for individuals with pancreatic cancer was based on personal and family history as well as the age of onset. The gene(s) or multi-gene panels performed for patients are based on the individuals' personal and family history at the time of the initial genetic counseling.

Table 1 Clinicohistopathologic features of the pancreatic acinar cell carcinoma patients in this dataset (*n* = 11)

Characteristic	Number of patients (%)
Male	11 (100)
Median age at diagnosis, yr	65 (56.5-74.0)
Median tumor size, cm	7.0 (2.7-16.4)
Histology	
Pure acinar	6 (55)
Mixed acinar-neuroendocrine	3 (27)
Mixed acinar-ductal	2 (18)
Primary tumor site	
Head/uncinate	4 (37)
Body	2 (18)
Tail	5 (45)
Stage	
Resectable	2 (18)
Locally advanced	2 (18)
Metastatic	7 (64)
1st line treatment	
Surgery only	1 (9)
Surgery and chemotherapy	2 (18)
Preoperative chemoradiotherapy and surgery	1 (9)
Chemotherapy only	7 (64)
Palliative chemotherapy	
Modified FOLFIRINOX	6 (60)
Gemcitabine with Nab-paclitaxel	3 (30)
Gemcitabine	1 (10)

Seven patients in our case series had clinical germline testing. Four patients did not have germline testing, as they did not meet eligibility criteria based on family history at the time of their diagnosis. Germline PV/LPV was identified in four patients [2 *BRCA2* (18%), 1 *ATM* (9%), 1 *CDKN2A* (9%)]. Two (18%) patients had somatic testing with whole-genome sequencing and RNA sequencing as part of clinical trial participation. Identified somatic variants were SND1-*BRAF* fusion in one patient and *KRAS*, *SMAD4*, *CDKN2A*, *ATM*, *TP53*, *TGFBR2*, and *KDM6A* in another patient.

In terms of actionability, we identified two patients with *BRCA2* PV/LPV (18%) and one *SND1-BRAF* fusion (9%). The first patient carrying a germline *BRCA2* LPV is described in this case report. The second patient carrying a germline *BRCA2* PV had advanced acinar neuroendocrine carcinoma. Briefly, he was commenced on a combination of 5 FU and Oxaliplatin with a dose reduction (30%) due to comorbidities. Despite this, the evaluation CT scan following 8 cycles of chemotherapy showed a partial response of the primary tumor (63% smaller than baseline) as per Response Evaluation Criteria in Solid Tumors (RECIST) 1.1. He continued additional 4 cycles with further tumor regression, followed by resection. The tumor was pathologic near complete treatment response.

The patient harboring an *SND1-BRAF* fusion was commenced on first line GnP. The evaluation CT scans following 6 cycles of chemotherapy showed a significant partial response (55% decrease than baseline) of the primary tumor and lymph nodes as per RECIST1.1. After 16 cycles of GnP, he developed progressive disease and was switched to single-agent Cobimetinib as part of clinical trial participation. Molecular profiling was negative for other key driver mutations *KRAS, TP53, CDKN2A, SMAD4*, and *BRCA* in this patient. Full mutational profiles of the patients and treatment history are outlined in Table 2.

Table 2 Germline and somatic molecular profiles of the pancreatic ductal adenocarcinoma patients in this study

Year of diagnosis	Age at diagnosis	Germline testing	Somatic testing	Driver mutation	Personal or family history of malignancy	Disease staging	Surgery and perioperative chemotherapy	TTP after surgery (mo)	First line systemic therapy	Second line systemic therapy	Best treatment and clinical status	OS (mo)
2014	65	*BRCA1, BRCA2* negative	Not performed MMR IHC intact	Nil	Family history of leukemia, colorectal, ovarian, and prostate cancers	T3N0M0 R1	Neoadjuvant mFFX with radiotherapy followed by Whipple procedure	1.5	Gemcitabine for 4 cycles	BSC	PD, DOD	28.2
2014	63	Not performed	Not performed	Nil	Nil	T3N1bM0 R0	Whipple procedure with perioperative mFFX for 12 cycles	10.4	BSC	Nil	PD, DOD	23.2
2014	74	*CDKN2A* pathogenic variant c.159G>C, p.Met53Ile	Not performed	Nil	Family history of melanoma and PDAC Personal history of malignant melanoma	Stage IV	Nil	NA	mFFX for 3 cycles	BSC	PD, DOD	4.6
2015	60	Not performed	Not performed	Nil	Thoracic cancer	T2N0M0 R0	Distal pancreatectomy with adjuvant Gemcitabine for 2 cycles which were discontinued due to toxicities	10.7	GnP 18 mo	N/A	SD, DOD	36.0
2017	*66*	WGS and RNA seq	WGS and RNA seq *KRAS, SMAD4, CDKN2A TP53, ATM, TGFBR2,* and *KDM6A* MMR IHC intact	*KRAS, SMAD4*	Nil	Stage IV	Nil	NA	mFFX for 8 cycles	BSC	SD, DOD	13.4
2018	64	Not performed	Not performed	Nil	Nil	T3N0M0 R0	Whipple procedure. No adjuvant therapy	10.6	GnP for 4 cycles	BSC	PD, DOD	32.3
2020	61	91 gene panel	WGS and RNA seq *SND1-BRAF* fusion MMR IHC intact	*BRAF*	Family history of PDAC	Stage IV	Nil	NA	GnP for 16 cycles	Cobimetinib 60 mg OD PO (enrolled on CAPTUR trial	PR, AWD	20.4 ongoing
2021	70 (case	12 gene panel	Not performed	*BRCA2*	Family history of	Stage IV	Nil	NA	mFFX for 11 cycles	Maintenance	PR, AWD	11.5

	described)	*BRCA2* Likely pathogenic variant c.4356delinsCA, p.Gln1452Hisfs*8	MMR IHC intact		ovarian cancer Personal history of renal cell cancer and NSCLA					Olaparib 150 mg twice daily		ongoing
2021	65	91 gene panel *ATM* Pathogenic variant c.8418+5_8418+8del	Not performed	Nil	Family history of head and neck cancer	Stage IV	Nil	NA	mFFX for 14 cycles, followed by maintenance FOLFIRI	RP-3500 in combination with Gemcitabine (enrolled on RP-3500-01 trial)	SD, ADW	13.4 ongoing
2021	71	*BRCA1, BRCA 2* *BRCA2* pathogenic variant c.8904delC, p.Val2969Cysfs*7	Not performed	*BRCA2*	Family history of breast cancers	Stage IV	Nil	NA	5FU with Oxaliplatin for 12 cycles, downsized to Whipple procedure	N/A	PR, AWD	12.1 ongoing
2021	57	12 gene panel negative	Not performed	Nil	Family history of non-Hodgkins Lymphoma	Stage IV	Nil	NA	Ongoing mFFX; had 16 cycles	N/A	PR, AWD	12.3 ongoing

TTP: Time to progression; OS: Overall survival; MMR IHC: Mismatch repair immunohistochemistry; mFFX: Modified FOLFIRINOX; GnP: Gemcitabine with Nab-paclitaxel; BSC: Best supportive care; PD: Progressive disease; DOD: Dead of disease; SD: Stable disease; PR: Partial response; AWD: Alive with the disease; WGS: Whole genome sequencing; RNA seq: RNA sequencing; PDAC: Pancreatic ductal adenocarcinoma; NSCLA: Non-small cell lung adenocarcinoma.

FINAL DIAGNOSIS

These findings were compatible with PACC with panniculitis, hepatic metastases, and indeterminate bony involvement. Histology revealed no concurrent existence of ductal adenocarcinoma, neuroendocrine or mixed tumor of the pancreas.

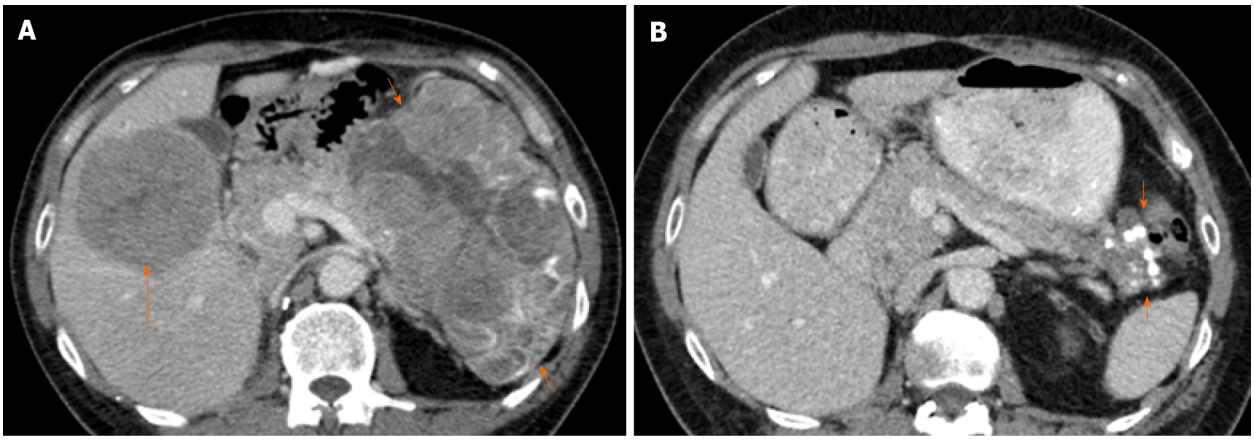
TREATMENT

The initial plan was to treat the patient with GnP in consideration of his poor PS. However, this decision was changed to the mFFX regimen following the documentation of the germline *BRCA2* LPV. mFFX was administered every 2-wk with an additional 20% dose reduction of Oxaliplatin and Irinotecan (Oxaliplatin 65 mg/m^2, Irinotecan 120 mg/m^2, Fluorouracil 4200 mg/m^2 and Folinic acid 400 mg/m^2). The chemotherapy calculations were based on a body surface area of 1.77 m^2.



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Figure 1 Panniculitis is a skin manifestation that can be detected in up to 45% of patients before the recognition of pancreatic disease. A: The widespread ill-defined erythematous tender skin nodules and/or plaques that develop on the shins and around the ankles of our patient as the initial clinical presentation; B: Complete resolution of the panniculitis in our patient after 4 cycles of modified FOLFIRINOX, suggestive of early clinical response.



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Figure 2 Comparison of serial computed tomography images of our patient during chemotherapy. A: Initial axial post-contrast computed tomography (CT) scan shows a large heterogeneous solid mass in the pancreatic tail (short arrows) and a hypoattenuating metastatic liver mass (long arrow); B: Post-chemotherapy axial CT scan performed after 11 cycles of chemotherapy demonstrates marked interval reduction in the size of the pancreatic mass (arrows) and metastatic liver mass (not visible in this image).

OUTCOME AND FOLLOW-UP

After the first cycle of mFFX, the patient was hospitalized due to fever, confusion, and worsening polyarthritis. A full septic screen revealed no clear infectious etiology. CT brain showed no brain abnormality. X-rays of several joint areas including sacroiliac joints showed no radiographic evidence of osteomyelitis or septic arthritis. A left knee joint aspiration revealed an inflammatory synovial fluid with elevated white blood cell count, but no growth of infectious organisms and negative for crystal arthropathy. Rheumatoid factor and anti-cyclic citrullinated peptide levels were negative. The rheumatology team believed the patient's inflammatory seronegative arthritis was paraneoplastic in nature. The patient also displayed clinical adverse events consistent with steroid-induced psychosis, due to the concurrent prednisolone and dexamethasone use. He was started on Naproxen with a tapering dose of prednisolone (from 15 mg daily). His condition improved within a week time and chemotherapy was resumed. Two months after starting mFFX, CT evaluation (post 4 cycles of chemotherapy) showed a 56% partial response based on RECIST1.1. The patient had a marked improvement in symptomatology and panniculitis (Figure 1B). His PS also improved to 0. He continued to tolerate mFFX with grade 1 peripheral sensory neuropathy of hands and feet. Another CT (post 8 cycles of chemotherapy) showed further tumor shrinkage in the primary tumor and hepatic metastases. The sclerotic bone lesion was unchanged in the interval. As the patient was getting a deepening partial response and tolerating mFFX, the chemotherapy was repeated for a total of 11 cycles. A CT imaging at that time point showed further tumor regression in the pancreatic tumor and the hepatic metastases, totaling a 70% decrease

from baseline (Figure 2B). The biochemical response was also seen with a CA19-9 level of 17 kU/L.

After 11 cycles of mFFX, we decided to stop chemotherapy due to the accumulative neurotoxicity. Considering the germline *BRCA2* LPV, we elected a therapeutic switch to Olaparib, a polyadenosine diphosphate-ribose polymerase inhibitor (PARPi), as maintenance therapy. He was started on Olaparib 150 mg twice daily dosing that was adjusted for his renal function. At the time of this writing, the patient experienced disease stability for 5 mo with Olaparib, which is ongoing. He tolerates Olaparib with grade 1 fatigue but has no major side effects. He is on monthly follow-ups.

DISCUSSION

PACC typically presents in the younger population with a median age of 62 years old. It is more frequent in males, with a male-to-female ratio of 2.3:1[6-8]. The majority (50%-60%) present at an advanced stage, with a median tumor size of 7 cm, and lesions smaller than 2 cm are rarely detected[6-10]. Some cases present with mixed differentiation including mixed acinar-ductal and mixed acinar-neuroendocrine subtypes. As the tumor is predominantly found in the tail of the pancreas, patients do not usually present with biliary obstruction, and elevation of CA19-9 is not typically seen[3,11]. However, there have been reports of elevated alpha-fetoprotein in younger patients[7,8]. In extreme cases, up to 10%, of patients have lipase hypersecretion which leads to systemic fat necrosis with eosinophilia, erythematous subcutaneous nodules, and polyarthralgia[6,7,9,10]. This paraneoplastic syndrome, also known as Schmid's triad, is often associated with a poor prognosis[12-15]. The prognosis of PACC is slightly better than that for PDAC[6]. In comparison, 5-year OS for PACC was 42.8% *vs* PDAC 3.8% [16]. In this study, we analyzed the full clinical characteristics, therapeutic outcomes, and mutational signatures of 11 patients with PACC treated at our center. Based on our analysis, the median OS across all stages is 20.4 mo and 30.3 mo among the resected patients.

Available literature suggests that over one-third of PACC patients harbor potentially druggable alterations such as *BRCA2, PALB2, ATM, BRAF,* and *JAK1*[17]. We observed only one PACC with somatic *KRAS* mutation (9%). This result may be limited by the incomplete somatic testing rate in this study. In distinction to PDAC which is associated with *KRAS* driver mutations in more than 93% of cases, *KRAS* mutations occur at a much lower prevalence in the acinar/mixed neuroendocrine tumor (9%)[18-21]. While it is difficult to generalize as pancreatic carcinoma is a complex heterogeneous disease, a strong argument can be made that the lack of mutated *KRAS* identifies a cohort rich in targetable alterations including fusions, and should have access to integrative germline and somatic sequencing[22].

Multiple studies including a large series reported by Chmielecki *et al*[2] involving 44 PACCs reported a 45% deficiency of DDR pathway genes[3,4,23]. These are inclusive of deficiencies in the *BRCA* pathway and mismatch repair. Combined results suggested that approximately 23% of PACCs are enriched with fusion rearrangements involving *BRAF* or *RAF1* genes[2,19]. It appears that PACC subgroups that are lacking *RAF1* rearrangements (*i.e.,* fusion-negative tumors) were significantly enriched for deficiency in HRD, and both tumor types are mutually exclusive[2]. Conceptually, these "fusion-negative" tumors can serve as a beneficial demarcation in over two-thirds of PACC patients who may be candidates for platinum-based chemotherapy. PACC with *BRCA1/2* variants have greater sensitivity to platinum-based chemotherapy and demonstrate significantly better OS than when treated with non-platinum agents[24]. Platinum chemotherapy drugs exert their cytotoxic effect by binding directly to DNA, causing crosslinking of DNA strands and thereby inducing DNA double-strand breaks, which also are ineffectively repaired in cells lacking functioning *BRCA1/2*. Both the patients in our case series with germline *BRCA2* PV/LPV had substantial radiographic regression despite dose reduced Oxaliplatin. Although our patient described in the case report was very unwell with poor PS at presentation, raising the question of suitability for mFFX, the knowledge of the *BRCA2* LPV as predictive for platinum sensitivity guided our decision to use this regime and resulted in his improved outcome. The other patient was successfully downsized to enable the Whipple procedure for curative intent. Notably, we identified one patient with *SND1-BRAF* kinase fusion in our case series. Germline and somatic testing were negative for *BRCA1* or *BRCA2* in this patient. This particular variant fusion joins *SND1* exons 1-10 with *BRAF* exons 11-18 and maintains the reading frame. It is worth noting that this particular configuration is the most prevalent gene fusion described in melanoma, thyroid, and lung cancers. It has also been reported in PACC[2]. This novel fusion is potentially targetable with MEK inhibitors, such as Trametinib and Cobimetinib[2,25].

Germline testing and tumor sequencing results are invaluable in identifying PACC patients for treatment regime determination and predictive biomarkers for investigational targeted therapies[14,22,23,26,27]. Newly diagnosed patients with PACC should undergo germline genetic testing and somatic profiling where appropriate, given the high frequency of pathogenic germline *BRCA* alterations in PACC. This should be made available to patients regardless of clinical presentation, the pattern of metastases, and pre-existing co-morbidities. This is also consistent with NCCN American Society of Clinical Oncology guidelines which recommend all PDAC patients have upfront germline testing as part of the evolving precision strategy and screening strategies[28]. Similar to numerous studies, our

patients with pathogenic *BRCA1/2* variants have an increased risk of pancreatic, ovarian, breast, and other cancers (Table 2). The lifetime risk for pancreatic cancer in *BRCA1* and *BRCA2* mutant carriers is 1% and 4.9%, respectively[29,30]. Unlike breast and ovarian cancers, germline *BRCA1/2* mutations alone do not pose a significant risk of pancreatic cancers. Recent literature review shows *BRCA2* confer to 5%-17% of familial pancreatic cancers (FPC) and *BRCA1* is not as highly prevalent[31-34]. Studies show that germline susceptibility gene mutations were not found in 80% of pancreatic cancer individuals with strong family history[31,35]. Therefore, comprehensive genome sequencing is needed to identify new possible deleterious genes associated with FPC.

There are no current clinical practice algorithms for PACC, and it is treated in the same way as PDAC. Although FOLFIRINOX represents the standard treatment with the highest efficacy in PDAC, it is not well studied in PACC[36]. Since 2010, there is a recognized OS benefit to platinum-based agents compared to Gemcitabine or Capecitabine-based regimens, and current therapeutic approaches of metastatic PACCs utilize more FOLFOX or FOLFIRINOX. Furukawa *et al*[37] described a PACC patient with a *BRCA2* PV who received Cisplatin after a recurrence of liver metastasis and had a complete remission of the recurring tumor. Ploquin *et al*[38] reported a PACC patient with a *BRCA2* PV who experienced a 14-year complete remission following nine cycles of GEMOX, without surgical intervention. Therefore, Cisplatin and GEMOX may be alternatives in patients harboring deficiencies in DDR genes who are unfit for FOLFIRINOX.

Due to accumulative neurotoxicity after 11 cycles of mFFX, our patient decided to stop systemic chemotherapy completely and de-escalated to Olaparib as a maintenance approach. The use of PARPi in PACC patients with germline *BRCA1* or *BRCA2* PV/LPV is anecdotal[2,23,26]. Furthermore, the updated analysis of the POLO trial showed a lack of OS benefit and quality of life improvement in their Olaparib-treated patients compared to the placebo arm[39]. Despite the aforementioned, we believe that metastatic PACC patients with confirmed HRD phenotype and demonstrated strictly defined platinum sensitivity that involved exceptional response after 16 wk of chemotherapy should be considered for the benefit of PARPi, as the case described.

Like PDAC, surgery offers the best treatment approach for improved long-term survival[11,16,40]. The combination of surgical approach and perioperative chemotherapy in PACC is mainly adapted from the PDAC practice[40-42]. As mentioned, our patient with metastatic germline *BRCA2* PV had remarkable tumor downstaging following mFFX, underwent curative surgery, and achieved a pathologic near complete treatment response. Optimizing treatment approaches from this standpoint, with growing access to germline and somatic profiling, should also be further explored in PACC.

CONCLUSION

Although it is a rare disease, it is important to identify both common and rare actionable variants in PACCs. In PACC patients with *BRCA* variants, the maintenance treatment of PARPi after effective platinum-based chemotherapy should be explored further. Surgical resection may provide the chance of cure after induction chemotherapy in very well-selected patients, particularly in patients with *BRCA* variants. Further large-scale studies are required to verify these therapeutic strategies for PACC patients.

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REVIEW

Recent progress in molecular mechanisms of postoperative recurrence and metastasis of hepatocellular carcinoma

Zhao-Shan Niu, Wen-Hong Wang, Xiao-Jun Niu

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Abstract

Hepatectomy is currently considered the most effective option for treating patients with early and intermediate hepatocellular carcinoma (HCC). Unfortunately, the postoperative prognosis of patients with HCC remains unsatisfactory, predominantly because of high postoperative metastasis and recurrence rates. Therefore, research on the molecular mechanisms of postoperative HCC metastasis and recurrence will help develop effective intervention measures to prevent or delay HCC metastasis and recurrence and to improve the long-term survival of HCC patients. Herein, we review the latest research progress on the molecular mechanisms underlying postoperative HCC metastasis and recurrence to lay a foundation for improving the understanding of HCC metastasis and recurrence and for developing more precise prevention and intervention strategies.

Key Words: Hepatocellular carcinoma; Recurrence; Metastasis; Mechanisms; Postoperation; Intervention

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Core Tip: Surgical resection is currently a vital treatment option for patients with early and intermediate hepatocellular carcinoma (HCC). Unfortunately, the prognosis of HCC patients remains unsatisfactory due to high rates of postoperative metastasis and recurrence. Therefore, studies on the molecular mechanisms of postoperative HCC metastasis and recurrence will help develop effective intervention measures to prevent or delay HCC metastasis and recurrence and to improve the long-term survival of HCC patients. Herein, we review the latest research progress on the molecular mechanisms underlying postoperative HCC metastasis and recurrence to lay a foundation for improving the understanding of HCC metastasis and recurrence and for developing more precise prevention and intervention strategies.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is extremely metastatic and invasive, with postoperative metastasis and recurrence causing much of its high mortality. HCC metastasis can be roughly divided into intrahepatic and extrahepatic metastasis. Intrahepatic metastasis chiefly results from the direct invasion of cancer cells or tumor thrombus shedding to form lesions (Figure 1A), while extrahepatic metastasis includes hematogenous, lymphatic and implant metastases, which can reach many organs and tissues throughout the body. Surgical resection is currently the preferred therapy for patients with early- and mid-stage HCC, but the rate of postoperative HCC recurrence is high, and no effective preventive scheme is available. Thus, the prognosis of HCC remains poor. Numerous clinical studies have indicated that the 5-year recurrence rate of HCC after surgery is as high as 70%[1,2]. Moreover, the 5-year recurrence rate of small HCC tumors ranges from 50% to 60%[3]. Thus, HCC metastasis and recurrence have become the greatest obstacles to improving therapeutic efficacy and the long-term prognosis of HCC. Currently, the clinical detection and diagnosis of HCC metastasis and recurrence rely mainly on imaging examinations and the detection of traditional tumor markers, but the early detection of metastasis or recurrence is difficult. The molecular and biological characteristics of tumors are the key determinants of HCC metastasis and recurrence. Thus, elucidating the molecular mechanisms underlying HCC metastasis and recurrence and searching for early-warning molecules and metastasis-related intervention targets will have important implications for prolonging the survival of HCC patients after surgery.

HCC metastasis and recurrence are closely correlated with a series of molecular changes whose mechanisms may involve oncogenes, tumor suppressors, the tumor microenvironment (TME), epithelial-mesenchymal transition (EMT), liver cancer stem cells (LCSCs), circulating tumor cells (CTCs) and epigenetic modifications (Table 1). To date, the mechanisms of HCC metastasis and recurrence have not been completely elucidated. Therefore, research on the molecular mechanisms of postoperative HCC metastasis and recurrence will facilitate the development of effective intervention measures to prevent or delay HCC metastasis and recurrence and to improve the long-term survival of HCC patients. Herein, we review the latest research progress on the molecular mechanisms underlying postoperative HCC metastasis and recurrence to lay a foundation for improving the understanding of HCC metastasis and recurrence and for developing more precise prevention and intervention strategies.

ONCOGENES AND SUPPRESSORS

Aberrant expression of cancer-related genes regulates tumor cell growth, differentiation, invasion and metastasis. The activation of oncogenes and the inactivation of tumor suppressor genes can lead to uncontrolled growth of cancer cells, enabling invasion and metastasis. In addition, the abnormal expression of certain cancer-related genes can lead to functional alterations in HCC cells and contribute to metastasis and recurrence. The main genes closely connected with HCC metastasis and recurrence are described below.

Oncogenes

H-ras: H-ras proto-oncogenes encode G proteins. The 12th codon of Ras interacts with GTPase-activating protein. After binding with guanosine triphosphate (GTP), H-ras participates in intracellular information transmission. In normal cells, H-ras almost completely binds to guanosine diphosphate and is in an inactive state. However, H-ras is abnormally highly expressed in tumor tissues[4]. The abnormal

Table 1 Molecular mechanisms associated with postoperative hepatocellular carcinoma metastasis and recurrence in this review

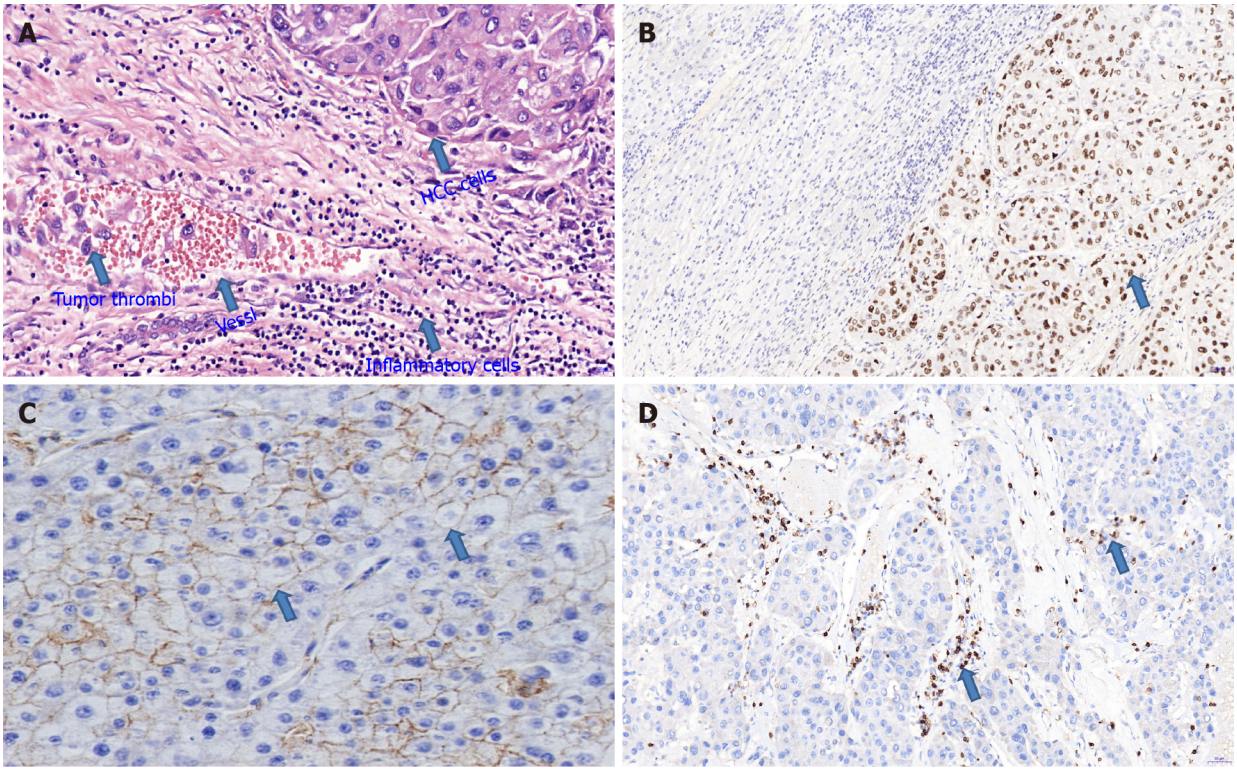
Molecular marker	Association with HCC metastasis and recurrence	Ref.
Oncogenes and tumor suppressors		
Oncogens		
H-ras	Increased expression	Ma *et al*[9]
OPN	Increased expression	Yu *et al*[22]; Zhu *et al*[25]
S100A9	Increased expression	Chiou and Lee[29]; Liao *et al*[31]
Tumor suppressors		
Mutated p53	Increased expression	Nikolova *et al*[40]; Chaudhary *et al*[44]
Nm23	Decreased expression	Khera *et al*[50]
KAI1	Decreased or lost expression	Xu *et al*[55]
Tumor microenvironment		
Tumor stromal cells		
Immune cells		
T lymphocytes	Decreased expression	Cai *et al*[68]; Gabrielson *et al*[70]; Li *et al*[71]
NK cells	Decreased expression	Wu *et al*[85]; Lee *et al*[90]
DCs	Decreased expression	Shi *et al*[108]
M2-TAMs	Increased expression	Yao *et al*[128]; Park *et al*[133]; Liao *et al*[134]
MDSCs	Increased expression	Kalathil and Thanavala[143]; Lu *et al*[147]
Tregs	Increased expression	Xiong *et al*[155]; Trehanpati and Vyas[159]
N2-TANs	Increased expression	Zhou *et al*[172]; Yang *et al*[175]
HSCs	Increased expression	Barry *et al*[198]; Cheng *et al*[200]
CAFs	Increased expression	Gao *et al*[217]; Zhang *et al*[220]
TECs	Increased expression	Tahmasebi *et al*[236]; Dong *et al*[239]
ECM		
Adhesion enhancement in tumor cell-ECM		
ICAM-1	Increased expression	Chen *et al*[255]
E-Cadherin	Decreased or lost expression	Ren *et al*[267]; He *et al*[269]
Integrins		
Oncogenic integrins	Increased expression	Zhang *et al*[276]; Huang *et al*[279]
Tumor-suppressive integrins	Decreased expression	Zhang *et al*[282]
Selectins		
P-selectin	Increased expression	Xu *et al*[288]
CD44V6	Increased expression	Chen *et al*[292]
ECM degradation		
uPA	Increased expression	Song *et al*[293]
MMP-2 and MMP-9	Increased expression	Liu *et al*[296]; Fu *et al*[297]
Cytokines		
IFN-γ	Decreased expression	Li *et al*[302]
Interleukin		
IL-6	Increased expression	Jiang *et al*[315]
IL-8	Increased expression	Zhang *et al*[327]

IL-1β	Increased expression	Duan *et al*[332]
TNF-α	Increased expression	Hammam *et al*[338]
Chemokines		
CCL2/CCR2 axis	Increased expression	Avila and Berasain[348]
CXCL12-CXCR4 axis	Increased expression	Li *et al*[355]
Growth factors		
VEGF	Increased expression	Zhang *et al*[365]
TGF-β	Increased expression	Sun *et al*[375]
Exosomes	Increased expression	Watanabe and Tanaka[385]; Luo *et al*[404]
EMT		
E-cadherin	Decreased or lost expression	Xu *et al*[413]; Helal *et al*[417]
N-cadherin and Vimentin	Increased expression	Xu *et al*[413]; Helal *et al*[417]
LCSCs		
CD133	Increased expression	Liu *et al*[363]
EpCAM	Increased expression	Tsuchiya *et al*[435]; Krause *et al*[437]
CD90	Increased expression	Yamashita and Kaneko[440]; Hwang *et al*[441]
CTCs	Increased expression	Schulze *et al*[463]
Epigenetic modifications		
DNA methylation		
Tumor suppressors	Hypermethylation	He *et al*[476]; Qian *et al*[478]
Oncogenes	Hypomethylation	Zhou *et al*[479]; Chen *et al*[480]
Noncoding RNAs		
LncRNAs		
Oncogenic lncRNAs	Increased expression	Shi *et al*[484]
Tumor-suppressive lncRNAs	Decreased expression	Song and Qiu[486]
CircRNAs		
Oncogenic circRNAs	Increased expression	Gu *et al*[499]
Tumor-suppressive circRNAs	Decreased expression	Sun *et al*[501]
miRNAs		
Tumor-suppressive miRNAs	Decreased expression	Yao *et al*[508]
Oncogenic miRNAs	Increased expression	Wang *et al*[510]

HCC: Hepatocellular carcinoma; OPN: Osteopontin; NK: Natural killer; DCs: Dendritic cells; TAMs: Tumor associated macrophages; MDSCs: Myeloid-derived suppressor cells; Tregs: T regulatory cells; TANs: Tumor-associated neutrophils; HSCs: Hepatic stellate cells; CAFs: Cancer-associated fibroblasts; TECs: Tumor-associated endothelial cells; ECM: Extracellular matrix; ICAM-1: Intercellular adhesion molecule-1; MMP-2: Matrix metalloproteinase 2; MMP-9: Matrix metalloproteinase 9; uPA: Urokinase-type plasminogen activator; IL-6: Interleukin-6; IL-8: Interleukin-8; CCL2/CCR2: Chemokine ligand 2/Chemokine receptor 2; CXCL12-CXCR4: Chemokine (C-X-C motif) ligand 12/Chemokine (C-X-C motif) receptor 4; VEGF: Vascular endothelial growth factor; TGF-β:Transforming growth factor-β; EMT: Epithelial-mesenchymal transition; LCSCs: Liver cancer stem cells; CTCs: Circulating tumor cells; lncRNAs: Long non-coding RNAs; CircRNAs: Circular RNAs; miRNAs: MicroRNAs.

activation of H-ras has been implicated in the invasiveness of multiple malignancies[5,6]. In HCC, H-ras mutations are closely associated with invasion and metastasis[7]. In addition, the EMT process is regulated by various signaling molecules, particularly transforming growth factor-β1 (TGF-β1)[8]. H-ras can induce EMT in HCC through the TGF-β1 pathway and thereby participate in HCC invasion and metastasis[9]. Thus, inhibition of H-ras activity may be a therapeutic target for HCC metastasis and recurrence.

Osteopontin: Osteopontin (OPN) is a glycoprotein and a secretory calcium-binding phosphoprotein with multiple biological activities encoded on chromosome 4q13. OPN can exert diverse functions by



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Figure 1 Microscopic image. A: Microscopic image of intrahepatic hematogenous metastasis. The presence of tumor thrombi in the vessels indicates that the Hepatocellular carcinoma (HCC) cells have invaded the surrounding blood vessels and lymphatic vessels, which is a high-risk factor for HCC recurrence and metastasis (arrow, hematoxylin and eosin × 400); B: Microscopic image of mutant p53 expression in HCC cells. TP53 is highly expressed in HCC tissues (Right), but not in benign liver tissues (Left) (arrow, immunohistochemical staining × 200); C: Microscopic image of KAI1 expression in HCC cells. KAI1 expression is down-regulated or absent in HCC cells (arrow, immunohistochemical staining × 200); D: Microscopic image of CD^{3+} T cells in HCC tissues and stromal tissues. CD^{3+} T cells are densely distributed in tumor stroma, while a single CD^{3+} T cell is occasionally distributed among HCC cells (arrow, immunohistochemical staining × 200). HCC: Hepatocellular carcinoma.

interacting with integrins or CD44 receptors to regulate cell adhesion and cell chemotaxis, regulate signal transduction, and facilitate tumor formation and progression[10]. OPN is also considered a metastasis-related gene given its high expression in metastatic cancer and tumors with high metastatic potential[11,12]. OPN overexpression is closely associated with tumor occurrence, metastasis and recurrence[13-15] and has become a molecular signature for the metastasis and recurrence of various malignancies.

OPN is overexpressed in HCC[16], in which it modulates overall signal transduction and performs a crucial role in invasion, metastasis and recurrence by binding to its receptors CD44 and integrin[17-20]. OPN promotes HCC metastasis and recurrence through several molecular mechanisms: (1) OPN inhibits apoptosis and promotes angiogenesis. Specific splice variants of OPN (OPN-b, OPN-c) inhibit tumor cell apoptosis, and OPN binding to the integrin receptors α9β1 and αvβ3 can induce angiogenesis and promote HCC metastasis[21]; (2) OPN induces EMT and increases the metastatic ability of HCC cells by directly upregulating the expression of Twist (an E-cadherin gene transcriptional regulator)[22]; (3) OPN increases the stemness of HCC cells. For example, it increases the stemness of CD133+/CD44+ cancer stem cells (CSCs) by regulating DNA methylation reorganization in HCC cells[23]; (4) OPN drives extracellular matrix (ECM) remodeling. OPN degrades the ECM by activating matrix metalloproteinase (MMP)-2 and urokinase-type plasminogen activator (uPA), resulting in HCC metastasis[24]; and (5) OPN mediates immune evasion. HCC cells secrete OPN, and serum OPN levels are elevated in HCC patients. OPN and programmed cell death ligand 1 (PD-L1) are linked in HCC, which implies that OPN is involved in immune evasion[25] and thus facilitates HCC cell infiltration and metastasis. Therefore, OPN may be a target for regulating HCC metastasis and recurrence.

S100A9: S100A9 belongs to the S100 family and contains an amino acid sequence that can bind calcium ions with high affinity and selectivity[26]. The gene encoding S100A9 is located on chromosome lq21. This region is easily affected by the internal and external environment, certain cytokines and other factors. Therefore, S100A9 exhibits poor stability and is prone to chromosomal deletion and translocation, which are connected to the growth, differentiation and metastasis potential of various malignancies[27,28]. High S100A9 levels in the preoperative serum of HCC patients are closely

associated with metastasis and recurrence[29]. The mechanisms through which S100A9 mediates HCC metastasis are currently unclear. The possible mechanisms are as follows: (1) S100A9 in HCC tissues induces the migration and aggregation of bone marrow-derived myeloid-derived suppressor cells (MDSCs), a heterogeneous population composed of immature myeloid cells with immunosuppressive properties, and thereby the formation of a powerful immunosuppressive environment[30], which is conducive to HCC invasion and metastasis; (2) Immune neutrophils/macrophages in the HCC microenvironment induce cancer cells to produce S100A9, which acts as a proinflammatory factor associated with a high risk of HCC recurrence[31,32]; and (3) S100A9 is mainly expressed in epithelial cell adhesion molecule (EpCAM)-positive cells in HCC tissues, thereby promoting HCC recurrence[33].

Suppressors

p53: The tumor suppressor gene p53 encodes a transcription factor and stress sensor that can specifically bind to DNA sequences and is very important for maintaining genomic stability[34]. Compared with other tumor suppressor genes, which only lose their tumor suppressor function after mutation (nonsense mutation), p53 not only loses its original tumor suppressor function but also acquires a new oncogenic function after mutation (missense mutation)[35]. Mutated p53 facilitates cancer cell migration, invasion and metastasis through the activation of receptor tyrosine kinase signaling[36]. Mutant p53 is a valuable predictor of local recurrence in multiple malignancies[37,38].

TP53 alteration is the most common genetic alteration in HCC and participates in HCC metastasis (Figure 1B). The *p53* gene has the potential to regulate HCC invasion and metastasis[39]; *p53* gene mutations are closely related to vascular invasion and metastasis and serve as predictors of HCC metastasis, recurrence and poor prognosis[40-44]. Mounting evidence indicates that *p53* gene mutation is related to loss of immune cells in the HCC microenvironment[45,46], which promotes the survival of HCC cells and their evasion of immune surveillance and thereby contributes to HCC metastasis. Thus, targeted therapy for *p53* gene mutation may help improve the prognosis of HCC patients.

Nm23: Nm23 (synonym: NME) is a tumor metastasis-related suppressor gene located in the q22 region of chromosome 17. Nm23 has two isoforms, nm23-H1 and nm23-H2, which are regulated by two independent regulatory systems. Of the two, nm23-H1 is more closely related to tumor metastasis, with nucleoside diphosphate kinase and histidine protein kinase (PDK) activities[47]. Nm23-H1 has the potential to inhibit the metastasis of various malignancies[48,49]. In HCC, nm23-H1 participates in the regulation of metastasis: Low nm23-H1 expression reduces nm23-H1-mediated tumor suppression, which may be an important reason for HCC metastasis[50,51]. These findings suggest that reduced expression of nm23-H1 is related to HCC metastasis and recurrence and that nm23-H1 may be a therapeutic target for HCC.

KAI1: The *KAI1* gene, also known as CD82, is a specific tumor suppressor gene[52]. The transmembrane domain of the KAI1 protein regulates tumor invasion and metastasis by relying on cell–cell or cell–ECM interactions or by regulating the expression of cell surface adhesion molecules[53,54]. The rate of KAI1/CD82-positive expression in HCC patients without intrahepatic metastasis is greater than that in patients with intrahepatic metastasis[55], which suggests that loss of KAI1/CD82 expression is linked to HCC local invasion and metastasis. In addition, KAI1 expression is downregulated or absent in HCC tissues (Figure 1C), resulting in decreased adhesion of tumor cells; this decreased adhesion allows the tumor to separate easily from the primary lesion, causing metastasis[55]. These findings suggest that the downregulation or absence of KAI1 expression may be a cause of HCC metastasis and that KAI1 may be a biomarker of HCC metastasis and recurrence. The regulation and activation of KAI1/CD82 expression may therefore be useful for HCC treatment.

TME

The TME is a local steady-state environment that is required for tumor occurrence, progression and metastasis during the growth process. The HCC TME mainly consists of HCC cells, tumor stromal cells, ECM and exosomes[56]. In the TME, interactions occur at different levels, and links between tumor cells and intercellular substances are observed. Tumor cells can reshape the TME. The remodeled TME can affect the onset and progression of cancer. The interaction between tumor cells and TME promotes the proliferation, invasion, metastasis, angiogenesis, immune evasion of tumor cells[57,58]. Other nontumor components of the microenvironment also participate in HCC metastasis and recurrence. In recent years, the tumor immune microenvironment (TIME) has gradually been recognized as a decisive factor in HCC metastasis. Mounting evidence indicates that the gene expression profiles of nontumor cell components in peritumoral tissue can predict HCC metastasis and recurrence[59-61], and the HCC TME can regulate the TIME. Cytokine release and changes in angiogenesis and other pathways promote HCC cell growth, invasion and metastasis. These findings reveal that the microenvironment of HCC cell growth has an important influence on the potential for metastasis and recurrence. Therefore, research on the dynamic relationship between the TME and HCC metastasis and elucidation of the molecular

mechanisms of different factors in the microenvironment during metastasis are key to inhibiting HCC metastasis.

Tumor stromal cells

Stromal cells are the major cellular components in the HCC microenvironment. HCC stromal cells mainly include immune cells, hepatic stellate cells (HSCs), cancer-associated fibroblasts (CAFs), and vascular endothelial cells (VECs). Interactions occur between tumor cells and stromal cells in the TME. Various stromal cells can secrete a variety of cytokines and chemokines, which act on nearby cancer cells in a paracrine manner to enhance tumor invasion and metastasis. In turn, the behavior patterns of stromal cells are influenced and modified by tumor cells.

Immune cells

The immune cells in the TME constitute the TIME and are mainly divided into effector immune cells and inhibitory immune cells. The former include T lymphocytes, natural killer (NK) cells, and dendritic cells (DCs), while the latter encompass tumor-associated macrophages (TAMs), MDSCs, regulatory T cells (Tregs), and tumor-associated neutrophils (TANs). The existing evidence indicates that the TME is in an immunosuppressive state and that the infiltrated immune effector cells show varying degrees of low immune function or even severe defects. Tumor cells establish immunosuppressive networks in the TME[62]. Under the severe metabolic and nutritional stress of the TME, immune cells are often in a disordered state, which impairs the antitumor immune response and thereby accelerates cancer metastasis and recurrence. Currently, considerable evidence indicates that the TIME is key to promoting immune evasion, metastasis and recurrence in HCC[63]. In brief, in addition to existing treatments, TIME regulation to delay postoperative HCC metastasis and recurrence warrants further consideration.

T lymphocytes: T lymphocytes are the main components of immune cells, and the detection of cellular immune-related indicators has important clinical value for evaluating the development and prognosis of tumors[64]. CD^{3+}, CD^{4+}, and CD^{8+} T cells are the main subsets of T lymphocytes, and their levels most strongly reflect the immune status of the human body. CD^{3+} T-cell numbers represent overall cellular immunity. CD^{4+} T cells can mediate the immune tolerance of tumor cells[65] and can differentiate into cytotoxic T lymphocytes (CTLs), which are stimulated by inflammatory factors and thus mediate a killing effect on tumor cells[66,67]. Therefore, dysregulation of the CD^{3+} (Figure 1D), CD^{4+} and CD^{8+} T-cell balance may participate in HCC cell growth and metastasis. The levels of T-cell infiltration decrease sequentially during the malignant progression of HCC, which suggests that decreased T-cell infiltration is associated with HCC occurrence and metastasis[68]. An increase in T-cell infiltration in HCC patients predicts a better prognosis, whereas a decrease implies a poorer prognosis[69]. Specifically, HCC patients with increased tumor-infiltrating CD^{3+} or CD^{4+} T cells are less likely to relapse[70,71]. A decreased number of CD^{8+} T cells in HCC tissues indicates a reduced killing effect, which contributes to HCC cell growth and metastasis[72]. Some studies have indicated that HCC patients with increased numbers of tumor-infiltrating CD^{8+} T cells are unlikely to relapse[71,73], suggesting that patients with elevated CD^{8+} T-cell numbers have a better prognosis[74,75]. However, other research has shown that HCC patients with increased CD^{8+} T-cell numbers are more likely to relapse[76,77]. Thus, the effects of CD^{8+} T cells on HCC metastasis and recurrence remain controversial, and the specific roles and mechanisms of these cells need to be further explored.

NK cells: NK cells are important innate immune cells that exert antiviral and antitumor effects in the body. Their cell surfaces contain activated receptors that stimulate NK cells to generate killing effects[78,79], directly kill tumor cells, activate downstream immune cells, and exert antitumor effects through immune clearance and surveillance[80]. Tumor cells can weaken the immune surveillance and clearance functions of NK cells by restraining the activation of activating receptors and facilitating the activation of inhibitory receptors[81,82]. NK-cell dysfunction, which enables tumor cells to evade the immune surveillance of NK cells, has been noted in many cancer patients[83,84].

The activation level of NK cells is strongly related to HCC metastasis and recurrence. Peripheral blood and HCC tissues often show reduced counts or inhibited functions of NK cells[85-87], which prevents the cells from performing their normal immune surveillance functions, resulting in the escape of HCC cells from immune surveillance and ultimately promoting HCC metastasis and recurrence[85,88-90]. Recent studies have found that the HCC microenvironment cannot recognize and kill tumor cells because of its functional inactivation by a variety of factors. For example, the upregulation of NK-cell immune checkpoint molecules, including programmed cell death protein 1 (PD-1), T lymphocyte immunoglobulin and mucin domain 3 (TIM-3), and cytotoxic T lymphocyte antigen 4, can restrain NK-cell function in the HCC microenvironment[91,92]. In addition, cytokines produced by HCC cells promote NK-cell dysfunction and suppress the killing function of NK cells[93], and interleukin (IL)-35 or IL-10 can inhibit NK-cell activity in the HCC microenvironment[94,95]. These factors negatively regulate NK-cell activity in HCC, which may be a significant mechanism through which NK cells participate in the immune evasion of HCC cells. Thus, blocking the regulation of NK cells mediated by these factors is an important strategy for improving NK-cell function.

Interestingly, liver-resident NK (LRNK) cells have been found to exert powerful immune killing effects. In early HCC, LRNK cells can effectively eliminate cancerous liver cells through surface receptors, such as NKG2D, TRAIL and FASL, and maintain the homeostasis of the normal liver microenvironment[96]. However, with the progression of HCC, the gradually formed HCC microenvironment affects the antitumor activity of LRNK cells. The expression of the immune checkpoint protein TIM-3 is significantly upregulated on the surface of tumor-infiltrating LRNK cells, decreasing cytokine secretion by LRNK cells and weakening cytotoxicity[97]. In addition to TIM-3, LRNK cells in HCC tissues also highly express PD-1, LAG-3, TIGIT and other immune checkpoint molecules, and HCC patients with higher proportions of immune checkpoint–expressing LRNK cells in tumors are more likely to develop tumor thrombi and to lack capsules[97,98], features that are closely related to HCC recurrence[99,100]. In summary, the immune function impairment caused by upregulated expression of immune checkpoint molecules on LRNK cells is conducive to HCC metastasis and recurrence. Thus, blocking the regulation of these immune checkpoint molecules, particularly inhibitory receptors, is an important strategy for improving the function of LRNK cells.

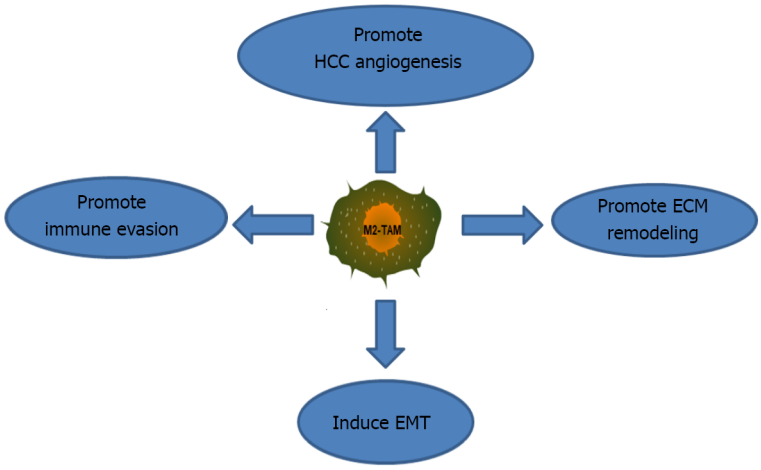
DCs: DCs typically function as antigen-presenting cells. After exposure to antigens in tumor patients, DCs present antigen information to T cells, stimulate T-cell proliferation and activation, and generate large amounts of tumor cell–killing cytotoxic T cells; these cells help completely remove some tiny cancer foci and thus delay and reduce tumor recurrence[101-103]. DCs that infiltrate tumor tissues are called tumor-infiltrating DCs (TIDCs). The immunosuppressive environment in the TME induces an abnormal state in TIDCs, including maturation disorder, functional abnormalities, and phenotypic changes. Thus, these cells cannot effectively present tumor antigens and activate T cells to kill tumor cells, resulting in tumor cell immune evasion[104,105]. Current evidence suggests that the expression of TIDCs is reduced in multiple malignancies[106,107], which indicates that cancer cells can achieve immune evasion by decreasing their TIDC levels.

In HCC patients, DCs are present in reduced numbers and exhibit abnormal differentiation and impaired maturation in the tumor regions and lymph nodes. These DCs fail to activate antigen-specific T-cell responses[108]. DC infiltration into tumors reflects the host's immune defense mechanism. Greater levels of DC infiltration are associated with a lower tumor recurrence rate and a lower metastasis rate[109]. CD1a is typically considered a marker of immature DCs but is also expressed in mature DCs[110]. CD1a+ DC infiltration is closely associated with tumor metastasis and recurrence in a variety of malignancies, including HCC, and the degree of CD1a+ DC infiltration in tumors with distant metastasis is significantly lower than that in nonmetastatic tumors[110,111]. The potential mechanism involves DCs in tumors that ingest and present tumor antigens and subsequently induce the host's antitumor immunity. This immune state is helpful for completely eliminating some residual tiny lesions, micrometastases and even early recurrent lesions after tumor resection, which ultimately delays and reduces tumor recurrence.

Plasmacytoid DCs (pDCs) are an interesting subgroup of DCs in human blood[112]. The infiltration of pDCs after HCC resection is a poor prognostic indicator. The infiltration of pDCs into tumors may induce the formation of an immune inflammatory TME *via* Tregs and IL-17. This condition may lead to a state of tolerance and promote tumor immune evasion[113,114], thereby promoting HCC invasion and metastasis.

TAMs: TAMs are the main infiltrating immune cells in the TME and exhibit phenotypes and functions that differ from those of other macrophages during inflammatory generation. Under the action of different stimulatory factors, TAMs may differentiate into M1-type TAMs with tumor-inhibitory activity or M2-type TAMs with tumor-promoting activity[115,116]. Most of the high-density TAMs located within or around tumors exhibit signatures of M2-type TAMs that promote tumor metastasis by regulating matrix remodeling, promoting neovascularization, and inhibiting local immunity[117-120].

Current evidence suggests that M2-type TAMs act on HCC cells through complex signaling pathways, promoting HCC cell proliferation, angiogenesis, and intrahepatic metastasis and thus resulting in poor prognosis in HCC patients[121,122]. M2-type TAMs promote HCC metastasis and recurrence through several mechanisms (Figure 2): (1) M2-type TAMs promote tumor angiogenesis. TAMs are significantly correlated with angiogenesis in HCC patients[123]. TAMs secrete large amounts of vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), platelet-derived growth factor (PDGF), and TGF-β under stimulation by hypoxia or a lack of nutrients in tumor cells, ultimately inducing VEC proliferation and promoting HCC angiogenesis[124,125]. In addition, proteolytic enzymes and MMPs secreted by TAMs can promote chemotaxis to induce the abovementioned proangiogenic cytokines to travel to tumor tissues, accelerate the process of angiogenesis, and subsequently promote HCC invasion and metastasis[126]; (2) M2-type TAMs induce ECM remodeling. The ECM acts as a scaffold and barrier for tumor cell migration, and its degradation is a focus in the field of tumor metastasis research. TAMs can secrete a variety of proteolytic enzymes in HCC, particularly MMP-2 and MMP-9, which act on the ECM, degrade the basement membrane (BM) and promote tumor cell invasion and metastasis[127]; (3) M2-type TAMs induce EMT, a key event in HCC metastasis. TAMs are critical cells in the HCC microenvironment and promote EMT progression in HCC cells through various pathways. For example, M2-TAMs induce EMT by activating the Toll-like receptor 4



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Figure 2 Schematic diagram of molecular mechanisms by which M2-type tumor associated macrophages promote hepatocellular carcinoma metastasis and recurrence. HCC: Hepatocellular carcinoma; M2-TAMs: M2-type tumor associated macrophages; ECM: Extracellular matrix; EMT: Epithelial mesenchymal transition.

(TLR4)/STAT3 pathway and thus promote HCC metastasis[128]. In addition, TAMs secrete various factors, including IL-8, TNF-α and TGF-β1, that promote the activation of downstream signaling pathways and thereby induce EMT in HCC[129-131]. Furthermore, some proteases secreted by TAMs, such as MMPs, are also involved in the progression of EMT. For example, MMP17 secreted by TAMs induces EMT to facilitate HCC metastasis[132]; and (4) M2-type TAMs promote immune evasion. TAMs play a pivotal role in tumor-induced immunosuppression. TAMs in HCC can also express PD-1, an extremely important immune checkpoint target; these PD1+ TAMs show the characteristics of the M2 phenotype. PD1+ TAMs directly interact with PDL1+ cells to produce large amounts of IL-10, which inhibits CD^{8+} T-cell proliferation, destroys the CD^{8+} T-cell antitumor immune effect, and promotes HCC invasion and metastasis[133-135]. In addition, TAMs induce the HCC microenvironment to become immunosuppressive. TAM-enriched sites show decreased numbers and functional defects of infiltrated CD^{8+} T cells and inhibited function of NK cells and DCs among killer immune cells. Moreover, TAMs activate Tregs[136-138]. These phenomena all play key roles in tumor immune evasion. Some cytokines secreted by TAMs, particularly TGF-β and IL-10, are also significant factors in the formation of the immunosuppressive HCC microenvironment[139].

In summary, the above findings suggest that reducing the density or effects of M2-type TAMs can inhibit HCC metastasis and recurrence. Thus, M2-type TAMs are expected to be potential targets for HCC therapy.

MDSCs: MDSCs, one of the fundamental cell types in malignancies, can be recruited into the TME to help establish an immunosuppressive TME and promote tumor immune evasion[140]. In addition to immunosuppressive activity, MDSCs exhibit tumor immune evasion–supporting and nonimmunosuppressive effects, promoting tumor metastasis and recurrence *via* multiple molecular mechanisms[141].

Emerging evidence indicates that MDSCs in the TME are strongly connected to HCC invasion, metastasis and recurrence[142-144]. MDSCs accelerate HCC metastasis and recurrence through several main molecular mechanisms: (1) MDSCs promote the formation of a premetastatic niche (PMN). Tumors can recruit MDSCs to target organs by secreting cytokines or chemokines[144]. In addition, MDSCs secrete related inflammatory factors to form an immunosuppressive PMN, which can further promote HCC metastasis[140,145]; (2) MDSCs promote immunosuppression. MDSCs restrain T-cell proliferation or facilitate Treg expansion by secreting arginase-1, inducible nitric oxide synthase, reactive oxygen species (ROS), and IL-6 to exert immunosuppressive effects[114,143]. In addition, MDSCs effectively suppress the proliferation and function of CD^{8+} CTLs in the HCC microenvironment [146]. These changes allow tumor cells to escape immune surveillance and attack and ultimately promote HCC progression and metastasis; (3) MDSCs promote HCC angiogenesis. MDSCs can induce tumor angiogenesis and further modify the TME. For example, Bv8 produced by MDSCs has been shown to induce HCC angiogenesis[147] and thereby promote HCC growth and invasion; and (4) MDSCs induce ECM remodeling. MDSCs promote ECM degradation by secreting proteases, such as MMP9, this degradation enhances HCC cell invasion[148].

Taken together, these findings indicate that MDSCs are essential for the promotion of HCC metastasis and recurrence and may be potential targets for antitumor therapy. Notably, targeting MDSCs alone will be insufficient; a combination of different treatment strategies will be needed. However, combining therapies targeting MDSCs with existing treatments may be a new integrated strategy for HCC patients in the future.

Tregs: Tregs are a class of T lymphocytes with powerful negative immunomodulatory functions. Tregs restrain the activity of effector T and B cells, which are key to the maintenance of immune balance and tolerance. Massive infiltration of Tregs in the TME not only inhibits the immune system response to cancer cells, leading to tumor cell immune evasion and inhibiting the function of antitumor immunity [149], but also facilitates tumor cell proliferation, invasion and metastasis[150]. Treg infiltration in malignancies also predicts poor outcomes and is associated with postoperative local metastasis and recurrence[151].

The Treg density in the peripheral blood of HCC patients increases progressively during the malignant progression of HCC, which suggests that an increased density of Tregs is closely related to HCC invasiveness, metastasis and recurrence[152-154]. Tregs facilitate HCC invasion and metastasis through several molecular mechanisms: (1) An association exists between Treg infiltration and driver gene mutations. TP53 mutation is strongly associated with HCC metastasis and recurrence, and HCCs with TP53 mutations have higher Treg infiltration than those without TP53 mutations[155]; (2) Treg infiltration induces EMT. Tregs promote HCC invasion and migration by promoting TGF-β1 expression to induce EMT in HCC cells[156]; (3) Treg infiltration promotes immune evasion. Tregs promote HCC cell immune evasion through multiple pathways. (a) The recruitment of Tregs to the TME is related to tumor cell evasion of immune surveillance and clearance, and the powerful inhibitory function of Tregs promotes the immune tolerance of tumor cells and inhibits T-cell activity[157]; (b) Tregs upregulate the checkpoint inhibitors PD-L1 and PD-1 to suppress the production of T effector cells in the TME and thereby weaken the immune killing effect on HCC cells[158]; and (c) Tregs directly contact and/or secrete immunosuppressive factors, including IL-2, IL-10, TGF-β and IL-35. These immunosuppressive factors form an immunosuppressive microenvironment by inhibiting the activity of naïve T cells. The tumor cells then achieve immune evasion, resulting in HCC spread and even metastasis[159]; and (4) The increased numbers of Tregs may inhibit CD^{4+} and CD^{8+} T-cell activity, disrupting the immune balance in the TME and generating an immunosuppressive microenvironment; these effects enable HCC cells to escape attack by immune cells[160].

In summary, the Treg population is associated with HCC metastasis and recurrence. Thus, therapeutic strategies targeting Tregs or combined therapies can selectively inhibit Tregs on the premise of maintaining immune balance and can support antitumor immunity mediated by effector T cells to achieve effective treatment.

TANs: TANs are neutrophils that infiltrate the TME. As important inflammatory cells, TANs participate in the formation and maintenance of the tumor inflammatory microenvironment and regulate tumor progression[161]. Increasing amounts of evidence suggest that neutrophil plasticity and diversity underlie the dual potential of TANs in the TME. Depending on the different stimulating factors in the TME, TANs can be activated into the tumor-suppressive N1 type and the tumor-promoting N2 type. The N1 phenotype has cytotoxic and proinflammatory activities, whereas the N2 phenotype has strong immunosuppressive ability[161]. N2-TANs mainly promote tumor proliferation, invasion, angiogenesis and metastasis by releasing neutrophil extracellular traps (NETs), suppressing the immune response, and producing derivatives, such as cytokines and proteases[162-164].

Three types of granules (primary, secondary and tertiary granules) and secretory vesicles are present in neutrophils. These granules are composed of various proteases, including MMPs, cathepsins, and neutrophil elastase[165]. MMPs play various roles in tumor progression, not only mediating ECM degradation but also controlling tumor angiogenesis and affecting tumor cell growth. MMPs are principal regulators of tumor invasion and metastasis[166]. For example, MMPs induce HCC cells to break through the histological barrier composed of the BM and ECM, invade their neighboring tissues and metastasize to distant tissues by destroying the degradation balance of the matrix[167]. In addition, MMPs can induce and promote EMT in the HCC microenvironment, facilitating HCC metastasis[168]. Cathepsin is also closely related to HCC metastasis[169].

HCC cells can further activate chemokine ligand (CCL) 2 and CCL17 by activating TANs. These chemokines induce the migratory activity of tumor cells by affecting TAMs and Tregs[170]. These recruited immune cells promote tumor progression, facilitating HCC cell invasion and metastasis by releasing cytokines[171]. Moreover, TANs can accelerate HCC invasion and metastasis by inducing and enhancing the stemness traits of HCC cells and thus inducing increased secretion of CXC chemokine ligand 5 (CXCL5) to induce TAN intratumoral infiltration and form a positive feedback pathway consisting of TANs, HCC cells, and stem cell-like HCC cells[172]. Recent studies have confirmed that TANs form NETs by releasing chromatin structures from cells, blocking contact with immune cells by covering tumor cells, and protecting tumor cells from cytotoxicity mediated by CD^{8+} T cells and NK cells [173]. TANs also "wake up" and "trap" dormant cancer cells and serve as cancer cell adhesion substrates that promote tumor occurrence and metastasis[174]. The ability to form NETs is significantly enhanced in HCC patients, and NETs ultimately promote HCC metastasis by capturing CTCs and enhancing the migration and angiogenesis capabilities of CTCs[175]. In addition, NETs promote HCC metastasis by establishing a PMN[176]. In conclusion, numerous studies have demonstrated the potential mechanisms by which TANs promote HCC metastasis and recurrence. The phenotypic plasticity and functional diversity of TANs suggest that TANs are potential therapeutic targets for HCC.

HSCs

HSCs are mainly distributed in the hepatic space of Disse[177] and have two phenotypes, quiescent and activated. Under physiological conditions, most HSCs are in a quiescent state and participate primarily in the dynamic balance of vitamin A metabolism. HSC activation is the central link in hepatic fibrosis caused by various etiologies, and activated HSCs play an important role in HCC metastasis and recurrence. Therefore, elucidating the molecular mechanisms of HSC activation is of great significance in preventing or reversing hepatic fibrosis and in preventing postoperative HCC recurrence and metastasis. The mechanisms of HSC activation are complicated. In addition to cytokines, transcription factors and oxidative stress can activate HSCs, and noncoding RNA (ncRNA) and exosomes have also been found to be involved in HSC activation in recent years. In brief, the mechanisms of HSC activation are as follows: (1) Cytokines participate in HSC activation. When the liver is injured, hepatocytes, Kupffer cells, sinusoidal endothelial cells, macrophages and platelets all secrete cytokines that play decisive roles in HSC activation, such as TGF-β and PDGF. The mechanisms of action by which cytokines activate HSCs are quite complex. Rather than acting in isolation, cytokines form a network through autocrine and paracrine interactions, thereby activating HSCs. TGF-β can activate HSCs by binding to specific receptors of HSCs and activating both small mother against decapentaplegic (SMAD)-dependent and non-SMAD pathways[178]. TGF-β can also activate HSCs through HAS2, TLR4he and Notch1[179]. PDGF is mainly produced by platelets, Kupffer cells and sinusoid endothelial cells in the liver. After PDGF binds to the receptor on the membrane of HSCs, it promotes HSC proliferation and activation mainly through phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3), and mitogen-activated protein kinase (MAPK)[180-182]; (2) Transcription factors participate in HSC activation. Transcription factors related to HSC activation mainly include nuclear factor-κB (NF-κB) and peroxisome proliferator-activated receptor γ (PPAR-γ). NF-κB is an important intracellular mediator of the liver inflammatory response. NF-κB activation can promote HSC activation by promoting the generation of proinflammatory factors such as IL-8, IL-1β, TNF-α and IL-6[183,184]. PPAR-γ is a class of ligand-activated nuclear receptor transcription factors that are members of the nuclear hormone receptor superfamily. PPAR-γ exerts anti-inflammatory effects by inhibiting the transcription of inflammatory cytokines and has a regulatory effect on maintaining HSC quiescence and inhibiting HSC activation. It has been shown that PPAR-γ inhibits HSC activation mainly by inhibiting the activity of the TGF-β1/Smad pathway or by downregulating the expression of potent fibrogenic factors such as PDGF, connective tissue growth factor (GF), and TGF-β1[185,186]; (3) Oxidative stress is an important factor in HSC activation. When oxidative stress occurs, excessive production of ROS *in vivo* not only directly damages hepatocytes but also activates various signaling pathways, such as nuclear factor-related factor-2, TGF-β, NF-κB and other related cytokines, signaling molecules and their downstream signaling pathways, that further aggravate liver tissue injury and activate the production of various inflammatory factors. This process further stimulates the continuous activation of NF-kB and regulates apoptosis-related proteins, IL-1β, TNF-α and IL-6, which play important roles in promoting HSC activation and proliferation[187,188]; (4) NcRNAs are also involved in HSC activation. NcRNAs include microRNAs (miRNAs), long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs), which can regulate HSC activation through a variety of mechanisms. Among them, oncogenic ncRNAs facilitate HSC activation. For example, miRNA-503 overexpression can enhance HSC activation by activating the TGF-β/Smad pathway[189]; the upregulation of lncRNA SNHG7 promotes HSC activation by sponging miR-29b, which enhances DNA methyltransferase (DNMT) 3A expression[190]; and the upregulation of circUbe2k facilitates HSC activation by sponging miR-149-5p, which enhances TGF-β2 expression[191]. In contrast, tumor suppressive ncRNAs inhibit HSC activation. For example, overexpression of miR-489-3p and miR-122-5p suppresses HSC activation by restraining the uneven regulation of the jagged canonical Notch ligand 1/notch homolog protein 3 (NOTCH3) signaling pathway[192]; upregulation of lncRNA Meg8 inhibits HSC activation by suppressing the Notch pathway[193]; and increased expression of hsa_circ_0070963 suppresses HSC activation by enhancing LEM domain containing 3 expression by sponging miR-223-3p [194]; and (5) Exosomes regulate HSC activation through various mechanisms. For example, high expression of exosomal miR-192 derived from hepatitis C virus (HCV)-infected hepatocytes can stimulate TGF-β1 expression and thus induce HSC activation[195]. In contrast, NK-cell-derived exosomes can inhibit TGF-β1-induced HSC activation[196].

Activated HSCs subsequently secrete soluble proteins that cause interstitial changes, providing structural support to the microenvironment for HCC invasion and metastasis. HSCs play important roles in promoting HCC invasion and metastasis *via* multiple mechanisms: (1) The direct effects of activated HSCs on HCC cells promote HCC metastasis. Activated HSCs secrete numerous related proteins, such as vimentin, ECM protein and α-smooth muscle actin (α-SMA)[197], which subsequently promote the secretion of a variety of inflammatory factors and cytokines, aggravate the formation of the inflammatory microenvironment, and participate in various processes that affect the progression of HCC[198,199]. In addition, TGF-β1 promotes α-SMA expression in activated HSCs, upregulates the expression of IL-1 and VEGF in HCC, and thereby promotes HCC invasion and metastasis[200]; (2) HSCs also promote HCC invasion and metastasis by participating in ECM remodeling. When HSCs are activated, the secreted TGF-β1 can facilitate the degradation of MMPs and upregulate tissue inhibitor of metalloproteinase (TIMP) expression, breaking the balance between the two and resulting in reduced

ECM degradation. The deposited ECM can promote HSC activation[201], forming an HSC–ECM–HSC positive feedback loop and promoting the occurrence and development of HCC. TGF-β1 can further encourage HSCs to synthesize large amounts of ECM and thus to promote HCC occurrence, development, invasion and metastasis[200]; (3) HSCs promote tumor angiogenesis by secreting cytokines and thus facilitating HCC metastasis. Activated HSCs can produce large amounts of proangiogenic factors, such as VEGF and angiopoietin, which promote tumor angiogenesis by binding with homologous receptors on the surfaces of endothelial cells[202] and facilitate HCC invasion and metastasis[203]; (4) Activated HSCs exert important immunosuppressive effects and thus promote HCC metastasis[203]; and (5) HSCs secrete hepatocyte GF to facilitate *STMN1* expression in HCC cells, and the upregulation of this gene accelerates the activation of HSCs and results in the acquisition of the CAF phenotype[204].

In summary, HCC cells can induce the activation of HSCs, and activated HSCs can counteract HCC cells in the HCC microenvironment. The two-way interaction creates a cascade amplification effect that forms a microenvironment conducive to immune evasion and thus ultimately promotes tumor invasion and metastasis. Therefore, combining current HCC therapies with targeted therapies for HSCs in the tumor stroma is a promising strategy for the treatment of HCC.

CAFs

Fibroblasts are abundant and widely distributed in the stroma. CAFs do not exist in healthy individuals, but once tumors develop *in vivo*, tumor cells can induce fibroblasts to transform into CAFs. CAFs further promote tumor cell proliferation and thereby form a vicious cycle[205-207]. CAFs are regulators of tumor cell metastasis to distant sites, which can generate PMNs in distant organs and trigger subsequent metastatic events. Thus, these cells are also known as metastasis-associated fibroblasts[208]. Numerous types of pro-invasion molecules and inflammatory mediators act in a paracrine manner in CAFs through cell-to-cell interactions and participate in the remodeling of the ECM, induction of EMT, and promotion of tumor angiogenesis, which increases tumor invasion and metastasis[209-213].

CAFs play a significant role in promoting HCC invasion and metastasis by secreting cytokines. In patients with high expression of CAF-specific molecular markers, HCC is prone to metastasis and recurrence[214]. Current evidence shows that CAFs may facilitate HCC metastasis through several mechanisms: (1) CAFs promote tumor angiogenesis. In HCC, CAFs promote angiogenesis by releasing proangiogenic factors[215]. For example, placental growth factor (PlGF) is highly expressed in CAFs, and PlGF overexpression is highly correlated with tumor angiogenesis. CAFs secrete PlGF to promote HCC angiogenesis[216]. In addition, CAFs form a network of cytokines with HCC cells, secreting GFs and MMPs that can induce tumor cell invasion through autocrine or paracrine signaling and secreting proangiogenic factors to recruit endothelial cells to stimulate tumor angiogenesis[217]. These findings further demonstrate the role of CAFs in HCC angiogenesis; (2) CAFs induce EMT. CAFs can enhance HCC invasion and distant metastasis by inducing EMT in HCC cells[218,219]; (3) CAFs induce ECM remodeling. CAFs alter the ECM structure, reshape the stroma, and subsequently affect HCC cell growth, migration and invasion[220,221]; (4) CAFs promote HCC metastasis by facilitating the stemness acquisition of tumor cells and maintaining the stemness of CSCs. CSCs are the "seeds" of tumor metastasis and recurrence, and maintaining the stemness of CSCs requires the support of the TME in which they are located. CAFs play a significant role in the transformation of the stemness phenotype of cancer cells, in the maintenance of the stemness of CSCs, and in increasing the number of CSCs[222, 223]. CAFs enhance the stem cell-like characteristics of HCC cells by activating the IL-6/STAT3/Notch pathway[224]. Moreover, CAFs maintain and promote the stem-like biological characteristics of CD^{24+} HCC stem cells by secreting HGF and IL-6 to activate the STAT3 pathway[225]; (5) CAFs promote immune evasion. CAFs regulate peripheral immune cells by secreting chemokines and thus participate in tumor immune regulation. CAFs derived from HCC induce myeloid-derived immunosuppressive cells, such as TANs, to mediate immunosuppression and participate in HCC immune evasion[226]. CAFs in HCC recruit normal DCs and induce their transdifferentiation into regulatory DCs by activating the IL-6-mediated STAT3 pathway; these cells rarely present antigens and can also secrete inhibitory cytokines to form an immunosuppressive microenvironment[227]; (6) CAFs mediate HCC metastasis by secreting cytokines. Cytokines secreted by CAFs, such as CCL2, IL-6, and IL-8, promote HCC cell migration and metastasis[228,229]. CAFs also promote HCC metastasis *via* the chemokine-activated Hedgehog and TGF-β pathways; specifically, CCL2, CCL5, and CCL7 secreted by CAFs promote HCC metastasis by coactivating the Hedgehog and TGF-β pathways[230]; and (7) CAF-mediated HCC metastasis is partly related to the exosomes secreted by CAFs. CAFs produce numerous exosomes to regulate the microenvironment and biological characteristics of tumor cells and to facilitate tumor invasion and metastasis[231]. Dysregulated expression of exosomal miRNAs or lncRNAs secreted by CAFs is an important mechanism affecting tumor initiation, growth, invasion and metastasis; it is also an important feature of TME imbalance and an important cause of tumor metastasis and recurrence. Abnormal expression of exosomal miRNAs or lncRNAs secreted by CAFs may also occur in the HCC microenvironment. For example, miR-320a, miR-150-3p and miR-29b are miRNAs in CAF-secreted exosomes in HCC that act as tumor suppressors, and loss of their expression significantly facilitates HCC cell proliferation and metastasis and is an important risk factor for HCC recurrence[232-234].

In recent years, in-depth exploration of CAFs and their secreted cytokines has provided increasing amounts of evidence that CAFs should be new targets for anti-HCC therapy.

VECs

VECs control the entry of nutrients into tissues, maintain blood flow, and regulate leukocyte transport [235]. VECs in the TME can lead to endothelial dysfunction due to factors such as hypoxia and chronic GF stimulation, resulting in irregular shapes and sizes, wrinkled edges, and other abnormal morphologies in tumor endothelial cells (TECs)[236]. Various interactions exist between tumor cells and VECs. For example, tumor cells can induce endothelial cells to form neovascularization networks around tumor cells by secreting GFs[237]. Conversely, endothelial cells in the tumor neovasculature, as an important component of the TME, also synthesize and secrete certain cytokines to facilitate tumor cell migration and invasion and to promote tumor cell metastasis to distant tissues[238].

Tumor VECs promote the entire process of HCC metastasis through various mechanisms: (1) VECs promote HCC metastasis through interaction with cancer cells[236]; (2) VECs promote HCC metastasis by forming cancer nests. Unique small, spherical cancer cell nests surrounded by endothelial cells have been found in some cases of HCC. These spherical cancer nests can enter the blood circulation through fusion with microvessels and metastasize[239]. Unlike single HCC cells, cancer nests metastasize in small groups with higher metastasis efficiency; and (3) VECs promote HCC metastasis *via* rapid neovascularization. Neovascularization in tumors is an important channel for tumor cells to achieve metastasis. Secretory CXCL12 can rapidly induce neovascularization by regulating C-X-C chemokine receptor 4 (CXCR4) in endothelial cells and promote HCC cell metastasis[240]. Based on the above findings, TECs can be directly and selectively inhibited or killed to suppress or prevent HCC metastasis and recurrence.

ECM

The ECM is a glycoprotein network that constitutes the main component of the BM and forms a natural barrier to tumor metastasis[241]. Collagen protein, proteoglycans (glycosaminoglycan, proteoglycans and hyaluronic acid) and glycoproteins (such as elastin, fibronectin and laminin) are the major components of the ECM. The ECM connects the inside and outside of the cell as a whole through membrane integrins and thereby determines the cell shape and controls cell differentiation and migration[242,243]. Whether cancer cells infiltrate and metastasize is related to the cancer cells themselves and to the ECM. A series of dynamic changes occur between cancer cells and the surrounding ECM during cancer invasion and metastasis. The remodeled ECM creates a loose microenvironment for cancer cell proliferation and differentiation, leading to cancer cell infiltration and metastasis. Moreover, cancer cells adhere to the ECM and induce the production of proteases to degrade the ECM, which allows cancer cells to break away from their primary site, enter blood vessels or lymphatic vessels, circulate, survive, and then escape the BM and ECM to form secondary metastases [244-246].

Tumor cell–ECM adhesion enhancement in HCC metastasis

The first step of tumor metastasis involves changes in cell adhesion characteristics. These changes weaken adhesion between tumor cells and enhance adhesion between tumor cells and the ECM involving cell adhesion molecules (CAMs)[247], which are transmembrane glycoproteins that mediate cell–cell and cell–ECM adhesion. The CAMs discovered thus far can be roughly divided into five categories: Immunoglobulin superfamily (IgSF) members, cadherin family members, integrin family members, selectin family members and CD44V6[248]. Abnormal expression or functional changes in CAMs decrease (homogeneous) adhesion between tumor cells and enhance (heterogeneous) the adhesion of ECM to nontumor cells, which is the initial step of tumor invasion and metastasis[249].

IgSF: IgSF-like cell adhesion molecules are a class of molecules that contain specific immunoglobulin-specific domains, which mediate both homogenous and heterogeneous adhesion. This superfamily mainly includes intercellular adhesion molecules (ICAMs; ICAM-1, 2, 3), vascular intercellular adhesion molecules-1 and nerve cell adhesion molecules[250]. Among the IgSF members, ICAM-1 is the most closely associated with HCC metastasis and recurrence. ICAM-1 is a glycoprotein expressed on the cell surface. High ICAM-1 expression reduces adhesion between tumor cells; thus, the tumor cells can detach, enter the blood and combine with lymphocytes to escape the killing effects of immune cells. The tumor cells easily become trapped and implant in capillaries or lymph sinuses, forming metastatic foci and completing tumor invasion and metastasis[251-253]. In addition to mediating adhesion, ICAM-1 exists in serum in a soluble form called sICAM-1. sICAM-1 retains the extracellular structure of ICAM-1, binds to ligands (lymphocyte function-associated antigen-1, very late antigen-4) on the surfaces of immune cells, and thus prevents immune cells from effectively recognizing and killing the tumor cells, enabling immune evasion[254], which is conducive to tumor cell invasion and metastasis.

HCC patients with high levels of sICAM-1 have higher distant metastasis and recurrence rates than HCC patients with normal or low levels of sICAM-1[255]. These findings suggest that the level of sICAM-1 in serum or plasma can be used as an important auxiliary indicator to predict and monitor HCC metastasis and recurrence. When combined with clinical and imaging examinations, sICAM-1 detection can improve the early diagnosis rate of HCC recurrence.

Cadherin family: A cadherin is a type of CAM containing an immunoglobulin domain that is dependent on calcium ions and can mediate signaling through a signal transduction pathway. The cadherin family contains E-cadherin, N-cadherin, P-cadherin, and T-cadherin[256]; among these, E-cadherin is most closely associated with tumor invasion and metastasis. E-cadherin is a tumor invasion suppressor that inhibits tumor metastasis in multiple malignancies[257,258], prevents the shedding of primary tumor cells and inhibits tumor metastasis. E-cadherin is a critical protein in the process of EMT, and its low expression or loss can lead to EMT. Tumor cell numbers significantly increase after the loss of contact inhibition features, resulting in local tumor cell detachment and eventually leading to tumor cell infiltration into the surrounding tissues and distant metastasis[259,260]. E-cadherin is the most important molecule in mediating homogeneous adhesion between cells of the same type and is the key negative regulatory adhesion factor for metastasis[261]. Abnormal expression of E-cadherin is associated with tumor metastasis and recurrence[262,263].

A reduction in or loss of E-cadherin expression is a significant factor leading to high aggression and metastatic ability of HCC cells. Loss of E-cadherin expression may lead to weakening or loss of adhesion between homologous cells, which allows cancer cells to spread and metastasize to other sites[264,265]. The loss of E-cadherin in HCC cells can induce EMT and thus facilitate HCC invasion and metastasis [266]. In addition, E-cadherin can be regulated by the Wnt/β-catenin or PI3K/AKT/mammalian/mechanistic target of rapamycin pathway, leading to participation in HCC invasion and metastasis [267,268]. In particular, HCC patients with metastasis have lower E-cadherin expression than patients without metastasis, which suggests that a decrease in or loss of E-cadherin expression is strongly associated with HCC metastasis and recurrence after radical resection[269]. Therefore, E-cadherin is expected to be a useful index for predicting HCC metastasis and recurrence.

Integrins: The integrins are a group of 24 heterodimers formed by the noncovalent combination of at least 18 α and 8 β subunits[270]. Integrins can preferentially recognize and bind to different ECM ligands, cell surface ligands and water-soluble ligands, and the Arg-Gly-Asp sequence in the ECM is a common binding motif of integrins[271]. The binding of integrins to the ECM generates bidirectional signals and triggers a variety of signal transduction pathways that mainly regulate cell adhesion, invasion, migration, proliferation, survival and apoptosis[272,273]. Integrins, which are highly expressed in numerous types of malignant cells, reduce the homogeneous adhesion between tumor cells and promote the shedding of tumor cells from the primary site into the blood circulation. Moreover, integrins can enhance the adhesion between tumor cells and heterogeneous cells or protein molecules and promote tumor invasion and metastasis[274,275]. Therefore, integrins play a major regulatory role in the TME, tumorigenesis, metastasis and recurrence.

Integrins also play a critical role in HCC cell proliferation, invasion and metastasis: They are target genes of miRNAs, and they regulate intracellular signaling pathways. For example, integrin-β1 (ITGB1), an oncogene of HCC, facilitates HCC cell growth, EMT and metastasis as the target gene of miR-3653 and accelerates HCC cell invasion as the target gene of miR-1226-3p[276,277]. ITGB5, another oncogene of HCC, accelerates HCC cell invasion and metastasis as the target gene of miR-221-5p and miR-134[278, 279]. In addition, the HCC oncogenes ITGB1 and integrin alpha 7 (ITGA7) promote HCC cell migration, invasion, and stemness by regulating intracellular TGF-β/Smad and protein tyrosine kinase 2-PI3K-Akt signaling, respectively[280,281]. Conversely, the tumor-suppressive ITGA9 prevents HCC cell aggressiveness and metastasis *via* focal adhesion kinase/c-Src tyrosine kinase-Ras-related C3 botulinum toxin substrate 1/Ras homolog family member A signaling[282].

Selectins: Selectins are members of a class of Ca^{2+}-dependent transmembrane glycoproteins with a lectin-like domain that are mainly expressed in bone marrow-derived and endothelial cells[283]. Under normal circumstances, selectins are in an inert state, and their main physiological function is to mediate the recruitment of leukocytes to inflammatory sites or lymphoid tissues[284]. Selectins facilitate the hematogenous metastasis of cancer cells, and cancer cells mimic the selectin-mediated exudation of leukocytes from VECs to aid cancer cell extravasation and even accelerate cancer cell metastasis *via* the formation of tumor thrombi through adhesion between cancer cells and platelets/leukocytes[285].

Selectins are mainly expressed on the surfaces of platelets and VECs. These proteins bind with ligands on the surfaces of tumor cells to modulate the adhesion of tumor cells to platelets and VECs and thus promote tumor metastasis[286,287]. Hematogenous metastasis is the main mode of HCC metastasis. P-selectin plays a major role in this process, and the most frequent sites of extrahepatic metastasis are the lungs. The expression of P-selectin in HCC and lung metastatic tissues is significantly higher than that in adjacent normal tissues, and the mechanism may involve the adhesion of CTCs to the surface of the vascular endothelium in a manner similar to that of inflammatory cells under the action of P-selectin. This regulatory mechanism of tumor cells is very conducive to HCC infiltration and metastasis[288]. This finding suggests that the clinical characteristics of HCC with easy infiltration and hematogenous metastasis may be tightly correlated with abundant P-selectin expression and that P-selectin can be used as an index to predict HCC extrahepatic hematogenous metastasis.

CD44V6: The CD44 gene, located on chromosome 11P13, encodes a highly glycosylated cell surface transmembrane protein. As a matrix hyaluronic acid receptor, CD44 has many biological functions; for

example, it activates lymphocytes, participates in signal transduction and promotes specific cell-cell and cell-matrix adhesion. CD44 has a standard form (CD44s) and variant forms (CD44vs), and CD44V6 is one of the variant forms of CD44. CD44v6 overexpression has been implicated in the occurrence, development, invasion and metastasis of multiple malignancies[289,290]. CD44v6 can facilitate HCC progression by inducing the adhesion and infiltration of cancer cells and enhancing the ability of cancer cells to break through to BM tissue. High CD44V6 expression indicates highly invasive HCC. HCC patients with low differentiation exhibit higher levels of CD44V6 expression than patients with high differentiation[291]. In addition, CD44V6 expression is related to HCC invasion and metastasis[292]. These findings suggest that higher CD44V6 expression levels in HCC are associated with a higher degree of malignancy and with stronger HCC invasion and metastasis and that CD44V6 can be used as a marker for predicting HCC metastasis and recurrence.

ECM degradation and HCC metastasis

The degradation of the ECM and BM is a crucial step in the process of tumor invasion and metastasis. Tumor cells adhere to various components of the ECM through their surface receptors and then degrade the ECM and BM by themselves or by inducing host cells to produce a variety of proteolytic enzymes. These effects lead to the formation of a local lytic area, which constitutes the metastatic pathway of tumor cells. Cancer cells pass through the ECM and BM and enter the blood circulation to form metastatic foci. The enzymes that are currently known to be most closely related to tumor invasion and metastasis include ECM-degrading enzymes, mainly uPA and MMPs.

uPA acts primarily by binding to uPA receptors located on the cell surface. After binding, uPA can hydrolyze and shear inactive serine plasminogen to transform it into active plasmin, which can degrade ECM proteins. High expression of uPA and its corresponding regulatory molecules has been observed in HCC, and these proteins are tightly linked to HCC metastasis and recurrence[293,294].

MMPs are a superfamily of Zn^{2+}- and Ca^{2+}-dependent endopeptidases that can specifically degrade the ECM and vascular BM and thereby enhance vascular permeability and promote tumor invasion and metastasis. In the MMP family, MMP-2 and MMP-9 exhibit the closest relationships with tumor invasion and metastasis. MMP-2 is a type IV collagenase in MMPs that can degrade structural proteins and the BM in the ECM as well as type IV collagen in the BM, which promotes the breakthrough of exfoliated HCC cells into the ECM and through the BM barrier to form metastases[295-297]. MMP-9 can degrade the ECM, promote cancer cell penetration of the BM, and participate in multiple sequential steps of HCC invasion and metastasis[298].

Cytokines

After deadhesion between tumor cells, adhesion between tumor cells and the ECM, and ECM degradation, tumor cells need to be activated by cytokines and move deeply into the site of metastasis. The cytokine family is mainly composed of a large class of soluble small-molecule peptides or proteins. This family of proteins participates in the development of diseases because of the leading roles of these proteins in cellular immunity. The major cytokines consist of interferons (IFNs), ILs, TNF, chemokines, and GFs. After the removal of lesions in HCC patients, the patients' immune function is suppressed because cytokines accumulate in the TME, and malignancies interfere with the normal expression of various cytokines to inhibit normal immune function. Thus, the immune system cannot identify and kill tumor cells during hematogenous metastasis. Hence, immune evasion is achieved, and tumor growth and metastasis are promoted[299]. In addition, prolonged exposure to inflammatory cytokines may also induce angiogenesis, DNA damage and other conditions favorable for tumor occurrence and invasion [300].

IFN-γ: The IFN family of cytokines has antitumor effects that regulate immune function. Thus far, three types of cytokines have been found, among which IFN-γ exhibits the strongest immunostimulatory activity. In HCC, IFN-γ inhibits tumor occurrence and development by inhibiting tumor cell proliferation, inhibiting tumor neovascularization, affecting the TME and promoting tumor cell apoptosis [301]. The reduced serum IFN-γ levels in HCC patients are related to metastasis and recurrence[302]. One probable reason for this link is that a low level of IFN-γ facilitates HCC cell proliferation and inhibits HCC cell apoptosis, thus promoting HCC occurrence and development[303,304]. Another reason is that T helper (Th) cells are vital immunomodulatory cells. Th1 and Th2 cells are the main subsets of Th cells. Th1 cells are the main immune regulators of tumors and primarily mediate the cellular immune response by secreting cytokines such as TNF-γ and they play a role in tumor immune defense[305]. Th2 cells primarily mediate humoral immunity, which can inhibit the Th1-cellular immune response. It has been demonstrated that a Thl/Th2 imbalance (increase in Th2 cells and decrease in Thl cells) occurs in the HCC microenvironment. Thus, the HCC microenvironment is in an immunosuppressive state[306], which enables immune evasion by tumor cells, leading to HCC metastasis and recurrence.

ILs: ILs are a class of cytokines. Numerous types of ILs with extensive regulatory functions have been discovered, and among these, IL-6, IL-8, and IL-1β play important roles in HCC metastasis and recurrence.

IL-6 is a multipotent cytokine that is closely associated with the occurrence, progression, invasion and metastasis of various malignancies[307-310]. Mechanistically, IL-6 exerts its biological effects mainly by activating the IL-6/STAT3 and IL-6/JAK2/STAT3 signaling pathways[311]. In HCC, members of the abnormally activated IL-6/STAT3 and IL-6/JAK2/STAT3 pathways, namely, p-JAK2 and p-STAT3, can cause abnormally high expression of downstream related genes and thereby promote HCC angiogenesis, invasion and metastasis[312-315]. In addition, abnormal activation of IL-6/STAT3 signaling enhances immune evasion or establishes immune tolerance through various mechanisms, and the inflammatory microenvironment further promotes HCC angiogenesis, growth, invasion and metastasis[316,317]. High serum IL-6 levels in HCC patients are related to early postoperative recurrence[317,318]. We conclude that IL-6 acts as an activator of proinflammatory cytokines in the HCC microenvironment. With the progression of HCC, IL-6 is continuously activated, and the persistently high level of serum IL-6 may reflect the severe local inflammatory response of the tumor. Tumor inflammatory infiltration may cause tumor cells to break through and eventually spread to the outside of the liver capsule. The tumor is then in the expansion stage, which is closely associated with rapid progression, heightened metastasis and an increased risk of recurrence of HCC[319,320].

IL-8 is a chemotactic inflammatory cytokine that belongs to the CXC (C is cysteine; X is any amino acid) chemokine family. The binding of IL-8 to its two corresponding receptors, CXCR1 and CXCR2, causes biological effects through the G protein signal transduction chain and induces neutrophils and lymphocytes to aggregate at the injured site, which promotes tumor cell proliferation, differentiation, metastasis and angiogenesis[321,322]. High expression of IL-8 in the tissues and plasma of HCC patients is associated with HCC cell proliferation, migration, invasion and metastasis[323,324]. Moreover, the IL8 levels in nonmetastatic HCC are markedly lower than those in metastatic cancer[325]. This finding may be due to the "squeezing" of normal hepatocytes by invasively growing HCC cells, which results in liver injury, necrosis, and a strong inflammatory reaction in the liver and directly stimulates immune cells to secrete large amounts of IL-8. Another explanation is that most HCC cells synthesize and secrete IL-8, which may affect the biological behavior and metastasis of HCC through autocrine and paracrine signaling[326]. In addition, IL-8 plays major roles in the regulation of tissue cell homeostasis and immune regulation as well as in the stimulation of angiogenesis, which is closely related to HCC intrahepatic metastasis[327]. As an inflammatory factor, IL-8 activates a variety of signaling pathways to initiate or accelerate the occurrence of EMT and ultimately promote HCC invasion and metastasis[129]. In addition, IL-8 upregulates the expression of integrin to facilitate HCC invasion and metastasis[328].

IL-1β is generated by activated mononuclear macrophages, and its cleavage by IL-1β convertase yields a mature biologically active molecule that binds to the corresponding receptors and exerts biological effects. IL-1β not only promotes the inflammatory response but also participates in biological behaviors such as migration and invasion in a variety of cells[329]. IL-1β participates in tumorigenesis, angiogenesis, invasion and metastasis by mediating multiple pathways[330,331]. IL-1β expression is increased in HCC and is related to HCC metastasis[200,332]. IL-1β participates in HCC metastasis through a variety of mechanisms. For example, IL-1β promotes HCC metastasis by upregulating homeobox C10 expression in HCC cells[333]. In addition, IL-1β, as an inflammatory cytokine, can induce the aggregation of MDSCs and TAMs, which accounts for the finding that most IL-1β is located in the TME. Thus, IL-1β plays a significant role in inducing HCC cell angiogenesis, immune regulation and metastasis[334].

TNF-α: TNF-α is a polypeptide cytokine with multiple biological activities that is produced by activated mononuclear phagocytes and is tightly associated with the immune response of the body. TNF-α, as a proinflammatory cytokine, acts as an inflammatory mediator and immune regulator and is clearly expressed in multiple malignancies. TNF-α expression is closely correlated with tumor occurrence, development, invasion and metastasis[335,336].

TNF-α facilitates HCC invasion and metastasis through several main mechanisms: (1) TNF-α is related to tumor immunity. In HCC patients, the continuous stress response can cause immune disorders. These disorders lead to continuously increased TNF-α levels and thereby affect T lymphocyte differentiation[337]; (2) TNF-α promotes the production of multiple inflammatory cytokines and chemokines, directly participates in tumor angiogenesis, creates favorable conditions for tumor growth, and promotes HCC invasion and metastasis[338]; (3) TNF-α induces EMT and maintains the stemness of HCC stem cells. TNF-α, which is clearly expressed in M2 macrophages infiltrating HCC tissues, can induce EMT and cancer stemness of HCC cells and thus promote HCC invasion and metastasis[130,339]; and (4) In some cases, TNF-α promotes HCC metastasis by activating endothelial CAMs or transcription factors. For example, the expression of endothelial CAMs induced by TNF-α can promote adhesion between tumor cells and the vascular wall and infiltration into tissues at the early stage of HCC metastasis[340]. In addition, the proinflammatory effect of TNF-α occurs mainly through the activation of NF-κB to facilitate HCC metastasis and recurrence[315]. However, some studies have shown that high concentrations of TNF-α inhibit HCC metastasis and recurrence, whereas low concentrations of TNF-α promote HCC metastasis and recurrence[341]. Thus, the effects of TNF-α on HCC cells should be further studied.

In general, TNF-α, as an important inflammatory factor, maintains the inflammatory microenvironment by acting as the main triggering factor and master switch for the transformation from inflam-

mation to cancer and thereby facilitates tumor invasion and metastasis. Exploring the mechanisms of HCC metastasis mediated by TNF-α will help to identify therapeutic and preventive targets.

Chemokines and their receptors: Chemokines are a series of protein signaling cytokines secreted by cells, so named due to their ability to induce reactive cells to move directionally *via* chemotaxis. Chemokines can be divided into four categories, CXC, CC, C, and CX3C, depending on the location of the N-terminal conserved cysteine residues. Chemokine receptors are G protein-coupled transmembrane receptors that are divided into CXCR, CCR, CR4 and CX3CR proteins based on their ligands. The interaction between chemokines and their receptors is a key link in the TME and promotes tumor cell proliferation, immune cell recruitment and remodeling of the ECM[342,343]. Tumor cells, stromal cells and chemotactic inflammatory cells in the TME release a variety of chemokines, which affect tumor cell proliferation, differentiation, migration, and invasion through autocrine and paracrine pathways and induce angiogenesis in the TME[344].

CCL2/CCR2 axis: CCL2 mainly binds to its receptor CCR2 and chemotactic CCR2-positive monocytes, macrophages and tumor cells through the CCR2-CCL2 axis. Numerous studies have shown that the CCR2-CCL2 axis facilitates tumor cell growth, survival, angiogenesis, invasion and metastasis[345,346]. CCL2/CCR2 promotes HCC metastasis through multiple main mechanisms: (1) CCL2/CCR2 promotes HCC metastasis directly or by recruiting TAMs. The chemokines CCL2 and CCR2 play significant roles in recruiting TAMs to participate in tumor invasion and metastasis[346]. High expression of the CCL2/CCR2 axis can promote HCC angiogenesis and metastasis, and the mechanism of action is related to the recruitment of TAMs by this axis[347,348]; and (2) EMT is induced by the activation of signaling pathways. EMT may be a key step in the promotion of tumor invasion and metastasis by the CCL2-CCR2 axis. The CCL2-CCR2 axis induces EMT by activating the Hedgehog pathway and promotes HCC metastasis[349].

CXCL12-CXCR4 axis: CXCR4, a specific G protein-coupled receptor (GPCR), has high affinity for its ligands. CXCL12 is the natural ligand of CXCR4. The CXCL12/CXCR4 axis can initiate a variety of signaling pathways, leading to gene transcription and mediating cell chemotaxis, cell survival and proliferation. The CXCL12-CXCR4 axis is related to the directional metastatic ability of malignancies [350-352] and is associated with neovascularization and distant metastasis in HCC[240,353]. High MMP-9 and MMP-2 expression levels are often used as indices to detect the invasion and metastasis of malignancies[354]. The interaction between CXCR4 and CXCL12 can mediate the secretion of MMP-9 and MMP-2, accelerate the degradation of the ECM, and promote HCC metastasis[355]. In addition, a synergistic effect has been noted between CXCR4 and MMP-9, and a positive correlation between the expression of these two proteins has been observed in HCC. High expression of both CXCR4 and MMP-9 indicates that HCC exhibits metastatic activity[356]. The combined detection of these molecules can be used as an important index for assessing the lymph node and distant metastasis of HCC.

The above findings indicate that the CCL2-CCR2 and CXCL12-CXCR4 axes are closely associated with HCC metastasis and recurrence and suggest that targeting these axes may be a new strategy for restraining HCC metastasis.

GFs: The HCC microenvironment contains many types of GFs; among these, VEGF and TGF-β are closely associated with HCC metastasis and recurrence.

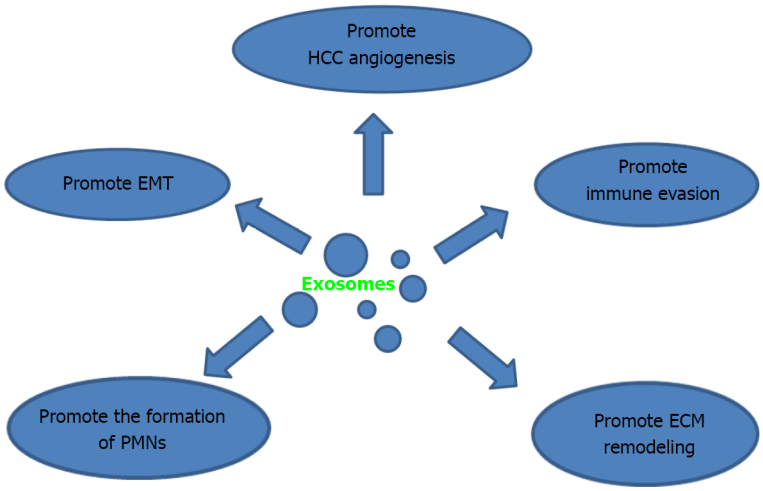
Neovascularization promotes tumor growth, infiltration and metastasis and causes tumor recurrence [357]. VEGF can increase vascular permeability and facilitate VEC proliferation and angiogenesis[358]. The increased expression of VEGF in malignancies can result in degradation of the matrix of VECs, weakening of the vascular barrier, vascular infiltration of cancer cells, and spread of cancer cells through the bloodstream, which results in the induction of tumor metastasis[359,360]. The proliferation, invasion and metastasis of HCC cells depend on massive neovascularization within the tumor. VEGF has been confirmed to be related to postoperative HCC metastasis and recurrence[361,362]. VEGF, as a crucial regulatory factor in angiogenesis, plays a regulatory role in the proliferation, migration and vascular construction of HCC VECs. In addition to promoting angiogenesis, VEGF can increase the permeability of the vascular endothelium, making it easier for tumor cells to invade and penetrate blood vessels and consequently facilitating tumor invasion and metastasis. Therefore, the increased expression of VEGF in HCC tissues is an important reason for the high vascular invasion and the high incidence of postoperative recurrence and metastasis of HCC[363-365]. Of note, VEGF not only acts as an important proangiogenic GF but also affects the functions of various immunosuppressive cells and participates in tumor immune evasion. VEGF is involved in mediating the immunosuppressive HCC microenvironment[366,367]. Mechanistically, the concentration of VEGF in the serum of HCC is proportional to the number of MDSCs[368]. VEGF secreted by HCC cells can act as a chemokine, guiding MDSCs to accumulate and infiltrate locally into the tumor. Moreover, VEGF can affect the antigen presentation of DCs by inhibiting DC differentiation and maturation and subsequently influencing the activation and expansion of CTLs and the sensitivity of tumor cells to CTL-mediated killing[369,370]. HCC cell immune evasion is an important factor promoting metastasis[371]. Therefore, the above findings indicate that VEGF-mediated immune evasion can promote HCC metastasis.

TGF-β: TGF-β is a polypeptide cytokine with extensive biological effects. TGF-β plays two different roles in tumor occurrence and development. Specifically, TGF-β exhibits tumor-inhibitory or tumor-promotive activity that modulates the expression of oncogenes and tumor suppressor genes[372]. TGF-β can exert its antitumor role at the early stage of HCC by promoting hepatocyte cell cycle arrest and apoptosis. In the late stage of HCC, TGF-β induces changes in the microenvironment through various mechanisms, *e.g.*, by acting as an autocrine or paracrine GF, and facilitates HCC metastasis and recurrence by promoting immune evasion, angiogenesis, and the acquisition of tumor stem cell-like characteristics by HCC cells and by inducing EMT. TGF-β can stimulate fibroblasts to transform into CAFs and can promote or induce HCC immune evasion by upregulating the expression of Tregs[373, 374], providing a good internal environment for HCC metastasis and growth and thus promoting and accelerating HCC growth and metastasis. The TGF-β pathway is closely associated with tumor angiogenesis; specifically, the TGF-β/VEGF-related pathway is an important pathway of tumor angiogenesis. In addition, TGF-β can significantly increase VEGF/VEGF-R expression, promote capillary formation and the migration of endothelial cells, and lead to tumor cell angiogenesis, thereby promoting HCC invasion and metastasis[375]. TGF-β can lead to HCC metastasis by inducing tumor cells to undergo EMT[376,377]. TGF-β enhances the ability of HCC cells to acquire hepatic stem cell-like characteristics and thus promotes HCC growth and invasion[378]. In summary, TGF-β is closely related to HCC invasion and metastasis and may be a target for controlling metastasis in HCC.

Exosomes

Exosomes are membranous vesicles containing all types of bioactive substances, such as proteins, lipids, and nucleic acids, and they closely participate in the exchange of information and substances between cells[379,380]. Tumor-derived exosomes are important carriers for bidirectional communication between the TME and tumor cells and participate in TME remodeling processes, particularly in tumor angiogenesis and metastasis[381,382]. In addition, tumor-derived exosomes can induce metastatic target organs to form PMNs and promote tumor metastasis by stimulating neovascularization after the colonization of CTCs[383,384].

HCC-derived exosomes facilitate HCC metastasis and recurrence through the following mechanisms (Figure 3): (1) HCC-derived exosomes promote HCC angiogenesis. Tumor-derived exosomes participate in TME remodeling and release bioactive components such as cytokines, thereby inducing angiogenesis and facilitating tumor metastasis[385]. For example, HCC-derived exosome transfer lysyl oxidase-like 4 can accelerate HCC angiogenesis[386]. Vasculogenic mimicry (VM) is a newly discovered tumor microcirculation pattern that does not involve endothelial cells. Tumor cells interact with the ECM through their own morphological changes to form a pipeline system that can transport blood and connect with host blood vessels to remodel the tumor microcirculation[387,388]. VM occurs widely in multiple human malignancies and is closely related to tumor progression, invasion, metastasis and recurrence[389,390]. HCC-derived exosomes can promote HCC angiogenesis and VM and thus facilitate HCC metastasis[391]; (2) HCC-derived exosomes inhibit the host immune response to promote immune evasion. The 14-3-3ζ protein leads to T-cell exhaustion *via* HCC-derived exosomes[392]. In addition, endoplasmic reticulum stress can induce HCC cells to release exosomes that are rich in miRNA-23a-3p and enhance PD-L1 expression in macrophages by inhibiting the phosphatase and tensin homolog (PTEN) pathway and activating the AKT pathway, which results in the inhibition of T cells and mediation of immune evasion[393]; (3) HCC-derived exosomes promote EMT. Tumor-derived exosomes act as transporters of EMT initiation signals and transmit metastatic signals to metastatic tumor cells, resulting in EMT and metastasis[394]. For example, the expression of exosomal circ-0004277 secreted by HCC cells is upregulated, which induces EMT through cellular communication and promotes HCC intrahepatic metastasis[395]. HCC-derived exosomal miR-1307-5p also promotes HCC metastasis by inducing EMT[396]. Furthermore, the tumor metastasis induced by a hypoxic microenvironment is closely related to exosomes because exosomes produced by tumor cells under hypoxic conditions are enriched with EMT signaling molecules. For example, HCC-derived exosomes produced in a hypoxic environment are rich in miR-1273f, which can activate the Wnt/β-catenin pathway and induce EMT [397]. The mechanisms through which metastasis is promoted are relatively clear. However, whether all HCC-derived exosomes can promote EMT remains to be further explored; (4) HCC-derived exosomes promote ECM remodeling. The most important feature of ECM remodeling is the change in the molecular composition of the ECM. The remodeled ECM creates a permissive microenvironment for tumor cell proliferation and differentiation, which leads to tumor cell growth, infiltration, and metastasis[241,398]. In addition, CAFs are important cells in ECM remodeling and participate in the homeostatic maintenance of tissue ECM synthesis and degradation through cell–cell communication [399]. Exosomes produced by HCC cells participate in ECM remodeling by activating CAFs. For example, HCC cell-derived exosomal miR-1247-3p promotes fibroblast activation by directly acting on its target B4GALT3, and activated CAFs facilitate the lung metastasis of HCC by secreting proinflammatory cytokines, such as IL-6 and IL-8[229]. In addition, HCC-derived exosomal miRNA-21 directly targets PTEN, resulting in the activation of the inositol 3-phosphate dependent PDK1/serine threonine kinase (Akt) signaling pathway in normal HSCs, which can be transformed into CAFs. Activated CAFs can increase the density of blood vessels, remodel the ECM, and subsequently promote tumor



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Figure 3 Schematic diagram of molecular mechanisms by which exosomes promote hepatocellular carcinoma metastasis and recurrence. HCC: Hepatocellular carcinoma; ECM: Extracellular matrix; EMT: Epithelial mesenchymal transition; PMNs: Pre-metastatic niches.

metastasis[215]; and (5) HCC-derived exosomes promote the formation of PMNs before metastasis. The PMN is a microenvironment that is ready for tumor colonization and spread in distant organs. Studies have shown that the construction of specific microenvironments in potential metastatic target organs is an important reason why tumor metastasis occurs in specific organs[400]. Currently, distal cell–cell communication mediated by primary tumor cells through their exosomes is generally believed to be fundamental to the formation of PMNs in potential metastatic target organs[401]. Recent evidence has revealed that exosomes can also promote the establishment of PMNs in HCC[402,403]. For example, miR-103, an HCC cell-derived exosomal miRNA, enhances vascular permeability by targeting various endothelial connexins in endothelial cells, and changes in vascular changes promote the establishment of PMNs and thereby facilitate HCC metastasis[404].

Numerous clinical studies have confirmed the potential utility of exosomes as indicators for predicting HCC metastasis and recurrence. HCC patients with increased serum levels of exosomal miR-638 exhibit increased metastatic potential and an increased risk of recurrence[403]. Exosome-derived miR-1307-5p is highly expressed in preoperative plasma and can be indicative of HCC metastasis and recurrence[396]. In addition, exosome-derived differentiation-antagonizing nonprotein-coding RNAs are linked to postoperative HCC recurrence[405].

The specific mechanisms of exosomes in HCC invasion and metastasis have not been clearly defined to date. Future research should focus on the mechanisms of exosome generation and the specific signal transmission process of exosomes in HCC to obtain new findings regarding the roles of exosomes in HCC metastasis and recurrence. Exosome treatment may be an effective HCC treatment strategy in the future.

EMT

EMT is a process through which cells change from an epithelial state to a mesenchymal state, leading to migration and invasive behavior. As the initial step mediating tumor invasion and metastasis, EMT is often accompanied by changes in various marker proteins and transcription factors. Functional loss of E-cadherin and cytokeratins and upregulated expression of N-cadherin and vimentin are frequently observed in tumor cells undergoing EMT[406]. Cells undergoing EMT exhibit alterations in morphology and function, loss of epithelial–basal polarity and reductions in cell–cell connections, and these cells can penetrate the BM and interstitial tissue as well as the circulatory system to travel to distant tissues[407]. Therefore, EMT is an important cellular event during tumor invasion and metastasis. Increasing evidence indicates that EMT is strongly associated with tumor migration, invasion, metastasis and recurrence and that tumor cells can acquire the ability to invade and metastasize through EMT[408-411].

EMT plays a leading role in HCC metastasis and recurrence. Loss of E-cadherin expression and increases in N-cadherin and vimentin expression are closely related to HCC metastasis[412-414]. E-cadherin and vimentin form a complex between epithelial cells that can prevent tumor cell metastasis and invasion. In contrast, EMT occurs in malignant tumor cells, allowing the cells to separate from each other and thereby improving the migration ability of the cells[415,416]. A lack of E-cadherin expression and an increase in vimentin expression are the two most important markers of EMT that can predict the recurrence of HCV-associated HCC[417]. EMT is also an important mechanism through which many

oncogenes promote HCC invasion, metastasis and recurrence. For example, the oncogenes vasodilator-stimulated phosphoprotein, thrombospondin 4, GPCR-kinase interacting protein-1, and lysyl oxidase promote HCC invasion, metastasis and recurrence by inducing EMT[418-420]. Studies have found that HCC cells with EMT are more likely to acquire stem cell characteristics and have stronger self-renewal and invasion capabilities than ordinary HCC cells and that these cells are closely associated with early HCC recurrence[421-423]. In addition, EMT can induce the release of CTCs from HCC cells, and CTCs are important indicators predicting HCC recurrence and poor prognosis[424,425].

In conclusion, EMT is a dynamic evolutionary process and an important initial stage of HCC metastasis. Elucidating the molecular mechanisms of EMT in HCC cells will be conducive to finding new targeted drugs to reverse EMT, which will provide new insights into the clinical targeted regulation of HCC.

LCSCs

CSCs are subsets of tumor cells with self-renewal ability and stem cell characteristics. CSCs have the ability to generate heterogeneous tumors, are highly invasive, and easily metastasize and develop multiple resistance properties, and they can accordingly drive tumor growth, metastasis and recurrence [426]. Increasing evidence indicates that LCSCs exist in HCC and drive HCC initiation, metastasis, drug resistance and recurrence. Currently, the molecules used to as LCSC markers include CD133, CD90, EpCAM, CD13, CD44, and acetaldehyde dehydrogenase[427]. Among them, CD133, EpCAM and CD90 are closely associated with HCC metastasis and recurrence. The expression of these markers indicates HCC with strong aggressiveness and metastasis[428].

CD133

CD133 is a transmembrane protein composed of five transmembrane regions and two large glycosylated cell outer rings located on the cell surface. CD133 is expressed on the surfaces of stem cells and various tumor stem cells[429,430]. $CD133^+$ tumor cells are closely associated with tumor occurrence, invasion, metastasis, and recurrence[431,432]. Currently, CD133 is widely regarded as a specific surface marker of LCSCs. $CD133^+$ LCSCs are strongly associated with HCC metastasis and early recurrence[55,363].

EpCAM

EpCAM, a transmembrane glycoprotein involved in cell adhesion, has the properties of an oncogene messenger and thus facilitates cell proliferation and tumor formation[433]. The expression level of EpCAM protein in HCC tissues, particularly in HCC metastases, is higher than that in normal liver tissues[434,435]. These findings suggest that EpCAM+ cells have strong characteristics of tumor stem cells, participate in HCC cell metastasis and invasion, and can be used as stem cell markers for the acquisition of metastatic ability by HCC cells. In addition, the positive expression rate of EpCAM protein in HCC patients with recurrence after surgery is significantly higher than that in patients without recurrence[436], and HCC patients with high EpCAM expression are more prone to relapse than those with low EpCAM expression[437]. These findings suggest that EpCAM is a predictor of postoperative HCC recurrence.

CD90

CD90, a surface marker of oval cells, is a glycosylphosphatidylinositol-anchored glycoprotein with a molecular weight of 25-37 kDa that is expressed in T cells, thymocytes, neurons, endothelial cells, fibroblasts and other cell types. In recent years, CD90 has been used as a marker of LCSCs. CD90 is associated with the high metastatic potential of HCC[438-440], and HCC patients with high CD90 expression have exacerbated pathological differentiation and an increased chance of postoperative recurrence[363,441], which suggests that CD90 may play a significant role in HCC metastasis and recurrence.

In summary, although studies have found that many LCSC-related markers are strongly associated with HCC metastasis and recurrence, the specificity remains low. In addition, although therapeutic methods targeting CSCs have great potential, specific markers are currently lacking. Therefore, improving the sorting and identification of LCSCs and continuing to identify and screen specific LCSCs represent future research directions in the field of LCSCs. In addition, given the complex and variable heterogeneity and plasticity of CSCs[442,443], obtaining a further understanding of the heterogeneity and plasticity of CSCs and the mechanisms through which they drive tumor metastasis and recurrence will greatly promote the development of new safe and effective therapeutic strategies, regimens and drugs targeting CSCs.

CTCs

CTCs are tumor cells that are shed from primary or metastatic foci and enter the bloodstream to reach distant organs, where they may trigger metastasis. The number, morphology and phenotype of CTCs are closely and fundamentally related to tumor metastasis and recurrence[444,445]. The formation of metastatic tumors is a process of "natural selection": Only a small proportion of potential cancer cells passively or actively detach from the primary tumor, enter the lymphatic or blood circulation to form CTCs, evade immune surveillance, and then reach target organs to form metastatic tumors. This process is complex, and although the mechanisms are not completely clear, the basic process has been outlined. First, an association between EMT and the TME exists. After cancer cells undergo EMT, upregulation of the expression of MMPs accelerates ECM degradation, resulting in the detachment of cancer cells from the lesion and the transformation of these cells into CTCs[446]. Then, CTCs typically penetrate the vascular system in the capillary bed and colonize the premetastatic microenvironment after they arrive at the target organ *via* the circulatory system. This process is also called metastatic dissemination, and the cells that colonize the premetastatic microenvironment are called disseminated tumor cells (DTCs) [447]. Under unfavorable conditions (such as when immune function remains intact), DTCs can be transferred and remain in a dormant state for a long time under the combined actions of many internal and external factors[448,449]. Dormant DTCs often exhibit CSC-like phenotypes and directly bind to other cells, such as CAFs and neutrophils. These cells then attach to the inner walls of blood vessels and not only resist mechanical damage or immune attack but also facilitate cancer cell dissemination through the vascular system and colonization of distal metastatic target organs at an appropriate time [450,451]. Under certain circumstances, dormant DTCs can be awakened and can rapidly and clonally grow to form metastatic tumors[452,453].

CTCs exhibit obvious phenotypic heterogeneity. Among the many phenotypes of CTCs, only a few have "seed" characteristics, that is, resistance to anoxic apoptosis, exudation, transformation into disseminated cells, high adaptability to PMNs, and the ability to induce angiogenesis and matrix formation during metastasis[454,455]. During the process of metastasis, CTCs undergo various phenotypic changes, among which EMT is the most important, to escape immune clearance or acquire the ability to resist anoxic apoptosis and adapt to the new living environment[456]. EMT is an important mechanism through which CTCs survive and acquire aggressive and metastatic abilities[457]. The EMT process can alter the tight connections and adhesion between tumor foci cells, and CTCs with invasive and metastatic potential often exhibit EMT characteristics[448]. CTCs with an EMT phenotype are closely related to HCC metastasis or recurrence[458,459]. Given the occurrence of EMT during HCC progression, EMT may be a crucial factor influencing the survival and metastatic ability of CTCs. Alternatively, CTCs, as cells with high activity and high metastatic potential that are present in the circulatory system, may possess CSC characteristics. CSCs persist in tumor tissues and are considered specific cell types that promote tumor metastasis and recurrence. The small fraction of tumor metastasis-initiating cells among CTCs that eventually lead to metastasis are a class of tumor stem cells or at least tumor cells with multiple stem cell characteristics[460]. Because CTCs exhibit EMT and CSC characteristics, interstitial CTCs are generally considered more capable of forming metastatic foci than other cell types. CTCs with CSC characteristics are closely related to HCC vascular invasion, metastasis and recurrence[461]. Given that CTCs have CSC characteristics during HCC progression, the CSC phenotype may be an important feature by which CTCs persist in the blood circulation for a long time and maintain high invasion and metastasis abilities. Hematogenous metastasis is a common form of HCC metastasis. The CTC levels in the blood of HCC patients are directly related to prognosis, and patients with large numbers of CTCs have a poor prognosis[462]. A total of 30.5%-93.3% of HCC patients have CTCs in the peripheral circulatory system that cause distant metastasis, which is the key event responsible for metastasis and recurrence[424,463,464]. Therefore, high levels of CTCs can be used to predict and evaluate the risk of HCC metastasis and recurrence.

The majority of previous studies on CTCs have concentrated on the characteristics of CTCs and ignored the overall relationships between CTCs and the primary tumor and between CTCs and the TME and internal environment. The regulation of the biological behavior of CTCs by an imbalance in the tumor environment may represent an important mechanism of HCC metastasis and recurrence. CTCs can survive in the blood circulation and eventually proliferate to form metastatic foci. This process is inseparable from the maintenance and regulation of GFs and corresponding signaling pathways, and research in this field may become key to further elucidating the mechanisms of HCC metastasis and recurrence.

EPIGENETIC MODIFICATIONS

Epigenetic modifications are heritable phenotypic changes unrelated to DNA mutations and are some of the key drivers of HCC metastasis and recurrence[465]. The main epigenetic regulatory mechanisms include DNA methylation, histone modification, genomic imprinting, chromatin remodeling, and ncRNA activity. Among these, DNA methylation and ncRNA-mediated modifications are the most

studied epigenetic modifications associated with postoperative HCC metastasis and recurrence. These factors provide clues supporting an in-depth understanding of the biological functions of epigenetic events implicated in HCC metastasis and supporting the development of novel approaches and strategies for the treatment of HCC.

DNA methylation

DNA methylation selectively adds methyl groups to cytosine residues in certain regions (CpG islands). Specifically, the cytosine 5' carbon position of the genomic CpG dinucleotide is covalently bonded to a one-carbon-unit methyl group provided by S-adenosylmethionine under catalysis by DNMTs[466,467]. An altered DNA methylation status characterized by a decrease in the overall methylation level and an increase in the local methylation level is prevalent in tumors. In tumor cells, oncogenes are activated in the state of hypomethylation, whereas tumor suppressor genes are inhibited in the state of hypermethylation[468]. In addition to point mutations and gene deletions, some tumor suppressor genes exhibit hypermethylation in the promoter region. This inhibits the transcription of tumor suppressor genes, resulting in inactivation of these genes and an inability to exert tumor-suppressive effects. Thus, hypermethylation has been implicated in tumor occurrence, invasion and metastasis[469, 470]. The hypomethylation of specific genes can induce the activation of oncogenes and facilitate tumor growth and metastasis[471]. DNA hypermethylation and hypomethylation are strongly correlated with tumor development, invasion and metastasis.

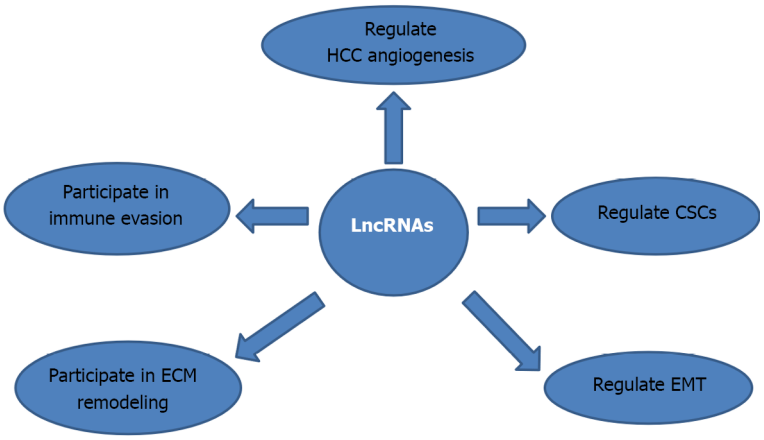
During DNA methylation, DNMTs are needed to maintain the methylation state and function as key enzymes. DNMT expression is typically increased in malignancies; thus, DNMTs are important causes of the hypermethylation of a series of tumor suppressor genes[472,473]. The epigenetic alterations mediated by DNMTs regulate HCC invasion and metastasis[474,475]. Emerging evidence confirms that abnormal methylation of many genes is closely correlated with HCC metastasis and recurrence after surgery. For example, hypermethylation of the tumor suppressor genes *SPG20*, *TERT* and *CCND2* is tightly associated with HCC metastasis and recurrence[476-478]. In contrast, hypomethylation of the oncogenes *GTPBP4* and *CTCFL* is closely related to HCC metastasis and recurrence[479,480].

Collectively, the evidence from studies on the methylation of HCC-related genes will provide new insights to help predict HCC metastasis and recurrence and to develop targeted therapies.

NcRNAs

LncRNAs: LncRNAs are a class of RNA molecules without protein-coding functions. LncRNAs can promote or inhibit tumor invasion and metastasis through multiple molecular mechanisms[481,482]. Tumor metastasis may alter lncRNA expression[482]. Therefore, lncRNAs that are dysregulated during tumor metastasis are potential molecular markers of tumor metastasis.

Mounting evidence suggests that lncRNAs are widely involved in HCC invasion and metastasis. LncRNAs can participate in regulating gene expression levels at various levels, such as epigenetic modification, transcription, posttranscription, and translation, and thereby affect HCC cell invasion and metastasis. LncRNAs play two different roles in HCC invasion and metastasis. Oncogenic lncRNAs promote invasion and metastasis[405], while tumor suppressor lncRNAs inhibit invasion and metastasis [483]. LncRNAs participate in HCC invasion and metastasis through multiple mechanisms (Figure 4): (1) LncRNAs regulate tumor angiogenesis, which is of great importance to tumor invasion and metastasis. For example, the oncogenic lncRNA OIP5-AS1 and the lncRNA noncoding RNA activated by DNA damage affect VEGF-A expression by competitively binding miR-3163 and miR-211-5p, respectively, ultimately inducing HCC angiogenesis and promoting HCC invasion and metastasis[484, 485]. In contrast, the tumor suppressor lncRNA miR503HG suppresses angiogenesis and thereby inhibits HCC invasion and metastasis[486]; (2) LncRNAs regulate CSCs. Specific lncRNAs are abnormally expressed in LCSCs, and the stemness characteristics of these lncRNAs are regulated at different molecular levels. For example, the oncogenic lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) can bind to miR-124 *via* a competing endogenous RNA (ceRNA) mechanism, which means that ncRNA with a miRNA response element can relieve the miRNA-based inhibition of the target mRNA by competitively combining with the miRNA, and thereby regulate the characteristics of HCC stem cells induced by HBx through the PI3K/Akt pathway[487]. Moreover, the lncRNA MALAT1 is clearly expressed in LCSCs and facilitates the display of CSC characteristics by HCC cells by sponging miR-375 to enhance Yes-associated protein 1 expression[488]. Furthermore, the oncogenic lncRNA H19 facilitates the transformation of HCC stem cells by sponging miR-193b to enhance MAPK1 expression and thereby promotes HCC metastasis[489]; (3) LncRNAs regulate EMT, a vital step in initiating tumor metastasis. After undergoing EMT, HCC cells are primed to break through the BM and invade blood vessels or lymphatic vessels to achieve invasion and metastasis. LncRNAs are key regulators of EMT in HCC. The regulation of EMT by lncRNAs is mainly realized by ceRNAs, which are key elements among EMT-related miRNAs, or *via* regulation of the activity of EMT-related transcription factors. For example, the expression of the oncogenic lncRNA SNHG3 can induce EMT through miR-128/CD151 activation and thus promote HCC invasion and metastasis[490]. Conversely, the expression of the tumor suppressor lnc-Ma301 is reduced in HCC cells, which reduces the probability of HCC lung metastasis by participating in the suppression of EMT progression and even reversing EMT progression in HCC cells[491]; (4) LncRNAs participate in ECM remodeling. For



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Figure 4 Schematic diagram of molecular mechanisms by which long non-coding RNAs regulate hepatocellular carcinoma metastasis and recurrence. HCC: Hepatocellular carcinoma; LncRNAs: Long non-coding RNAs; CSCs: Cancer stem cells; ECM: Extracellular matrix; EMT: Epithelial mesenchymal transition.

example, the oncogenic lncRNA LINC01615 and the lncRNA SNHG16 promote HCC invasion and metastasis by participating in ECM remodeling[492,493]; and (5) LncRNAs participate in HCC cell immune evasion. Mounting evidence suggests that lncRNAs participate in TIME formation and thus affect tumorigenesis and metastasis[494,495]. For example, the oncogenic lncRNA lnc-epidermal growth factor receptor facilitates Treg differentiation, inhibits CTL activity, and thus promotes HCC cell immune escape and tumor metastasis[496].

Research on lncRNAs remains in its infancy, and a large number of lncRNAs associated with HCC metastasis have not yet been identified. In addition, although existing evidence reveals the important roles and some mechanisms of lncRNAs in HCC invasion and metastasis, research on the mechanisms of such lncRNAs remains relatively limited. Most studies have tended to investigate the effect of a single pathway on HCC invasion and metastasis, but these processes are complex and involve interactions among multiple factors. The next step should be to systematically study the involvement of lncRNAs in multiple pathways to jointly regulate HCC invasion and metastasis. This information will promote the use of lncRNAs as promising markers for the early prediction of HCC metastasis and recurrence. In addition, lncRNAs offer multiple effective therapeutic targets for HCC; thus, lncRNA research can provide a new direction and new treatments to prevent HCC metastasis and recurrence.

CircRNAs: CircRNAs are closed circular covalent RNA structures whose 5' and 3' ends are connected by nonclassical splicing. CircRNAs are highly conserved and exhibit tissue specificity that is not affected by RNA exonuclease. Recent studies have shown that circRNAs promote or inhibit tumor metastasis through multiple mechanisms[497,498].

In HCC, circRNAs facilitate or inhibit invasion and metastasis through several main mechanisms: (1) CircRNAs affect the stemness transition. The oncogenic circRNA circIPO11 facilitates the self-renewal of LCSCs through activation of the Hedgehog pathway and thus accelerates HCC invasion and metastasis [499]; (2) CircRNAs affect EMT. The oncogenic circRNA hsa_circ_0000092 binds miR-338-3p to upregulate downstream HN1 expression by reducing miR-338-3p expression and thus accelerating the EMT process of HCC and promoting HCC migration, invasion and metastasis[500]. The expression of the tumor-suppressive circRNA circSETD2 is reduced in HCC tissues, and overexpression of this circRNA restrains EMT occurrence in HCC cells and thereby inhibits HCC cell invasion and metastasis [501]; (3) CircRNAs affect ECM degradation. The tumor suppressor cSMARCA5 (hsa_circ_0001445) inhibits ECM degradation by affecting TIMP3 expression and thereby inhibits HCC cell invasion and ultimately tumor metastasis[502]; (4) CircRNAs affect tumor angiogenesis. For example, exosomal circ-100338 enhances the angiogenic capacity and permeability of HCC cells and promotes HCC metastasis [391]. In contrast, the tumor suppressor circ_0004018 inhibits HCC angiogenesis and thus HCC metastasis[503]; and (5) CircRNAs regulate tumor immune evasion. Oncogenic circRNAs can help tumor cells evade immune surveillance through multiple pathways. Hsa-circ-0003288, which is highly expressed in HCC cells, induces tumor immune evasion and promotes HCC metastasis by upregulating PD-L1 expression[504]. Hsa_circ_0048674 may lead to immunosuppression by inhibiting the cytotoxicity of NK cells toward HCC cells and thereby promote HCC metastasis[505].

In summary, research on circRNAs in the field of HCC metastasis remains in its infancy. Although the existing evidence indicates that circRNAs are associated with HCC metastasis, clinical research results remain lacking. Further in-depth investigation of the mechanisms of circRNAs in HCC metastasis will provide new strategies to combat HCC metastasis.

MiRNAs: MiRNAs are a group of endogenous ncRNAs with a length of 18 to 24 nucleotides. MiRNAs play significant regulatory roles in the expression of numerous protein-coding genes and participate in cancer cell proliferation, differentiation, angiogenesis and metastasis[506,507].

Emerging evidence suggests that miRNAs play significant roles in HCC metastasis and recurrence. MiRNAs mainly affect HCC metastasis *via* several mechanisms: (1) MiRNAs regulate EMT occurrence in HCC cells: miRNAs can either inhibit or promote EMT-mediated HCC invasion and metastasis by regulating signaling pathways and transcription factors. For example, the tumor suppressor miR-3194-3p inhibits the occurrence of EMT by inactivating Wnt/β-catenin signaling and thereby inhibits HCC metastasis and recurrence[508]. The tumor suppressor miR-509-3p prevents EMT by decreasing the expression of the transcription factor JDP2 to inhibit HCC invasion and metastasis[509]; (2) MiRNAs affect tumor angiogenesis. The oncogenic miRNA miR-1290 facilitates HCC angiogenesis and thus promotes HCC invasion and metastasis[510]. In contrast, the tumor suppressor miR-325-3p restrains HCC angiogenesis and thereby inhibits HCC invasion and metastasis[511]; and (3) MiRNAs modulate ECM degradation. The tumor suppressor miR-324-5p inhibits ECM degradation by reducing MMP2 and MMP9 activity, inhibiting invasion and metastasis in HCC[512].

Although miRNAs have been confirmed to participate in HCC metastasis and recurrence, the specific mechanisms of these functions need to be further studied. MiRNAs are expected to become new targets for the prediction and treatment of HCC metastasis and recurrence.

CONCLUSION

Problems and perspectives

Postoperative HCC metastasis and recurrence are complicated processes involving multiple steps, links, factors, and molecules as well as interactions among the body, tumor tissues and the TME. Clinical studies have found that tumor size, number, vascular invasion, and disseminated foci are important prognostic factors of HCC, but clinical practice suggests that common clinicopathological features, such as the size and number of HCC tumors, cannot individually accurately predict the potential of tumor metastasis and recurrence. Rather, the biological characteristics of tumors may be the real key factors in metastasis and recurrence. Previous studies have reported numerous molecular markers associated with HCC metastasis and recurrence, but the current studies primarily involve single-factor and single-molecule research models and focus on the expression of a few genes and proteins. In addition, the sample sizes of these studies are limited, and accurately reflecting the molecular characteristics underlying HCC metastasis and recurrence is difficult. Thus far, there are no indicators that can be used to clinically predict HCC metastasis and recurrence. Therefore, additional experiments and clinical verification are needed to clarify the specific molecular mechanisms of HCC metastasis and recurrence. In the process of HCC treatment, some unavoidable objective factors, internal factors of the HCC tissue itself, and immunosuppression are encountered. These factors work together to make HCC metastasis and recurrence a bottleneck that severely restricts the efficacy of surgical resection. Detailed elucidation of the molecular mechanisms underlying HCC metastasis and recurrence will strengthen our understanding of HCC and help us to more accurately grasp the mechanisms of HCC metastasis and recurrence. Such knowledge will be crucial for the identification of new therapeutic targets.

FOOTNOTES

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REVIEW

Liquid biopsy leads to a paradigm shift in the treatment of pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most cancers. Its 5-year survival rate is very low. The recent induction of neoadjuvant chemotherapy and improvements in chemotherapy for patients with pancreatic cancer have resulted in improved survival outcomes. However, the prognosis of pancreatic cancer is still poor. To dramatically improve the prognosis, we need to develop more tools for early diagnosis, treatment selection, disease monitoring, and response rate evaluation. Recently, liquid biopsy (circulating free DNA, circulating tumor DNA, circulating tumor cells, exosomes, and microRNAs) has caught the attention of many researchers as a new biomarker that is minimally invasive, confers low-risk, and displays an overall state of the tumor. Thus, liquid biopsy does not employ the traditional difficulties of obtaining tumor samples from patients with advanced PDAC to investigate their molecular biological status. In addition, it allows for long-term monitoring of the molecular profile of tumor progression. These could help in identifying tumor-specific alterations that use the target structure for tailor-made therapy. Through this review, we highlighted the latest discoveries and advances in liquid biopsy technology in pancreatic cancer research and showed how it can be applied in clinical practice.

Key Words: Pancreatic cancer; Cell-free DNA; Circulating tumor DNA; Circulating tumor cells; Exosomes; MicroRNAs

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Core Tip: We focused on liquid biopsy technology for pancreatic ductal adenocarcinoma (PDAC), including circulating free DNA, circulating tumor DNA, circulating tumor cells, exosomes, and microRNAs. We equally described the characteristics of these technologies and reviewed the clinical significance according to the purpose of these biomarkers: early diagnosis, prognosis, prediction of recurrence, and therapeutic response. Although liquid biopsy still has many limitations to its widespread utilization in clinical practice, liquid biopsy has the potential to be applied from diagnosis to treatment. It is expected to improve the prognosis of PDAC radically.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the seventh most common cause of cancer-related death worldwide, and its incidence is increasing[1-3]. In the United States and Japan, it ranks as the fourth leading cause of cancer-related mortality[4,5]. Its 5-year survival rate remains as low as 6% in the United States[6]. Without novel diagnostic methods and/or treatments, it is expected to become the second leading cause of cancer-related deaths by 2030[7]. Due to its early metastatic nature, up to 20% of patients with PDAC are eligible for initial resection[6]. The poor prognosis results from the low resectability rate at diagnosis, with surgery being the only potentially curative treatment. However, even with radical resection, most patients relapse within a year. Moreover, due to the high resistance rate to chemotherapy, radiotherapy, and immunotherapy[8], non-operative treatment has a poorer prognosis with a median survival of 5-9 mo[9].

A variety of genetic and molecular alterations have been identified in PDAC, including mutations in *KRAS*, *p16*, *p53*, *BRCA2*, *Smad4*, *etc*[10]. However, translating this scientific knowledge into clinical treatment regimens has not yet been achieved. Tumor marker-adjusted treatments for PDAC have been discussed by several authors[11-16]. Carbohydrate antigen 19-9 (CA19-9) is considered the best tumor marker for patients with PDAC. CA19-9 values correlate with tumor size, stage, and burden[17,18]. Therefore, CA19-9 is commonly used to diagnose PDAC, assess resectability, monitor progression, and determine prognosis[19]. Preoperative and postoperative CA19-9 levels predict prognosis[20-24]. Although CA19-9 is a helpful prognostic factor of PDAC, its usefulness remains controversial[19,25-27]. Better tumor markers that correlate with tumor size, predict recurrence after surgery, reflect tumor progression and metastasis, and indicate response to chemotherapy are urgently needed to improve the prognosis of PDAC.

This ideal tumor biomarker should be disease-specific and sensitive. It should also have a high positive predictive value and allows for the detection of disease at very early stages. Furthermore, it should be easy to collect, and the test should be simple and economically feasible. To replace CA19-9 as an alternative biomarker, many researchers have reported the usefulness of liquid biopsy in various cancers. Liquid biopsy is used to diagnose cancer through the detection of circulating tumor cells (CTCs), cell-free and circulating tumor DNA (cfDNA and ctDNA), and microvesicles such as exosomes containing nucleic acids and proteins released from primary tumors and metastases into body fluids. In contrast to surgical or needle biopsy, liquid biopsy is a non-invasive diagnostic method. As a result, it can be detected in real-time and can provide valid information[28-33].

Through this review, we aimed at exploring cfDNA, ctDNA, CTCs, exosomes, and microRNAs (miRNAs) in liquid biopsy technology for early detection, prognostic evaluation, prediction of response to chemotherapy, development of acquired resistance, and early detection of disease relapse, and to evaluate their clinical utility.

CFDNA

cfDNA in plasma possibly originates from necrosis, apoptosis, and/or macrophage digestion of tumoral and healthy cells. Previous studies demonstrated that most of the plasma cfDNA molecules originate from the hematopoietic system in healthy individuals[34,35]. However, in certain physiological or pathological conditions, such as pregnancy, organ transplantation, and cancers, the related/affected tissues could release additional DNA into peripheral circulation[36-38]. In 1948, Mandel *et al*[39] first discovered circulating cfDNA. However, it was not until 1977 that the utility of cfDNA was appreciated when Leon *et al*[36] discovered that significantly elevated levels were detected in patients with cancer compared to healthy controls. In 1989, Stroun *et al*[40] found that some of the cfDNA in the plasma of

cancer patients was derived from cancer cells (called ctDNA). Subsequent studies revealed that cancer cells release ctDNA fragments into blood and other biological fluids such as urine, saliva, and cerebrospinal fluid. ctDNA is highly fragmented, with a shorter fragment length found in cancer patients (134-145 bp) than that of healthy individuals (165-167 bp)[41]. ctDNA is derived from various tumor sites and can provide much more comprehensive genomic and epigenomic information than single-site biopsies. Thus, ctDNA overcomes the issue of tumor heterogeneity, a significant limitation of conventional tissue biopsy. Furthermore, its non-invasive nature allows for continuous real-time monitoring of the molecular status of cancer.

CTCS

CTCs are defined as cells derived from primary, recurrent, or metastatic tumors. CTCs were reported in 1869, and their significance in peripheral blood has been extensively studied in various malignancies[42-46]. It potentially correlated with tumor metastasis and recurrence in breast cancer[42,43], prostate cancer[44], lung cancer[45], and colorectal cancer[46]. Multiple techniques have been developed to explore CTCs, tumor-specific epitopes that are not present in normal blood cells, changes in physical properties such as size, density, and electromechanical properties, or high-throughput imaging to uncollected blood cell preparations[47]. CTCs can travel in the interstitium and bloodstream as single cells as well as clusters[48,49]. Several studies have depicted that CTC clusters correlate with high metastatic potential and poor outcomes. CTC clusters were more significantly associated with distant metastasis than single CTCs[50,51]. Its predictive value has been explored in recent studies in patients with lung, breast, and colon cancer[48,52,53].

EXOSOMES

In 1967, Peter Wolf first discovered that platelets release numerous vesicles[54]. At the time, these vesicles were regarded as cell fragments with no associated biological function. In 1983, exosomes were first observed in sheep reticulocytes, and Johnstone named them "exosomes" in 1987[55]. They are one of the extracellular vesicles (EVs) and have attracted attention in tumor biology recently. Exosomes are 40-150 nm in diameter containing transmembrane proteins, heat shock proteins, nucleic acids (DNA, mRNA, miRNA, long and short non-coding RNA), and enzymes (GAPDH, ATPase, pgk1, RAB)[56-58]. The molecular content of exosomes reflects the nature and state of the cell from which they originate, and their content can alter the function of the recipient cell[59]. In 1996, B lymphocyte-derived exosomes were found to exhibit antigen-presenting properties and induce T-cell responses[60]. Similarly, antigen-presenting exosomes derived from dendritic cells retarded the progression of cancer[61]. These lipid-bilayer vesicles apparently regulate tumor drug resistance, metastasis, and suppression of immune responses. From tumor growth to cellular metastasis, a complex exosome communication network between tumor and non-tumor cells directs all stages[62]. Tumor cells develop exosome-based mechanisms that promote a favorable microenvironment to support tumor growth by facilitating cell metastasis, evading apoptosis, establishing a premetastatic niche, and transporting information from cell to cell[62]. Tumor-derived exosomes can efficiently be captured by various separation methods and gives substantial information about the tumor. In addition, since exosomes are ubiquitously present in body fluids, diffuse through them, and fuse with cell membranes to exert their effects, they are potential tumor therapeutic drug carriers.

MIRNAS

miRNAs are non-coding RNAs that are involved in regulating gene expression. Most miRNAs are transcribed from DNA sequences to become primary miRNAs, then processed into precursor miRNAs and finally mature miRNAs. In most cases, miRNAs interact with 3′ untranslated region (3′ UTR) of the target mRNA to induce mRNA degradation and translational repression. However, interactions with other regions, such as the 5′ UTR, coding sequences, and gene promoters, have also been reported. Under certain conditions, they activate translation or regulate transcription. The interaction of miRNAs with their target genes is dynamic. It depends on many factors, including the intracellular location of the miRNA, the amount of miRNA and target mRNA present, and the affinity of the miRNA-mRNA interaction. MiRNAs are secreted into the extracellular fluid and transported to target cells *via* vesicles such as exosomes or binding to proteins such as Argonaute. Extracellular miRNAs mediate cell-to-cell communication as chemical messengers. The first miRNAs were reported in the nematode, *C. elegans* by the Ambros group at Harvard University in 1993[63]. Ambros *et al*[63] screened for mutants that affect the timing of cell fate switching during nematode development. They identified two genes*: lin-4 and let-7*. Surprisingly, these genes did not code for proteins but for small RNAs, later called miRNAs. So far,

over 38000 miRNAs have been identified and catalogued in the public database, miRBase (www.mirbase.org). miRNAs are involved in a wide range of processes, including metabolism, cell proliferation, apoptosis, and developmental timing[64]. Overexpressed miRNAs act as regulators of oncogenes (through downregulation of tumor suppressor genes) and/or cellular processes such as cell differentiation and apoptosis. Thus, miRNAs are associated with the development of various types of cancer, including colorectal, breast, ovarian, and endometrial cancer[65-68], but their exact pathways are not fully understood. MiRNAs are involved in cell growth and differentiation regulators and have been proposed to be good candidates for diagnosis and treatment of cancers[69].

CLINICAL SIGNIFICANCE OF LIQUID BIOPSY IN PANCREATIC CANCER

Early diagnosis

KRAS mutations in tumoral tissues have been detected in 90% of PDAC[9,70,71] and the heterogeneity of *KRAS* mutations between primary tumor and metastasis in patients with PDAC[72,73]. Due to high penetration of *KRAS* mutations in PDAC patients, identification of *KRAS* mutations in cfDNA could be a suitable cancer biomarker. CfDNA, however, is immediately removed from the circulatory system by nuclease action and urinary excretion. In addition, uptake by the liver and spleen and degradation by macrophages may also affect its removal from the circulation[74,75]. This short half-life, a few hours at most[74], makes it complicated to detect cfDNA and ctDNA in early-stage cancer.

CTCs was not highly observed in early-stage PDAC, with a sensitivity of < 50%[76,77]. The detection rate of CTCs is 0.0%, 60.7%, 78.6%, and 96.3% of American Joint Commission on Cancer stages I, II, III, and IV patients, respectively, and increases dependence on cancer progression[76]. On the other hand, CTCs have also been detected in patients with early-stage PDAC[78-80], with relatively high sensitivity of over 70%. Kulemann *et al*[81] reported that no difference in the detection rate of CTCs between early-stage and advanced PDAC, which may suggest that CTCs are disseminated from the primary tumor in the early stages of the disease and may be used to diagnose PDAC in the initial stages.

Exosomes have shown promise for early detection of PDAC and proved to be a useful tool clinically [33,82]. Melo *et al*[33] found glypican-1 (the cell surface proteoglycan glypican-1) on tumor-derived exosomes as a diagnostic biomarker in PDAC, which enabled to distinguish healthy persons from patients with benign diseases and patients with early- and late-stage pancreatic cancer with 100% of the sensitivity and specificity. Another study, however, failed to show significant differences in GPC1 between healthy, PDAC and chronic pancreatitis samples while a combination of detection with high levels of exosomal miR-10b, miR-21, miR-30c, and miR-181a and low levels of miR-let7a succeeded to distinguish PDAC from healthy and chronic pancreatitis samples[83]. The EV is also a potent biomarker for early detection. Liang *et al*[84] identified ephrin type-A receptor 2 (EphA2) to distinguish PDAC patients from pancreatitis patients and healthy control. In addition, it discriminated against PDAC patients with early disease (stage I/II), who were potentially benefit from curative surgical resection, from normal healthy control (NC) and pancreatitis cases. The blood levels of EphA2-EV before treatments distinguished stage I/II PDAC patients from NC and pancreatitis accurately.

Differences in expression of miRNA are considered precursors to prominent diagnostic biomarkers of PDAC[85-89]. High expressions of serum exosomal miR-17-5p and miR-21 are likely observed in PDAC patients in connection with metastasis and the advanced stage of PDAC[85]. Studies of miR-10b[86,87] also showed increased levels in exosomes isolated from the plasma of PDAC patients compared to those with chronic pancreatitis or normal controls. Peng *et al*[90] conducted the meta-analysis including 46 studies and found that the sensitivity, specificity, and AUC of circulating miRNAs for discriminating early-stage PDAC patients (0-IIa) from non-PDAC controls were 0.78 (0.76-0.81), 0.78 (0.75-0.80) and 0.85 (0.82-0.88), respectively.

cfDNA, ctDNA, and CTCs are highly detectable in patients with advanced PDAC, but less noticeable in early-stage PDAC while exosomes are secreted into cells from early stages and miRNAs are abundant and relatively easy to detect. These biomarkers, however, are not yet appropriate for early diagnosis in clinical practice and liquid biopsy (especially cfDNA and ctDNA) needs to be improved for the detection capability as a screening tool for PDAC (Table 1).

Prognosis assessment

Numerous articles have been published regarding the prognosis of PDAC using liquid biopsy. It is challenging to detect cfDNA in early-stage PDAC due to small amount of cfDNA at the instance. On the other hand, cfDNA is detected with high probability in plasma from patients with advanced PDAC and high levels of cfDNA are significantly associated with poor prognosis[91-93]. Higher levels of plasma DNA (> 62 ng/mL) is significantly associated with poor outcome in overall survival (OS), presence of vascular encasement and metastasis[91]. Fragment size of cfDNA and cfDNA are investigated in patients with advanced PDAC, where a pretreatment cfDNA fragment size of 167 bp or less and a high pretreatment cfDNA level are associated with shorter progression-free survival (PFS) and OS[92]. Regarding ctDNA, *KRAS* mutations in plasma from PDAC patients were detected in 1999 in connection with poor survival[94]. Then, several studies reported the feasibility of detecting circulating mutant

Table 1 Liquid biopsy in the early diagnosis of pancreatic cancer

Ref.	Journal	No. of patients	Biomarker	Method	Main findings
Ankeny *et al*[76], 2016	*Br J Cancer*	72	CTC	Microfluidic NanoVelcro CTC chip	Detection rate of PDAC: 54/72 (sensitivity = 75.0%, specificity = 96.4%)
Rhim *et al*[77], 2014	*Gastroenterology*	51	CTC	Microfluidic geometrically enhanced differential immuno-capture	CTC (≥ 3) in 33% of patients with cystic lesions and no clinical diagnosis of cancer, 73% with PDAC, and 0% of controls
Xu *et al*[78], 2017	*Int J Mol Sci*	40	CTC	NE-iFISH	The positive rate of diagnosis of PDAC is nearly 97% in combination with CA19-9
Poruk *et al*[79], 2017	*Clin Cancer Res*	60	CTC	ISET method & immunofluor-escence	The positive rate is 12% in stageI PDAC
Gao *et al*[80], 2016	*J Exp Clin Cancer Res*	25	CTC	SE-iFISH platform	Sensitivity of 88 % and specificity of 90 % in PDAC
Kulemann *et al* [81], 2015	*Pancreas*	11	CTC	ScreenCell; Cyto kit	No difference in the rate of CTC detection between early-stage and advanced-stage diseases (P = 0.71)
Melo *et al*[33], 2015	*Nature*	56	Exosome	FACS analysis	The sensitivity and specificity of GPC1+ circulating exosomes in diagnosing PDAC were both 100%
Buscail *et al*[82], 2019	*Cancers*	30	Exosome and CTC	FACS, Cellsearch and RosetteSepTM	Combining quantification of GPC1-positive exosomes and CTC detection identified all the PDAC patients, showed a negative predictive value of 100%, and an overall diagnostic accuracy of 91%
Lai *et al*[83], 2017	*Cancer Lett*	40	Exosome and miRNA	LC-MS & RT-qPCR	High levels of exosomal miR-10b, miR-21, miR-30c, and miR-181a and low levels of miR-let7a differentiated PDAC from healthy and chronic pancreatitis samples
Liang *et al*[84], 2017	*Nat Biomed Eng*	23	EV	nPES assay	Pre-therapy EphA2-EV blood levels accurately distinguished stage I/II pancreatic cancer patients from NC (AUC = 0.96) and pancreatitis patients (AUC = 0.93)
Que *et al*[85], 2013	*World J Surg Oncol*	49	miRNA	RT-PCR	Serum exosomal miR-17-5p was higher in PDAC patients than in non–PDAC patients and healthy participants
Cote *et al*[86], 2014	*Am J Gastroenterol*	215	miRNA	RT-PCR	Increased expression of miRNA-10b, -155, and -106b in plasma appears highly accurate in diagnosing PDAC
Ouyang *et al*[87], 2015	*Oncogene*	42	miRNA	RT-PCR	Plasma miR-10b levels significantly increased in comparison with normal controls
Slater *et al*[88], 2014	*Transl Oncol*	59	miRNA	Real-time PCR	A combination test of miRNA-196a and miRNA-196b, whose expression is upregulated from the PanIN state, can identify patients with PanIN 2/3
Madhavan *et al* [89], 2015	*Int J Cancer*	220	Exosome and miRNA	miRNeasyMinikit, RT-PCR, qRT-PCR and flow cytometry	The selected miR-1246, miR-4644, miR-3976 and miR-4306 were significantly upregulated in 83% of PDAC serum-exosomes, but rarely in control groups

AUC: Area under the curve; CA19-9: Carbohydrate antigen 19-9; CTC: Circulating tumor cell; EphA2: Ephrin type-A receptor 2; EV: Extracellular vesicles; FACS: Fluorescence-activated cell sorting; ISET: Isolation by size of epithelial tumor cells; LC-MS: Liquid chromatography-tandemmass spectrometry; miRNA: microRNA; nPES: Nanoplasmon-enhanced scattering; NC: Normal healthy control; NE-iFISH: Negative enrichment immunofluorescence and in situ hybridization of chromosome 8; PDAC: Pancreatic ductal adenocarcinoma; RT-qPCR: Quantitative reverse transcription polymerase chain reaction; SE-iFISH: Subtraction enrichment and immunostaining-fluorescence in situ hybridization.

KRAS genes in the blood of PDAC patients, as well as the prognostic relevance of *KRAS* genes[95-103]. Hadano *et al*[96] assessed the prognosis of patients who underwent curative pancreatoduodenectomy for PDAC according to the presence of *KRAS*-mutated ctDNA before surgery. The median OS of patients with PDAC with ctDNA was significantly worse than that of those without ctDNA (13.6 and 27.6 mo, respectively). ctDNA before surgery is not associated with prognosis or recurrence[97,98]. Bernard *et al*[99] validated the significance of change in ctDNA and exosome DNA (exoDNA) during neoadjuvant chemotherapy in resectable PDAC patients and found that increased exoDNA levels after neoadjuvant chemotherapy were significantly associated with disease progression, whereas ctDNA showed no correlation with outcome. They also elucidated the significance of the presence of ctDNA and exoDNA before chemotherapy in unresectable PDAC patients demonstrating that detection of ctDNA and exoDNA mutant allele frequency (MAFs) ≥ 5% at baseline status were a significantly poor prognosis. Kinugasa *et al*[100] reported that *KRAS* mutations were observed in 62.5% of serum samples of 75 patients with PDAC at all stages using droplet digital polymerase chain reaction (PCR) and were correlated with worse OS. *KRAS* mutations in ctDNA were an independent negative predictor of survival in unresectable pretreatment PDAC patients[101-103].

CTCs serve as prognostic markers in several studies. A recent 9-cohort meta-analysis of separate studies using CellSearch and reverse transcription PCR detection methods involving 623 PDAC patients found an association between detection of CTCs and poor prognosis. Among the 623 patients, 268 patients (43%) with CTCs showed poor PFS and OS compared to the those without CTCs[104]. The different methods of enrichment and detection of CTCs demonstrate that the abundance of CTCs[76,105-108], circulating tumor microemboli (CTMs)[109], could be predictive of worse survival. Results have varied depending on the isolation techniques, detection methods, and the population of patients. Bidard *et al*[110] investigated the CTC detection rate using CellSearch® in a subgroup of 79 patients with locally advanced PDAC enrolled in the LAP 07 trial; 11% with CTCs had worse OS. Using the CellSearch enrichment method, Kurihara *et al*[111] investigated the significance of CTCs as a biomarker of clinical outcomes in 26 patients. Eleven of 26 patients (42%) showed CTCs. PDAC patients without CTCs exhibited significant longer median survival times of 375.8 d than those with CTCs (110.5 d, $P < 0.001$). de Albuquerque *et al*[112] also reported a worse median PFS in patients with CTC (47% of patients). Detection of CTCs using immunomagnetic epithelial cell adhesion molecule and mucin1 demonstrated longer PFS of 138.0 d in patients without CTCs than in those with CTC (66.0 d). Intriguingly, CTC enumeration is not correlated with clinicopathological features of the disease, including metastasis status and tumor stages. The detection of CTCs alone makes no difference in the prognosis of PDAC patients[113].

The exosomal miRNA-mediated cell-to-cell signaling in the tumor microenvironment plays a significant role in the progression of cancer[114,115]. The proliferation and invasive properties of surrounding cancer cells were shown to be modulated by PDAC exosomal miR-222[116], which was related to poor outcome in PDAC patients[116]. PDAC cells releases exosomes enriched with miR-301a under hypoxic conditions, where circulating exosomal miR-301a-3p levels were positively associated with depth of invasion, lymph node metastasis, late TNM stage, and poor prognosis of PDAC patients [117]. Exosomal miRNAs have been studied to assess PDAC growth, migration, and invasion; further longitudinal studies are essential to identify exosomal miRNAs as prognostic biomarkers for PDAC. Exosomal proteins also play a significant role in PDAC diagnosis. The levels of GPC1 are associated with tumor size and disease burden of PDAC[118]. Macrophage migration inhibitory factor (MIF) is highly expressed in PDAC-derived exosomes and its blockade prevented liver pre-metastatic niche formation and metastasis[119]. MIF was markedly higher in exosomes from patients of stage I PDAC, who later developed liver metastasis, in comparison with advanced PDAC patients, suggesting that increased levels of exosomal MIF could be a biomarker for developing liver metastasis in PDAC patients (Table 2).

Recurrence monitoring

A liquid biopsy is a promising tool to detect minimal residual disease in various gastrointestinal malignancies. Predicting cancer recurrence earlier improves prognosis and provides more options for early consideration of anticancer drugs and operations. Longitudinal monitoring of ctDNA demonstrated to show tumor dynamics in colorectal cancer due to its short half-life[74], which could be available for PDAC.

The monitoring of ctDNA in PDAC patients after surgery reflects early recurrence[97,98,103,120-123]. Multivariate analysis revealed that detection of *KRAS* mutations in postoperative serum was an independent prognostic factor for disease-free survival (DFS), which is associated with recurrence ($P = 0.027$)[97]. Increase of *KRAS* mutated ctDNA after surgery, during periods of adjuvant chemotherapy and observation, was a highly predictive dynamic marker of early relapse[120]. We reported that detection of *KRAS* mutated ctDNA after surgery was associated with OS regardless of recurrence ($P = 0.005$) while increase of CA19-9 was associated with recurrence ($P < 0.001$) but not OS ($P = 0.692$)[98]. Yamaguchi *et al*[124] reported that detection of ctDNA before surgery was significantly associated with poor DFS. Detection of ctDNA before surgery is likely to be associated with recurrence comparing to the monitoring of ctDNA after surgery[125-128]. *KRAS* mutated ctDNA before surgery, especially *KRAS* G12D mutation, serves as a biomarker for early relapsein resectable PDAC patients[125]. Lee *et al*[126]

Table 2 Liquid biopsy in the predicting prognosis of pancreatic cancer

Ref.	Journal	year	No. of patients	Biomarker	Method	Main findings
Singh *et al*[91], 2015	*Cancer Invest*	2015		cfDNA		Higher level of plasma DNA (> 62 ng/mL) was found to associate significantly with lower overall survival time (P = 0.002), presence of vascular encasement (P = 0.030) and metastasis (P = 0.001)
Lapin *et al*[92], 2018	*J Transl Med*	2018	61	cfDNA	2100 Bioanalyzer	Pre-treatment cfDNA levels could independently predict prognosis for both PFS (HR = 3.049, P = 0.005) and OS (HR = 2.236, P = 0.028)
Wang *et al*[93], 2021	*Pancreas*	2021	97	cfDNA	PCR	The 1- and 5-year survivals for those with high cfDNA were poorer; 70.2% and 21.2%, respectively, as compared with 93.4% and 23.7% for those with low cfDNA level
Castells *et al*[94], 1999	*J Clin Oncol*	1999	47	ctDNA	PCR-RFLP and SSCP	Plasma *KRAS* mutations were identified as the only independent prognostic factor (odds ratio, 1.51; 95%CI: 1.02 to 2.23)
Ako *et al*[95], 2017	*Pancreatology*	2017	40	ctDNA	ddPCR	*KRAS* mutation at G12V in the plasma or serum conferred a significantly poorer prognosis than without the mutation (P < 0.01)
Hadano *et al*[96], 2016	*Br J Cancer*	2016	105	ctDNA	ddPCR	Patients who were preoperative ctDNA+ had a significantly poorer prognosis with respect to OS (P < 0.0001)
Nakano *et al*[97], 2018	*Br J Cancer*	2018	45	ctDNA	PNA directed, PCR clamping	There were no significant differences in DFS and OS between patients with and without *KRAS* mutations from preoperative serum
Watanabe *et al* [98], 2019	*PLoS One*	2019	78	ctDNA	ddPCR	No effect of the presence of *KRAS*-mutated ctDNA before surgery on RFS (median: 16.9 mo *vs* 32.4 mo) was observed
Bernard *et al*[99], 2019	*Gastroenterology*	2019	34	ctDNA and exosome DNA	ddPCR	Increased exosome DNA levels after neoadjuvant therapy were significantly associated with disease progression (P = 0.003)
Kinugasa *et al* [100], 2015	*Cancer*	2015	75	ctDNA	ddPCR	*KRAS* mutations in plasma correlated with poor OS (P = 0.002)
Tjensvoll *et al* [101], 2016	*Mol Oncol*	2016	14	ctDNA	PNA clamp PCR	Kaplan-Meier survival analyses indicated that patients with a positive ctDNA before or after initiation of chemotherapy had shorter PFS and OS
Chen *et al*[102], 2010	*Eur J Surg Oncol*	2010	91	ctDNA	Direct sequencing	*KRAS* codon 12 mutation from plasma DNA was an independent negative prognostic factor (HR, 7.39; 95%CI: 3.69-14.89)
Sausen *et al*[103], 2015	*Nat Commun*	2015	101	ctDNA	Next-generation sequencing and digital PCR	ctDNA was an independent prognostic marker of OS in advanced disease, with OS of 6.5 mo *vs* 19.0 mo for ctDNA-positive and negative patients, respectively
Khoja *et al*[105], 2012	*Br J Cancer*	2012	54	CTC	Cellsearch and ISET	The PFS and OS for patients without *vs* those with CTCswas 140 d *vs* 94 d (P = 0.13) and 164 d *vs* 127 d (P = 0.26), respectively
Earl *et al*[106], 2015	*BMC Cancer*	2015	45	CTC	Cellsearch	A Cox regression analysis showed a significant difference in OS for CTC positive *vs* negative patients with a HR of 3.0 (P = 0.023)

Zhang *et al*[107], 2015	*Int J Cancer*	2015	61	CTC	The EpCAM-independent method	CTCs positive pancreatic cancer patients exhibit a worse (P = 0.0458) survival rate
Okubo *et al*[108], 2017	*Eur J Surg Oncol*	2017	65	CTC	Cellsearch	A multivariate analysis identified the presence or absence of CTCs as an independent prognostic factor (P = 0.049)
Ankeny *et al*[76], 2016	*Br J Cancer*	2016	100	CTC	Microfluidic NanoVelcro CTC chip	A cut-off of 3 CTCs in 4 mL venous blood was able to discriminate between local/regional and metastatic disease (AUROC = 0.885; 95%CI: 0.800-0.969; and P < 0.001)
Chang *et al*[109], 2016	*Clin Chem*	2016	63	CTM	anti-EpCAM conjugated supported lipid bilayer-coated microfluidic chips	CTM was an independent prognostic factor of OS and PFS (P 0.0001 and P = 0.003, respectively)
Bidard *et al*[110], 2013	*Ann Oncol*	2013	79	CTC	Cellsearch	CTC positivity was associated with poor tumor differentiation (P = 0.04), and with shorter OS in multivariable analysis (P = 0.01)
Kurihara *et al* [111], 2008	*J Hepatobiliary Pancreat Surg*	2008	47	CTC	Cellsearch	MST of the CTC-positive and -negative patients were 110.5 and 375.8 d (P < 0.001)
de Albuquerque *et al*[112], 2008	*Oncology*	2012	74	CTC		Median PFS time was 66.0 d for patients with baseline CTC positivity and 138.0 days for CTC-negative patients (P = 0.01)
Kulemann *et al* [81], 2015	*Pancreas*	2015	21	CTC	ScreenCell	The presence of CTC did not adversely affect MST: 16 mo in CTC-positive (n = 18) *vs* 10 mo in CTC-negative (n = 3) patients
Li *et al*[116], 2018	*Cell Physiol Biochem*	2018	73	miRNA	Arraystar Human miRCURYTM LNA Array	Multivariate analyses showed that exosomal miR-222 was independent risk factors for PDAC survival (P = 0.046)
Wang *et al*[117], 2018	*Cancer Res*	2018	50	miRNA	qRT-PCR	Exosomal miR-301a-3p overexpression predicted late TNM stage and poor survival in human PDAC (P = 0.0182)
Frampton *et al* [118], 2018	*Oncotarget*	2018	43	GPC1+ circulating exosomes	ELISA	Patients with high crExos GPC1 levels have significantly larger PDACs (> 4 cm; P = 0.012)
Costa-Silva *et al* [119], 2015	*Nat Cell Biol*	2015	55	Exosome	ELISA	Increased levels of MIF in exosomes isolated from patients with PDAC with progression of disease post-diagnosis compared with PDAC patients with no evidence of disease five years post-diagnosis (P < 0.01) and with healthy controls (P < 0.01), but not patients with liver metastasis

AUROC: Area under the curve; cfDNA: Cell-free DNA; ctDNA: Circulating tumor DNA; CTC: Circulating tumor cell; CTM: Circulating tumor microemboli; DFS: Disease-free survival; ddPCR: Droplet digital polymerase chain reaction; ELISA: Enzyme-linked immuno-sorbent assay; EPCAM: Epithelial cell adhesion molecule; ISET: Isolation by size of epithelial tumor cells; HR: Hazard ratio; MST: Median survival time; MIF: Macrophage migration inhibitory factor; miRNA: microRNA; OS: Overall survival; PFS: Progression-free survival; PCR: Polymerase chain reaction; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; PNA: Peptide nucleic acid; PDAC: Pancreatic ductal adenocarcinoma; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; RFS: Recurrence-free survival; SSCP: Single-strand conformation polymorphism.

reported that *KRAS* mutated ctDNA before and after surgery was associated with shorter recurrence-free survival in resectable PDAC patients and all patients with ctDNA had a recurrence. Recurrence after surgery is likely identified earlier by ctDNA than imaging study of computed tomography[103, 121], suggesting that ctDNA may serve as postoperative surveillance.

While preoperative CTCs have been reported to predict tumor recurrence, postoperative CTC monitoring has been rarely evaluated. Park *et al*[129] reported that 12 of 40 patients with CTC (33.3%) in preoperative blood showed a significantly frequent rate of systemic recurrence (distant metastases and peritoneal dissemination). Multivariable logistic regression analysis showed the detection of CTC was an independent risk factor for early recurrence and systemic recurrence.

The detection of exosome-derived *KRAS* with MAFs of > 1% is associated with worse DFS after resection in patients with localized PDAC[130]. Exosomal miR-451a in plasma of PDAC patients is linked to recurrence after surgery[131]. miRNA derived from portal vein blood exosomes (miR-4525, miR-451a, and miR-21), as well as CTCs, can be utilized for the evaluation of PDAC recurrence[132] (Table 3).

Therapeutic effect monitoring

An accurate real-time understanding of tumor dynamics during chemotherapy helps to select adequate drugs and avoid unnecessary side effects. For this purpose, more sensitive and novel biomarkers are required to overcome disadvantages of conventional biomarkers including CA19-9; however, only few studies have described monitoring using liquid biopsy during chemotherapy in unresectable PDAC, and most of them reported that the prognosis is poor when each marker is detected [78,84,98,99,108,133-137].

The importance of long-term monitoring of ctDNA its predictive ability for the prognosis of PDAC [98,133,134]. In 2018, Kruger *et al*[134] highlighted the cut-off value of ctDNA for detecting early drug response in metastatic PDAC patients who underwent chemotherapy: an increase in ctDNA at day 14 correlated with disease progression determined by subsequent imaging study with a sensitivity of 83% and specificity of 100%. Yin *et al*[138] evaluated ctDNA in patients with PDAC with pathologic complete response (pCR) to neoadjuvant chemotherapy and found its associations with the outcome. They reported that ctDNA existed even in patients with PDAC with pCR to neoadjuvant chemotherapy, thereby predicting early recurrence and reduced survival. These data were obtained using somatic mutations in tumor tissues and CTCs in addition to ctDNA.

Change in number of CTCs was reported in response to neoadjuvant chemotherapy in patients with advanced stage PDAC[138-140]. In a study of 57 PDAC patients who underwent surgery, patients who received neoadjuvant chemotherapy had significantly lower number of CTC than those who were chemotherapy-naive at the time of surgery[139]. Another study, however, demonstrated no differences in 16 patients who received neoadjuvant chemotherapy[140]. Wei *et al*[135] evaluated CTCs in patients with PDAC who underwent surgery and chemotherapy (modified FOLFIRINOX; oxaliplatin, leucovorin, irinotecan, and fluorouracil, or gemcitabine plus nab-paclitaxel chemotherapy). They reported that the number of CTC decreased or remained same in 12 (92.3%) patients with drug response (either tumor shrinkage or stable tumor burden). Negative enrichment, immunofluorescence, and *in situ* hybridization of chromosome 8 was applied to capturing CTC and determine the role of chromosomal instability in patients with PDAC. Improvement in detection rate of CTCs by using this system revealed the significance of triploid CTCs for the prediction of drug response to chemotherapy in these patients. In addition, CTMs correlated with poor response to chemotherapy in patients having stage IV PDAC [78].

The abundance of exosomal miRNAs was applied to assess therapeutic response. The expression levels of serum miRNAs (miR-221) was significantly upregulated at an earlier time, 3-6 wk of chemotherapy in patients with PDAC without drug response to lapatinib and capecitabine, compared to those without drug response. The expression levels of miRNA (specifically miR-221) increased in PDAC patients who did not respond to lapatinib and 5-fluorouracil (the active form of capecitabine), indicating that the increased level of specific serum miRNAs was associated with resistance to lapatinib and capecitabine treatment[136]. The levels of exosomes in serum at serial time points throughout chemoradiotherapy correlated with treatment resistance in 10 patients with locally advanced PDAC[137]. The levels of EphA2-EV in plasma were strongly associated with treatment response described in the early diagnosis section, reflecting the drug response to neoadjuvant therapy in 23 PDAC patients[84] (Table 4).

CONCLUSION

Liquid biopsy is gaining attention as a non-invasive methodology and is involved in obtaining important tumor information *via* blood-based biomarkers for early diagnosis and treatment of cancer. Tumor components such as CTCs, cfDNA, ctDNA, miRNAs, and exosomes in liquid biopsy have promising value for diagnosis, prognosis, and treatment prediction of surgery and chemotherapy. In particular, the most significant advantage of liquid biopsy over tissue biopsy is its ability to monitor disease progression and treatment efficacy longitudinally in "real time." However, liquid biopsy is not yet considered a standard means of confirming or diagnosing various diseases, including cancer.

The main limitation of liquid biopsy is its lack of sensitivity and accuracy in identifying various types of tumor compared to tissue biopsy. In addition, the ability to detect liquid biopsy is difficult because CTCs, ctDNA, and RNA are relatively scarce compared to other blood components. Furthermore, there are no standard separation, enrichment, and detection methodologies. Applying different techniques and assays to detect CTCs and ctDNA will result in varying sensitivity and specificity[141,142]. Usually, enriched CTCs are identified using tumor-associated biomarkers, either at the protein or mRNA level. However, in patients with epithelial tumors, epithelial markers are downregulated in the process of epithelial mesenchymal transition, making it difficult to identify them, leading to false-negative results

Table 3 Liquid biopsy in recurrence monitoring of pancreatic cancer

Ref.	Journal	No. of patients	Biomarker	Method	Main findings
Nakano *et al* [97], 2018	*Br J Cancer*	45	ctDNA	PNA-directed PCR clamping	Multivariate analysis revealed that *KRAS* mutations in postoperative serum are an independent prognostic factor for DFS (P = 0.027). Furthermore, the change from not detecting mutant *KRAS* in preoperative to mutant *KRAS* in postoperative cfDNA was an independent prognostic factor for OS (P = 0.004)
Hussung *et al* [120], 2021	*BMC Cancer*	25	ctDNA	ddPCR, PCR	An increased *KRAS* mutated ctDNA during adjuvant chemotherapy and follow-up was a highly predictive dynamic marker of early relapse and poor OS
Watanabe *et al*[98], 2019	*PLoS One*	78	ctDNA	ddPCR	Detection of mutant *KRAS* on postoperative ctDNA was associated with OS regardless of recurrence (P = 0.005)
Groot *et al* [121], 2019	*Clin Cancer Res*	59	ctDNA	ddPCR	ctDNA detected during follow-up predicted clinical recurrence (sensitivity 90%, specificity 88%) with a median lead time of 84 d
Sausen *et al* [103], 2015	*Nat Commun*	20 (surgery group)	ctDNA	Next-generation sequencing and digital PCR	Patients with detectable ctDNA after surgical resection (n = 10) were more likely to relapse and die from disease compared with those with undetectable ctDNA (P = 0.0199)
Jiang *et al* [122], 2020	*Front Oncol*	27	ctDNA	Next-generation sequencing	Patients with ctDNA-positive status postoperatively had a markedly reduced DFS compared to those with ctDNA-negative status (P = 0.019)
Kim *et al* [123], 2018	*Clin Chem*	106	ctDNA	ddPCR	Patients who had increased *KRAS* MAF values at 6 mo had a shorter OS (P = 0.036) than those who had decreased values
Yamaguchi *et al*[124], 2021	*Ann Surg Oncol*	97	ctDNA	ddPCR	The multivariate analysis showed that the presence of preoperative ctDNA was associated with poorer OS (P = 0.008) and that postoperative ctDNA was not associated with either RFS or OS
Guo *et al* [125], 2020	*Br J Cancer*	113 and 44 (discovery and validation cohorts)	ctDNA	ddPCR	Survival analysis showed that plasma *KRAS* mutations, especially *KRAS* G12D mutation, had significant association with OS and RFS of resectable PDAC. Plasma *KRAS* G12D mutation showed a strong correlation with early distant metastasis
Lee *et al* [126], 2019	*Ann Oncol*	42	ctDNA	PCR-based-SafeSeqS assays	Preoperative ctDNA detection was associated with inferior RFS (P = 0.002) and OS (P = 0.015). Detectable ctDNA following curative intent resection was associated with inferior RFS (P < 0.0001) and OS (P = 0.003)
Pietrasz *et al* [127], 2017	*Clin Cancer Res*	31	ctDNA	Next-generation sequencing	The presence of ctDNA was associated with a shorter DFS (4.6 mo *vs* 17.6 mo; P = 0.03) and shorter OS (19.3 mo *vs*.32.2 mo; P = 0.027)
Okada *et al* [128], 2020	*J Gastroenterol*	66 (surgery group)	ctDNA	Digital PCR	Patients with preoperative ctDNA MAF > 0.45% exhibited significantly shorter disease-free survival than those with lower MAF (HR 3.179, 95%CI: 1.025-9.859; P = 0.0452)
Park *et al* [129], 2021	*Sci Rep*	40	CTC	CD-PRIM kit	On multivariable logistic regression analysis, CTC positivity was an independent risk factor for early recurrence (P = 0.027) and systemic recurrence (P = 0.033)
Allenson *et al* [130], 2017	*Ann Oncol*	142 and 121 (discovery and validation cohort)	Exosome and ctDNA	Electron microscopy, flow cytometry and particle analysis and ddPCR	Higher exosome *KRAS* MAFs were associated with decreased disease-free survival in patients with localized disease (P = 0.031)
Takahasi *et al* [131], 2018	*J Hepatobiliary Pancreat Sci*	50	miRNA	qRT-PCR	In cox proportional hazards model analysis, exosomal miR-451a showed significance to OS and DFS (P = 0.001, P = 0.004)
Kawamura *et al*[132], 2019	*J Hepatobiliary Pancreat Sci*	55	miRNA	qRT-PCR	miR-4525, miR-451a, and miR-21 from portal vein can be utilized for the evaluation of pancreatic cancer recurrence (P = 0.002, 0.001 and 0.002, respectively)

ctDNA: Circulating tumor DNA; cfDNA: Cell-free survival; CTC: Circulating tumor cell; DFS: Disease-free survival; ddPCR: Droplet digital polymerase chain reaction; HR: Hazard ratio; MAF: Mutant allele frequency; miRNA: microRNA; OS: Overall survival; PNA: Peptide nucleic acid; PCR: Polymerase chain reaction; PDAC: Pancreatic ductal adenocarcinoma; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; RFS: Recurrence-free survival; SafeSeqS: Safe-sequencing system.

Table 4 Liquid biopsy in the therapeutic effect monitoring of pancreatic cancer

Ref.	Journal	No. of patients	Biomarker	Method	Main findings
Del Re *et al*[133], 2017	*Sci Rep*	27	ctDNA	ddPCR	There was a statistically significant difference in PFS and OS in patients with increase *vs* stability/reduction of ctDNA in the sample collected at day 15 (P = 0.03 and P = 0.009, respectively)
Kruger *et al* [134], 2018	*Ann Oncol*	54	ctDNA	BEAMing	An increase in ctDNA at day 14 correlated with disease progression on subsequent imaging with a sensitivity of 83% and specificity of 100%
Watanabe *et al* [98], 2019	*PLoS One*	39	ctDNA	ddPCR	The emergence of *KRAS* ctDNA in longitudinal tests was associated with prognosis (P < 0.005)
Wei *et al*[135], 2019	*Cancer Lett*	13 (chemotherapy group)	CTC	Vimentin or EpCAM immobilized microfluidic chip	In patients exhibiting a response, their CTC counts decreased or remained the same, except for one case
Okubo *et al* [108], 2017	*Eur J Surg Oncol*	65	CTC	Cellsearch	The overall survival rate was significantly lower in patients with than in those without CTCs even after chemotherapy and chemoradio-therapy (P = 0.045)
Xu *et al*[78], 2017	*Int J Mol Sci*	83	CTC	NE-iFISH	The proportion of triploid CTC detected by the NE-iFISH was significantly decreased after chemotherapy (P < 0.001)
Tian *et al*[136], 2016	*Oncol Lett*	17	microRNA	RT-qPCR	Significant upregulation of serum miRNAs (miR-21, miR-210, miR-221 and miR-7), at earlier time points (3-6 wk) was observed in non-responders of chemotherapy compared to responders
Bernard *et al* [99], 2019	*Gastroenterology*	104 (chemotherapy group)	exosome and ctDNA	ddPCR	In the longitudinal analysis in chemotherapy group, a MAF peak above 1% in exosome DNA was significantly associated with radiologic progression (P = 0.0003)
An *et al*[137], 2017	*J Proteome Res*	10	exosome	iTRAQ	They analyzed exosomes before treatment, after one cycle of induction gemcitabine-based chemotherapy, and at 3 wk after starting chemoradiation therapy and compared these samples to serum derived from healthy volunteers. They identified eight proteins that changed during a course of therapy in all patients
Liang *et al*[84], 2017	*Nat Biomed Eng*	23 (neoadjuvant chemotherapy group)	EV	nPES	EphA2-EVs were also informative in detecting early responses to neoadjuvant therapy (P < 0.05)
Yin *et al*[138], 2021	*Clin Cancer Res*	36	somatic mutations, CTCs, and ctDNA	Next-generation sequencing & ISET	Somatic mutations, CTCs, and ctDNA existed even in patients with PDAC with pathologic complete response to NAT, which could possibly predict early recurrence and reduced survival
Poruk *et al*[140], 2016	*Ann Surg*	50	CTC	ISET	The detection of CTCs expressing both vimentin and cytokeratin was predictive of recurrence (P = 0.01)
Gemenetzis *et al* [139], 2018	*Ann Surg*	57	CTC	ISET	Patients who received neoadjuvant chemotherapy had significantly lower total CTCs (tCTCs, P = 0.007), eCTCs (P = 0.007), and mCTCs (P = 0.034), compared with untreated patients eligible for upfront resection

ctDNA: Circulating tumor DNA; CTC: Circulating tumor cell; CR: Complete response; ddPCR: Droplet digital polymerase chain reaction; eCTCs: Epithelial circulating tumor cell; EpCAM: Epithelial cell adhesion molecule; EphA2: Ephrin type-A receptor 2; EV: Extracellular vesicles; iTRAQ: Isobaric tag for relative and absolute quantitation; ISET: Isolation by size of epithelial tumor cells; MAF: Mutant allele frequency; mCTCs: Mesenchymal circulating tumor cell; nPES: Nanoplasm-enhanced scattering; NAT: Neoadjuvant chemotherapy; NE-iFISH: Negative enrichment immunofluorescence and in situ hybridization of chromosome 8; OS: Overall survival; PFS: Progression-free survival; RT-qPCR: Quantitative reverse transcription polymerase chain reaction; tCTC: Total circulating tumor cell.

[143]. Another limitation is the low specificity due to the presence of cfDNA from normal tissue. Furthermore, cfDNA is released from normal cells as a diluent for trace amounts of ctDNA, so an additional pre-step is needed in the analysis to avoid an increase in non-neoplastic cfDNA[144]. Establishing blood noncoding RNA as a biomarker is more complicated than in other common biomarkers due to its lack of suitable housekeeping noncoding RNA reference analytes, and high intra-patient variability. Thus, these limitations can lead to a lack of consistency between biomarkers identified in different studies[145]. Exosomes are more manageable to isolate than CTCs or cfDNA in tumors. Therefore, more studies focus on exosomes in the early diagnosis of cancer. However, they still have limitations in clinical application, such as low targeting efficiency and easy phagocytosis by the immune system. In addition, methods for isolating and purifying exosomes are time and labor-consuming. More multicentered, large-scale, and long-term studies, including clinical trials, are urgently needed to make liquid biopsy clinically available.

Similar to other tumors, cfDNA, ctDNA, CTCs, exosomes, and miRNAs are promising new biomarkers in the treatment of PDAC. However, the use of liquid biopsies in the same manner as conventional tumor markers of PDAC is not clear yet. These liquid biopsies are at least as effective as currently used tumor markers. Furthermore, cost-effectiveness is significant when the marker is used for clinical practice. With the advent of *KRAS*12c inhibitors, liquid biopsy will be performed many times during treatment. It is also possible that oncocytic carcinomas may acquire somatic mutations, such as *EGFRT 790M* in lung cancer, which must be monitored. Although liquid biopsy is minimally invasive, new and innovative technologies are needed to reduce the time and effort required for multiple analyses. With advances in cancer genomic medicine, new base mutation-specific inhibitors have been developed or new genetic mutations directly linked to drug resistance have been identified. So, strategies for various cancers may change at an unprecedented pace. Since it is practically difficult to make next-generation sequence analysis mandatory many times during treatment, it will be important in the future to assemble an appropriate analytical system that is as minimal as necessary.

FOOTNOTES

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MINIREVIEWS

Nanotechnology for colorectal cancer detection and treatment

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Abstract

Colorectal cancer (CRC) is the third most diagnosed cancer and the second leading cause of cancer-related mortality in the United States. Across the globe, people in the age group older than 50 are at a higher risk of CRC. Genetic and environmental risk factors play a significant role in the development of CRC. If detected early, CRC is preventable and treatable. Currently, available screening methods and therapies for CRC treatment reduce the incidence rate among the population, but the micrometastasis of cancer may lead to recurrence. Therefore, the challenge is to develop an alternative therapy to overcome this complication. Nanotechnology plays a vital role in cancer treatment and offers targeted chemotherapies directly and selectively to cancer cells, with enhanced therapeutic efficacy. Additionally, nanotechnology elevates the chances of patient survival in comparison to traditional chemotherapies. The potential of nanoparticles includes that they may be used simultaneously for diagnosis and treatment. These exciting properties of nanoparticles have enticed researchers worldwide to unveil their use in early CRC detection and as effective treatment. This review discusses contemporary methods of CRC screening and therapies for CRC treatment, while the primary focus is on the theranostic approach of nanotechnology in CRC treatment and its prospects. In addition, this review aims to provide knowledge on the advancement of nanotechnology in CRC and as a starting point for researchers to think about new therapeutic approaches using nanotechnology.

Key Words: Nanoparticles; Colorectal cancer; Carbon nanotubes; Dendrimers; Quantum dots

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Core Tip: Colorectal cancer (CRC) is the third most diagnosed cancer and the second leading cause of cancer-related mortality in the United States. Genetic and environmental risk factors play a significant role in the development of CRC. This review discusses the recent insights into nanotechnology-based methods for screening, detection and treatment of CRC. This review aims to provide knowledge on the advancement of nanotechnology in CRC and as a starting point for researchers to think about new therapeutic approaches using nanotechnology.

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INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer-related deaths in the United States, after lung cancer. The mortality rate of a cancer depends on its type and the sex, race and other social factors of the patient. Death from CRC affects more males than females[1]. While it is predicted that more than 1 million individuals will be diagnosed with CRC every year[2], there is a substantial geographical difference in the global distribution of CRC. The countries with the highest incidence rates of CRC are Australia, New Zealand, Canada, the United States and parts of Europe. The lowest-risk countries include China, India and parts of Africa and South America[3]. Risk factors of CRC are associated with body mass index, smoking cigarettes, red meat consumption, family history of CRC and irritable bowel disease[4], while published literature also suggests that risk of CRC decreases with physical activity, hormone therapy in postmenopausal women, aspirin/nonsteroidal anti-inflammatory drug use, fruit consumption and vegetable consumption. Thus, CRC is a disease that the management of our daily habits can prevent. Although a hereditary risk factor is involved with CRC, effective early diagnosis strategies enable earlier initiation of therapy, thereby reducing the cancer's mortality rate.

Recently, nanotechnology has gained global consideration with its great potential for diagnosing and treating CRC. Nanotechnology utilizes nanoparticles (NPs) for specific identification of tumors and cancer biomarkers, biologically targeted contrast agents, drug delivery systems and novel treatment approaches[5]. This review discusses the recent insights into nanotechnology-based methods for screening, detection and treatment of CRC.

CRC CAUSES AND STAGES

The growth of CRC incidence worldwide is related to genetic and environmental risk factors, in addition to sex, age, ethnicity, gut microbiota and socioeconomic factors[6]. Environmental risk factors include obesity, a sedentary lifestyle, poor diet, alcohol addiction, long-term smoking[7] and genetic factors due to inherited mutations or familial CRC[8]. Diet significantly impacts the microbiome environment and the risk of CRC development. Consumption of high-fiber diets and diet supplementation with polyunsaturated fatty acids, polyphenols and probiotics could potentially reduce the risk of CRC[9]. About 70% of CRC cases are sporadically seen among patients over age 50, mainly due to dietary and environmental factors[10]. In addition, a family history of CRC and a personal history of chronic inflammatory bowel disease, type 2 diabetes, or genetic diseases like familial adenomatous polyposis or Lynch syndrome are related to augmented risk[11]. Acquisition of genetic and epigenetic mutations in specific oncogenes and tumor suppressor genes in normal epithelial cells leads to the sequential transformation of the normal colorectal epithelial cell into adenocarcinoma[12]. Chromosomal instability, microsatellite instability and CpG island methylator phenotype are primary pathways influencing development of CRC[13].

The stages of CRC determine the survival rate. Around 90% of CRCs are stage I, while 10% of the cases are diagnosed with stage IV metastasis, implying that early diagnosis leads to high chances of survival[14]. The tumor stage determines whether the therapy is beneficial and how successful the treatment might be against CRC[15]. The stages of CRC range from stage 0 to IV depending on the progression of cancer across the layers of the colon and rectum, effect on lymph nodes or nearby tissues, or its metastasis to distant organs (Table 1)[16]. Out of all the stages, stage IV CRC is not often curable, but it can be managed based on the cancer's growth and spread[17]. The sequential steps in CRC carcinogenesis are presented in Figure 1.

Table 1 Stages of colorectal cancer development and progression

Stage	Progression	Metastasis
0	Unusual abnormal cells arise from mucosa of the colonic wall, eventually becoming cancerous and known as carcinoma *in situ* or intramucosal carcinoma	No
I	Cancerous cells spread from the mucosa to submucosa and may also extended until the muscularis propria	No
II		
IIA	Cancerous cells spread out from the submucosa to serosa (outermost layer) of the colon wall	No
IIB	Cancerous cells spread through serosa but are not extended to nearby organs	No
IIC	Cancerous cells spread to nearby organs	No
III		
IIIA	In the first case, cancerous cells grow through the mucosa into submucosa and also may extended until the muscularis propria. The cancer spread to 1 to 3 nearby lymph nodes or into the areas of fat near lymph nodes but not to distant sites In the second case, the cancer has spread through the mucosa into submucosa and has spread to 4 to 6 nearby lymph nodes	No
IIIB	In the first case, cancerous cells have spread through the muscularis propria of colon and/or rectum up to serosa or through the serosa to tissues and visceral peritoneum. They have also spread to 1 to 3 nearby lymph nodes or cancer cells are present in nearby tissues of lymph nodes but have not spread to distant sites Second case, the cancer has spread into the muscularis propria or into the outermost layers of colon or rectum and has spread to 4 to 6 nearby lymph nodes but not to distant sites Third case, the cancer has spread through the mucosa into submucosa and it might also have grown into the muscularis propria of colon and/or rectum and has spread to 7 or more nearby lymph nodes but not to distant sites	No
IIIC	First case, the cancer has grown through the serosa colon and/or rectum to visceral peritoneum but has not reached nearby organs. It has spread to 4 to 6 nearby lymph nodes but not to distant sites Second case, the cancer has grown into the outermost layers of the colon and/or rectum or through visceral peritoneum but has not reached nearby organs. It has spread to 7 or more nearby lymph nodes but not to distant sites Third case, the cancer has grown through the wall of the colon and/or rectum and is attached to or has grown into other nearby tissues or organs. It has spread to at least one nearby lymph node or into areas of fat near the lymph nodes but not to distant sites	No
IV		
IVA	The cancer has spread to one area or organ that is not near the colon and/or rectum, may be liver, lung, ovary, or a distant lymph node	Yes
IVB	The cancer has spread to more than one area or organ that is not near the colon and/or rectum, such as the liver, lung, ovary, or a distant lymph node	Yes
IVC	The cancer has spread to the tissue that lines the wall of the abdomen and may have spread to other areas or organs	Yes

CURRENTLY AVAILABLE METHODS OF CRC DETECTION

The United States Preventive Services Task Force recommends colorectal screening for adults aged 45 to 75. Several techniques are used to detect polyps or CRC, but colonoscopy is a must to confirm the screening process and other screening tests. The most used methods for screening CRC are stool test, flexible sigmoidoscopy, colonoscopy, computed tomography colonography and double-contrast barium enema[18-28] in Table 2. Detection of DNA biomarkers is a promising technique for CRC screening. Stool-based DNA markers are convenient and easy to use. For early detection of CRC, biomarkers such as *ITG4* methylation, *SFRP2* methylation, miR-21, miR-92a and miR-135b, as well as CologuardÒ (Exact Sciences Corp., Madison, WI, United States), can be used. For malignancy detection, biomarkers such as *SFRP2* methylation, *VIM* methylation, *TFPI* methylation, miR-21, miR-92a and miR-223 can be used[29].

Clinical trials reported that sigmoidoscopy[30] and colonoscopy[31] reduce the chances of incidence and mortality of CRC. Sigmoidoscopy has a sensitivity and specificity of 92%-97% for the detection of polyps and expanded CRC and can be used to diagnose and treat CRC in patients who are ineligible for surgery (according to the severity of the tumor or comorbidities)[29]. The mentioned screening techniques detect either abnormal blood, antibodies, hemoglobin, DNA markers in the stool, or any polyps in the colon or rectum wall. Besides this, circulating tumor cell (CTC) detection-based diagnostic technology is also available, including CellSerch® assay (Janssen Diagnostics, LLC, South Raritan, NJ, United States), followed by faster and more efficient diagnostic systems like MagSweeper (Illumina, San Diego, CA, United States), Cynvenio (Cynvenio Biosystems Inc., Westlake Village, CA, United States), IsoFlux (Fluxion Biosciences, Alameda, CA, United States), VerIFASt, AdnaGen and magnetic sifters[5]. Most current screening methods of CRC are costly and not accessible at the point of care. Therefore, developing a more sensitive, fast, low-cost and specific screening test for CRC is essential.

Table 2 Screening methods for colorectal cancer

Ref.	Screening methods	
[18-20]	Stool tests	Kit-based technique; guaiac-based fecal occult blood test uses guaiac to detect heme; FIT uses antibody to detect hemoglobin in stool; FIT-DNA test detects hemoglobin and DNA biomarkers in the stool
		Non-invasive, low cost, colon cleansing is not required, non-bleeding tumors cannot be detected
[21, 22]	Flexible sigmoidoscopy	Flexible tube with light, lens for viewing and tool for removing tissue; rectum and lower third of the colon screened to detect polyps, cancer or other abnormalities
		Sedation is required; accurate detection if polyps and cancers are present; unable to detect polyps or any abnormalities present in cecum, ascending colon hepatic flexure, or on transverse colon; colon cleansing is required
[23, 24]	Colonoscopy	Flexible tube light with a lens for seeing and a tool for excising the abnormal tissue; entire colon and rectum are screened for cancer by inserting the colonoscope through anus
		Sedation is required; visualization of entire inner lining of the colon and rectum; minute polyps can be detected; sedation may lead to bleeding or tear of the intestinal wall; sedation may lead to bleeding or tear of the intestinal wall
[25-27]	Computed tomography colonography	X-ray based computed tomography scanner captures two- and three-dimensional images of the entire colon; computer assembles these pictures into detailed images
		Sedation is not required in this method; non-invasive; colon cleansing is not essential; may miss small polyps
[28]	Double-contrast barium enema	X-ray images are then captured by introducing barium sulphate enema
		Examination of the whole colon and the rectum; sedation is not required; for the patient who cannot undergo standard colonscopy, this method is useful; colon cleansing is very necessary for this method; otherwise, it will give false positive results

FIT: Fecal immunochemical test.

CURRENTLY AVAILABLE THERAPIES FOR CRC TREATMENT

CRC care entails a multidisciplinary approach as the treatment options depend on factors such as the patient's age, comorbidities, overall health, type and stage of cancer, possible side effects of treatment regimens, *etc*[32]. Based on these characteristics and risk factors, patients are grouped into the following four categories for treatment guidance: (1) Group 0, no metastatic disease or relapse; (2) Group 1, potentially resectable metastatic disease; (3) Group 2, disseminated unresectable disease; and (4) Group 3, unresectable disease and unavailability of intensive or sequential treatment.

Surgery

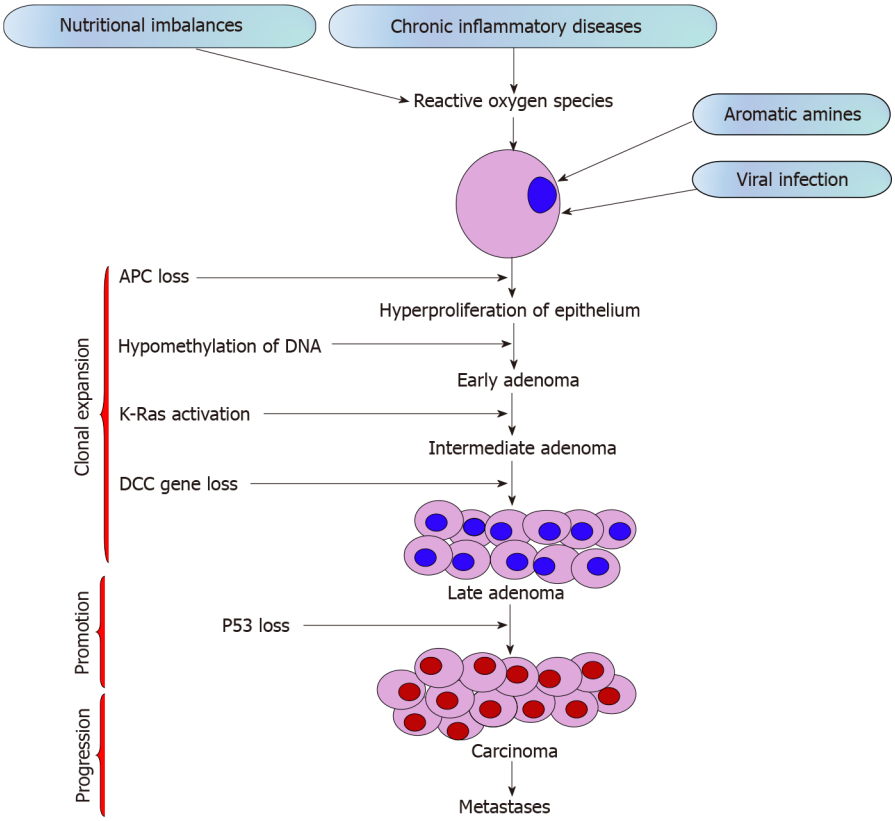
Surgery is considered the most common treatment for CRC. It demarcates the removal of the tumor clinically during an operation. This is also called surgical resection. Group 0 patients fall under this category. Depending upon the size and location of the tumor, the surgical options include laparoscopic surgery, colostomy and radiofrequency ablation or cryoablation. Laparoscopic surgery is an effective method that is carried out by passing several scopes into the abdomen *via* smaller incisions, whereas colostomy is a surgical opening for waste removal (into a pouch) by connecting the colon to the abdominal surface. When radiofrequency waves are used to heat or freeze the malignant tumors in the liver and lungs of CRC patients, then it is called radiofrequency ablation or cryoablation, respectively [33].

Radiation therapy

In radiation therapy, the size of the tumor is reduced and cancer cells are destroyed using high-energy radiations such as X-rays, radio waves and protons. This therapy is primarily used in patients at risk of tumor recurrence. Different types of radiation therapies include external-beam, stereotactic, intraoperative and brachytherapy[34]. The difference lies with the dose and the location of the cancer. For instance, external-beam radiation therapy is usually given 5 d a week for several weeks, whereas stereotactic radiation therapy delivers precise radiation to a small area. Stereotactic radiation therapy is given in malignant cancers that spread from the colorectal region to the liver or lungs. Further, intraoperative radiation therapy and brachytherapy are specialized radiation therapies applicable only in small areas of cancer that cannot be removed by surgery. In intraoperative radiation therapy, a single and high dose of radiation is given, whereas radioactive "seeds" are used and placed inside the body in brachytherapy. Lastly, neoadjuvant therapy shrinks the tumor for easier removal through surgery[35].

Therapies using medications

Medications are given as treatment plans to destroy cancer cells or reduce the tumor size. It can be delivered systemically *via* the bloodstream or directly through intravenous injections, pills, or capsules.



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Figure 1 Schematic diagram representing the sequential steps in colon carcinogenesis. APC: Adenomatous polyposis coli; DCC: Deleted in colon carcinoma; Ras: Rat sarcoma.

Medications include chemotherapy, targeted therapy and immunotherapy[36]. Chemotherapy is generally given to group 1 patients. It is considered a central treatment strategy for all types of cancers. The United States Food and Drug Administration (FDA)-approved drugs to treat CRC are capecitabine (XelodaÒ), fluorouracil (5-FU), irinotecan (CamptosarÒ), oxaliplatin (EloxatinÒ) and trifluridine/tipiracil (LonsurfÒ). For combined therapies, the combination of 5-FU with leucovorin (folinic acid), or leucovorin and oxaliplatin, or leucovorin and irinotecan, or capecitabine with irinotecan or oxaliplatin are used in group 3 patients for the treatment of CRC[37]. Different medications have different mechanistic pathways through which they act upon the cancer cells, *viz.*, inhibition of DNA replication, interference in chromosomal separation in the cell cycle, or cytotoxicity. However, chemotherapy may attack healthy cells. Chemotherapy is often given with radiation therapy to increase the effectiveness of the treatment regimen. A combination of chemotherapy and radiation is known as chemoradiation therapy. Chemoradiation therapy is often given to reduce the risk of colostomy or cancer recurrence.

Target therapy

In targeted therapy, specific genes, proteins, or a niche is targeted to hinder the growth and survival of cancer cells. It limits damage to the healthy cells in group 1 and 2 patients. The targeted therapies for treating CRC include antiangiogenesis therapy and epidermal growth factor receptor inhibitors[38,39]. As the name suggests, the role of antiangiogenesis therapy is to stop angiogenesis, *i.e.*, the formation of new blood vessels. In the tumor niche, the nutrients are provided by blood vessels for the growth of tumor cells. However, these antiangiogenesis therapies lead to the starvation of tumor cells. For example, bevacizumab (AvastinÒ), regorafenib (StivargaÒ), ziv-aflibercept (ZaltrapÒ) and ramucirumab (CyramzaÒ) are currently used targeted drugs. The FDA approved bevacizumab and chemotherapy as the first-line treatment for CRC in 2004[40].

On the other hand, epidermal growth factor receptor inhibitors such as cetuximab (ErbituxÒ) and panitumumab (VectibixÒ) are considered adequate for group 2 patients[41]. These drugs are specific for genes like *Ras* and *Raf* and are ineffective in cases with mutations[42,43]. Other nonspecific cancerous medications such as larotrectinib (VitrakviÒ) and entrectinib (RozlytrekÒ) are also used. These medications act on a specific genetic change called an neurotrophic tyrosine receptor kinase fusion. The currently available treatment methods for CRC and their side effects are detailed[32-34,36,38,39,44-47] in Table 3.

Table 3 Currently available colorectal cancer treatment methods

Treatment methods	Types/medication details	Side-effects	Stages	Ref.
Surgery	Laparoscopic surgery Colostomy for rectal cancer Radiofrequency ablation or cryoablation	Pain; tenderness; irritation and itching; constipation or diarrhea	I, II, III, IV	[32, 44]
Radiation therapy	External-beam radiation therapy Stereotactic radiation therapy Intraoperative radiation therapy Brachytherapy	Fatigue; mild skin reactions; bleeding in stools; constipation or diarrhea; infertility	II, III, IV	[33, 34]
Chemotherapy	Capecitabine (Xeloda) Fluorouracil Irinotecan (Camptosar) Oxaliplatin (Eloxatin) Trifluridine/tipiracil (Lonsurf)	Vomiting; nausea; diarrhea; mouth sores; neuropathy; fatigue; hair loss	II, III, IV	[36, 39]
Targeted therapy	Anti-angiogenesis therapy Epidermal growth factor receptor inhibitors Combined targeted therapies Tumor-agnostic treatment	Rashes	IV	[38, 45]
Immunotherapy	Pembrolizumab (Keytruda) Nivolumab (Opdivo) Dostarlimab (Jemperli) Nivolumab and ipilimumab (Yervoy) combination	Fatigue; rashes, pain and itching; diarrhea; nausea; fever; vomiting; shortness of breath	III, IV	[46, 47]

NANOTECHNOLOGY FOR CRC DIAGNOSIS AND TREATMENT

NPs were first reported as drug carriers in 1986 and it was shown that they accumulate in tumoral tissues. This passive accumulation of NPs, known as the ''enhanced permeability and retention'' effect, was one of the turning points of cancer treatment using NPs. Therefore, due to their high specificity, accumulation in tumor sites and prolonged blood circulation time, NPs are considered promising for cancer therapeutics[48,49] (Figure 2). Various families of organic and inorganic NPs are available today with a vast variety of sizes, structures and compositions[49] (Figure 3). Some of the common NPs that are used for CRC diagnosis and treatment are listed here.

Iron oxide nanocrystals

Iron oxide is popular as a diagnostic and therapeutic tool for its use in oncology. The superparamagnetic properties of iron oxide NPs have attracted attention for their biomedical applications, arising due to their biocompatibility and non-toxicity[50,51]. The diameter ranges from 1-100 nm with a magnetic core to the iron oxide nanocrystals and a polymer covering containing the various medicinal molecules. These NPs have diverse biological properties due to their relatively small dimension, minimal deposition rate, efficient surface region and ease of cellular transport. Iron oxide NPs loaded with 5-FU with magnetic hyperthermia effectively reduce tumor growth in heterotopic human colon cancer in mice[52]. Epirubicin-5TR1 aptamer-superparamagnetic iron oxide NP (SPION) tertiary complex could efficiently deliver epirubicin to C26 murine colon carcinoma cells and allows tumor detection by magnetic resonance imaging (MRI)[51]. Chitosan-coated iron oxide enhanced reactive oxygen species' production in a human colorectal carcinoma cell lines (HCT 116) and cell death was caused *via* caspase 9/3 activation[53].

Quantum dots

Quantum dots are nanocrystal particles of semiconductors ranging from 2-10 nm, with fluorescence emission affected by particle size. With its optical and chemical advantages, quantum dot-based

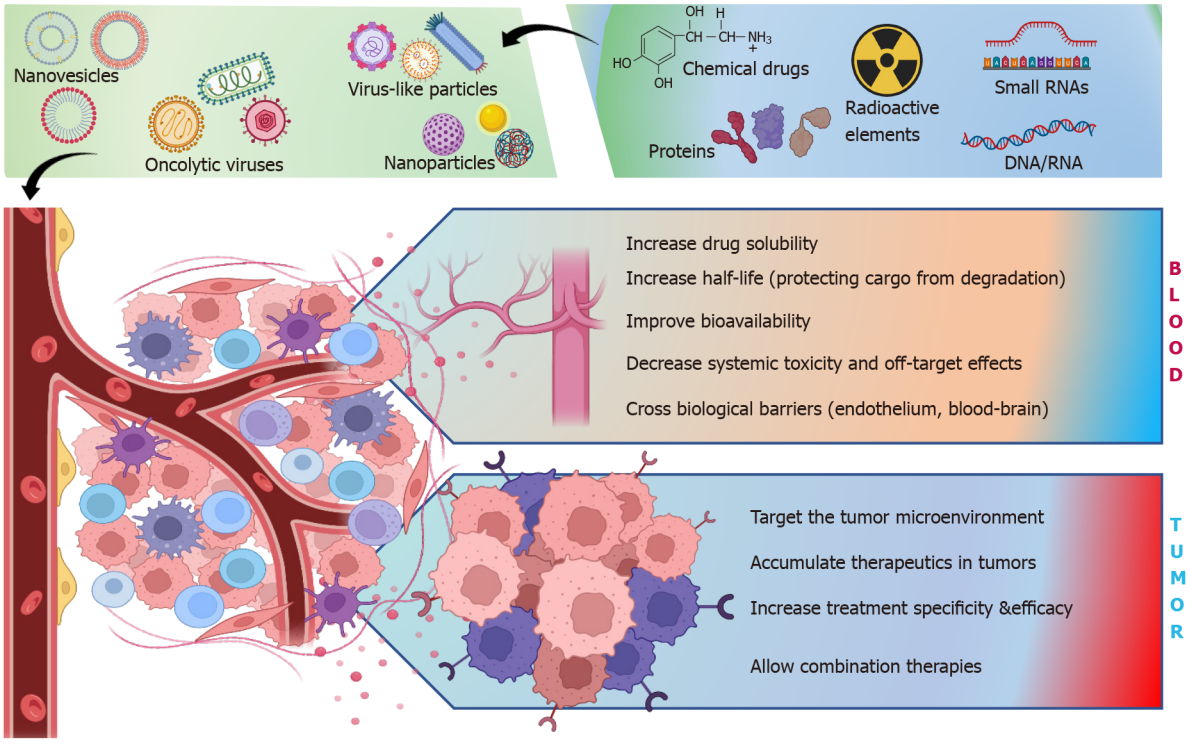


Figure 2 Advantages of nanoparticles in cancer drug delivery. Nanoparticles along with extracellular vesicles or viruses improve the clinical efficacy of various therapeutic molecules (nucleic acids, antibodies, drugs, radionuclides) with efficient delivery of these molecules to the tumor with minimal off-targeting. Nanoparticles enhance the transport of these molecules by increasing their solubility, bioavailability and half-life in the bloodstream. The tumor delivery of therapeutic molecules is also enhanced by nanoparticles, leading to their accumulation and thus potentiating the use of various combination therapies for colorectal cancer. Citation: Briolay T, Petithomme T, Fouet M, Nguyen-Pham N, Blanquart C, Boisgerault N. Delivery of cancer therapies by synthetic and bio-inspired nanovectors. *Mol Cancer* 2021; 20: 55. Copyright© The Author(s) 2021. Published by Springer Nature. The authors have obtained the permission for figure using from the Springer Nature Publishing Group (Supplementary material).

nanotechnology is a growing platform for cancer research, particularly CRC. Quantum dots are preferred for biomedical imaging due to their high quantum efficiency, photostability, extended excitation wavelengths and narrow emission band[54,55]. Various *in vitro* and *in vivo* studies have used quantum dots as fluorescent markers for cancer, with their *in vivo* use limited to nontargeted or xenograft labeling[56,57]. Vascular endothelial growth factor receptor 2 (VEGFR2) is upregulated in CRCs and QDot655 targeted to VEGFR2 (QD655-VEGFR2) showed its ability to detect VEGFR2-expressing tumors *in vivo*[58].

Poly (lactic-co-glycolic acid) NPs/nanocells

Amongst the natural and synthetic polymeric NPs, poly (lactic-co-glycolic acid) (PLGA) has been used for oral drug delivery applications approved by the United States FDA. PLGA NPs are physically and chemically stable, have higher stability in biological fluids and protect encapsulated drugs from enzymatic degradation. In addition, PLGA NPs can entrap small macromolecules, enhance thermal stability of the molecules and help in sustained release[59,60]. Due to its biodegradability and biocompatibility characteristics and its ability to encapsulate hydrophobic and hydrophilic drugs, PLGA is often used as a drug carrier[61,62]. PEGylated-PLGA nanocapsules loaded with docetaxel and SPIONs treated tumor growth in CT26 colon cancer[63]. Encapsulated 5-FU into PLGA NPs reduced the proliferation rate of the colon cancer cell line HT-29 by increasing the intracellular concentration of drugs in cancer cells[64]. EGF-functionalized PLGA NPs loaded with 5-FU and perfluorocarbons inhibited colon tumor growth[65]. The single-step surface-functionalizing technique was used to prepare PLGA/PLA-PEG-FA NPs to incorporate 17-AAG (NP-PEG-FA/17-AAG), which improved the oral bioavailability of 17-AAG and effectively treated ulcerative colitis and associated cancer[66]. 5-FU-loaded PHBV/PLGA NPs are a promising nanodrug delivery system for the treatment of colon cancer[67].

Dendrimers

Dendrimers are highly branched spherical molecules having three-dimensional chemical structures, being enormously useful in nanopharmaceuticals due to their biodegradable backbones. The versatility of dendrimers in the delivery of anticancer drugs and theranostic applications in cancer therapy has been well established. Anticancer conjugated dendrimers can deliver the drug intracellularly, bypassing the efflux transporter and improving the bioavailability of loaded molecular cargo. They are also used

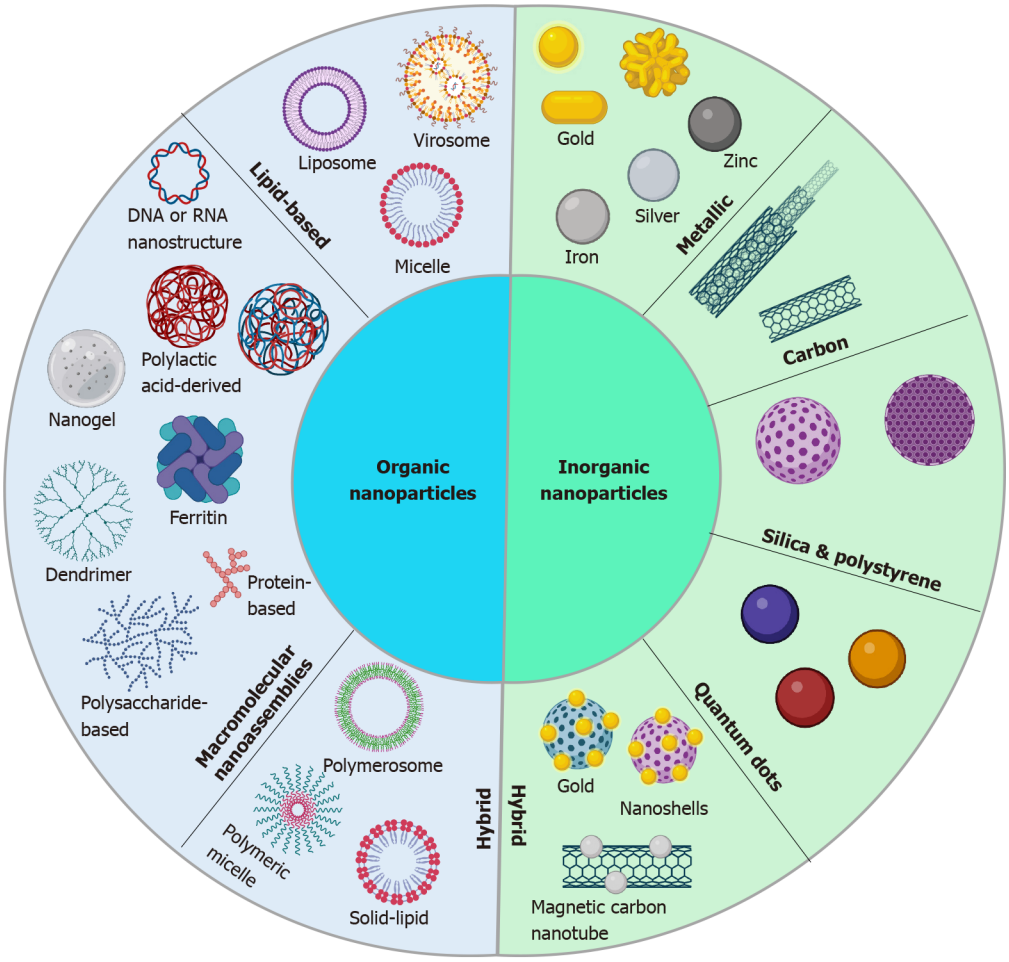


Figure 3 Inorganic and organic nanoparticles for cancer detection and therapy. The nanoparticles are divided into inorganic and organic nanoparticles. Inorganic nanoparticles, such as quantum dots, silica, carbon and metallic nanoparticles, are characterized by intrinsic electrical and optical properties suitable for cancer imaging, high stability and low biodegradability for theranostics. Organic nanoparticles, such as macromolecular assemblies or lipid-based nanoparticles, have lower stability but higher biocompatibility and multiple possibilities to add functional groups for drug delivery. Hybrid nanoparticles combine the qualities of both organic and inorganic nanoparticles to enhance their strength and biocompatibility. Citation: Briolay T, Petithomme T, Fouet M, Nguyen-Pham N, Blanquart C, Boisgerault N. Delivery of cancer therapies by synthetic and bio-inspired nanovectors. *Mol Cancer* 2021; 20: 55. Copyright© The Author(s) 2021. Published by Springer Nature. The authors have obtained the permission for figure using from the Springer Nature Publishing Group (Supplementary material).

for delivering diagnostic agents for tumor-targeted imaging[68,69].

The chemotherapeutic approach to treat CRC is not proven to be effective, as only a tiny fraction of the drug reaches the tumor target site at an effective concentration[70]. The CTCs are cancer cells that migrate and are primarily responsible for tumor metastasis. Given the importance of CTCs as an indicator of poor prognosis, several technologies (such as size-based filtration, microfluidics-based, *etc*) were utilized to isolate and capture CTCs from large populations of interfering cells but were not truly successful. Various methods were employed to detect colorectal CTC using dendrimers conjugated with antibodies, such as PAMAM dendrimers conjugated with Sialyl Lewis X antibodies to capture colon cancer HT29 cells[71].

Apart from diagnostic use, dendrimers are also reported for use in anticancer therapy *in vitro*. G4 PAMAM dendrimers conjugated with capecitabine reported decreased tumor size and reduced side effects of capecitabine[72]. Gold NPs inside PAMAM dendrimer conjugated with curcumin showed higher cellular uptake, internalization and cytotoxicity in C26 and HT29 colorectal cells[73]. Camptothecin-loaded PEGylated PAMAM dendrimer functionalized with AS1411 (anti-nucleolin aptamers) for site-specific targeting of CRC cells[74] and L-lysine dendrimers with polyoxazoline conjugated with SN-38 (the active metabolite of irinotecan) increased efficacy and it minimized adverse side effects[75]. PAMAM G4 dendrimers with oxaliplatin enhanced targeting efficacy towards folic acid receptor-expressing CRC cells *in vitro*[76]. Gemcitabine-loaded YIGSR-CMCht/PAMAM dendrimer NPs induced targeted mortality on HCT-116 cancer cells[48].

Carbon nanotubes

Carbon nanotubes (CNTs) are carbon nanomaterials with multifaceted roles in diagnosis, gene therapy, immunotherapy and a carrier in the drug delivery system. They have excellent optical properties,

thermal conductivity, chemical stability and functionalization. CNTs are tiny tubular carbon atoms ordered to form a honeycomb nanostructure with exceptional physicochemical properties[77]. Based on the sheets of carbon atom numbers, CNTs are divided into single-wall CNTs or multi-wall CNTs. Various researchers have reported the effectiveness of CNT in cancer treatment and diagnosis. CpG-conjugated CNTs elevate the CpG uptake in mouse colon cancer cells and activates nuclear transcription factor-kappa B signals. CpG-CNT conjugation successfully attenuated local xenograft tumor growth and liver metastasis[78]. Fluorescein functionalized single-walled CNT/II-NCC hybrids showed higher intrinsic activity against colon cancer cells (Caco-2) compared to the non-functionalized counterpart [79]. Single-wall CNT-conjugated antibody C225 binds to epidermal growth factor receptor-expressed CRC cells *via* receptor-mediated endocytosis[80]. Photodynamic therapy enhanced the ability to kill colon cancer cells by single-walled CNT nanobiocomposites[81].

Liposomes

Liposomes are artificial and non-toxic lipid-based vesicular carriers. Historically, liposomes were first introduced as nanocarriers in 1961 as an FDA-approved drug delivery system. It comprises a phospholipid bilayer structure with a small and spherical aqueous core[82]. The main characteristic and advantage of NPs is their smaller size, which favors the particle for effective and targeted delivery of the drug used to diagnose and treat the disease[83]. Moreover, these particles have minor side effects. Among several NPs, liposomes are primarily used for the delivery system for nucleic acids, proteins and peptides. Depending on the acting properties of liposomes, there are three types, as follows: (1) Stealth or long-circulating; (2) Active targeting; and (3) Thermo-sensitive/pH-sensitive/magnetic liposomes[84]. For example, doxorubicin (Doxil), DaunoXome and Marqibo® are FDA-approved liposomal drugs and Thermodox® is a thermo-sensitive liposome used to treat CRC[85].

Silica NPs

Silica NPs possess a beehive-like porous structure with adjustable sizes of cavities ranging from 50-300 nm and 2-6 nm[86]. The advantageous characteristics of silica NPs include a highly porous framework, less toxicity, biocompatibility, pH sensitivity and easy functionalization[87]. It is highly recommended for precise drug delivery for anticancer activity. For instance, a mesoporous silica NP-protamine hybrid system and conjugated hyaluronic acid to silica NPs load more medicine and increase the drug's efficacy in the tumor niche. For CRC, silica NPs, along with photosensitizer chemicals, are used. The photosensitizers become activated when exposed to light, releasing reactive oxygen molecules to kill the cancer cells[88].

Gold NPs

Gold NPs are considered the most stable among all noble NPs. They can be nanostructured in the form of cubes, spheres, rods, flowers, branches, wires, pyramids, shells and cages. With accurate surface coating, the gold NPs are safer, efficient for drug delivery and specific for cancer targeting[89]. Gold NPs enhanced cisplatin delivery efficacy and effectively decompressed CRC vessels[90]. Like nanoemulsion, gold NPs can also be conjugated with several molecules, such as antibodies, nucleic acids, proteins, enzymes and fluorescent dyes. These factors augment the properties of gold NPs, including stability, biocompatibility and functionalization in the medical field[91].

Nanoemulsion system

As the name suggests, a nanoemulsion system consists of oil, water and surfactant, formulating a transparent colloidal solution. It possesses the characteristics of less toxicity, high stability, thermo-sensitivity, pH-sensitivity and better efficacy[82]. Nowadays, antiangiogenic drugs have been used to target CRC cells. However, these agents cause toxicity and resistance and are affected by barriers. In the tumor niche, vascularized tissue is a barrier that nanoemulsions can easily overcome to target cancer cells[92]. Nanoemulsion systems are used to deliver water-insoluble drugs with a hydrophobic core. For effective drug delivery and treatment activity, nanoemulsions are conjugated with different antibodies for selective and specific targeting. Other than antibodies, nanoemulsions are also conjugated with polyethylene glycol. Polyelectrolyte complex micelles and DNA complexes enhance their ability as cancer therapeutics. Tween-80 is a widely accepted and used emulsifier[93].

Other NPs

In traditional cancer treatments, the therapeutic agents affect the immune system adversely, eliciting several side effects. Nanodrug delivery systems encapsulate the therapeutic agents, followed by targeted drug delivery in the tumor niche, which decreases the side effects. These systems not only lower the toxicity in the body but are highly stable, biocompatible and efficient [*e.g.*, polypeptide-based copolymers, poly(trimethylene carbonate)-block-poly(L-glutamic acid) derived polymersomes, plitidepsin-unloaded polymersomes, near-infrared fluorescent proteinoid-poly(L-lactic acid), P(EF-proteinoid-poly(L-lactic acid)] random copolymer, proteinoid-proteinoid-poly(L-lactic acid) copolymer, *etc*. Several copolymers are effective against CRC cells and do not elicit any side effects. Nanoformulation of curcumin in micelles, nanogels, liposomes, NPs and cyclodextrins has been documented for

treating CRC[94].

COMBINED NANOTECHNOLOGY-BASED APPROACHES FOR CRC DETECTION AND TREATMENT

With the advent of nanotechnology-based approaches, cancer detection and treatment strategies have changed in recent years. However, the clinical applications of nanotechnology-based formulations are limited due to their complex pharmacokinetics. The following are the combinatorial usage of nanotechnology-based approaches in the theranostics of CRC.

Enhancement of imaging techniques

Nanotechnology-based contrasting agents are used to enhance the capability of cancer diagnostic imaging modalities such as positron emission tomography (PET), MRI and other optical imaging techniques. The primary aim of this new technology is to detect the smallest possible number of cancer cells. PET is a powerful tool in diagnosing cancer due to its high sensitivity. A variety of relevant biomarkers are used for molecular and metabolic imaging with PET, such as fluorodeoxyglucose, 9mTc-labeled polyethylene glycol, [18F] DCFPyL, *etc*. Recently, NP-based contrasting agents including 56Fe and 14C conjugated with SPIONs, 99mTc-radiolabeled nanosilica system conjugated with a trastuzumab half-chain and MUC1 aptamer conjugated mesoporous silica NP have been used as radiolabeled tracers for PET. PET scan does not provide anatomical information; thus, other modalities such as computed tomography or MRI are used or have been merged with PET. In the case of MRI, SPIONs, gadolinium-encapsulated silicon microparticles and gadolinium-ion-doped upconversion NPs are mainly used as contrast agents. At the same time, 2-deoxy-d-glucose labeled gold NPs and iodinated gold nanoclusters (AuNCs-BSA-I) are used in computed tomography. The advent of nanotechnology has made advancements in the imaging modalities for early detection of cancer and thus timely treatment for cancer[95].

Combined drug delivery

The primary functional role of nanotechnology in the cancer field is drug delivery. The issues of multidrug resistance, stability, efficacy and biocompatibility have been improved by formulating the drug delivery system using NPs. Traditionally, chemotherapy and other therapies have significant side effects, which are also reduced with the implementation of the nanotechnology-based formulation. For instance, 5-FU nanoencapsulation, forming a combinatorial nanomedicine agent with thiolated chitosan, is non-toxic and has enhanced chemotherapeutic efficacy in CRC patients. The use of nanomedicine has also led to a reduction in the dose quantity. In conventional therapy of 5-FU, the dose was much higher and toxic compared to nanoencapsulation of 5-FU[96].

CONCLUSION

There has been significant advancement in the field of nanomedicine over the last decade. Nanotechnology is gaining immense importance because of its highly efficient delivery system. The enormous use of nanomaterials for disease diagnosis and treatment is on-trend currently. The NP-based approaches for colon cancer diagnosis are summarized[97] in Figure 4. The combined therapy with diagnosis, *i.e.*, theranostic application of nanomaterials, makes treatment easier and faster. CRC is mainly associated with lifestyle, sex and race, suggesting that certain groups are highly vulnerable. Thus, regular screening of CRC seems to be highly recommended to prevent the occurrence of CRC. Early diagnosis of CRC increases the chances of curing cancer and survival. Theranostic application of nanomaterials against CRC is still in development. Several researchers have already reported the successful treatment of CRC *in vitro* in cancer cell lines and *in vivo* in various CRC model animals. However, new strategies are required to improve the pre-existing therapeutics and hopefully novel therapeutics using nanomaterials to treat CRC will be available soon for clinical application.

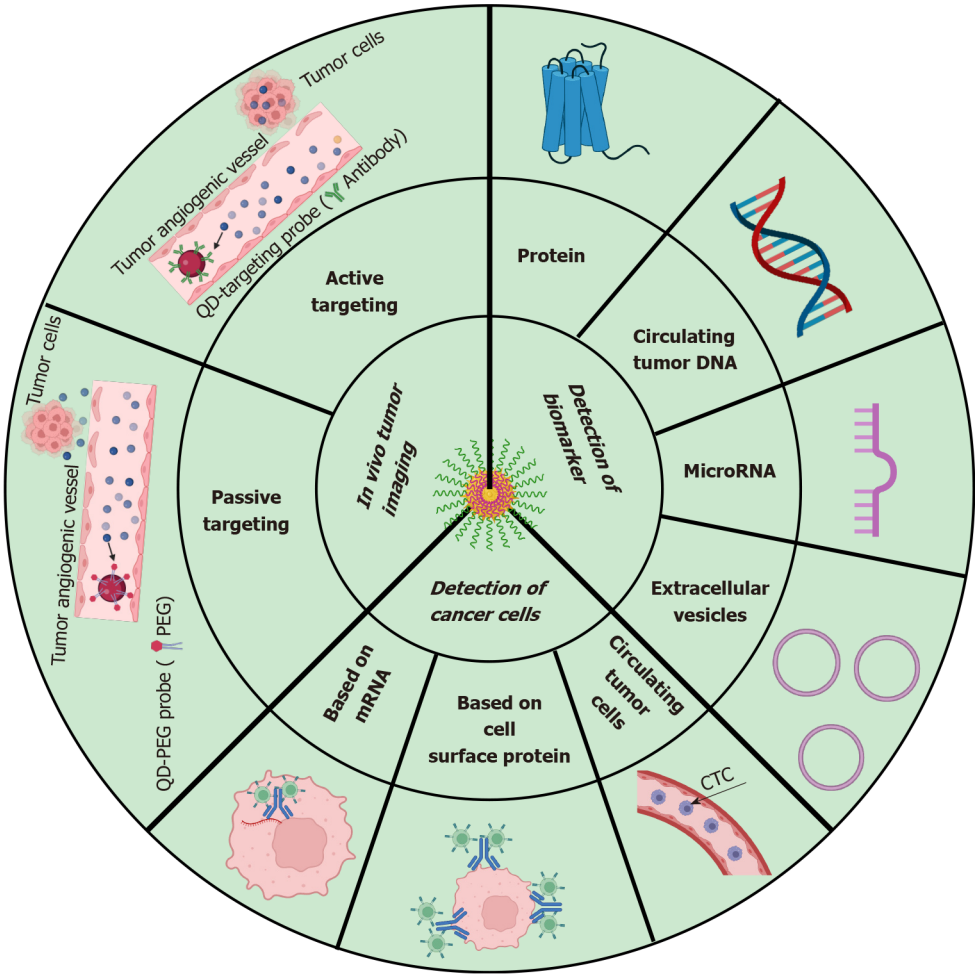


Figure 4 Schematic diagram representing the nanotechnology-based applications in colon cancer diagnosis. CTC: Circulating tumor cell; PEG: Polyethylene glycol; QD-PEG: Quantum dots-polyethylene glycol. Citation: Zhang Y, Li M, Gao X, Chen Y, Liu T. Nanotechnology in cancer diagnosis: progress, challenges and opportunities. *J Hematol Oncol* 2019; 12: 137. Copyright© The Author(s) 2019. Published by Springer Nature. The authors have obtained the permission for figure using from the Springer Nature Publishing Group (Supplementary material).

FOOTNOTES

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MINIREVIEWS

Factors other than fibrosis that increase measured shear wave velocity

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Abstract

Shear wave elastography (SWE) is now becoming an indispensable diagnostic tool in the routine examination of liver diseases. In particular, accuracy is required for shear wave propagation velocity measurement, which is directly related to diagnostic accuracy. It is generally accepted that the liver shear wave propagation velocity reflects the degree of fibrosis, but there are still few reports on other factors that increase the shear wave propagation velocity. In this study, we reviewed such factors in the literature and examined their mechanisms. Current SWE measures propagation velocity based on the assumption that the medium has a homogeneous structure, uniform density, and is purely elastic. Otherwise, the measurement is subject to error. The other (confounding) factors that we routinely experience are primarily: (1) Conditions that appear to increase the viscous component; and (2) Conditions that appear to increase tissue density. Clinically, the former includes acute hepatitis, congested liver, biliary obstruction, *etc*, and the latter includes diffuse infiltration of malignant cells, various storage diseases, tissue necrosis, *etc*. In any case, it is important to evaluate SWE in the context of the entire clinical picture.

Key Words: Liver; Shear wave elastography; Propagation velocity; Viscoelasticity; Artifact; Ultrasound

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Core Tip: Shear wave elastography (SWE) has become an indispensable diagnostic tool for diagnosing liver disease patients. The shear wave propagation velocity usually reflects the degree of fibrosis, but we must keep in mind other (confounding) factors. The confounding factors due to viscosity include acute hepatitis, congestive liver, and biliary stasis. The other confounding factors due to an increase of tissue density include diffuse infiltration of malignant cells, various storage diseases, and tissue necrosis. It is important to judge SWE results in the context of the entire clinical picture.

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INTRODUCTION

Percussion examination has been historically performed as part of physical examinations and is clinically important[1]. It is a method of investigating the mechanical characteristics of internal organs through the changes in reflected sound during percussion to recognize how internal organs respond to vibrations. This phenomenon indicates that the sound of reflection is related to tissue stiffness, and it provides useful diagnostic information of tissues. Elastography (stiffness measurement technique) scientifically confirmed this subjective experience-based phenomenon. In recent years, various ultrasound (US) and magnetic resonance elastography techniques for evaluating tissue elasticity have been developed and applied in the clinical setting[2,3]. The basic principle of these techniques is to use external vibration (or focused US) to create shear waves (SWs) in the tissue and to measure the SW propagation velocity as a function of time and distance[4,5]. Many algorithms then calculate the SW propagation velocity, based on the assumption that the tissue is unstressed, homogeneous, isotropic, and purely elastic. Under these conditions, the SW velocity is directly related to the elastic modulus of the tissue[6,7].

Presently, magnetic resonance elastography[2,3] and US elastography[8,9] are primarily used. The former is used for detailed examination due to its excellent stability and reproducibility[2,3]. However, it is bulky and costly. US elastography has attained preferential use in the clinical setting because of its lower cost and easier manipulability of the instrument[10,11].

Currently, there are three kinds of US-based SW elastography (SWE), namely transient elastography [12], point SWE[13], and two-dimensional SWE (2DSWE)[14,15]. Among these methods, 2DSWE has emerged as the most frequently used diagnostic technique due to its ability to sample a large area in the liver, change the sampling area quickly under B-mode observation, and display color mapping of SW values over the B-mode image, which gives the operator a sense of security. Recently, various 2DSWE devices have been developed by a number of US companies[16,17], and there have been many studies on the relationship between 2DSWE and liver histology that have shown a high degree of agreement, with the area under the receiver operating characteristic curve of 2DSWE performance of more than 0.9 in the prediction of fibrosis staging[18,19]. This indicates that 2DSWE can be a useful and accurate tool for evaluating liver stiffness. The precise definition of "increased SW propagation velocity" is not strictly determined, but the reported optimal cutoff values to differentiate liver cirrhosis are approximately 9 kPa (1.7 m/s) in most cases[20]. Thus, measurements above this threshold are considered increased values.

There are two ways to quantify relative tissue stiffness as SW propagation velocity expressed as m/s and as kPa. The SW propagation velocity is converted automatically to kPa, using the equation 1 kPa = 3 × P × (SW propagation velocity)^2, with the assumption that the examined tissue is always homogeneous, and P (tissue density) is defined as 1.00 kg/m^3.

THEORETICAL BASIS OF 2DSWE

Existing studies regarding 2DSWE of the liver included various manufacturers and models[21]. A detailed explanation of the complex working principles of recent SWE devices in each company is beyond the scope of this review. Despite variations in employed algorithm, the fundamental operating principles of these devices are similar[21].

The push-pulse produces small tissue movements in the push-pulse plane. These tissue movements produce SWs that propagate and produce minimal tissue movements in the horizontal plane of the push-pulse. The tissue movements in the horizontal plane are called "SWs" and further propagate through the tissue in a sideway direction, away from the push-pulse. The SW movements are tracked by the regular-interval tracking conventional US pulses, which are used to measure the arrival time of SWs

(Figure 1). The simple formula to determine the SW velocity is arrival time of SW/distance from the push-pulse. This measurement is possible because the SW propagation velocity is very low (1-10 m/s) compared to the velocity of US pulses (1540 m/s)[22].

MEASUREMENT OF SW PROPAGATION VELOCITY IN LIVER TISSUE

For SW propagation velocity measurements, assumptions are made that the tissue is homogeneous, perfectly elastic (no viscous component), and the SW propagates in a straight direction[23,24]. The normal liver is the closest to this condition of all organs, and it is not surprising that a large number of papers have been focused on liver disease[9]. Generally speaking, the factors that increase the SW propagation velocity include an increase in cell density and heterogeneous tissue structure[23,24].

Reports on fiber components and SW propagation in muscles have observed that the SW propagation velocity increases when SWs propagate along the fiber running in the muscle[25,26]. This is an important factor in the increase in SW propagation velocity due to SW propagation of fibrosis in the liver tissue.

OTHER FACTORS LEADING TO INCREASED HEPATIC SW PROPAGATION VELOCITY

In SWE, tissue stiffness is estimated by measuring the SW propagation velocity[23,27]. Fibrotic tissues are typically stiffer than normal tissues. Therefore, the SW propagation velocity in fibrotic tissue is naturally faster than in normal tissue. Presently, there are many successful demonstrations of SWE in assessing the severity of liver fibrosis showing a significantly high correlation between the progress of fibrosis and increase in SW propagation velocity in patients with chronic viral hepatitis[9]. Although there are a relatively small number of publications, increased hepatic SW propagation velocity has been reported due to factors other than fibrosis, including acute, hepatitis[28], cholestasis[29,30], hepatic congestion[31], storage disease[32], hepatic necrosis[33], and diffuse infiltration of malignant tumors [34].

As mentioned in the previous section, the current SW propagation velocity measurement is performed on the assumption that the tissue to be measured is a perfect elastic body. However, the liver is indeed "viscoelastic," and current SWE measures elastic components and viscous components without distinction. This is thought to be the main cause of increased SW propagation velocity seen in acute hepatitis, hepatic congestion, and biliary congestion[28,30,31]. On the other hand, increased SW propagation velocity in storage disease, necrosis (Figure 2), or diffuse infiltration of malignant tumors are related to increased tissue density[34].

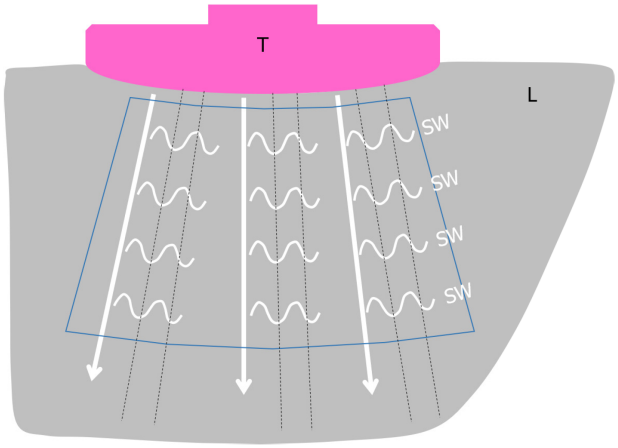
INCREASED HEPATIC SW PROPAGATION VELOCITY IN CHOLANGITIS AND SIMILAR PATHOLOGIC CONDITIONS

The main target of current hepatic SWE is various chronic hepatic diseases, particularly viral hepatitis [9]. Presence of parenchymal fibrosis is considered to be the main cause of increased SW propagation velocity. However, it indicates that similar phenomena can be observed in other pathologies with increased fibrosis within the liver parenchyma.

In patients with chronic cholangitis, it has been reported that the SW propagation velocity in the peripheral liver tissue, in which the tubular structure is invisible on B-mode imaging, is increased[35, 36]. It is presumed that fibrotic peripheral bile ducts are distributed throughout the liver and form an environment similar to fibrosis of chronic hepatitis for SW propagation. Increased SW propagation velocity can be observed in patients with von Meyenburg complex (Figure 3). Von Meyenburg complex is a benign developmental ductal plate malformation affecting the small peripheral bile ducts[37]. In addition, cases of thickened-wall peripheral small vessels can increase SW propagation velocity in patients with hereditary hemorrhagic telangiectasia (also known as Rendu-Osler-Weber disease)[38] (Figure 4). Hepatic involvement in hereditary hemorrhagic telangiectasia is characterized by diffuse development of small vascular shunts in the hepatic periphery, which likely increases SW propagation velocity[38].

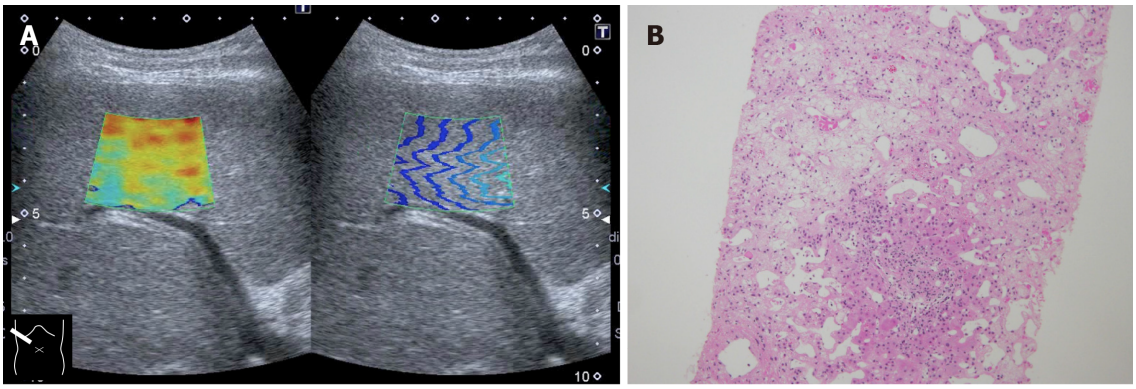
ARTIFACTUAL INCREASE IN SW PROPAGATION VELOCITY DUE TO EXAMINATION TECHNIQUES

Measurement of SW propagation velocity can be easily performed using 2DSWE, but it is well known that the results change according to the level of expertise of the ultrasound operator[39]. The literature



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Figure 1 Schematic illustration of two-dimensional shear wave elastography (commonly referred to as two-dimensional shear wave elastography) in a healthy subject. Multiple push-pulses (white arrow lines) radiate from the transducer to create shear waves (small wavy lines). The shear wave movements are tracked by the regular interval tracking conventional ultrasound pulses (black dotted lines). T: Transducer; L: Liver; SW: Shear wave. White arrow line: Push-pulse; Black dotted line: Tracking pulse; Blue line enclosure: Region of interest.



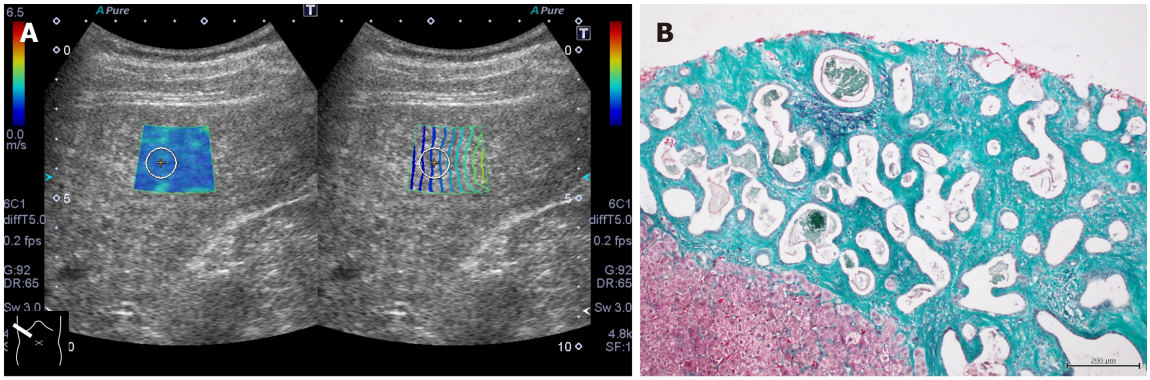
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Figure 2 Representative case of massive hepatic necrosis. A: Shear wave propagation velocity was 3.01 m/s (27.2 kPa); B: Biopsy specimen showed that hepatocytes in the central venous zone were completely lost due to necrosis and were replaced by reticular fibers. Hepatocytes remained in the portal vein area, which was one-half to one-third of the total, indicating sub extensive hepatic necrosis. Hematoxylin-eosin staining histologic finding (× 20).

includes reports of artifactual factors that give rise to pseudo-increased SW propagation velocity, including a reverberation artifact (Figure 5A), motion artifact due to cardiac motion (seen in the left lobe of the liver) (Figure 5B), and excessive probe compression during subcostal scanning (Figure 5C). However, the possibility of misinterpretation of these artifacts by an experienced SWE practitioner is extremely rare because the patterns produced by the artifacts are characteristic and recognizable.

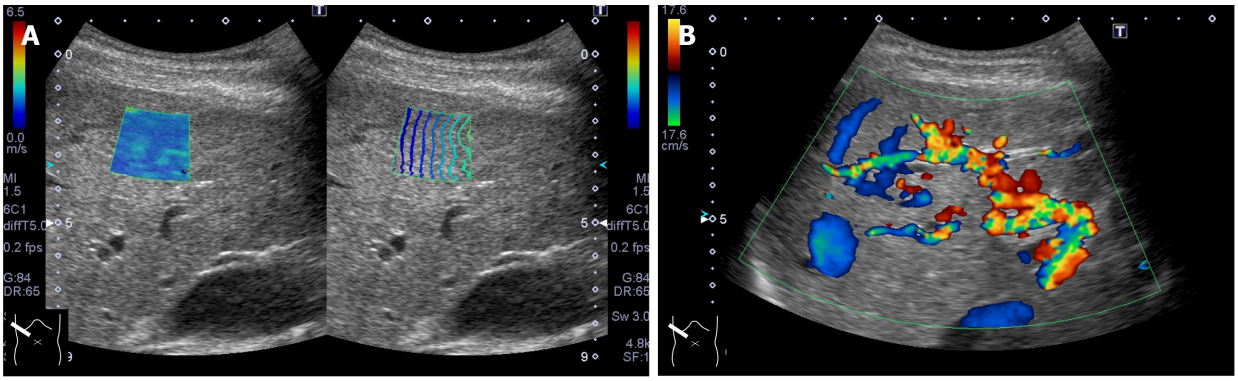
For example, there are some studies in the literature comparing SWE results of the left lobe and the right lobe of the liver[40]. However, current guidelines, including the World Federation for Ultrasound in Medicine and Biology and the European Federation for Ultrasound in Medicine and Biology, recommend SW measurement through the right intercostal space only because SW measurements obtained in the left lobe are usually affected by cardiac movement[8,41].

Recent clinical studies have used SWE to evaluate fatty liver (FL) stiffness. Unfortunately, there is a lack of understanding of the biomechanics of SWE to assess patients with FL due to the complexity of the structure of FL. Unlike the normal liver whose structure may be modeled as isotropic, FL has a heterogeneous structure. The complex structure and mechanical properties present a challenge in obtaining accurate results of SW propagation velocity[42]. It is not so rare to encounter artifactually (pseudo) increased SW propagation velocity when observing FL (Figure 5D). The various factors that affect propagation of SW waves in FL need to be properly understood before SWE can be used for accurately assess the severity of FL.



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Figure 3 Representative case of von Meyenburg complex. A: The liver showed a coarse parenchymal texture on ultrasound, and its shear wave propagation velocity was 1.86 m/s (10.3 kPa); B: Ultrasound-guided liver biopsy revealed bile duct proliferation with irregularly dilated small nuclei at the margins and fibrous stroma around the ducts. The fibrous stroma stained green on elastic Masson staining and was confirmed to be vitreous, confirming the diagnosis of von Meyenburg complex (ElasticaMasson staining, × 20).



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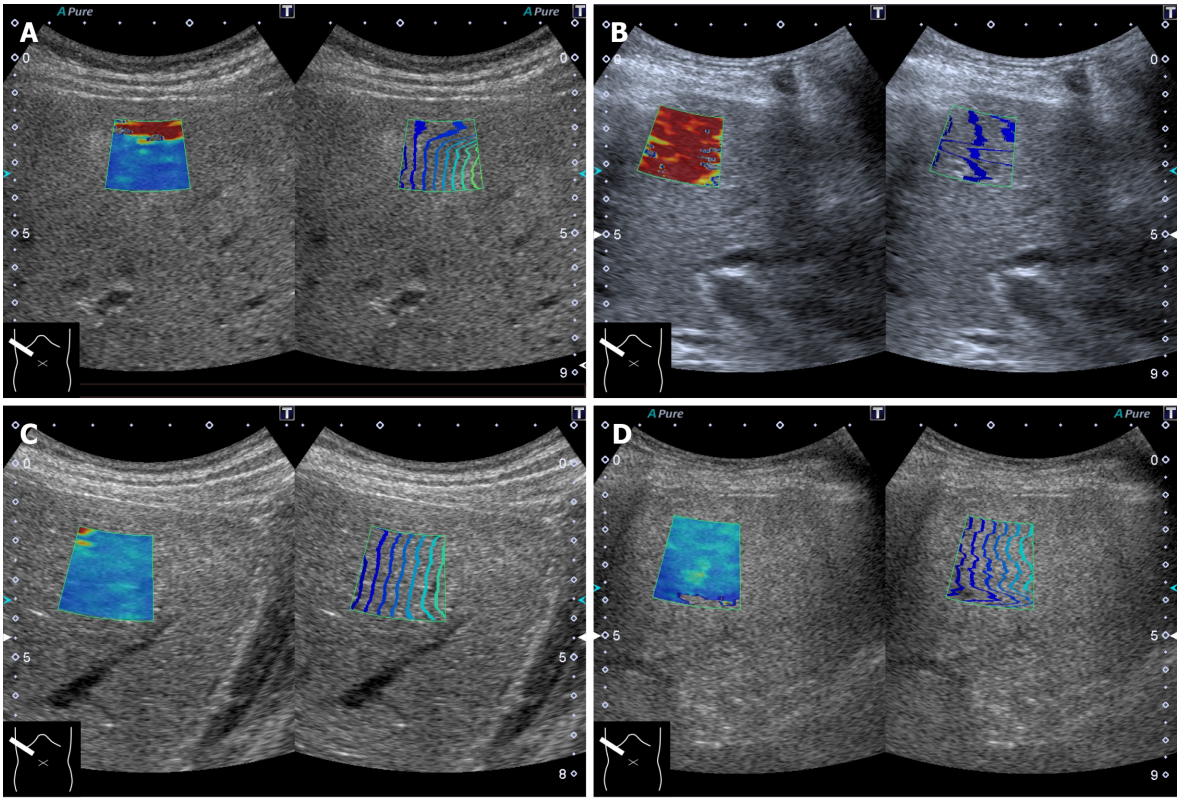
Figure 4 Representative case of hereditary hemorrhagic telangiectasia. A: The liver included many vascular shunts, and intrahepatic vessels were dilatated; B: The hepatic shear wave propagation velocity was 1.88 m/s (10.6 kPa).

OTHER REPORTED PHYSIOLOGICAL FACTORS THAT INCREASE SW PROPAGATION VELOCITY

Although the precise mechanisms are undetermined, studies have shown that a reduction in blood flow typically results in a decrease in SW propagation velocity in the liver[43]. In contrast, SW propagation velocity tends to increase with increasing organ perfusion. Other reported factors that change SW propagation velocity include water intake[44], breathing phases[45], eccentric exercise, patient positioning[46], and high calorie meal intake[47]. These factors can potentially contribute to minimal fluctuations in SW measurements, and care should be taken to account for these fluctuations to reduce the degree of uncertainty.

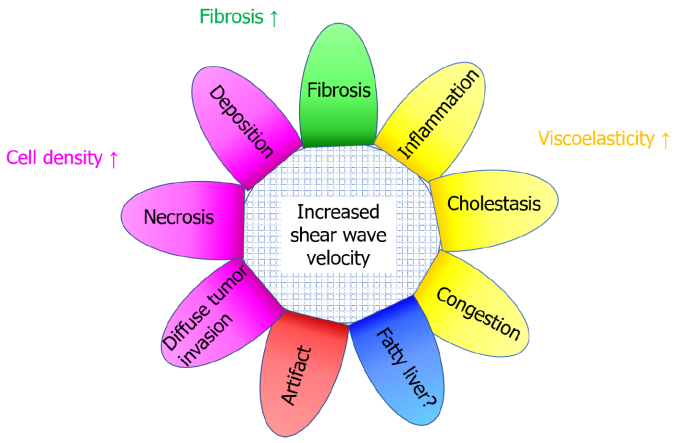
DISCUSSION

Presently, SWE is routinely used for providing a quantitative evaluation of liver elasticity[7]. The SW propagation velocity measured is reflective of the biomechanical properties of liver tissue where a higher value usually reflects stiffer (*i.e.*, fibrotic) parenchyma[8,9]. SWE algorithms assume that the liver is purely elastic and homogeneously structured. In most cases these assumptions are true. However, other factors may increase SW propagation velocity. They include acute hepatitis, cholestasis, hepatic congestion, storage disease, hepatic necrosis, and diffuse infiltration of malignant cells and others (Figure 6). Among them, the problem of viscosity (related to acute hepatitis, cholestasis, and hepatic congestion) is the most important because most commercial SWE systems assume soft tissues to be purely elastic and neglect the effects of viscosity when evaluating liver tissue stiffness.



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Figure 5 Artifactual pseudo-increase of shear wave propagation velocity. A: Reverberation of push-pulse; B: Motion artifact; C: Excessive probe compression (1.86 m/s, 10.3 kPa); D: Shear wave elastography image of fatty liver (2.10 m/s, 13.2 kPa).



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Figure 6 Summary of confounding factors that increase shear wave propagation velocity.

However, the liver tissue is a viscoelastic environment that results in a counterbalance of liver tissue stiffening[48,49]. When the liver tissue is modeled as a viscoelastic material, the SWs experience frequency dispersion. The SW propagation velocity and SW attenuation increases with increasing frequency rather than remaining constant, and the rate of change (slope) is positively correlated with viscosity[50-52]. As a result, modeling a viscoelastic material as linear elastic tends to overestimate the SW propagation velocity. When the effects of viscosity are considered, the scope of the diagnostic technique can be further broadened, and SW dispersion provides an estimation of liver viscosity, which may provide additional information of the underlying liver parenchyma.

Recent studies have reported that viscosity alone can serve as a parameter to diagnose several medical conditions such as inflammation and congestion[48,49]. Although only one company has developed this type of SW dispersion device, it is expected to be an area of great potential for further development. An increase in tissue density is another confounding factor that often poses a challenge in

obtaining reliable and accurate SWE readings. However, many problems remain that must be clarified in future studies.

Currently, automated diagnostics incorporating artificial intelligence are spreading rapidly worldwide[53-55]. SWE is a likely area in which artificial intelligence could be easily utilized. The adoption of artificial intelligence for SWE measurements is expected to correct slight variances in reference values (normal range) among manufacturers and enable prediction of liver fibrosis with higher probability. Artificial intelligence may also be able to detect when the probability of contamination by a confounding factor is high.

CONCLUSION

Although many confounding factors are recognized, our review emphasized that the interpretation of 2DSWE results must incorporate knowledge of these factors. As the use of 2DSWE becomes widespread, the problems related to confounding factors need clarification and solutions.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

3,6-dichlorobenzo[b]thiophene-2-carboxylic acid alleviates ulcerative colitis by suppressing mammalian target of rapamycin complex 1 activation and regulating intestinal microbiota

Qiong-Zi He, Peng Wei, Jun-Zhi Zhang, Tong-Tong Liu, Kun-Qun Shi, Huan-Huan Liu, Jing-Wei Zhang, Shi-Jia Liu

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Abstract

BACKGROUND

3,6-dichlorobenzo[b]thiophene-2-carboxylic acid (BT2) is a benzothiophene carboxylate derivative that can suppress the catabolism of branched-chain amino acid (BCAA)-associated mammalian target of rapamycin complex 1 (mTORC1) activation. Previous studies have demonstrated the therapeutic effects of BT2 on arthritis, liver cancer, and kidney injury. However, the effects of BT2 on ulcerative colitis (UC) are unknown.

AIM

To investigate the anti-UC effects of BT2 and the underlying mechanism.

METHODS

Mouse UC models were created through the administration of 3.5% dextran sodium sulfate (DSS) for 7 d. The mice in the treated groups were administered salazosulfapyridine (300 mg/kg) or BT2 (20 mg/kg) orally from day 1 to day 7. At the end of the study, all of the mice were sacrificed, and colon tissues were removed for hematoxylin and eosin staining, immunoblot analyses, and immunohistochemical assays. Cytokine levels were measured by flow cytometry. The contents of BCAAs including valine, leucine, and isoleucine, in mouse serum were

detected by liquid chromatography-tandem mass spectrometry, and the abundance of intestinal flora was analyzed by 16S ribosomal DNA sequencing.

RESULTS

Our results revealed that BT2 significantly ameliorated the inflammatory symptoms and pathological damage induced by DSS in mice. BT2 also reduced the production of the proinflammatory cytokines interleukin 6 (IL-6), IL-9, and IL-2 and increased the anti-inflammatory cytokine IL-10 level. In addition, BT2 notably improved BCAA catabolism and suppressed mTORC1 activation and cyclooxygenase-2 expression in the colon tissues of UC mice. Furthermore, high-throughput sequencing revealed that BT2 restored the gut microbial abundance and diversity in mice with colitis. Compared with the DSS group, BT2 treatment increased the ratio of *Firmicutes* to *Bacteroidetes* and decreased the abundance of *Enterobacteriaceae* and *Escherichia-Shigella*.

CONCLUSION

Our results indicated that BT2 significantly ameliorated DSS-induced UC and that the latent mechanism involved the suppression of BCAA-associated mTORC1 activation and modulation of the intestinal flora.

Key Words: 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid; Ulcerative colitis; Mechanistic target of rapamycin complex 1; Intestinal flora; Dextran sodium sulfate; Cyclooxygenase-2

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Core Tip: Gut microbiota dysbiosis and hyperactivated mammalian target of rapamycin complex 1 (mTORC1) make great contributions to the pathogenesis of ulcerative colitis (UC). In our study, 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid (BT2) exerted significant therapeutic effects on the amelioration of dextran sodium sulfate-induced acute colitis. Our work reported that BT2 played an anti-UC role through suppression of branched amino acid-associated mTORC1 activation and modulation of the intestinal flora. Overall, BT2 could represent a potential candidate drug in the design of treatment strategies for UC.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic idiopathic intestinal disease characterized by persistent mucosal inflammation extending from the proximal rectum[1]. Due to colonic mucosal damage and intestinal inflammation, UC patients generally experience abdominal pain, diarrhea, and bloody stools and are at a high risk for colorectal cancer[2,3]. UC is traditionally regarded as a disease of Western nations, but recent data have shown that the incidence of UC is increasing worldwide, including in Asia and the Middle East, which indicates its emergence as a global public health challenge[4,5]. Although the explicit etiology remains unclear, UC is primarily considered the result of gut microbiota dysbiosis, dysregulated immune responses, and disrupted intestinal epithelial function. The intestinal flora is involved in intestinal maturation, mucosal physiological functions, nutrient production, nutrient metabolism, and other processes. Patients with UC exhibit clearly disordered intestinal flora compared with healthy people[6-8], which reveals that gut flora imbalance plays an essential role in the pathogenesis of UC.

Mammalian target of rapamycin (mTOR), a crucial regulatory center of cell proliferation and growth, functions *via* two complexes called mTOR complex 1 (mTORC1) and mTOR complex 2. Previous studies have shown that mTORC1 makes a great contribution to the pathogenesis of UC and promotes the cyclooxygenase-2 (COX-2)-mediated inflammatory infiltration of T helper 17 cells to aggravate colitis[9-12]. A wide range of intracellular signals such as amino acids, growth factors, energy, and hypoxia activate the mTORC1 pathway. Notably, high levels of branched-chain amino acids (BCAAs) including valine, leucine and isoleucine can maintain the persistent activation of mTORC1, leading to a series of diseases such as liver cancer[13-15]. In general, the first step in BCAA catabolism is the branched-chain

aminotransferase (BCAT)-mediated transamination reaction, which generates branched-chain α-keto acids (BCKAs). The BCKAs are then oxidatively decarboxylated by the branched-chain α-keto acid dehydrogenase complex (BCKDC) to produce substrates for the tricarboxylic acid cycle[16]. 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid (BT2) is a potent inhibitor of branched-chain α-keto acid dehydrogenase kinase (BCKDK), which can specifically phosphorylate and inactivate BCKDC. BCKDC is highly dephosphorylated and activated in the heart, muscle, kidney, and liver of mice treated with BT2, which results in reductions in plasma BCAA levels[17]. Additionally, a previous study revealed that BT2 suppressed mTORC1 activation both *in vitro* and *in vivo* and caused no toxicity damage to mice following gavage administration[18,19]. However, the effects of BT2 on UC and its potential mechanism remain unknown.

In our study, we not only evaluated the anti-UC effect of BT2 but also emphasized its impact on the gut microbiota and the inhibition of BCAA-related mTORC1 activation in UC mice.

MATERIALS AND METHODS

Chemicals and reagents

Dextran sodium sulfate (DSS) was purchased from MP Biomedicals (Irvine, CA, United States), and salazosulfapyridine (SASP) was purchased from Xinyi Pharmaceutical Group (Shanghai, China). BT2 was obtained from Yuanye Biotechnology Co., Ltd. (Shanghai, China). The LEGENDplex™ Multi-Analyte Flow Assay Kit was supplied by BioLegend (San Diego, CA, United States). The BCA Protein Quantification Kit and Supper ECL Detection Reagent were purchased from Yeasen Biotechnology Co., Ltd. (Shanghai, China). The antibodies used for these experiments were as follows: Ribosomal protein S6 (S6) [1:1000, #2317; Cell Signaling Technology (CST), Danvers, MA, United States], phosphorylated $S6^{Ser235/236}$ (p-S6, 1:1000, 4858; CST), eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) (9644, 1:1000; CST), p-$4EBP1^{Thr37/46}$ (1:1000, 2855; CST), COX-2 (1:1000, 12282; CST), mTOR (1:1000, 66878-1-Ig; Proteintech, Rosemont, IL, United States), p-$mTOR^{Ser2448}$ (1:1000, 67778-1-Ig; Proteintech), branched-chain amino transferase 2 (BCAT2) (1:1000, 16417-1-AP; Proteintech), rabbit antibody (1:10000, SA00001-2; Proteintech), mouse antibody (1:10000, SA00001-1; Proteintech), BCKDK (1:500, 374424; Santa Cruz, Dallas, TX, United States), and branched-chain α–keto acid dehydrogenase (BCKDHA) (1:500, 271538; Santa Cruz).

Animals and ethics statement

Forty female C57BL/6 J mice (aged 7-8 wk; 18-20 g) were purchased from the Model Animal Research Center of Nanjing University, Nanjing, China (License No: 202124233). All experiments were performed in accordance with the regulations of the pharmacology laboratory and Animal Ethics Committee of the Affiliated Hospital of Nanjing University of Chinese Medicine (2022DW-16-02).

Induction of UC and experimental design

The animals were maintained five *per* cage and housed in a specific sterile environment with a temperature of 23 °C ± 2 °C, relative humidity of 50%, and 12-h light/dark cycle. After 2 wk of acclimatization, the mice were randomly divided into four groups: Control group, model group, SASP group, and BT2 group. A total of 10 mice were included in each group. The mice in the control group were given normal drinking water, and the mice in the other groups were given 3.5% DSS in drinking water to induce colitis. The mice in the BT2 group and SASP group were treated with 20 mg/kg BT2 and 300 mg/kg SASP, respectively, by oral gavage in a volume of 0.2 mL. The mice in the control group and DSS group were given 0.5% carboxymethylcellulose sodium. The daily body weight loss and stool characteristics were recorded, and the mice were sacrificed 7 d later. On the 8^{th} day, fecal samples and orbital blood samples were collected. The blood samples were clotted for at least 30 min and centrifuged for 20 min at 4 °C and 3000 r/min. Colon segments were extracted, and the lengths from the middle cecum to the end of the colon were measured. All abovementioned samples were stored at -80 °C until required for further investigation.

Multiple bead-based assay

The assay preparations were carried out according to the LEGENDplex™ Multi-Analyte Flow Assay Kit, and a flow cytometer (Beckman Coulter, Brea, CA, United States) was used for the detection of cytokine levels.

Assessment of the disease activity index score

The disease activity index (DAI) score comprehensively evaluates the severity of colonic inflammation from three aspects[20]: (1) Body weight loss, 1 point for every 5% decrease in the mouse body weight up to 4 points; (2) Stool consistency, the stool form was scored from 0 (normal) to 4 points (watery diarrhea); and (3) Hematochezia, scores of 0-4 points were given for negative hemoccult to gross bleeding.

Western blotting

Colon tissue samples were lysed with RIPA buffer (Solarbio, Beijing, China), protease inhibitor (Biosharp, Beijing, China), and phosphatase inhibitors (Biosharp). The protein concentration was detected using the BCA protein assay reagent, and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using 30 μg total protein samples at a constant voltage of 100 V. Separated proteins were blotted onto nitrocellulose membranes and then blocked in 5% skim milk for 1 h. The membranes were incubated overnight at 4 °C with primary antibodies diluted to appropriate concentrations and then incubated with secondary antibodies for 1 h at room temperature. The protein bands were visualized with enhanced chemiluminescence detection reagent and quantified using ImageJ software.

RNA extraction and quantitative polymerase chain reaction

Total RNA was extracted from colon tissues using the Total RNA Extraction Kit (Yeasen, Shanghai, China). The concentration and purity of the RNA samples were detected with an ultra-micro fluorescence UV spectrophotometer (DeNovix Inc., Wilmington, DE, United States). A kit (Yeasen) was used for the reverse transcription of total RNA into cDNA, and polymerase chain reaction (qPCR) was performed with a real-time fluorescence quantitative PCR detection system (Thermo Fisher Scientific, Waltham, MA, United States). The primers were as follows: β-actin forward, CTGTGCCCATCTACGAGGGCTAT and reverse, TTTGATGTCACGCACGATTTCC; COX-2 forward, GCGACATACTCAAGCAGGAGCA and reverse, AGTGGTAACCGCTCAGGTGTTG. All primers were provided by Tsingke Biotechnology Co., Ltd. (Beijing, China).

Immunohistochemical analysis

Colon tissue sections were dewaxed and dehydrated with different grades of alcohol and xylene. The tissue sections were placed in a repair box filled with citric acid (pH 6.0) antigen retrieval buffer for antigen retrieval in a microwave oven and then washed three times with phosphate-buffered saline (PBS). The sections were placed in 3% hydrogen peroxide to block endogenous peroxidase activity and then sealed with 3% bovine serum albumin for 30 min at room temperature. The sections were incubated with primary antibody overnight at 4 °C. The slices were washed three times with PBS, incubated with secondary antibody for 50 min, washed three times with PBS, and subjected to a diaminobenzidine color developing solution. The nuclei were counterstained with hematoxylin for 3-4 min. Images were collected by light microscopy and analyzed by expert pathologists. The immunohistochemistry score was calculated based on the proportion of cells with an unequivocal positive reaction (0: < 5%; 1: 5%-20%; 2: 21%-40%; 3: 41%-60%; 4: 61%-80%; and 5: > 80%).

Histopathological evaluation

Fresh colon tissues were fixed in fixing solution for more than 24 h and then embedded in paraffin blocks. Then the wax blocks were trimmed, cut into 4-μm-thick sections, and subjected to hematoxylin and eosin (H&E) staining. Histopathological changes were observed by a light microscope (Nikon, Tokyo, Japan). The histopathological score was divided into epithelial injury and inflammatory cell infiltration according to specific evaluation criteria described elsewhere[21].

16S ribosomal RNA sequencing of the gut microbiota

Total genomic DNA from fecal samples was extracted using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, United States) following the manufacturer's protocol. The DNA quality and concentration were detected using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The V3-V4 regions of bacterial 16S ribosomal DNA (rDNA) were amplified by PCR using the primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). The target bands were detected by 1% agarose gel electrophoresis, and the PCR products were purified by the magnetic bead method. Then microbial diversity sequencing library was established, and deep sequencing was performed with the Illumina MiSeq PE300 high-throughput sequencing platform at the Allwegene Company (Beijing, China). Pear (v0.9.6) software was used to filter and splice the data for the optimized sequence. Qualified reads were clustered into operational taxonomic units (OTUs) at a similarity level of 97% using the Uparse algorithm of Vsearch (v2.7.1) software. Taxonomic analysis at different levels (kingdom, phylum, class, order, family, genus, and species) was obtained using the Ribosomal Database Project classifier algorithm. QIIME1 (v1.8.0) software was used for α diversity index analysis (including the Shannon and Chao1 indexes), and nonmetric multidimensional scaling analysis (NMDS) was performed using the R package (v3.6.0).

Determination of BCAA concentrations

Serum amino acid concentrations were determined by liquid chromatography-tandem mass spectrometry with the Q-life Lab 9000 mass spectrometer at Shenzhen Institute of Life and Health Medicine Co., Ltd. (Jiangsu, China). Serum samples were loaded and separated online using the InfinityLab Poroshell 120 EC-C18 column (2.1 mm × 100 mm, 2.7 μm; Agilent, Santa Clara, CA, United States). The column temperature was set to 45 °C. The mobile phase consisted of 0.01% formic acid in

water (solvent A) and acetonitrile (solvent B). The flow rate was 0.3 mL/min, and the injection volume was 2 μL. Gradient elution was used as follows: 0-1.5 min, 8% B; 3.5 min, 16% B; 6.5 min, 20% B; 8.0 min, 95% B; and 8.01-11.0 min, 8% B. The electrospray ionization settings were as follows: Capillary voltage, 3.5 kV; spray voltage, 300 V; drying gas temperature, 250 °C; drying gas flow rate, 7 L/min; sheath gas flow rate, 11 L/min; and sheath gas temperature, 250 °C.

Statistical analyses

The experimental data are expressed as the mean ± SD, and one-way analysis of variance was used to evaluate the significance among multiple groups. $P < 0.05$ was considered statistically significant. Data analysis and picture drawing were performed using GraphPad Prism 7.0 software (GraphPad, San Diego, CA, United States).

RESULTS

BT2 ameliorated DSS-stimulated colitis

The effects of BT2 on DSS-induced UC mice were assessed based on the DAI score, body weight loss, and colon length. Oral 3.5% DSS treatment led to a significant reduction in body weight (Figure 1A), but the weight loss was reversed by BT2 and SASP treatment. The DAI score including weight loss rate, stool consistency and hematochezia was markedly increased in colitis mice, whereas the BT2- and SASP-treated groups showed improvements in this metric (Figure 1B). Then we examined the changes in the colon length. DSS treatment evidently shortened the colon length compared with that of the control group, whereas 7 d of BT2 and SASP administration extended the length (Figure 1C and D). These data showed that BT2 could improve the clinical symptoms of UC mice.

BT2 suppressed inflammation in UC mice

Representative H&E staining images and the pathological scores of colon tissues from each group are shown in Figure 1E and F. The mucosal epithelium of the mice in the control group was intact, and the morphology of the cells was normal and showed no inflammatory infiltration. The tissues of the mice in the DSS-treated group displayed evident epithelial damage and inflammatory infiltration. However, the histological injury score revealed that BT2 and SASP treatment reduced the severity of UC. Furthermore, the contents of the predominant proinflammatory cytokines including interleukin (IL-6), IL-2, and IL-9 were increased in the DSS group. By contrast, the level of an anti-inflammatory cytokine (IL-10) was decreased in the DSS group. By contrast, BT2 and SASP treatment clearly inhibited the increase in these proinflammatory cytokines and decreased the level of IL-10 (Figure 2). Together, these data revealed that BT2 could improve histopathological damage and reduce proinflammatory cytokine production in UC mice.

BT2 improved BCAA catabolism in UC mice

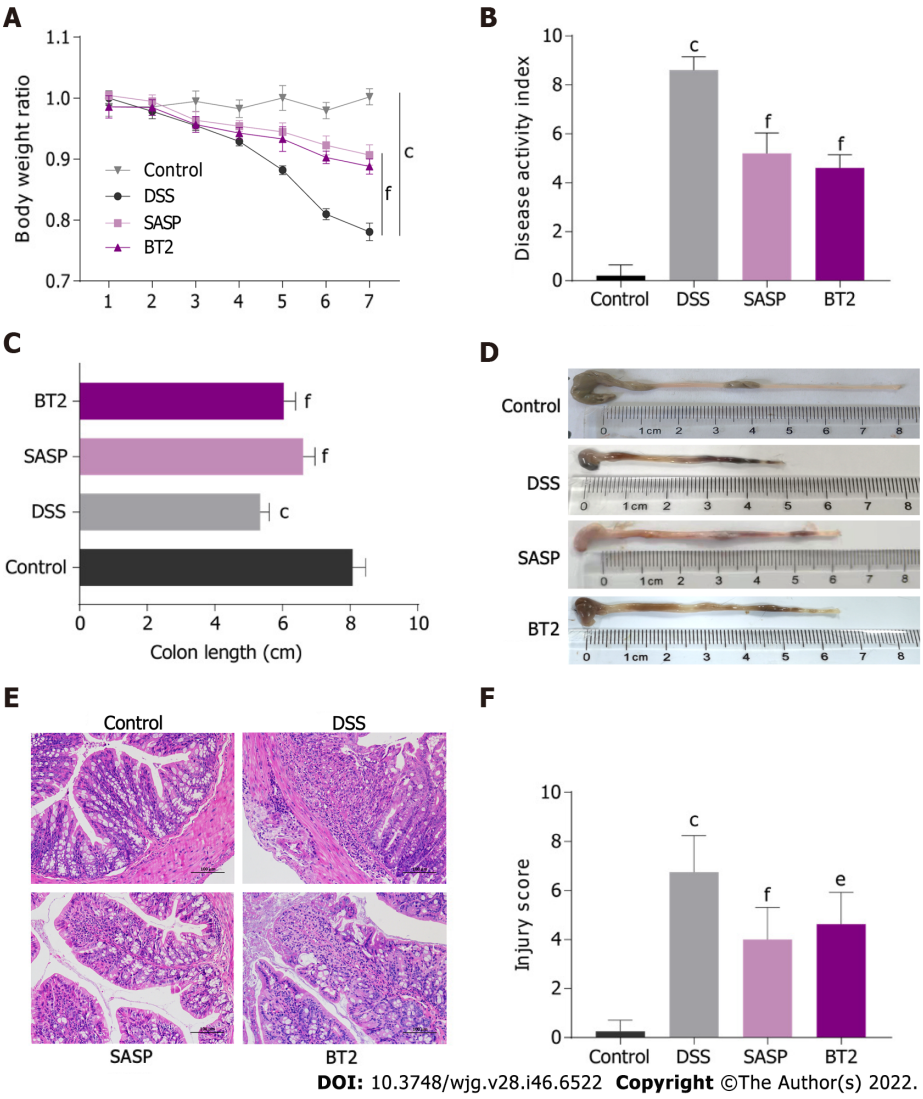
To verify the effects of BT2 on BCAA catabolism in DSS-induced UC mice, the expression levels of the catabolic enzymes BCKDHA, BCAT2, and BCKDK in colon tissues were detected by immunoblotting (Figure 3). The DSS group showed increased BCKDK expression and decreased expression of BCKDHA and BCAT2, but these changes were notably reversed by BT2 administration (Figure 3B-E). Consistently, immunohistochemistry analysis showed that BCKDK expression was increased in colon tissues with severe inflammation but reduced in BT2-treated mice (Figure 3A). Moreover, higher concentrations of leucine, isoleucine, and valine were observed in the serum of DSS-treated mice, whereas BT2 treatment resulted in downregulation of these amino acids (Figure 3F). In summary, BCAA catabolism was impaired in UC mice but rescued by BT2 treatment.

BT2 suppressed the mTORC1 signaling pathway in UC mice

Based on the beneficial effect of BT2 on BCAA metabolism, we hypothesized that BT2 could suppress BCAA-related mTORC1 activation in UC mice. In accordance with this assumption, the phosphorylation of mTOR and its characterized downstream substrates 4-EBP1 and S6 were explored by Western blot analysis. As shown in Figure 4A-D, the expression levels of these phosphorylated proteins were markedly elevated in the colon tissues from the DSS group but decreased in the colon tissues from the BT2 group. Additionally, immunohistochemical staining showed that BT2 treatment significantly reversed the upregulation of phosphorylated 4-EBP1 and p-S6 expression in colon tissues induced by DSS (Figure 4E). Collectively, these results suggested that BT2 could inhibit BCAA-related mTORC1 activation in the colon tissues of UC mice.

BT2 inhibited the expression of COX-2 in UC mice

The overexpression of COX-2 in experimental colitis has been widely reported; therefore, we investigated the effect of BT2 on COX-2 expression in colon tissues of UC mice. As shown in Figure 5A and B, COX-2 expression was markedly induced by DSS but reduced in the BT2 group. As depicted in



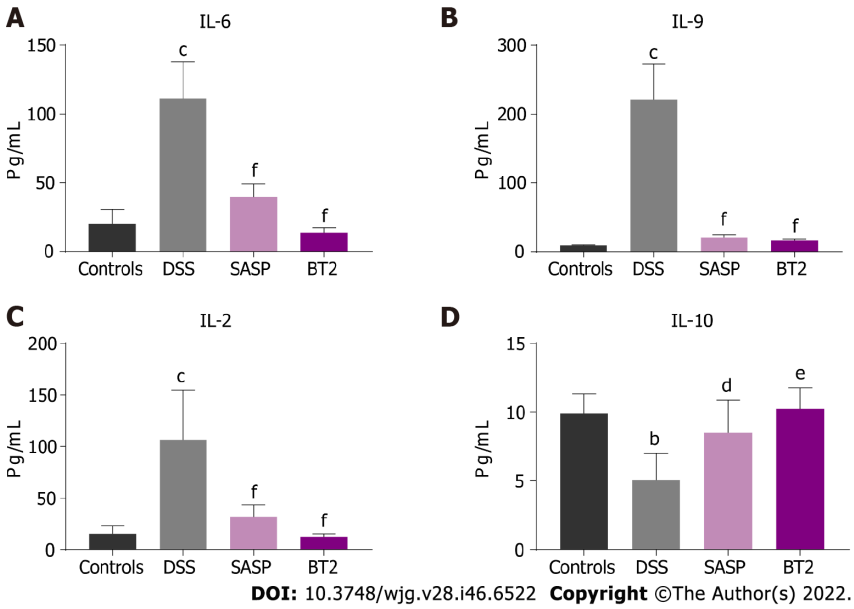
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Figure 1 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid improved colon inflammation in dextran sodium sulfate-treated mice. A: Changes in the body weight of mice; B: Disease activity index of each group of mice on day 8 (n = 5); C: The colon length of each group of mice was measured; D: Macroscopic observation of colons; E: Representative hematoxylin and eosin staining images of colon sections (200 × magnification); F: Injury scores. $^{e}P < 0.01$ and $^{f}P < 0.001$ *vs* the dextran sodium sulfate group; $^{c}P < 0.001$ *vs* the control group. The data are shown as the mean ± SD (n = 5-8). DSS: Dextran sodium sulfate; SASP: Salazosulfapyridine; BT2: 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid.

Figure 5C, mice in the DSS group showed higher mRNA levels of COX-2 but BT2 treatment reversed this effect.

BT2 restructured gut microbiota homeostasis in UC mice

The gut microbiota is involved in various physiological functions of the intestinal mucosa and is a new target for the prevention, diagnosis, and treatment of colonic inflammation. Therefore, 16S rDNA sequencing was performed to investigate whether BT2 regulates the composition of intestinal microflora in colitis mice (Figure 6). The rarefaction curve, which was used to estimate the sample size and the species richness, showed that the sequencing amount was reasonable (Figure 6D). NMDS analysis based on β diversity revealed that the flora structure was evidently different between the DSS group and the control group but that BT2 treatment improved this dissimilarity (Figure 6E). The Venn diagram was created to illustrate the number of common and unique OTUs among multiple samples. The overlapping part indicates the common OTUs, and the nonoverlapping areas represent the unique OTUs in each group. As shown in Figure 6A, 1443 OTUs were present in all groups, whereas 806, 319, and 363 OTUs were uniquely present in the control group, DSS group, and BT2 group, respectively. The α diversity index, including the Chao1 and Shannon indexes, showed that the gut microbiota diversity and richness were lower in colitis mice than in the control group. However, these alterations were normalized by BT2 treatment (Figure 6B and C).



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Figure 2 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid reduced the inflammatory cytokine levels in serum. The contents of interleukin (IL)-6, IL-9, IL-2 and IL-10 in serum were detected by flow cytometry. A: IL-6; B: IL-9; C: IL-2; D: IL-10. $^{d}P < 0.05$, $^{e}P < 0.01$, and $^{f}P < 0.001$ *vs* the dextran sodium sulfate group; $^{b}P < 0.01$ and $^{c}P < 0.001$ *vs* the control group. The data are shown as the mean ± SD ($n = 4$). DSS: Dextran sodium sulfate; SASP: Salazosulfapyridine; BT2: 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid.

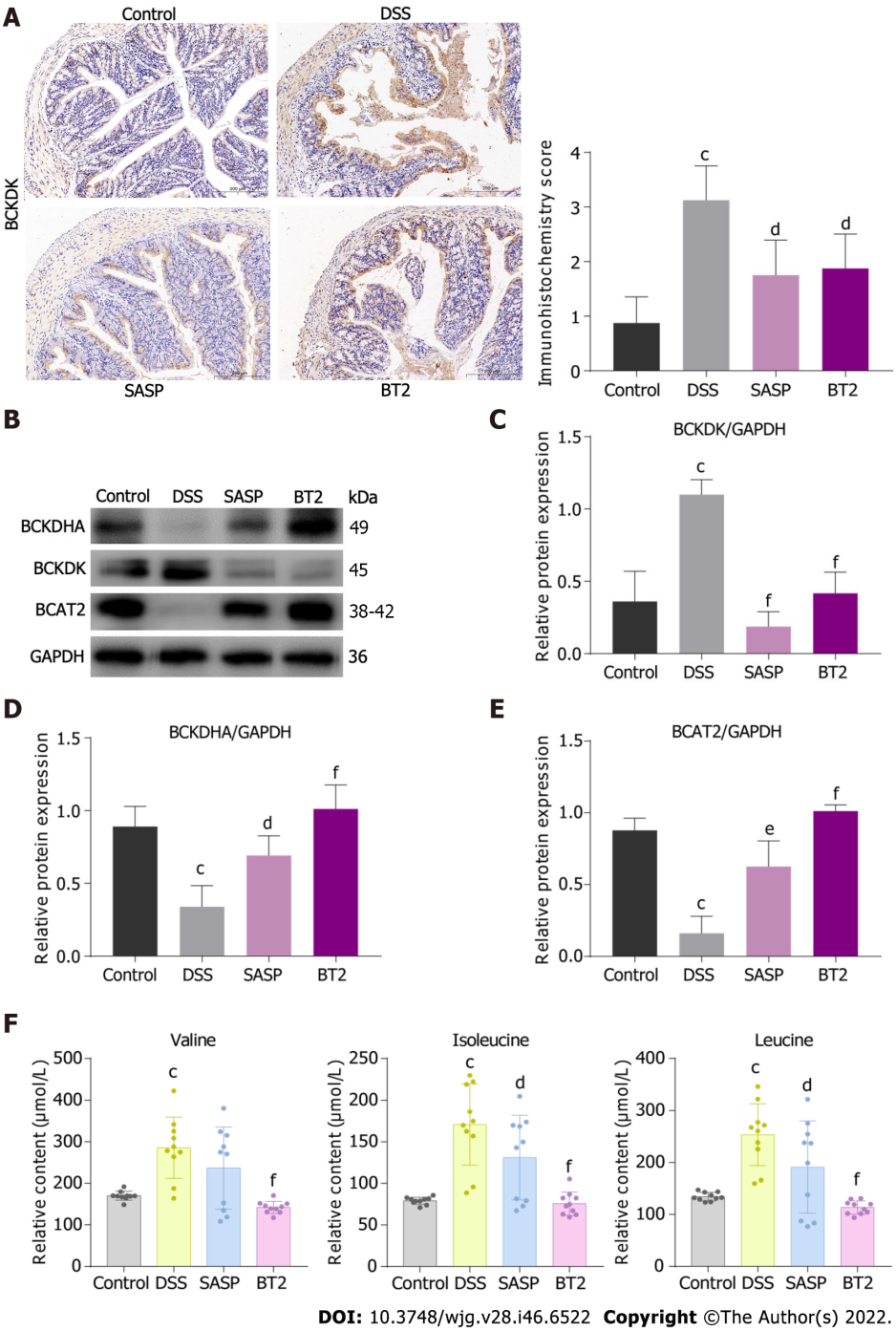
BT2 altered the gut microbiota composition and functionality in UC mice

As shown in Figure 7A, the intestinal flora mainly consisted of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* at the phylum level. The relative abundance levels of *Bacteroidetes* and *Proteobacteria* were increased in the DSS group, whereas the abundance of *Firmicutes* was reduced. However, BT2 intervention significantly restored the abundance of the three phyla. Moreover, the ratio of *Firmicutes* to *Bacteroidetes* (F/B) was decreased in the fecal samples from the DSS group relative to the control group, but this decrease was markedly reversed by BT2 (Figure 7B). At the family level, UC mice displayed evidently higher populations of *Clostridiaceae* and *Prevotellaceae*, which are related to the synthesis of BCAAs compared with normal mice. As expected, BT2 treatment notably increased the abundance of *Clostridiaceae* and *Prevotellaceae* (Figure 7C-E). Furthermore, the administration of BT2 prevented the increases in *Enterobacteriaceae* and *Escherichia-Shigella* caused by DSS and slightly restored the downregulation of *Oscillospiraceae* (Figure 7F-H).

DISCUSSION

UC has emerged as one of the most common chronic recurrent intestinal diseases in recent years and is treated with 5-aminosalicylic acid preparations, glucocorticoids, anti-tumor necrosis factor agents and thiopurines. These drugs can relieve colonic inflammation to a certain extent but are usually accompanied by adverse reactions, intolerance, and treatment limitations[22,23]. Therefore, research on new drug targets and therapeutics for UC has received increasing attention. A large number of studies have shown that disorder of the intestinal microbiota is crucial to the initiation and progression of UC. The metabolites produced by intestinal flora, such as BCAAs and short-chain fatty acids, can be absorbed by the host intestinal tract to affect its physiological and pathological functions, whereas an imbalance in the intestinal flora affects these functional metabolites[15]. Bosch *et al*[24] reported that the levels of leucine, isoleucine, and valine were significantly elevated in the feces of inflammatory bowel disease (IBD) patients. BT2 is a specific inhibitor of BCKDK that can improve BCAA catabolism and limit the tumor burden by inhibiting mTORC1 in mouse hepatocellular carcinoma models[18], but the impact of BT2 on UC has not been explored to date.

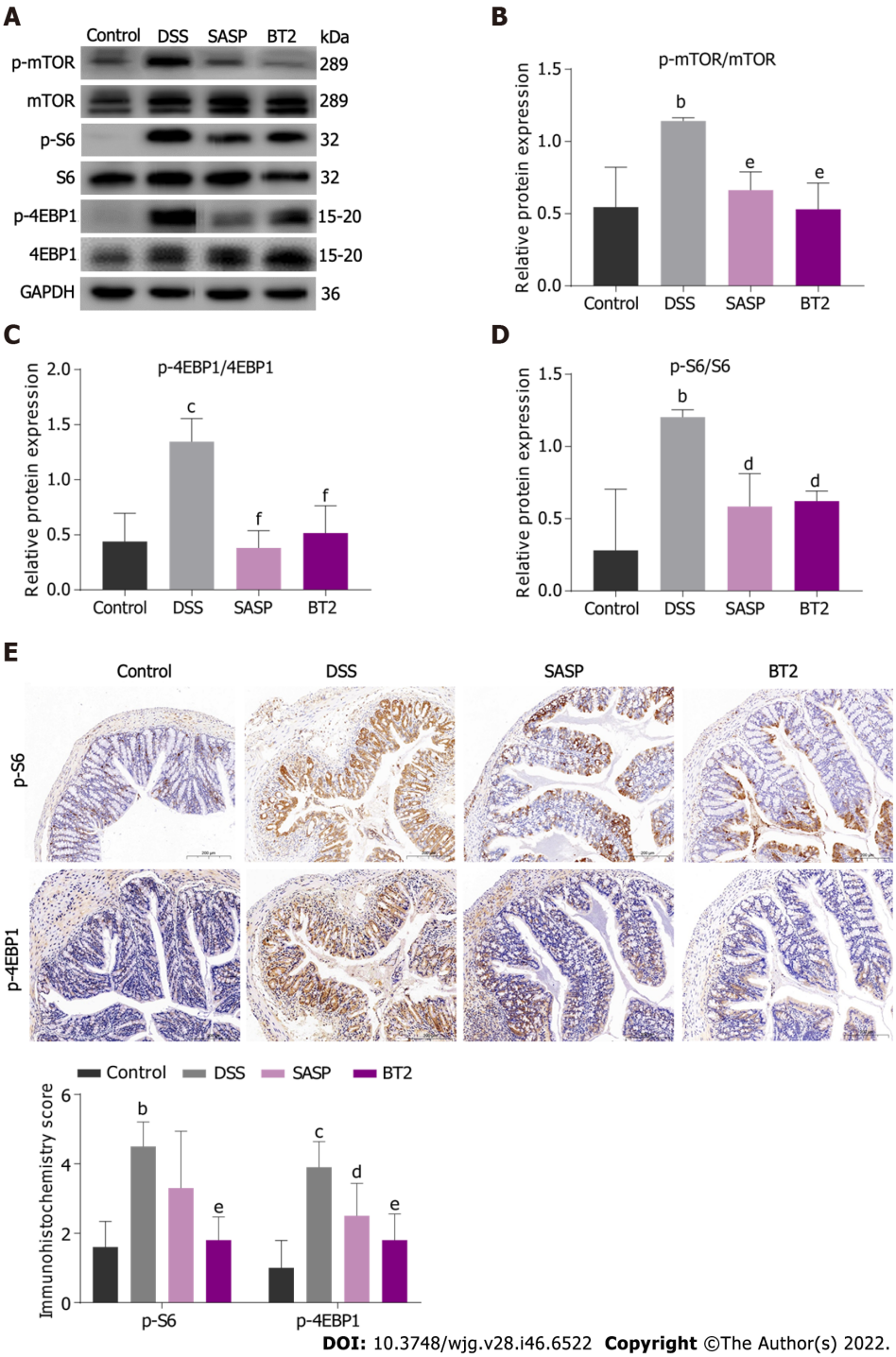
This study showed that BT2 exerted obvious therapeutic effects on DSS-induced UC. BT2 intervention led to a reduction in body weight loss; improved the pathological injury of colon tissues; decreased the content of proinflammatory cytokines including IL-6, IL-9 and IL-2; and decreased the level of IL-10 compared with the results obtained with DSS treatment. In particular, we found that BT2 could ameliorate the gut microbiota disruption caused by DSS. The diversity and richness of the fecal microbiota in the DSS group were decreased, but this downregulation was reversed in the BT2 group. At the phylum level, the fecal flora of mice was mainly composed of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. In addition, upregulation of the phylum *Proteobacteria* and downregulation of the F/B value in



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Figure 3 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid improved branched-chain amino acid catabolism in dextran sodium sulfate-induced colitis mice. A: The expression of branched-chain α-keto acid dehydrogenase kinase in colon sections was detected by immunohistochemical assays (n = 4, 200 × magnification); B-E: Western blot images and quantitative data of branched-chain amino acid (BCAA) catabolic enzymes in colon tissues (n = 3-4); F: The contents of BCAAs, including valine, leucine and isoleucine, in mouse serum were determined by liquid chromatography-tandem mass spectrometry (n = 10). ^{d}P < 0.05, ^{e}P < 0.01, and ^{f}P < 0.001 *vs* the dextran sodium sulfate group; ^{c}P < 0.001 *vs* the control group. The data are shown as the mean ± SD. BCKDK: Branched-chain α-keto acid dehydrogenase kinase; DSS: Dextran sodium sulfate; SASP: Salazosulfapyridine; BT2: 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid.

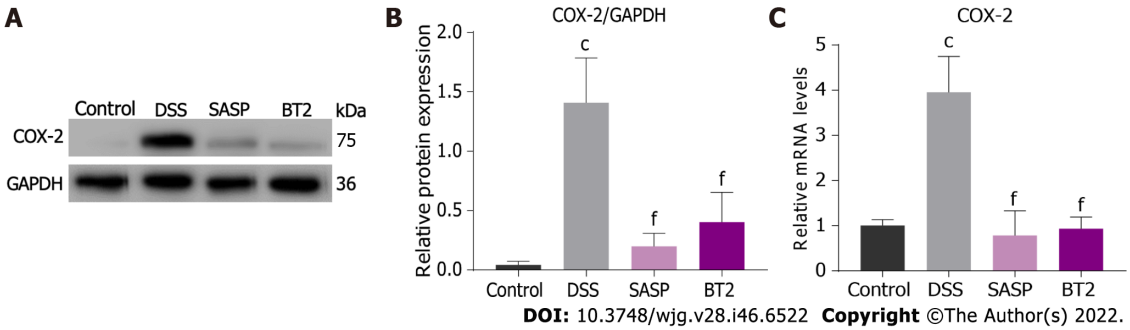
the DSS group were normalized by BT2 treatment. In accordance with previous reports, *Proteobacteria* represent the largest phylum in the bacterial domain and play a key role in driving proinflammatory changes and inducing IBD development due to their adhesiveness and invasiveness[25]. The F/B value has been used as an index for evaluating the disordered intestinal microbiome in UC and was significantly reduced in the fecal microbiota of IBD patients compared with controls[26,27]. At the family level, it is worth noting that two BCAA-producing bacteria, *Clostridiaceae* and *Prevotellaceae*, were increased in the DSS group but were downregulated in the BT2 group. Notably, *Prevotella copri* is a representative species that drives the synthesis of BCAAs, which are capable of inducing insulin resistance, aggravating glucose intolerance, and increasing the circulating level of BCAAs[28]. Intestinal microorganisms have great BCAA biosynthetic potential, and the increase in BCAA levels is related to



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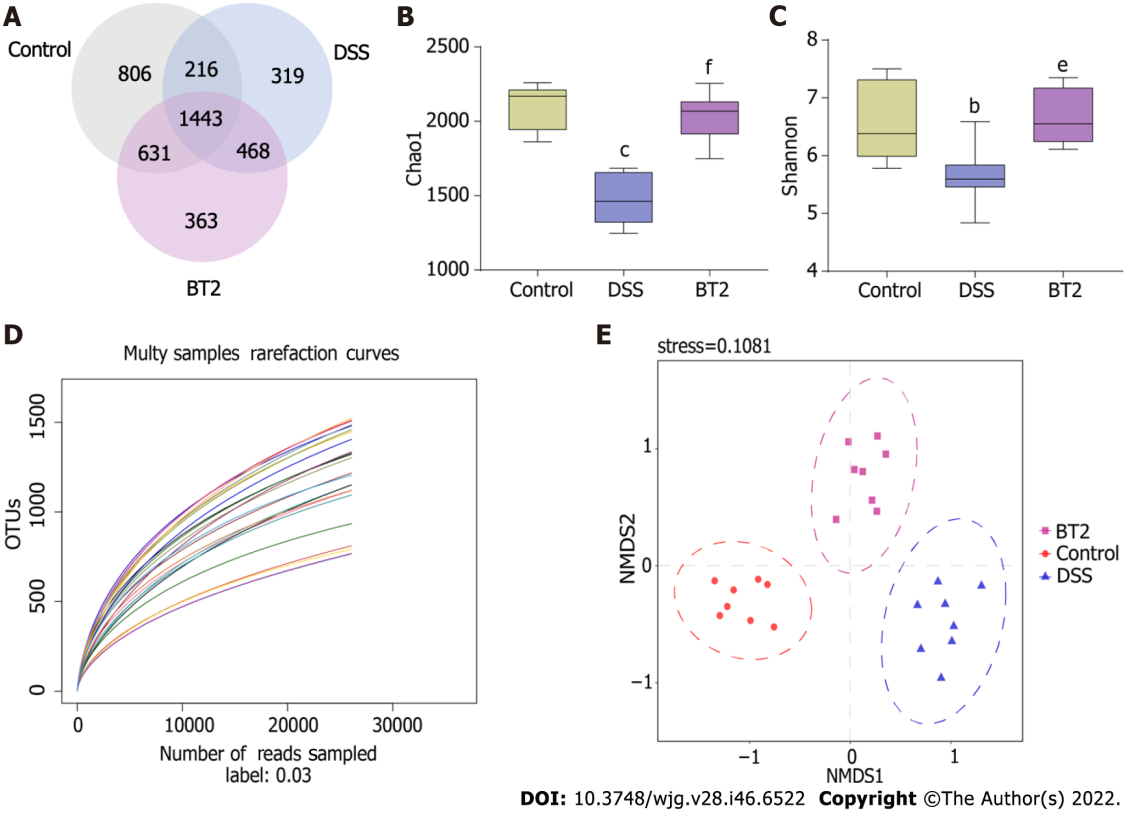
Figure 4 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid suppressed the activity of the mammalian target of rapamycin complex 1 activation. A-D: The total and phosphorylated proteins of S6, eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) and mammalian target of rapamycin were measured by Western blot analysis; E: Immunohistochemical analysis of the expression of phosphorylated S6 and phosphorylated 4EBP1 in colon tissues (200 × magnification). $^{d}P < 0.05$, $^{e}P < 0.01$, and $^{f}P < 0.001$ *vs* the dextran sodium sulfate group; $^{b}P < 0.01$ and $^{c}P < 0.001$ *vs* the control group. The data are shown as the mean ± SD (n = 3-5). mTOR: Mammalian target of rapamycin; S6: Ribosomal protein S6; 4EBP1: Eukaryotic translation initiation factor 4E binding protein 1; DSS: Dextran sodium sulfate; SASP: Salazosulfapyridine; BT2: 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid.

the change in bacteria involved in BCAA biosynthesis[28]. A previous study showed that the relative abundance of microbes associated with BCAA synthesis was increased in mice fed a high-fat diet including the order *Clostridiales*, family *Clostridiaceae*, and *Prevotellaceae*[29]. The abundance of *Clostridiaceae* is positively correlated with the concentration of serum BCAAs in mice[30]. We also found that the level of *Oscillospiraceae* was slightly increased in the BT2 group. *Oscillospiraceae* is widely present in the intestinal microorganisms of humans and animals and is associated with inflammatory diseases. A previous meta-analysis showed that *Oscillospiraceae* was significantly reduced in the intestines of patients with Crohn's disease but increased in those with gallstones[31]. Moreover, downregulation of



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Figure 5 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid reduced cyclooxygenase-2 expression. A and B: The level of cyclooxygenase-2 (COX-2) in colon tissues was detected by Western blotting; C: The mRNA level of COX-2 in colon tissues was detected by real-time polymerase chain reaction. $^{f}P < 0.001$ *vs* the dextran sodium sulfate group; $^{c}P < 0.001$ *vs* the control group. The data are shown as the mean ± SD ($n = 4$). COX-2: Cyclooxygenase-2; DSS: Dextran sodium sulfate; SASP: Salazosulfapyridine; BT2: 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid.

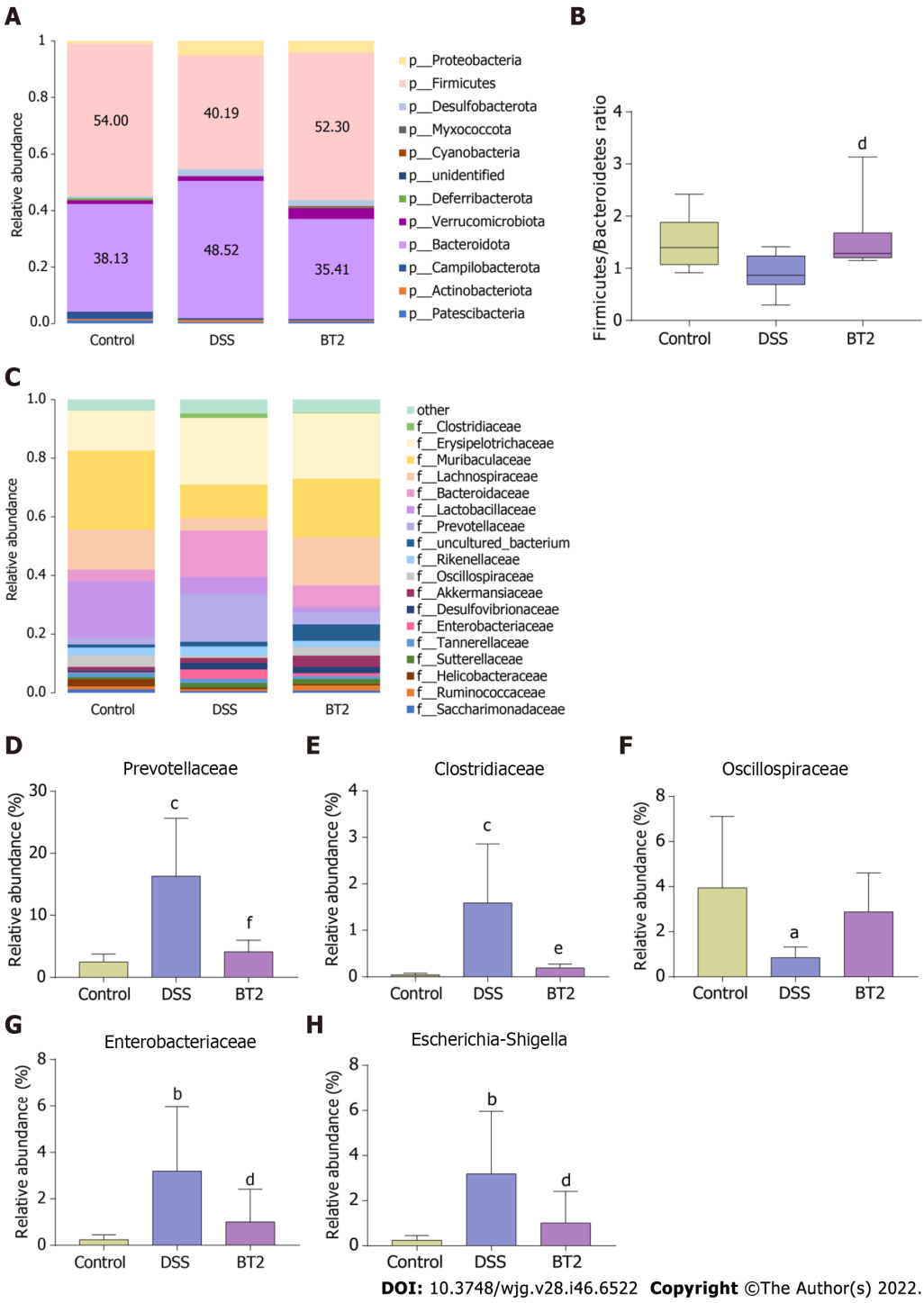


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Figure 6 Effects of 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid treatment on the overall structure of gut microbiota. A: The Venn diagram depicts the overlap of operational taxonomic units in each group; B: Chao1 index from fecal samples; C: Shannon index from fecal samples; D: The dilution curve indicates the rationality of the amount of sequencing data; E: Nonmetric multidimensional scaling analysis plot of fecal microbiota of each group. $^{e}P < 0.01$ and $^{f}P < 0.001$ *vs* the dextran sodium sulfate group; $^{b}P < 0.01$ and $^{c}P < 0.001$ *vs* the control group. The data are shown as the mean ± SD ($n = 8$). DSS: Dextran sodium sulfate; OUT: Operational taxonomic units; BT2: 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid.

Enterobacteriaceae and *Escherichia-Shigella* was also observed in the BT2 group. A recent report showed that host-induced inflammation disrupts gut microbes, leading to overgrowth of some aerobic bacteria, particularly *Enterobacteriaceae*, which is elevated in the intestinal tract of IBD patients and acts as an initiation inducer for inflammation[32]. *Shigella* is a gram-negative short bacillus that is highly contagious and regarded as a pathogen of IBD[33].

mTORC1 is a complex of mTOR that jointly promotes protein synthesis, cell growth, and proliferation by phosphorylating its direct downstream substrates S6 kinase 1 and 4E-BP1[34,35]. A growing number of findings have revealed the involvement of the mTORC1 pathway in the pathogenesis of UC. mTORC1 is hyperactivated in the colon tissues of UC patients and mice, and the overexpression of mTORC1 in colonic epithelial cells aggravates the inflammatory symptoms of UC mice[12]. Supplementation with mTORC1 inhibitors such as rapamycin, AZD8055, and everolimus markedly improves experimental UC by reducing leukocyte extravasation in the mucosa, suppressing T-cell proliferation



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Figure 7 Effects of 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid treatment on the species composition. A: Taxonomic analysis at the phylum level; B: BT2 treatment alters the *Firmicutes*/*Bacteroidetes* value in each group; C: Taxonomic analysis at the family level; D: Abundance of *Prevotellaceae*; E: Abundance of *Clostridiaceae*; F: Abundance of *Oscillospiraceae*; G: Abundance of *Enterobacteriaceae*; H: Abundance of *Escherichia-Shigella*. $^{d}P < 0.05$, $^{e}P < 0.01$, and $^{f}P < 0.001$ *vs* the DSS group; $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ *vs* the control group. The data are shown as the mean ± SD ($n = 8$). DSS: Dextran sodium sulfate; BT2: 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid.

and blocking the release of interferon from lymphocytes[36-38]. In our study, the activity of mTORC1 was enhanced in DSS-stimulated colitis mice, and BT2 significantly decreased mTORC1 activity. COX-2 is one of the isoforms of COX and is strongly associated with the development of IBD. COX-2 is highly expressed in the colon tissues of UC patients and animals and induces UC by producing prostaglandins (PGs), which promote inflammation and pain. The COX-2 inhibitor rofecoxib is potentially effective in improving experimental colitis by reducing PGE2 production, proinflammatory cytokines, and neutrophil infiltration[39,40]. In clinical UC samples, a positive correlation has been detected between COX-2 expression and mTORC1 activity[12]. Consistently, our study demonstrated that COX-2 expression was enhanced in UC mice but inhibited by BT2 intervention.

Amino acids, particularly BCAAs, serve as essential signaling factors for mTORC1 activation. The reduction of BCAA production by blocking BCAT1, a cytosolic aminotransferase for BCAAs, can significantly inhibit mTORC1 activation in chronic myeloid leukemia cells[41]. Rag guanine triphosphate (Rag GTPase) has been identified as a critical activator of mTORC1 in response to amino acid signals[42]. GATOR1, GATOR2, and Sestrin2 are three proteins involved in the regulation of recombination-activating genes. Sestrin2 is reportedly a leucine receptor for the mTORC1 signaling pathway[43]. Previous studies have shown that BCAA-induced mTORC1 activation is associated with the development of hepatocellular carcinoma, liver cancer, and prostate cancer[18,44,45]. Recently, Huangqin decoction relieves experimental colitis by regulating the metabolism of amino acids such as leucine and the mTOR pathway[46]. These results revealed that BCAA-mediated mTORC1 activation is a key research topic in the field of UC. Our data established an unreported link between BCAA-related mTORC1 activation and UC and thus elucidate a novel mechanism of BT2 against UC. Further investigations need to combine gene knockout mouse models and fecal microbiota transplantation to validate the specific mechanism of BT2-regulated BCAA metabolism in the anti-UC effect.

CONCLUSION

In summary, our data showed that the intestinal microbiota was disordered and BCAA catabolism was impaired in DSS-induced colitis mice, resulting in activation of the mTORC1 signaling pathway and overexpression of COX-2. Importantly, BT2 substantially reversed these phenomena and thus demonstrates potential value in the treatment of UC.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is a chronic inflammatory disorder caused by multiple factors. As a novel inhibitor, 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid (BT2) was recently shown to play an important role in improving amino acid metabolism and anti-inflammation. However, no relevant studies have reported the protective effect of BT2 on UC.

Research motivation

To investigate the therapeutic effect of BT2 on UC and the potential pharmacological mechanism.

Research objectives

To clarify the role of BT2 in UC treatment and its underlying mechanism.

Research methods

The acute colitis model was induced by 3.5% dextran sodium sulfate (DSS). The protective effect of BT2 was evaluated *via* clinical manifestations and colonic pathological changes. Flow cytometry was used to detect the contents of cytokines. Western blot analysis, immunohistochemical assays and liquid chromatography-tandem mass spectrometry were employed to estimate the mechanistic target of rapamycin complex 1 (mTORC1) activity and branched-chain amino acid (BCAA) catabolism. Moreover, the abundance of intestinal microbiota in feces was measured by 16S ribosomal DNA sequencing.

Research results

BT2 markedly alleviated colonic damage and reduced the release of proinflammatory cytokines in the DSS colitis model. BT2 also obviously improved BCAA catabolism in UC mice, as evidenced by the detection of related kinases in colon tissues and the targeted quantification of amino acids in serum. mTORC1 activity was enhanced in UC mice but inhibited by BT2 treatment. Moreover, 16S rRNA sequencing revealed that BT2 could prevent the decrease in the *Firmicutes*/*Bacteroidetes* ratio and the increase in harmful bacteria induced by DSS.

Research conclusions

BT2 relieved colitis by inhibiting BCAA-related mTORC1 activation and restoring gut microbiota metabolism homeostasis.

Research perspectives

BT2 showed significant efficacy on colitis and could serve as a promising therapeutic agent for UC treatment.

FOOTNOTES

Author contributions: He QZ and Wei P contributed equally to this work; He QZ, Wei P, and Liu HH conceived and performed experiments; He QZ, Zhang JZ, Liu TT, and Shi KQ analyzed the data; He QZ and Wei P wrote the paper; Liu SJ revised the manuscript and obtained funding for the project; All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Liver infiltration of multiple immune cells during the process of acute liver injury and repair

Yuan Xie, Ke-Bo Zhong, Yang Hu, Yong-Lun Xi, Shi-Xing Guan, Meng Xu, Yuan Lin, Feng-Yong Liu, Wei-Jie Zhou, Yi Gao

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Abstract

BACKGROUND

Immune cells, including neutrophils, natural killer (NK) cells, T cells, NKT cells and macrophages, participate in the progression of acute liver injury and hepatic recovery. To date, there has been no systematic study on the quantitative changes in these different immune cells from initial injury to subsequent recovery.

AIM

To investigate the infiltration changes of various immune cells in acute liver

injury models over time, and to study the relationship between the changes in leukocyte cell-derived chemotaxin 2 (LECT2) and the infiltration of several immune cells.

METHODS

Carbon tetrachloride- and concanavalin A-induced acute liver injury models were employed to mimic toxin-induced and autoimmune-mediated liver injury respectively. The quantitative changes in various immune cells were monitored at different time points. Serum samples were collected, and liver tissues were harvested. Ly6G, CD161, CD4, CD8 and F4/80 staining were used to indicate neutrophils, NK/NKT cells, $CD4^+$ T cells, $CD8^+$ T cells and macrophages, respectively. Lect2-KO mice were used to detect the function of LECT2.

RESULTS

During the injury and repair process, different types of immune cells began to increase, reached their peaks and fell into decline at different time points. Furthermore, when the serum alanine transaminase (ALT) and aspartate transaminase (AST) indices reverted to normal levels 7 d after the injury, the infiltration of immune cells still existed even 14 d after the injury, showing an obvious lag effect. We found that the expression of LECT2 was upregulated in acute liver injury mouse models, and the liver injuries of Lect2-KO mice were less severe than those of wild-type mice. Compared with wild-type mice, Lect2-KO mice had different immune cell infiltration.

CONCLUSION

The recovery time of immune cells was far behind that of serum ALT and AST during the process of liver repair. LECT2 could regulate monocyte/macrophage chemotaxis and might be used as a therapeutic target for acute liver injury.

Key Words: Immune cells; Liver injury; Liver repair; Leukocyte cell-derived chemotaxin 2

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Core Tip: In our study, we systematically described the infiltration changes of various immune cells during the process of acute liver injury and repair. We found that the recovery time of immune cells was far behind that of serum alanine transaminase and aspartate transaminase during the process of liver repair. Moreover, we found that leukocyte cell-derived chemotaxin 2 (LECT2) was upregulated in acute liver injury mouse models and the changes in LECT2 were related to the changes in several immune cells. LECT2 could regulate monocyte/macrophage chemotaxis and might be used as a therapeutic target for acute liver injury.

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INTRODUCTION

Acute liver injury is induced by a variety of causes, including viral infection, autoimmune diseases, alcohol or drug abuse and toxin intake, which can lead to liver failure and even death[1,2]. Various immune cells, including neutrophils, natural killer (NK) cells, T cells, NKT cells and macrophages, are directed and recruited to damaged sites and inflammatory lesions following acute injury[3]. Accumulated studies have indicated that these immune cells may participate in the progression of liver injury[4-6] and hepatic recovery[7-9]. A complete description of the changes in the number of various immune cells during the process of liver injury and repair could indicate the potential roles of these immune cells and may also help to treat acute liver injury by regulating these different immune cells at different time points. To date, there has been no systematic study on the quantitative changes in these different immune cells from initial injury to subsequent recovery. Here, we employed carbon tetrachloride (CCl_4)- and concanavalin A (ConA)-induced acute liver injury mouse models to mimic toxin-induced and autoimmune-mediated liver injury, respectively, and monitored the dynamic changes in various immune cells over time.

Leukocyte cell-derived chemotaxin 2 (LECT2) is a 16-kDa protein mainly produced by hepatocytes, first isolated in PHA-activated human T-cell leukemia SKW-3 cells in 1996[10]. Increasing evidence suggests that LECT2 is a pleiotropic protein, it not only functions as a cytokine to exhibit chemotactic properties but also plays multifunctional roles in some physiological conditions and pathological abnormalities[11], such as neuronal development[12], liver regeneration[13], home-ostasis of hematopoietic stem cells[14], liver fibrosis[15,16], insulin resistance[17] and renal amyloidosis[18]. We found that, during the repair of acute liver injury, the changes in LECT2 were related to the changes in several immune cells. Acute liver injury might be treated by inhibiting LECT2.

MATERIALS AND METHODS

Mice

C57BL6/J (wild-type) mice were purchased from Guangzhou University of Chinese Medicine. Lect2-KO mice were provided by Professor Jiong Chen from Ningbo University. The genomic DNA from the Lect2-KO mice was extracted for PCR amplification with the forward and reverse primers (Forward: 5′-CATAGCCAGGGGACTATGTTTTA-3′; Reverse: 5′-ATATAGTCATAGCTGCACACAGCA-3′). The PCR products were further used to confirm genotyping by Sanger sequencing. Mice were kept in a standard 12 h light–dark cycle under specific-pathogen-free conditions, and were allowed free access to water and food. All mice used for the study were male, healthy and immune-normal, weighing 22-25 g. Animal-related research protocols were consistent with the US Public Health Service Policy on Use of Laboratory Animals, and were approved by the Ethics Committee on Use and Care of Animals of Southern Medical University.

Acute liver injury models

CCl_4 (Maclin, C805332) and ConA (Solarbio, C8110) were separately used for the induction of acute liver injury model. For the CCl_4-induced acute liver injury model, mice were injected intraperitoneally with a CCl_4 mixture at a dose of 10 mL/kg body weight once (CCl_4 dissolved in olive oil at a volume ratio of 1:4). For the ConA-induced acute liver injury model, mice were injected intravenously with ConA solution at a dose of 10 mg/kg body weight once (ConA dissolved in PBS at a concentration of 2 mg/mL). Serum samples and liver tissues were collected and stored at -80 ℃ refrigerator for subsequent analyses. There were five mice per group.

Immunohistochemical staining

Harvested liver tissues were fixed in 4% neutral buffered formalin, and then dehydrated and embedded in paraffin. Sections of 3.5-mm thickness were dewaxed, and incubated in antigen retrieval solution for 5 min at 120 ℃, and then endogenous peroxidases were blocked with 3% H_2O_2 for 15 min. The slides were incubated with goat serum for 40 min at 37 ℃ to block nonspecific binding sites, followed by incubation with the appropriate primary antibodies overnight at 4 ℃, and then with HRP (horseradish peroxidase) anti-rabbit IgG for 30 min at 37 ℃. Color was developed by incubation with a DAB substrate kit (Zsbio, ZLI-9017). After washing with PBS, sections were counterstained with hematoxylin. Major primary antibodies used in the study included anti-Ly6G antibody (1:100, CST, 87048), anti-CD161 antibody (1:200, CST, 39197), anti-CD4 antibody (1:500, Abcam, ab183685), anti-CD8 antibody (1:1000, Abcam, ab209775) and anti-F4/80 antibody (1:200, CST, 70076). Images from ten random fields were taken for each section, under a 200 × light microscope. Cells positive for Ly6G, CD161, CD4 and CD8 were counted manually. The percentages of F4/80 positive areas were calculated automatically by ImageJ software.

qRT-PCR

Total RNA from mouse livers was extracted using TRIzol reagent (AG, 21102), and reverse transcribed using Evo M-MLV RT Premix (AG, 11706). Quantitative qRT-PCR was performed on an ABI 7500 Fast real-time PCR system with SYBR Green Master PCR Mix (AG, 11701), Rox Reference Dye (AG, 11710) and primers. The primers applied in qRT-PCR were as follows: *Lect2* forward (mouse), 5′-GGACGTGT-GACAGCTATGGC-3′; *Lect2* reverse (mouse), 5′-TCCCAGTGAATGGTGCATACA-3′; *GAPDH* forward (mouse), 5′-AGAAGGTGGTGAAGCAGGCATC-3′, *GAPDH* reverse (mouse), 5′-CGAAGGTGGAA-GAGTGGGAGTTG-3′.

ELISA

The serum levels of ALT, AST and TBil were detected by an Automated Chemical Analyzer (Mindray, BS-240VET) with standard diagnostic kits. The serum levels of LECT2 were tested by an ELISA kit (Cloud-Clone, SEF541Mu).

RNA sequencing

The wild-type and Lect2-KO mice were injected with CCl_4 or ConA, and then livers from these mice

were harvested after 2 d. Livers were also obtained from wild-type mice without CCl_4 and ConA treatment as standard controls. Total RNA was extracted from the tissue using TRIzol reagent according to the manufacturer's instructions (Invitrogen) and genomic DNA was removed with DNaseI (TaKaRa). Then RNA quality was determined and only a high-quality RNA sample was used to construct a sequencing library. RNA-seq transcriptome libraries were prepared following the TruSeq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, United States) using 1 μg of total RNA. Messenger RNA was isolated according to the poly (A) selection method by oligo (dT) beads and then fragmented by fragmentation buffer. Next, double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA, United States) with random hexamer primers (Illumina). The synthesized cDNA was subjected to end-repair, phosphorylation and 'A' base addition according to Illumina's library construction protocol. Libraries were size selected for cDNA target fragments of 300 bp, followed by PCR amplification with Phusion DNA polymerase (NEB) for 15 PCR cycles. After quantification by TBS380, the paired-end RNA-seq sequencing library was sequenced with an Illumina NovaSeq 6000 sequencer. All steps of RNA sequencing were performed by Majorbio BioPharm Technology Corporation, Shanghai.

Chemotaxis assay

Transwell inserts with 8-μm pores were used for the chemotaxis assay. THP-1 cells (2×10^6/mL, 200 μL) were suspended in RPMI 1640 medium containing 10% FBS and 4000 nmol/L PMA (Bioss, D10289s), cells were seeded in each upper chamber. LECT2 (Abcam ab188467) and MCP-1 (Proteintech, Ag24085) were added to the lower chambers filled with 500 μl serum free medium containing 4000 nmol/L PMA. After incubation at 37 °C in 5% CO_2 for 2 h, the Transwell inserts were removed. Six consecutive pictures were taken with 200 × light microscope for the cells that migrated to the lower chambers. The number of cells was counted by ImageJ software for each picture.

Statistical analysis

The results of the experiments are presented as the mean ± SD. The experimental data were analyzed by Student's *t* test. Statistical analysis and graph production were performed by GraphPad Prism software. A P value < 0.05 was considered to be statistically significant.

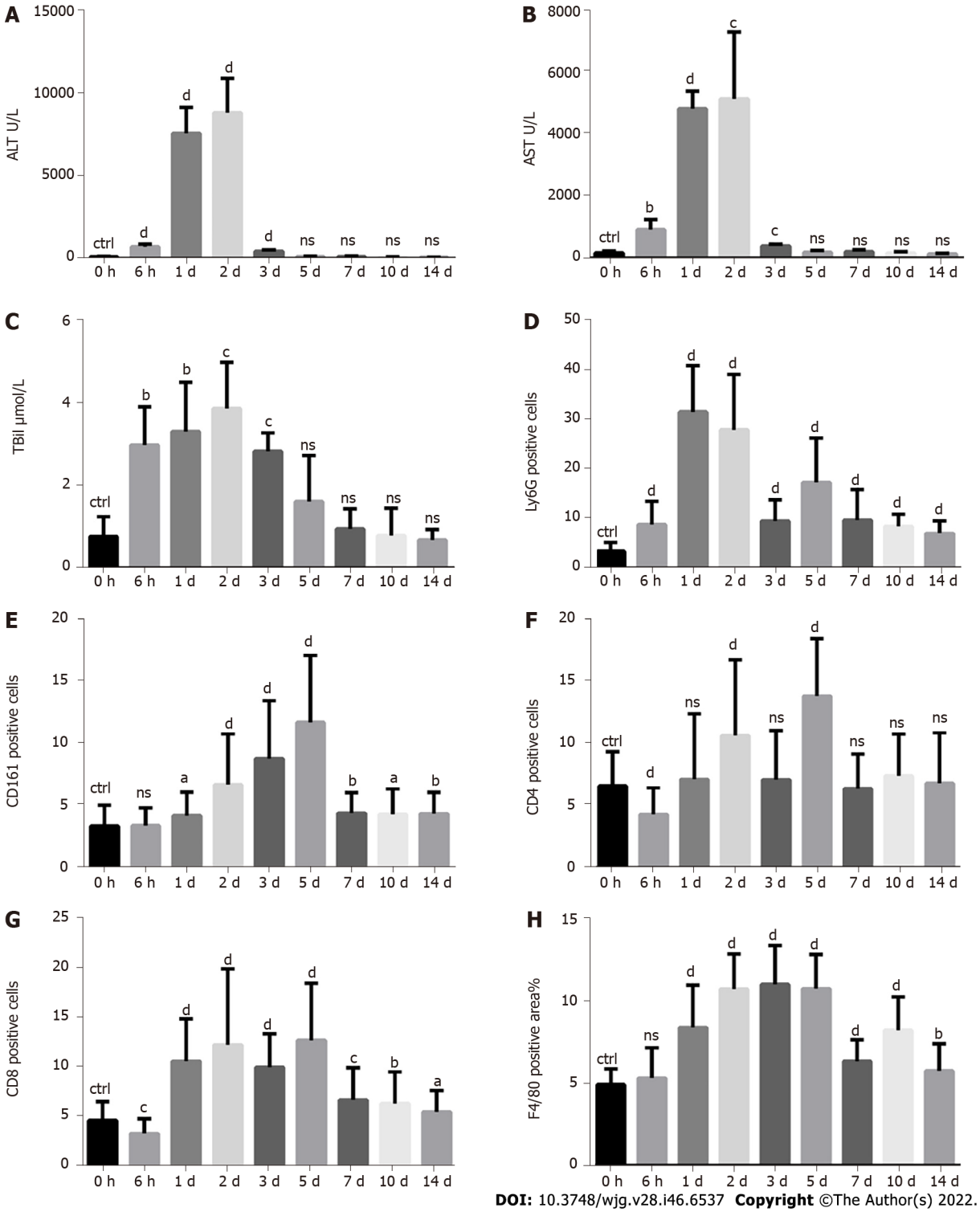
RESULTS

Immune cell infiltration during the process of CCl_4-induced liver injury and repair

A single injection of CCl_4 at a dose of 10 mL/kg body weight was used to mimic toxin-induced acute liver injury in mice. The levels of serum alanine transaminase (ALT), aspartate transaminase (AST) and total bilirubin (TBil) were measured to evaluate the degree of liver injury from 0 h to 14 d after CCl_4 treatment. The levels of serum ALT, AST and TBil increased as early as 6 h, followed by a sharp rise and a peak on the 1st and 2nd days, respectively, and then decreased to normal on the 5th day (Figure 1A-C). Ly6G, CD161, CD4, CD8 and F4/80 staining were used to indicate neutrophils, NK/NKT cells, $CD4^+$ T cells, $CD8^+$ T cells and macrophages, respectively. Neutrophils increased as early as 6 h, followed by a sharp rise lasting for 2 d (Figure 1D and Supplementary Figure 1). NK/NKT cells increased as early as the 1st day, followed by a continuous rise with a peak on the 5th day (Figure 1E and Supplementary Figure 2). $CD4^+$ T cells increased as early as the 2nd day, and peaked on the 5th day (Figure 1F and Supplementary Figure 3). $CD8^+$ T cells increased as early as the 1st day, and peaked on the 5th day (Figure 1G and Supplementary Figure 4). Macrophages increased as early as the 1st day, followed by a peak lasting for approximately 4 d (Figure 1H and Supplementary Figure 5). It should be noted that the number of infiltrated neutrophils, NK/NKT cells, $CD8^+$ T cells and macrophages on the 14th day after CCl_4 treatment was still higher than that of the control, compared with the fact that the levels of serum ALT, AST and TBil decreased to normal on the 5th day after CCl_4 treatment.

Immune cell infiltration during the process of ConA-induced liver injury and repair

A single injection of ConA at a dose of 10 mg/kg body weight was used to mimic autoimmune-mediated acute liver injury in mice. In contrast to CCl_4-treated mice, in ConA-treated mice, the levels of serum ALT, AST and TBil peaked as soon as 6 h, followed by a rapid decline on the 1st day, and then decreased to normal within 3 d (Figure 2A-C). In the ConA-induced acute injury model, neutrophils increased sharply and peaked as soon as 6 h (Figure 2D and Supplementary Figure 6). NK/NKT cells increased as early as the 1st day, and then peaked on the 2nd day (Figure 2E and Supplementary Figure 7). $CD4^+$ T cells increased as early as the 1st day, followed by a peak on the 2nd day (Figure 2F and Supplementary Figure 8). $CD8^+$ T cells increased gradually from 6 h to 2 d, and peaked on the 2nd day (Figure 2G and Supplementary Figure 9). Macrophages increased as early as 6 h, peaked on the 2nd day and lasted for approximately 4 d (Figure 2H and Supplementary Figure 10). Notably, the above immune cells could hardly revert to the normal count within 7 d.

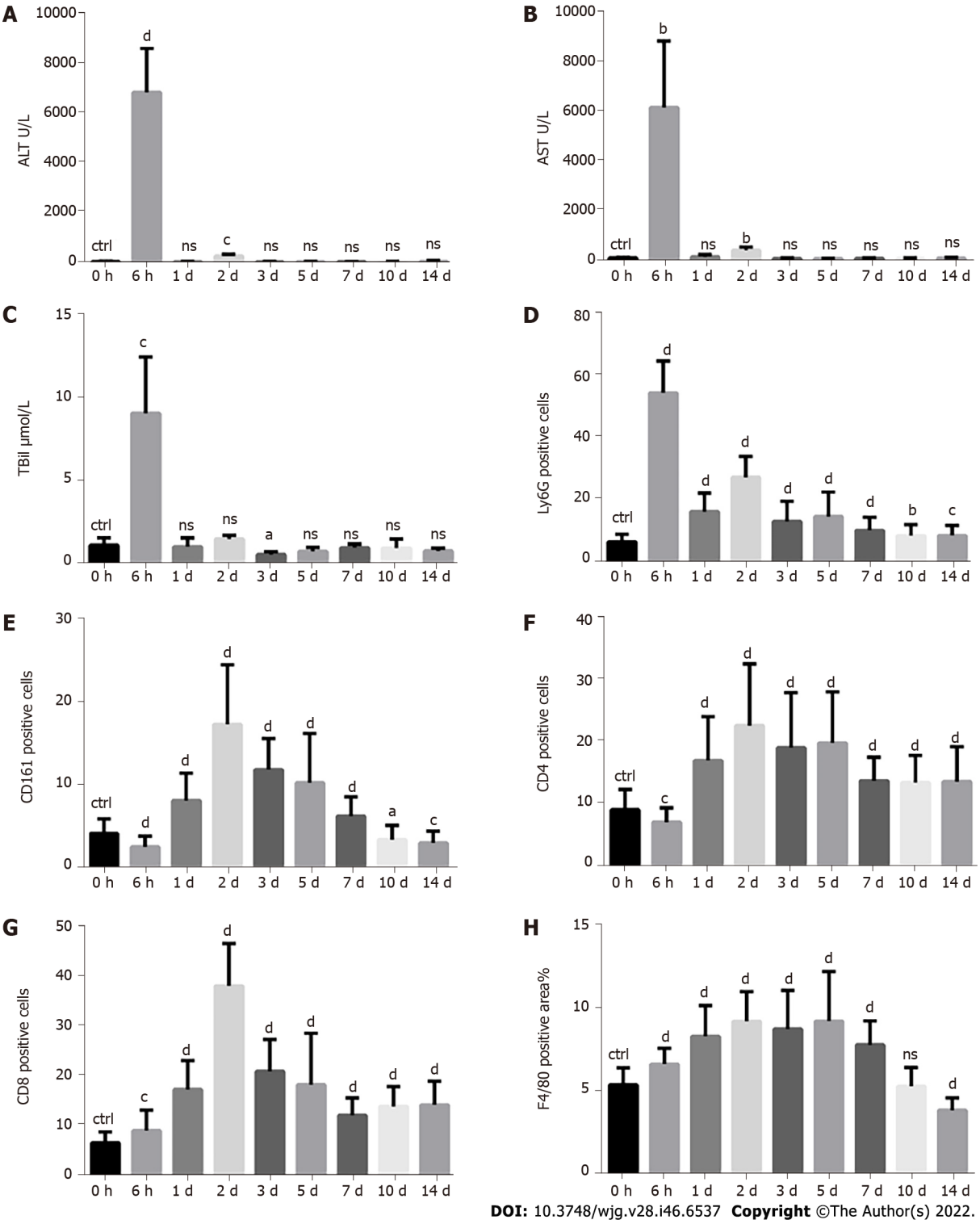


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Figure 1 Immune cell infiltration during the process of carbon tetrachloride-induced liver injury and repair. A-C: The levels of serum alanine transaminase, aspartate transaminase and total bilirubin in carbon tetrachloride (CCl_4)-treated wild-type mice; D-H: Quantitative changes in the infiltration of various immune cells in the livers of CCl_4-treated wild-type mice. The data represent the mean ± SD. $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$; $^dP < 0.0001$. ctrl: Control; ns: Not significant; ALT: Alanine transaminase; AST: Aspartate transaminase; TBil: Total bilirubin.

The expression of LECT2 was increased in acute liver injury models

We detected the dynamic changes in LECT2 expression in liver tissues and serum from 0 h to 14 d after CCl_4 and ConA treatment. We found that the expression of Lect2 mRNA was significantly increased on the 2nd day after CCl_4 treatment (Figure 3A), while increased levels of serum LECT2 could be observed on the 1st day and lasted for 2 wk, with a peak on the 2nd day (Figure 3B). In the ConA-induced acute injury model, the expression of Lect2 mRNA was significantly increased on the 3rd, 5th and 7th days (Figure 3C), while increased levels of serum LECT2 could be observed at as early as 6 h and lasted for 1 wk, with a peak at 6 h (Figure 3D).

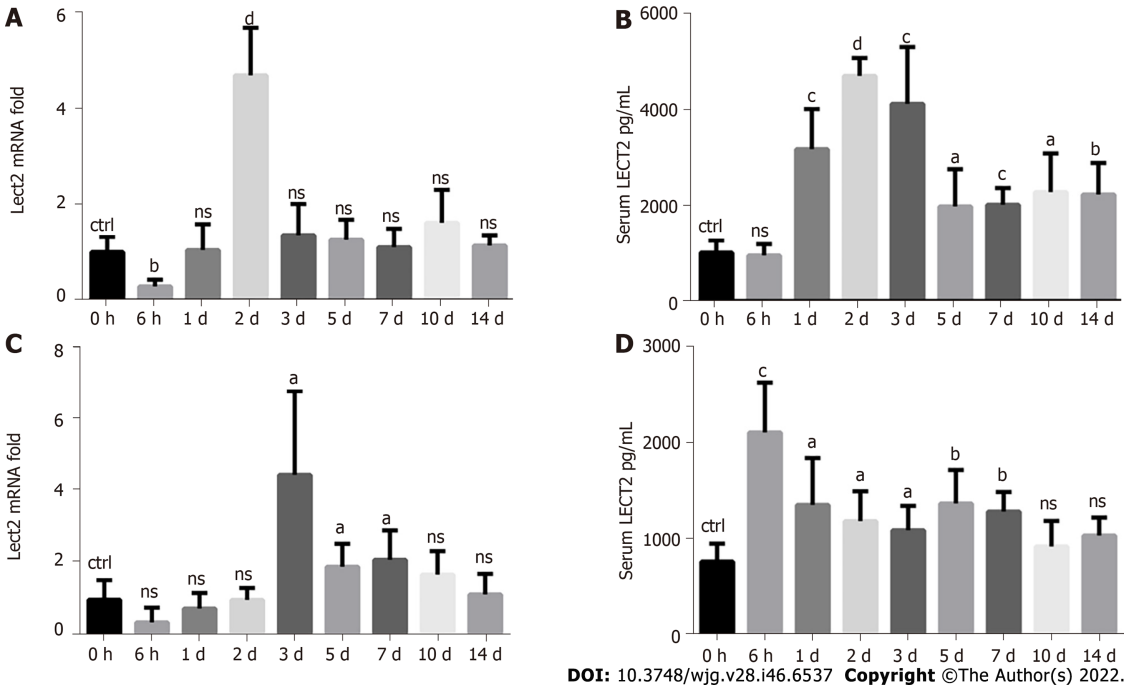


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Figure 2 Immune cell infiltration during the process of concanavalin A-induced liver injury and repair. A-C: The levels of serum alanine transaminase, aspartate transaminase and total bilirubin in concanavalin A (ConA)-treated wild-type mice; D-H: Quantitative changes in the infiltration of various immune cells in the livers of ConA-treated wild-type mice. The data represent the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. ctrl: Control; ns: Not significant; ALT: Alanine transaminase; AST: Aspartate transaminase; TBil: Total bilirubin.

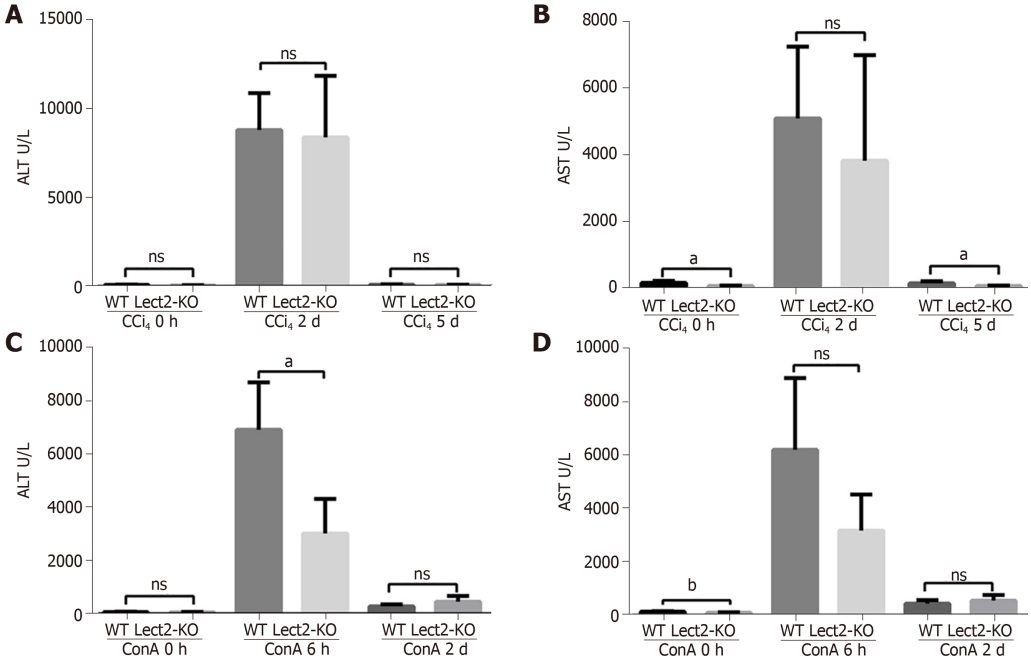
Lect2-KO mice showed less severe liver injuries

The levels of AST were significantly decreased in CCl_4-treated Lect2-KO mice on the 5th day compared with their WT (wild-type) counterparts (Figure 4A and B). The levels of ALT were significantly decreased in ConA-treated Lect2-KO mice at 6 h compared with their WT counterparts (Figure 4C and D).



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Figure 3 The expression of leukocyte cell-derived chemotaxin 2 was increased in acute liver injury models. A: The relative expression of Lect2 mRNA in the liver tissues of carbon tetrachloride (CCl_4)-treated wild-type mice; B The levels of serum leukocyte cell-derived chemotaxin 2 (LECT2) in CCl_4-treated wild-type mice; C: The relative expression of Lect2 mRNA in the liver tissues of concanavalin A (ConA)-treated wild-type mice; D: The levels of serum LECT2 in ConA-treated wild-type mice. The data represent the mean ± SD. ctrl: Control; ns: Not significant; $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$; $^dP < 0.0001$. ctrl: Control; ns: Not significant; LECT2: Leukocyte cell-derived chemotaxin 2.

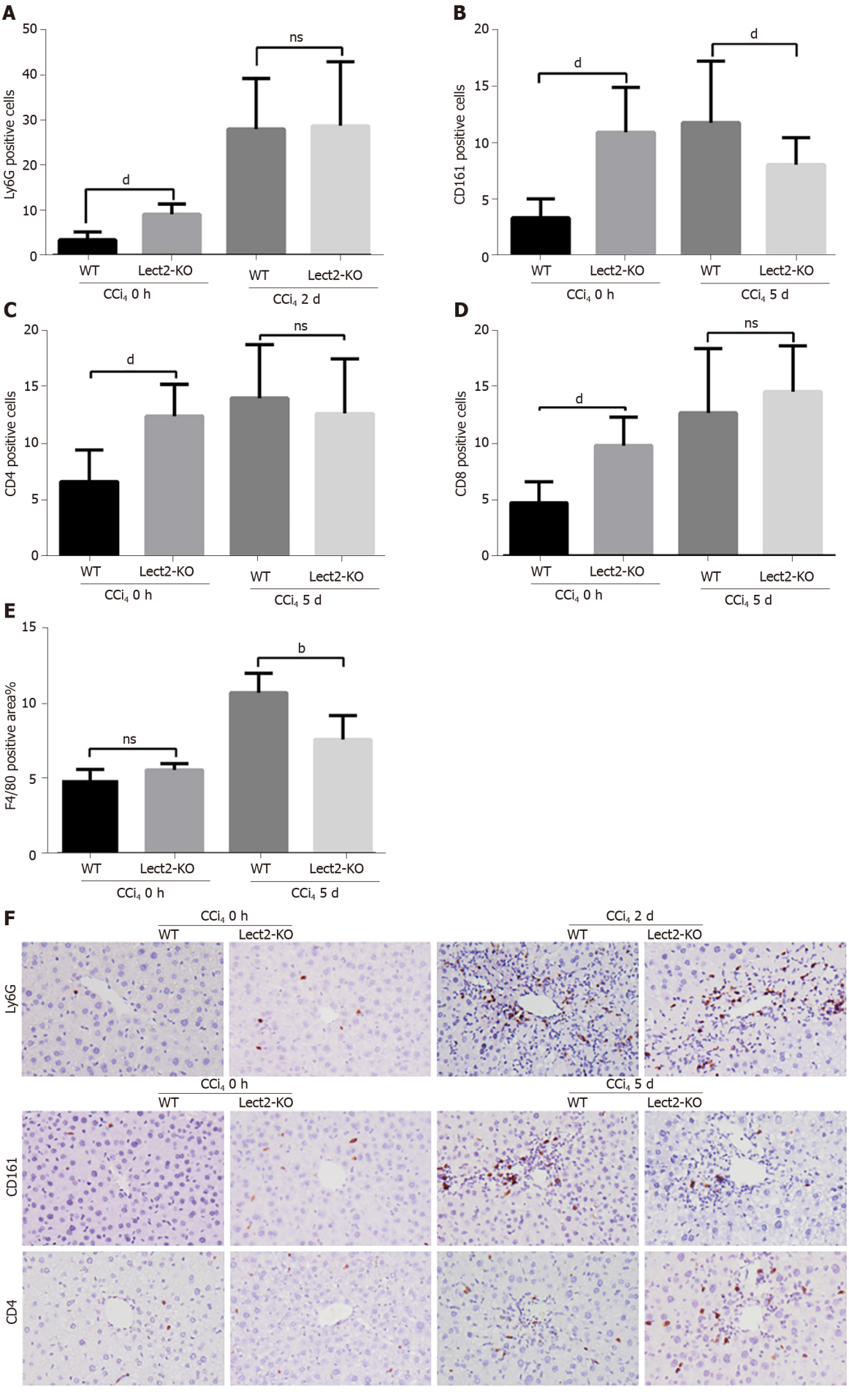


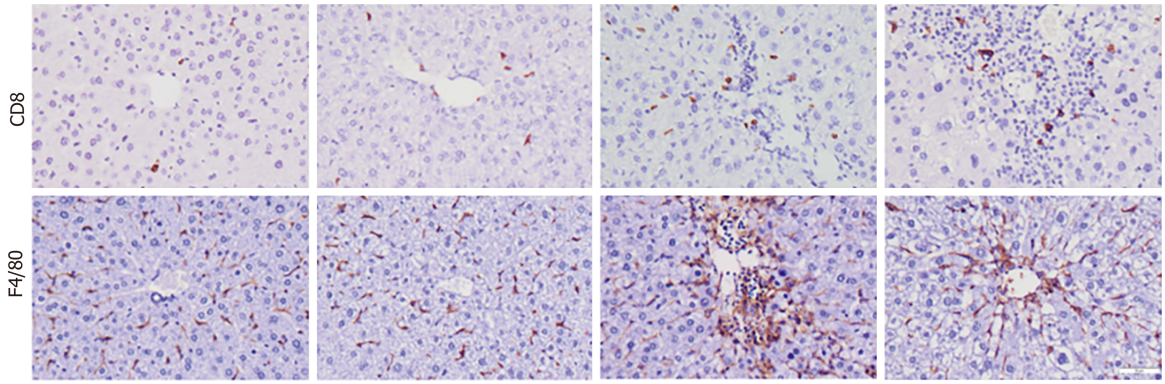
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Figure 4 Lect2-KO mice showed less severe liver injuries. A and B: The levels of serum alanine transaminase (ALT) and aspartate transaminase (AST) in the wild-type and Lect2-KO mice, with or without carbon tetrachloride treatment; C and D: The levels of serum ALT and AST in the wild-type and Lect2-KO mice, with or without concanavalin A treatment. The data represent the mean ± SD. $^aP < 0.05$; $^bP < 0.01$. ns: Not significant; ALT: Alanine transaminase; AST: Aspartate transaminase.

The effect of LECT2 on immune cell infiltration in acute liver injury models

We selected the peak time for different types of immune cells in the CCl_4 or ConA-induced acute injury model to observe the effect of LECT2 on the liver infiltration of various immune cells. WT and Lect2-KO mice were treated with CCl_4 and ConA, respectively, and livers were harvested on the 2nd and 5th days


A
Ly6G positive cells
50
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Lect2-KO
WT
Lect2-KO
CCi4 0 h
CCi4 2 d
B
CD161 positive cells
20
15
10
5
0
d
d
WT
Lect2-KO
WT
Lect2-KO
CCi4 0 h
CCi4 5 d
C
CD4 positive cells
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WT
Lect2-KO
WT
Lect2-KO
CCi4 0 h
CCi4 5 d
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CD8 positive cells
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Lect2-KO
CCi4 0 h
CCi4 5 d
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15
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ns
b
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Lect2-KO
WT
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CCi4 0 h
CCi4 5 d
F
CCi4 0 h
CCi4 2 d
WT
Lect2-KO
WT
Lect2-KO
Ly6G
CCi4 0 h
CCi4 5 d
WT
Lect2-KO
WT
Lect2-KO
CD161
CD4



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Figure 5 The effect of leukocyte cell-derived chemotaxin 2 on immune cell infiltration in the carbon tetrachloride-induced acute liver injury model. A-E: Quantitative changes in the infiltration of various immune cells in the livers of wild-type and Lect2-KO mice, with or without carbon tetrachloride (CCl_4) treatment; F: Representative images of infiltrated immune cells in CCl_4-treated wild-type and Lect2-KO mice, with or without CCl_4 treatment. The data represent the mean ± SD. ns: Not significant; $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$; $^dP < 0.0001$. Scale bar: 50 μm.

after CCl_4 treatment. For the mice treated with ConA, livers were harvested at the 6th hour and 2nd day. First, we compared the number of immune cells between Lect2-KO mice and WT controls at 0 h without CCl_4 and ConA treatment. We found that more neutrophils, NK/NKT cells, $CD4^+$ and $CD8^+$ T cells emerged in Lect2-KO mice (Figure 5 and Figure 6). Next, we compared the number of immune cells between Lect2-KO mice and WT controls when treated with CCl_4 or ConA. In the CCl_4-induced acute injury model, we found that the livers of Lect2-KO mice had less infiltration of NK/NKT cells and macrophages than those of WT mice. There was no difference in the number of neutrophils, $CD4^+$ T cells or $CD8^+$ T cells (Figure 5A-F). In the ConA-induced acute injury model, the livers of Lect2-KO mice had more infiltrated neutrophils, $CD4^+$ T cells and $CD8^+$ T cells but fewer macrophages than those of WT mice. There was no difference in the number of NK/NKT cells (Figure 6A-F).

Chemokine-related genes changed in Lect2-KO mice

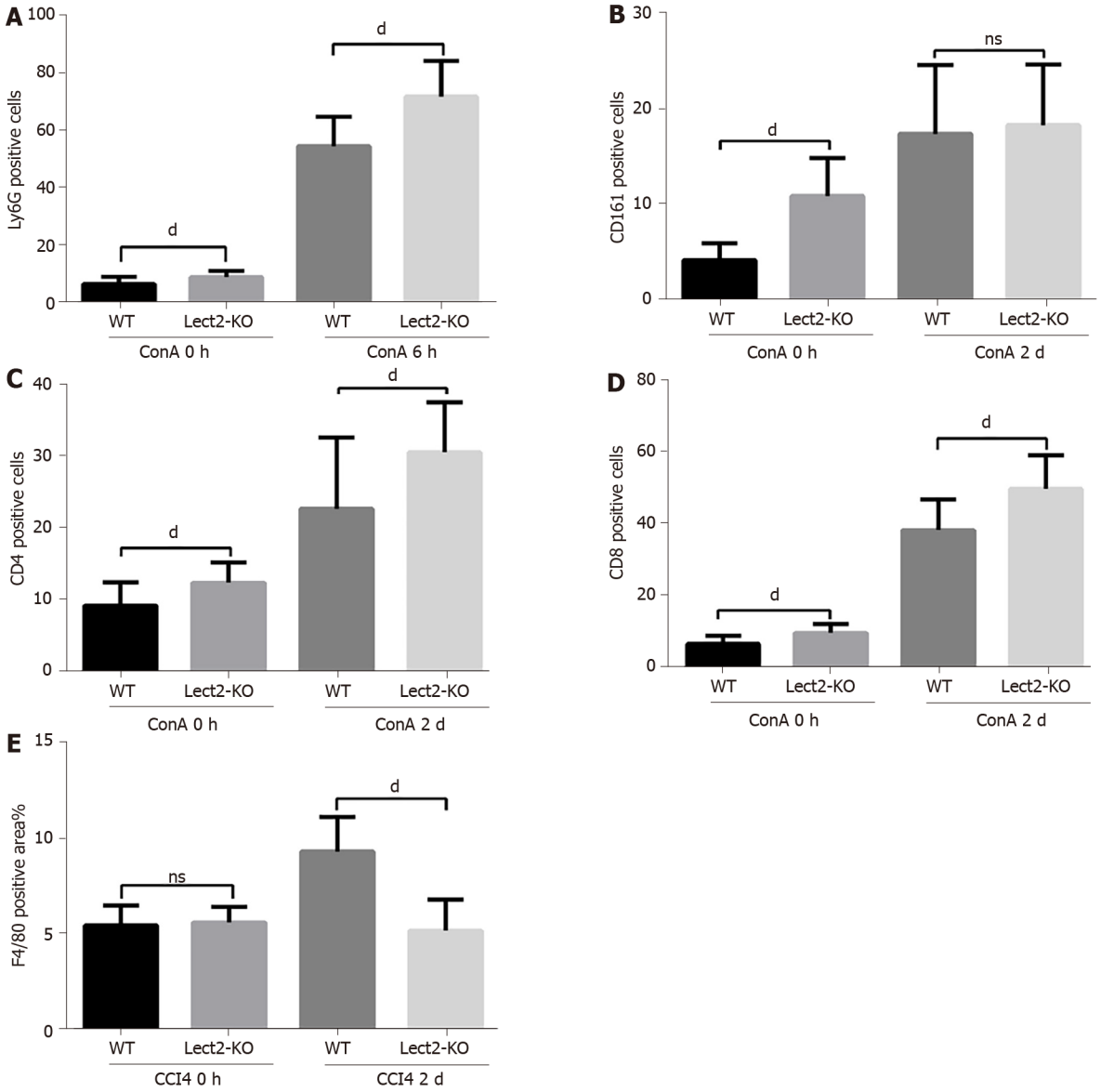
Chemokines are chemotactic cytokines that are capable of recruiting immune cells to sites of injury and inflammation. Next, we explored whether knockout of the Lect2 gene regulated the expression of chemokines in acute liver injury. RNA sequencing analysis was performed on injured liver samples derived from CCl_4 or ConA-treated Lect2-KO and WT mice. Differentially expressed genes were identified with a *p*-adjust value < 0.05 and fold change > 2. By Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis, we found that these changed genes could be partly categorized into chemokine signaling pathways (Figure 7A) and cytokine-cytokine receptor interactions (Figure 7B). Knockout of the Lect2 gene upregulated the expression of CXCL9, CXCL14, CCL6 and CCL22, and downregulated the expression of CCL19 in the CCl_4-treated mice (Figure 7C). In the ConA-treated mice, knockout of the Lect2 gene led to upregulated expression of CXCL2, CXCL3, CXCL5, CCL2 and CCL6 and downregulated expression of CCL21a, CCL21b and CCL27a (Figure 7D).

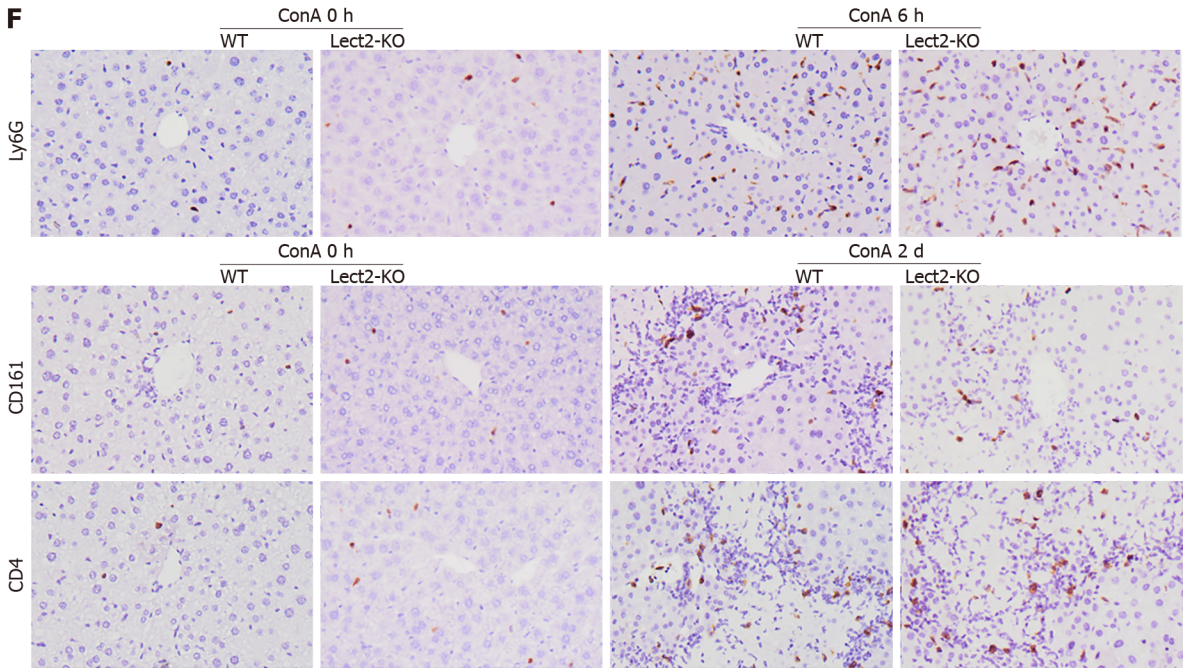
LECT2 promoted THP-1 monocyte chemotaxis

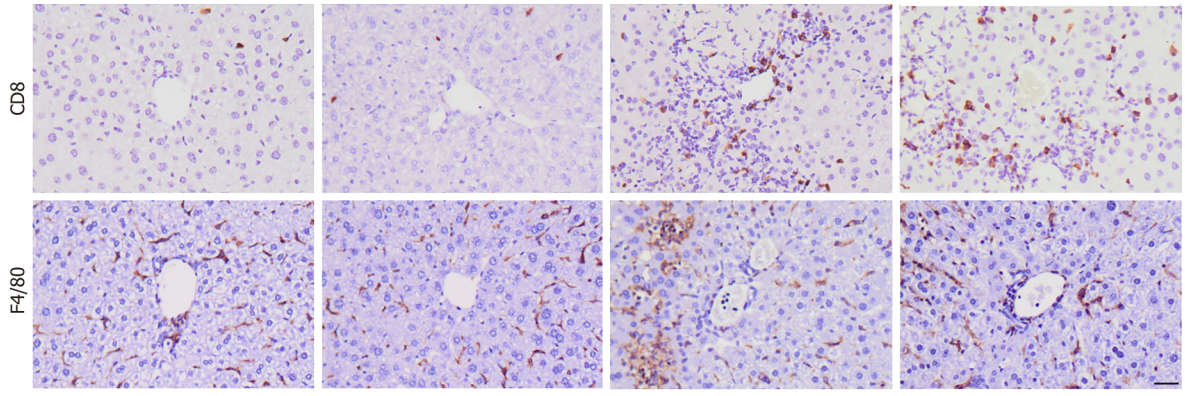
Since Lect2-KO mice displayed less macrophage infiltration than their WT counterparts in both CCl_4- and ConA-induced acute injury models, we explored whether LECT2 promoted monocytes/macrophage chemotaxis. The transwell assay showed that LECT2 could promote THP-1 monocytes chemotaxis (Figure 8A), but its chemotaxis was weaker than that of MCP-1, which is a well-recognized monocyte chemokine (Figure 8B).

DISCUSSION

The liver infiltration of various immune cell populations, including neutrophils, NK/NKT cells, T cells and monocytes/macrophages, is a critical pathological feature following acute liver injury and plays important roles in liver injury or repair[1-9]. Although there are many studies on the function of immune cells during liver injury or repair, there is still no study on the quantitative changes in immune cell infiltration at different time points from the beginning of injury to the end of repair. For the first time, employing CCl_4- and ConA-induced acute liver injury models, we monitored the infiltration of immune cells over time and found that different types of immune cells began to increase, reached their peaks and fell into decline at different time points, indicating that they played different roles in different injuries or repair stages. Therefore, appropriate regulation of immune cells at different time points may be a better strategy for the treatment of acute liver injury.

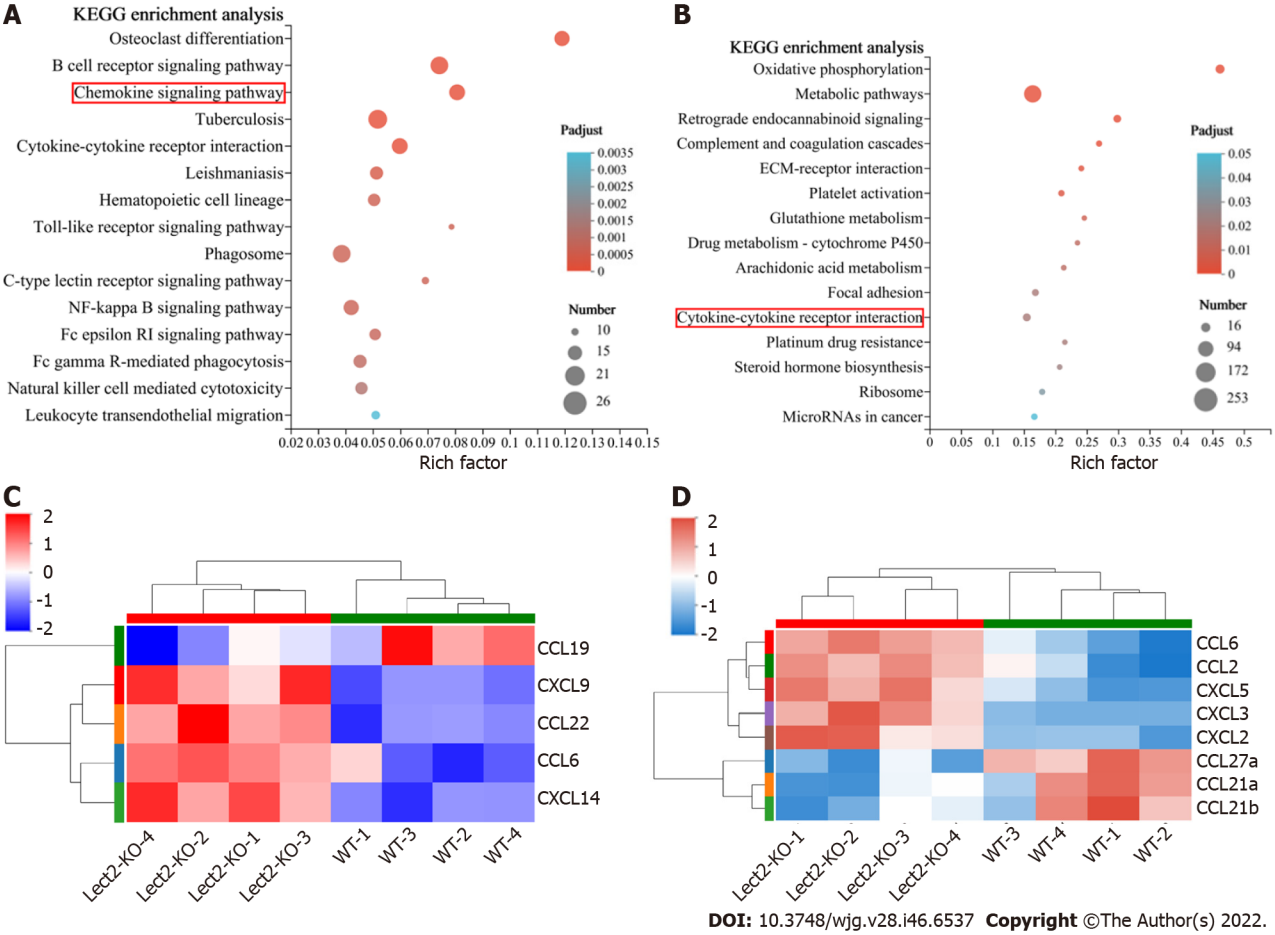

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Ly6G positive cells
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B
CD161 positive cells
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ConA 0 h
ConA 2 d
C
CD4 positive cells
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Lect2-KO
ConA 0 h
ConA 2 d
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CD8 positive cells
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Lect2-KO
ConA 0 h
ConA 2 d
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WT
Lect2-KO
CCI4 0 h
CCI4 2 d


F
ConA 0 h
ConA 6 h
WT
Lect2-KO
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Lect2-KO
Ly6G
ConA 0 h
ConA 2 d
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CD161
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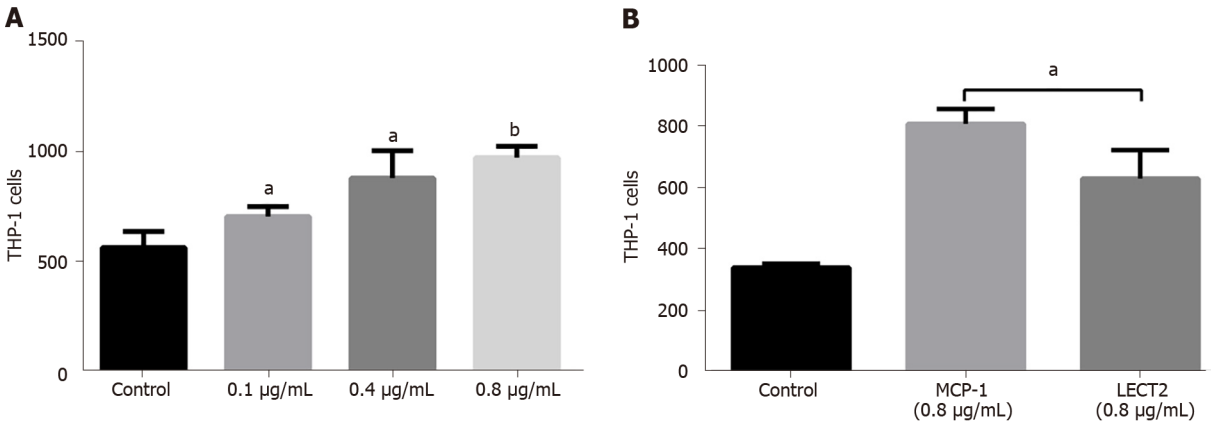
Figure 6 The effect of leukocyte cell-derived chemotaxin 2 on immune cell infiltration in the concanavalin A-induced acute liver injury model. A-E: Quantitative changes in the infiltration of various immune cells in the livers of wild-type and Lect2-KO mice, with or without concanavalin A (ConA) treatment; F: Representative images of infiltrated immune cells in ConA-treated wild-type and Lect2-KO mice, with or without ConA treatment. The data represent the mean ± SD. [a]*P* < 0.05; [b]*P* < 0.01; [c]*P* < 0.001; [d]*P* < 0.0001. ns: Not significant; Scale bar: 50 μm.



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Figure 7 Chemokine-related genes changed in Lect2-KO mice. A: Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis results for carbon tetrachloride (CCl_4)-treated Lect2-KO and wild-type mice; B: KEGG analysis results for concanavalin A (ConA)-treated Lect2-KO and wild-type mice; C: Heatmap of the differentially expressed chemokine-related genes between the Lect2-KO and wild-type mice after CCl_4 treatment; D: Heatmap of the differentially expressed chemokine-related genes between the Lect2-KO and wild-type mice after ConA treatment.

We were surprised to find that during the repair process, when the serum ALT and AST indices had reverted to normal levels 7 d after the injury, the infiltration of immune cells still existed even 14 d after the injury, showing an obvious lag effect. This suggests that the liver does not completely recover to normal as quickly as we previously thought. The recovery of serum indicators (ALT, AST, *etc.*) does not mean that the liver has recovered to normal, and the corresponding treatment may need to be extended



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Figure 8 Leukocyte cell-derived chemotaxin 2 promoted THP-1 monocyte chemotaxis. A: Chemotactic effects of Lect2 on THP-1 cells when treated with different doses; B: Comparison of chemotactic effects between Lect2 and MCP-1. The data represent the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$. ctrl: Control.

accordingly. Therefore, in addition to the serological indices, we suggest immunological indices to measure the repair of liver injury, which may have important value for the clinical treatment and prognosis of liver diseases. These remaining immune cells may continue to regulate liver repair and play a role in the response of the liver to rechallenges.

We previously reported that LECT2 regulated chronic liver fibrogenesis[15,16], while its role in acute liver injury is unclear. Here, we found that the expression of LECT2 was upregulated in acute liver injury models, and the liver injuries of Lect2-KO mice were less severe than those of wild-type mice. Compared with wild-type mice, Lect2-KO mice had different immune cell infiltration, suggesting that LECT2 may regulate acute liver injury and repair by regulating the infiltration of various immune cells. We demonstrated that LECT2 could indeed regulate monocyte/macrophage chemotaxis in vitro. Blocking LECT2 might be able to treat acute liver injury.

The limitation of this study is that we need further functional tests to verify the roles that different immune cells play in liver injury. In particular, when the liver is rechallenged, the roles of retained immune cells in the last injury need to be further studied. When the liver is rechallenged, these retained immune cells may cause greater damage to the liver, or may make the liver more tolerant to the same challenge.

CONCLUSION

In summary, we described the infiltration changes of various immune cells in acute liver injury models over time. We found that the recovery time of immune cells during the repair process was far behind that of serum ALT and AST. LECT2 could regulate monocyte/macrophage chemotaxis and might be used as a therapeutic target for acute liver injury.

ARTICLE HIGHLIGHTS

Research background

Immune cells, including neutrophils, natural killer (NK) cells, T cells, NKT cells and macrophages, participate in the progression of acute liver injury and hepatic recovery. leukocyte cell-derived chemotaxin 2 (LECT2) is a pleiotropic protein that not only functions as a cytokine to exhibit chemotactic properties but also plays multifunctional roles in some physiological conditions and pathological abnormalities.

Research motivation

To date, there has been no systematic study on the quantitative changes in these different immune cells from initial injury to subsequent recovery. LECT2 regulates chronic liver fibrogenesis, but its role in acute liver injury is unclear.

Research objectives

To investigate the infiltration changes of various immune cells in acute liver injury mouse models over time and to study the relationship between the changes in LECT2 and the infiltration of several immune

cells.

Research methods

Carbon tetrachloride (CCl_4)- and concanavalin A (ConA)-induced acute liver injury mouse models were employed to mimic toxin-induced and autoimmune-mediated liver injury, respectively. Immunohistochemical staining was used to indicate immune cells, and the quantitative changes in various immune cells were monitored at different time points. The levels of serum alanine transaminase (ALT), aspartate transaminase (AST) and total bilirubin (TBil) were measured by an automated chemical analyzer. The expression of LECT2 in liver tissues was detected by quantitative real-time polymerase chain reaction and the levels of LECT2 in serum were tested by enzyme-linked immunosorbent assay. Transwell assays were used to assess the chemotactic effect of LECT2 on THP-1 monocytes.

Research results

During the injury and repair process, different types of immune cells began to increase, reached their peaks and fell into decline at different time points. Furthermore, when the serum ALT and AST indices reverted to normal levels 7 d after injury, the infiltration of immune cells still existed even 14 d after injury, showing an obvious lag effect. The expression of LECT2 was up-regulated in acute liver injury models, and the liver injuries of Lect2-KO mice were less severe than those of wild-type mice. Compared with wild-type mice, Lect2-KO mice had different immune cell infiltration.

Research conclusions

The recovery time of immune cells was far behind that of serum ALT and AST during the process of liver repair. LECT2 could regulate monocyte/macrophage chemotaxis and might be used as a therapeutic target for acute liver injury.

Research perspectives

Our study indicated the potential roles of these immune cells and proposed a therapeutic concept that acute liver injury might be treated by regulating different immune cells at different time points.

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FOOTNOTES

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Hybrid XGBoost model with hyperparameter tuning for prediction of liver disease with better accuracy

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Abstract

BACKGROUND

Liver disease indicates any pathology that can harm or destroy the liver or prevent it from normal functioning. The global community has recently witnessed an increase in the mortality rate due to liver disease. This could be attributed to many factors, among which are human habits, awareness issues, poor healthcare, and late detection. To curb the growing threats from liver disease, early detection is critical to help reduce the risks and improve treatment outcome. Emerging technologies such as machine learning, as shown in this study, could be deployed to assist in enhancing its prediction and treatment.

AIM

To present a more efficient system for timely prediction of liver disease using a hybrid eXtreme Gradient Boosting model with hyperparameter tuning with a view to assist in early detection, diagnosis, and reduction of risks and mortality associated with the disease.

METHODS

The dataset used in this study consisted of 416 people with liver problems and 167 with no such history. The data were collected from the state of Andhra Pradesh, India, through https://www.kaggle.com/datasets/uciml/indian-liver-patient-records. The population was divided into two sets depending on the disease state of the patient. This binary information was recorded in the attribute "is_patient".

RESULTS

The results indicated that the chi-square automated interaction detection and classification and regression trees models achieved an accuracy level of 71.36%

and 73.24%, respectively, which was much better than the conventional method. The proposed solution would assist patients and physicians in tackling the problem of liver disease and ensuring that cases are detected early to prevent it from developing into cirrhosis (scarring) and to enhance the survival of patients. The study showed the potential of machine learning in health care, especially as it concerns disease prediction and monitoring.

CONCLUSION

This study contributed to the knowledge of machine learning application to health and to the efforts toward combating the problem of liver disease. However, relevant authorities have to invest more into machine learning research and other health technologies to maximize their potential.

Key Words: Liver infection; Machine learning; Chi-square automated interaction detection; Classification and regression trees; Decision tree; XGBoost; Hyperparameter tuning

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Core Tip: This article proposed the hybrid eXtreme Gradient Boosting model for prediction of liver disease. This model was designed by optimizing the hyperparameter tuning with the help of Bayesian optimization. The classification and regression trees and chi-square automated interaction detection models on their own are not accurate in predicting liver disease among Indian patients. The proposed model utilized different physical health status, *i.e.* level of bilirubin, direct bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, total proteins, albumin, and globulin in prediction of the liver disease. This work was aimed at designing a more accurate machine learning model in liver disease prediction.

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INTRODUCTION

The liver processes blood from the digestive tract. It is located on the right side of the abdomen, directly below the rib cage. The liver plays a critical role in the digestion of food and the elimination of toxins from the body. It is possible that liver illness is passed down *via* families (genetic). Viruses, alcohol, and obesity are all known to harm the liver, which can result in liver disease. Conditions that affect the liver can lead to scarring (cirrhosis), which can eventually lead to liver failure, a life-threatening condition[1]. The liver may be able to recover if therapy is started early enough.

One or more tests are recommended to correctly identify and determine the cause of liver illness. These may include: (1) Blood tests. Human blood is tested for the presence of liver enzymes. In addition, a blood-clotting test known as the international normalized ratio is used to measure liver function. Problems with liver function may be the cause of elevated levels[2]; (2) Imaging tests. Ultrasound, magnetic resonance imaging, and computed tomography scans can be used to examine a patient's liver for damage, scarring, or malignancies. FibroScan ultrasound can be used to measure scarring and fat accumulation in the liver, among other things; and (3) Liver biopsy. Small amounts of tissue are removed from the liver with the use of an ultrathin needle during the biopsy procedure. The tissue is examined for any indications of liver illness. The heart, lungs, stomach, skin, brain, cognitive function, and other elements of the nervous system can all be affected by liver disease. For a physical examination, the complete body may be examined. Blood tests can be used to determine the extent of liver inflammation and how well the organ is functioning[3].

There are several causes of liver disease. Cirrhosis can be caused by alcohol misuse. Nonalcoholic fatty liver disease and chronic hepatitis B and C are other possible causes. Having too much fat in the liver is known as nonalcoholic fatty liver disease. The liver may become inflamed as a result of the excess fat. Nonalcoholic steatohepatitis is a kind of nonalcoholic fatty liver disease that affects the liver. The liver is inflamed and damaged as well as full of fat. Other issues may be drug overdoses. Acetaminophen and other drugs might damage the liver if the patient uses them in large doses. Acetaminophen may appear in several medications the patient takes. Therefore, attention to the dosage directions on the label should be given. There is a risk of cirrhosis and liver scarring as a result of this

medication[4].

Dire complications of liver disease include acute liver failure when the patient does not have a long-term liver illness, but the liver shuts down in a matter of days or weeks. Acetaminophen overdosage, infection, or prescription medicine usage can be the cause for this. Another type is hepatic cirrhosis, which is the accumulation of scar tissue[5]. The more healthy liver tissue that is lost to scarring, the more difficult it is for the liver to function. It may not function as well as it should in the long run.

The so-called liver blood tests are generally a good indicator of liver damage as they show anomalies (*e.g.*, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase)[6]. It is common to refer to the liver blood tests as "liver function tests". This does not mean that liver dysfunction is the cause of all abnormalities in the blood tests, such as high bilirubin, lower-than-normal levels of albumin, and a prolonged prothrombin time. In addition, abnormalities in other liver blood tests may be indicative of liver damage. Hepatitis viruses, for example, may boost the levels of the alanine aminotransferase and aspartate aminotransferase enzymes in the blood, causing them to leak into the circulation[7].

This article aimed to fulfil various objectives. The first objective of this article was to identify the symptoms of liver disease and its impact on the patient's body. Then, it presented various machine learning approaches for predicting liver disease and evaluated the performance of decision-tree algorithms in the prediction of liver disease. Next, the article proposed a modified XGBoost model with a hyperparameter tuning mechanism. Finally, it validated the performance of the proposed model by comparing it with more traditional decision tree–based models.

MATERIALS AND METHODS

Dataset

There is a constant increase in the population encountering liver-related diseases due to an unhealthy breathing environment, excessive alcohol intake, contaminated elements in the diet, improper use of over the counter drugs, *etc*[8] (Table 1).

Table 1 depicts the description of the dataset used for the experimental purpose. The dataset used in this study constituted 416 people with liver problems and 167 with no such history, which was collected from the state of Andhra Pradesh, India[9]. The dataset population was divided into two sets depending on the disease status of the patient. This binary information was recorded in the attribute "is_patient". The aim was to correctly predict the value of this field so that the task can be automated and simplified for medical personnel. The dataset contained records for both male and female patients[10].

Data preprocessing

Data preparation is the most critical step before running various machine learning models. Machine learning does not perform as expected when datasets are not handled properly[11]. It is possible that the performance of a machine learning model during training and testing will diverge. Data errors, noise, and omissions can all contribute to this[12]. Prior to comparing data, preprocessing removes any duplicates, anomalies, and other inconsistencies. This ensures that the findings are more accurate[13].

Handling the null/missing values

Mean/median or mode are used to fill in the blanks in the dataset, depending on the type of data that are missing[14]: (1) Numerical data. Whenever a number is omitted, a mean or median value should be used instead. The outliers and skewness contained in the data pull the mean and median values in their respective directions, hence it is preferable to impute using the median value[15]; and (2) Categorical data. When categorical data are lacking, use the value that occurs the most frequently, *i.e.* by mode to fill in the blanks[16]. To manage the missing data, research can utilize three methods: (1) Delete rows with missing values[17]; (2) Impute missing values[18]; and (3) Predict the missing values[19]. In this work, the missing values were imputed for better performance of the machine learning models.

Handling the outliers

BoxPlot was used to identify outliers in a dataset. The outliers may be dealt with by limiting or transforming the data[20]. This can be done in two ways: (1) Capping the data. There are three ways to set data cap limitations[21]; and (2) Z-score approach. Outliers are any values that go outside of the normal range by a factor of 3 or more[22].

Chi-square automated interaction detection

Decision trees have existed for a long time, and chi-square automated interaction detection (CHAID) is the oldest one. The χ^2 test is used to determine the relevance of a feature[23]. The greater the statistical significance, the greater the value. CHAID uses decision trees to solve categorization issues. This implies that a category target variable is expected in the datasets[24]. Although ID3, C4.5, and classification and regression trees (CART) all employ information gain, CHAID uses χ^2 testing to determine

Table 1 Dataset description

No.	Attribute	Information	Type
1	Age	How old is the patient?	Integer
2	Sex	Patient's sex	String (male/female)
3	Tot_bilirbn	Level of bilirubin compound	Floating
4	Direct_bilirbn	Level of direct bilirubin compound	Floating
5	Alk_phos	Level of alkaline phosphatase compound	Floating
6	Al_amntrfrs	Level of alanine aminotransferase compound	Floating
7	As_amntrfrs	Level of aspartate aminotransferase compound	Floating
8	Total_prot	Level of total protein in the sample	Floating
9	Albmn	Level of albumin in the sample	Floating
10	Ag_ratio	Ratio of compound albumin to globulin	Floating
11	Is_patient	Categorizes the dataset into parts	Categorical

which characteristic is the most prominent. Karl Pearson introduced χ^2 testing because of its high accuracy, stability, and simplicity of interpretation. Tree-based learning algorithms are among the finest and most extensively used supervised learning methods[25]. The χ^2 statistic is used in the CHAID decision tree technique to determine the independent variable with the biggest χ^2 value for the dependent variable[26]. The manifestations of the dependent variable that has the most impact on the dependent variable become the new dependent variable[27]. The individual node in Figure 1 consists of category (0 or 1), % (accuracy level), and *n* (number of patients)[28].

Figure 1 demonstrates that the CHAID tree consists of multiple decision nodes (node 0 to node 8). The branch of the decision tree is leveled by three parameters (adjusted *P* value, χ^2, and df). These parameters play a very important role in decision making.

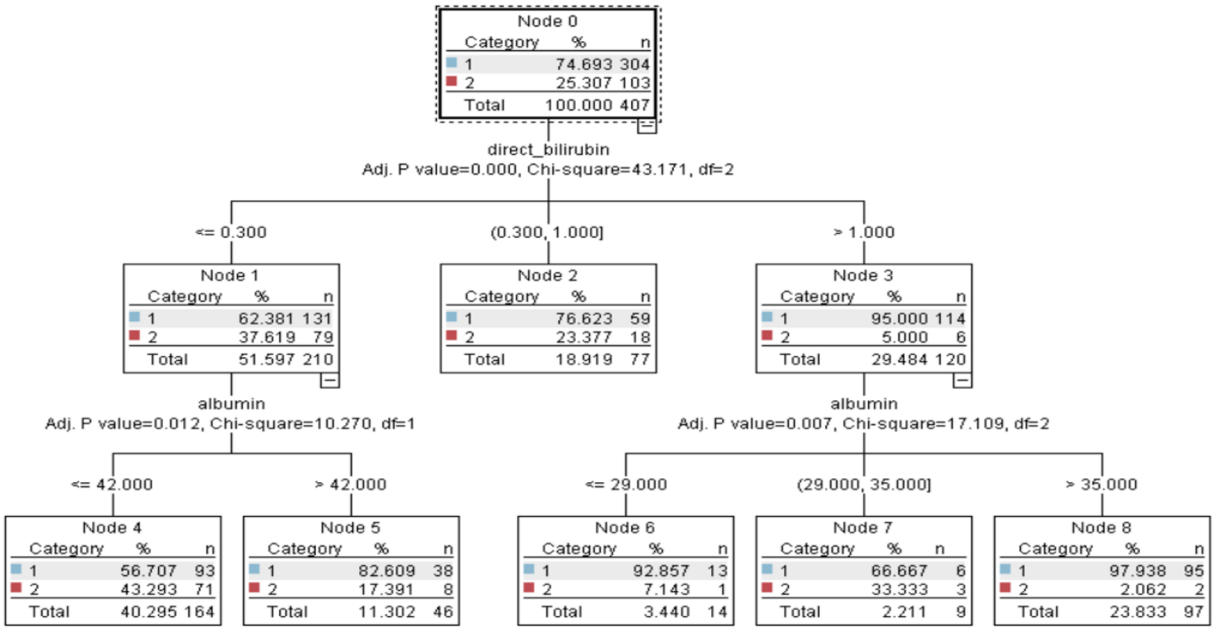
In the study presented here, the compound 'direct bilirubin' was adjudged as the most significant factor by the CHAID test, as shown in Figure 1, splitting the tree into three subtrees depending on the quantity of bilirubin found. It also showed the adjusted *P* values and χ^2 value calculated at each level marking the significant difference between the corresponding subcategories. Further, on the next level, the tree splits on the basis of next important factor, 'alkaline phosphate,' for the category represented by node 1, *i.e.* people with value ≤ 0.9 for compound direct bilirubin, and 'age' for node 2, which represents the direct bilirubin range of 0.9 to 4.1. The maximum height of the tree allowed is up to level 5 as the further splits do not significantly affect the result of the model. The significance value kept for splitting the records is 0.05, using Pearson's likelihood ratio for χ^2 and Bonferroni method for autoadjusting the significance value and actual *P* values. The threshold value for stopping the growth of tree is a minimum 2% records in the parent branch and 1% record in the child branch[29].

CART

The CART algorithm uses several different ways to divide or segment data into smaller subsets depending on the different values and combinations of predictors that are available. Splits are selected, and the procedure is repeated until the finest possible collection is discovered[30]. Binary splits lead to terminal nodes that may be characterized by a collection of rules, resulting in a decision tree. To benefit from the tree's visual appeal and an easy-to-understand layout, one does not have to be an expert data scientist[31] (Figure 2).

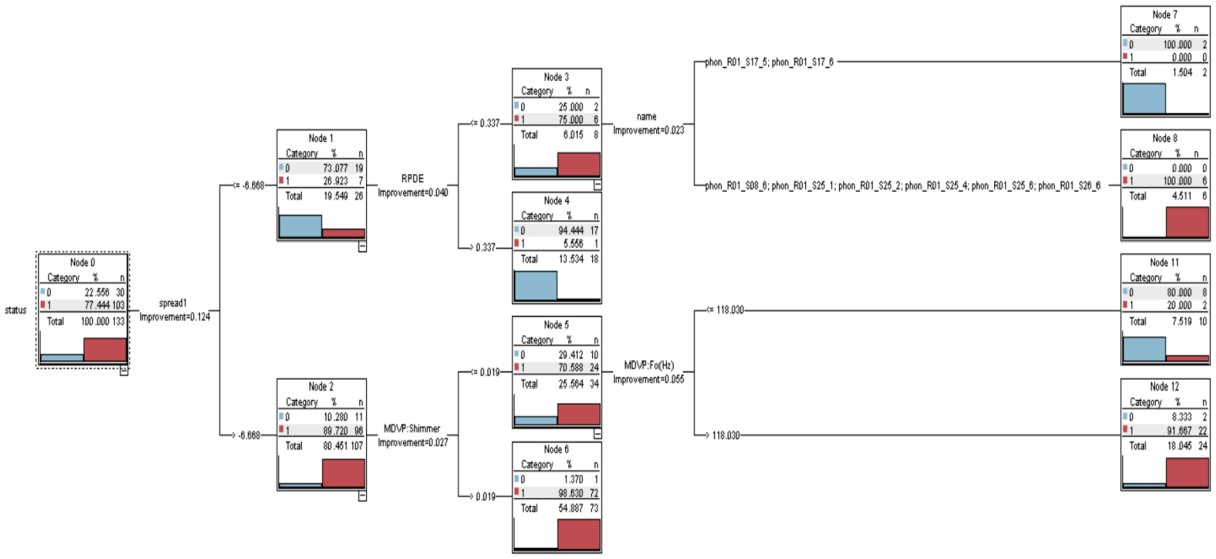
Figure 2 demonstrates that the CART algorithm consists of multiple decision nodes (node 0 to node 12). The CART approach can quickly uncover crucial associations that might otherwise go unnoticed when utilizing other analytical methods.

In this study, CART produced the binary tree classification for continuous variables and exhibited different sequences by adjudging compound total bilirubin as the least impure predictor[32]. The split-up value for total bilirubin was calculated to be 1.650 by the CART model at the first level, using the Gini impurity index method, with a gain of 0.047. Subsequently, the other important predictors were 'aspartate aminotransferase,' 'direct bilirubin,' and 'age' of the subjects, according to this model. The compound 'total bilirubin' was used at multiple levels for the split, which means that this compound produced maximum gain in the Gini index at multiple levels. The minimum value for recording change in impurity and making a split was set to '0.0001' after a series of run to maximize the efficiency of the model[33]. The maximum number of levels allowed here was '5' in the CART tree with the same criteria for stopping the growth, as used in the CHAID model[34].



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Figure 1 Chi-square automated interaction detection tree. Chi-square automated interaction detection tree consists of multiple decision nodes (node 0 to node 8). The branch of the decision tree is levelled by three parameters (adjusted *P* value, χ^2, and df). These parameters play a very important role in decision making.



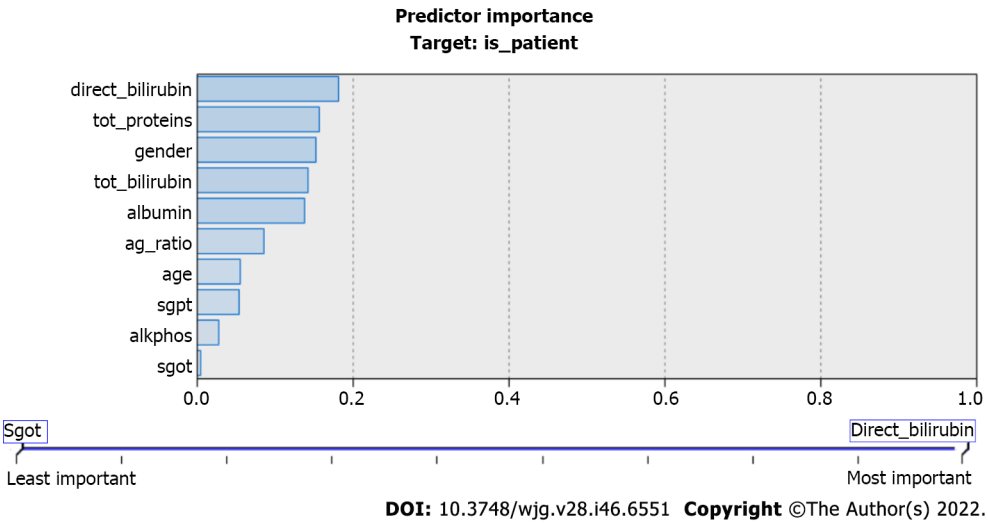
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Figure 2 Classification and regression tree. Classification and regression tree consists of multiple decision nodes (node 0 to node 12). The classification and regression tree approach can quickly uncover crucial associations that might otherwise go unnoticed when utilizing other analytical methods.

Figure 3 highlights the importance of predicators used in the CART model. They play important roles in liver disease prediction. CART is an innovative but highly desirable technique that incorporates automation, ease of use, performance, and accuracy, which sets it apart in the predictive analytics sector [35].

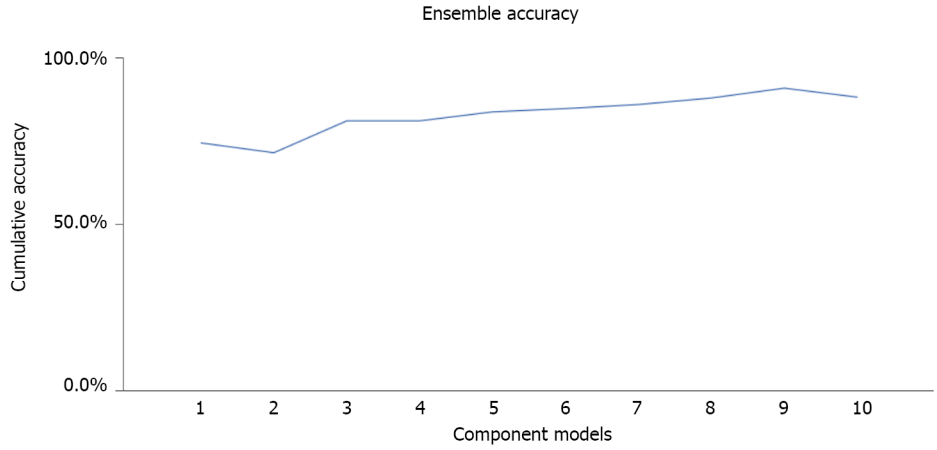
Ensemble with decision trees

The ensemble learning is used in techniques, such as boosting, for repeatedly training the model on different random samples of the dataset. Ensemble methods used in various advanced learning techniques, such as CART and XGBoost decision tees, also uses this approach to minimize the error and improve the accuracy[36]. The accuracy level of the decision tree may be enhanced by using the ensemble approach (Figure 4).



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Figure 3 Predictor importance in classification and regression trees. The values of the predicators used in the classification and regression tree model. These predictors play important roles in liver disease prediction. tot_proteins: Total protein; tot_bilirubin: Total bilirubin; ag_ratio: Ratio of compound albumin to globulin; alkphos: Alkaline phosphatase; sgpt: Serum glutamic pyruvic transaminase; sgot: Serum glutamic-oxaloacetic transaminase.



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Figure 4 Ensemble accuracy. It visually depicts ensemble accuracy that is improved gradually with each iteration. The ensemble approach increases the accuracy level of the decision tree models.

Figure 4 visually depicts ensemble accuracy that is improved gradually with each iteration. The ensemble approach increases the accuracy level of the decision tree models. If there is patient data, then a tree-like graph to model the choices can be used, and they can be continuous or categorical. As the patient answers each question, they will get a forecast of the data[37].

XGBoost

XGBoost is a collection of open-source functions and steps that uses supervised machine learning to estimate or predict a result. The result may be predicted using several decision trees in the XGBoost library. Batch learning is used to train the machine learning system, and then a model-based technique is used to generalize the results. Models for the link between predictor and outcome variables are constructed using all available data. These models are then generalized to the test data. In the context of computing, the term “extreme” implies its desire to push the boundaries of processing power. The concept of "gradient boosting", used to improve the performance of weak prediction models, is used in machine learning applications such as regression and classification[38].

Boosting

Because it can only predict the outcome variable slightly better than chance, a single decision tree is regarded as a weak or basic learner. Strong learners, on the other hand, are any algorithms that can be fine-tuned to attain maximum performance in supervised learning. To build a powerful learner,

XGBoost uses decision trees as its foundation learners. If you use many models (trees), the final prediction is called an ensemble learning approach as it incorporates the results of multiple models (trees)[39].

When a group of weak learners is combined to make a strong learner, it is called "boosting". Each weak prediction is weighted according to how well the weak learner performed, and XGBoost will repeatedly create a collection of poor models on subsets of the data. The weighted total of all base learners is used to make a prediction[40].

Building models with XGBoost

Although all the other features are used as predictors of the target variable *yi* in the training data, the target variable is provided. Decision trees are used to predict the values of *yi* based on *xi* using a set of trees. It would be difficult to predict the result variable with just one decision tree. Analysts may be able to generate more accurate predictions of *yi* if the decision trees are used collectively[41].

The learning process of an algorithm is governed by hyperparameters, which are specific values or weights. XGBoost, as previously mentioned, offers a wide range of hyperparameters. They may be fine-tuned to reach the highest level of accuracy possible. Auto-tuning of numerous learnable parameters allows for the XGBoost to recognize patterns and regularities in the datasets it analyzes. The learnable parameters in tree-based models like XGBoost are the decision variables at each node. A sophisticated algorithm like XGBoost has a lot of design choices and hence a lot of hyperparameters[42].

The main challenge faced in this stage is the optimized selection of parameters among multiple hyperparameters. It may be managed by efficient hyperparameter tuning. In this article, the Bayesian optimization was applied in the following four steps: (1) Initialize domain space for a range of values. Initially, the domain space is finalized considering the input values over what it is being searched. The input variables are max_depth, gamma, reg_alpha, reg_lambda, colsample_bytree, min_child_weight, and n_estimators; (2) Define objective function. In this step, the objective function is defined, which can be any function that returns a real number that must decrease in order to achieve the desired goal. The validation error of XGBoost with respect to hyperparameters should be minimized in this situation. To optimize accuracy, the other key value must be considered. It should return a value that is the opposite of the metric value; (3) Apply optimization algorithm. It is the method used to construct the surrogate objective function and choose the next values to evaluate. In this stage, the concept of Bayesian optimization in the tuning phase is used. The Bayesian optimization method is based on the Bayes theorem and provides an efficient and effective method for solving a global optimization issue. The real objective function is then used to evaluate the candidate samples; and (4) Obtain results. Results are scored by value pairs that the algorithm uses to build the XGBoost model.

For the XGBoost model, the objective function optimization is performed using the logistic model, as the target is a categorical variable. The tree building model used is 'auto' with 10 iterations for boosting at each level. The tree depth, with which maximum efficiency is obtained, is up to level 6, wherein minimum child weight is set as default '1,' and maximum delta step is set to no constraint with value '0' (Figure 5).

Figure 5 depicts the impact of the SHapley Additive exPlanations (SHAP) value on the XGBoost model. As the number of observations available is limited, the subsample space was set to '1' to consider all the datapoints during each iteration. Similarly, the parameter for column and level sampling are also set to value '1' (Figure 6).

Figure 6 depicts the mean SHAP values in the XGBoost model. The 'eta' parameter value is set to default '0.3' to keep the weights stable after each iteration, whereas the gamma value to specify the loss reduction required for the split up is '0.'

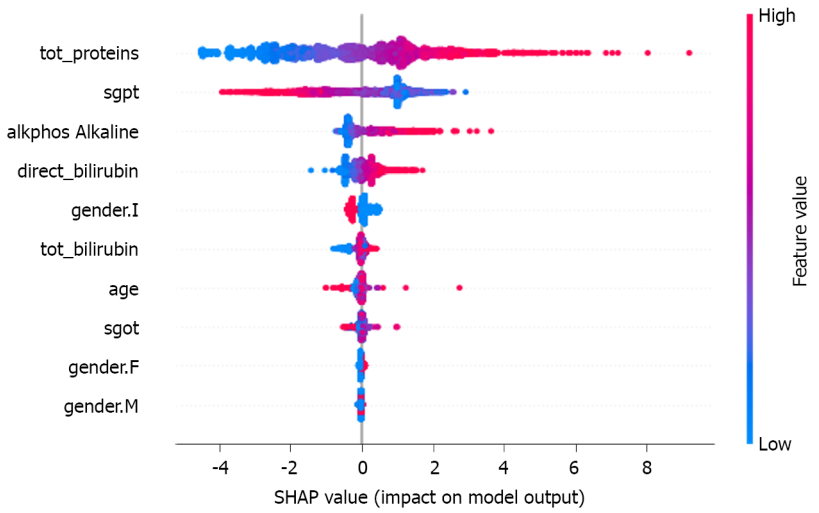
The value for the least square error and least absolute deviation, represented by parameter lambda and alpha, respectively, is set to value '1' and '0' and is used for regularizing the weights, making the model more stable, and controlling the overfitting[36] (Figure 7). The model is also executed with a hyperparameter tuning setup using the Bayesian optimization model and shows even better performance than the non-hyperparameter tuning setup.

Figure 7 depicts the model output value in the XGBoost model. These parameters make a direct impact on the output generated by the XGBoost model. The model is also executed with the hyper-parameter tuning setup using the Bayesian optimization model and shows even better performance than the non-hyperparameter tuning setup.

RESULTS

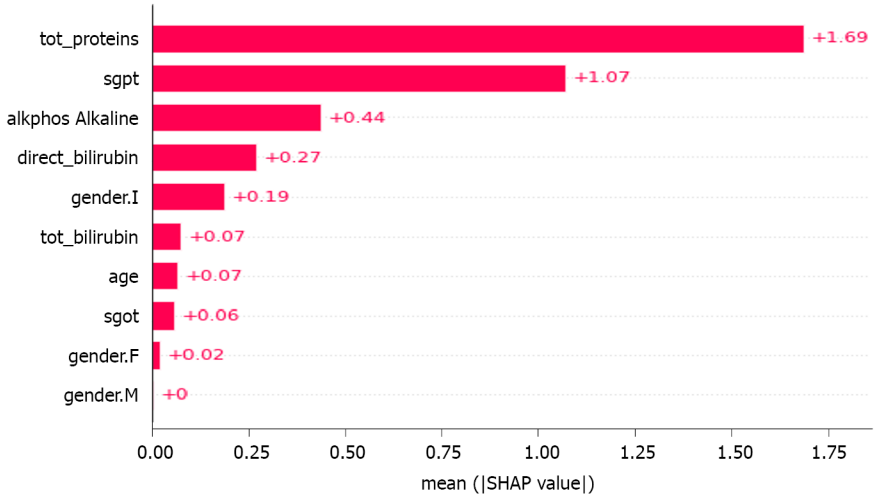
Results from individual decision trees

The output of the different prediction models presented in this work was shown in this section in the categorized tabular format. The Gini coefficient is a machine learning metric used to assess the efficiency of binary classifier models. In the range of 0 to 1, the Gini coefficient can be used. The higher the Gini coefficient, the better the model. There are two ways to measure precision: The number of properly categorized positive samples (true positives) and the overall number of positively classified samples



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Figure 5 Predicator impact on model output. The figure depicts the impact of the SHapley Additive exPlanations value on the eXtreme Gradient Boosting model. As the number of observations available is limited, the subsample space was set to '1' to consider all the datapoints during each iteration. tot_proteins: Total protein; tot_bilirubin: Total bilirubin; alkphos: Alkaline phosphatase; sgpt: Serum glutamic pyruvic transaminase; sgot: Serum glutamic-oxaloacetic transaminase; F: Female; M: Male.



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Figure 6 Mean SHapley Additive exPlanations value. The mean of the SHapley Additive exPlanations values in the eXtreme Gradient Boosting model. The 'eta' parameter value is set to default '0.3' to keep the weights stable after each iteration, whereas the gamma value to specify the loss reduction required for the split up is '0.' tot_proteins: Total protein; tot_bilirubin: Total bilirubin; alkphos: Alkaline phosphatase; sgpt: Serum glutamic pyruvic transaminase; sgot: Serum glutamic-oxaloacetic transaminase; F: Female; M: Male.

(either correctly or incorrectly). Using precision, we can see how reliable the machine learning model is when it comes to determining whether or not the model is positive.

The recall was computed by dividing the total number of positive samples by the number of positive samples that were correctly categorized as positive. The recall assesses the capability of the model to identify positive samples in a dataset. The more positive samples that are found, the higher the recall will be. Figure 8 depicts the accuracy of an individual decision tree and tabulates the values of various performance matrices.

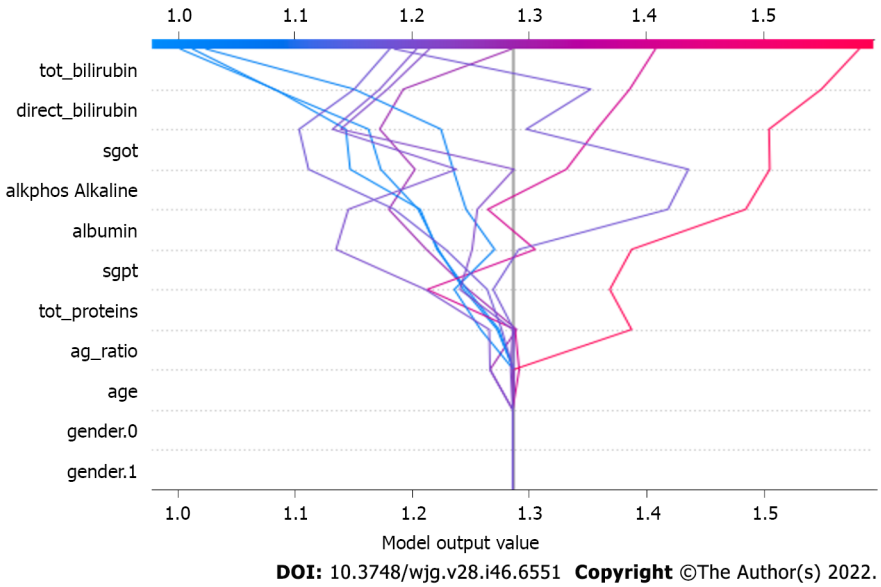
There were 583 patients in the Indian Liver Patient Dataset. A total of 500 patient records were used in the training process, and the remaining 83 records were used in the testing process. The CHAID model had 71.36% accuracy in predicting liver disease. The area under the curve and Gini value of this model were 0.746 and 0.493, respectively (Figure 8A). The model exhibited good discriminatory behavior according to the Gini coefficient value.

Of the 583 observations, 389 predictions were categorized as true positives, 38 as true negatives, and 156 as false in this model. The result for the CART model had a better accuracy, 73.24%, at predicting liver disease compared to the CHAID model. The area under the curve and Gini values of this model

Table 2 Result analysis

No.	Algorithm	Accuracy	AUC	Gini
1	CHAID model	71.36	0.746	0.493
2	CART model	73.24	0.724	0.448
3	Proposed model	93.55	0.987	0.974

AUC: Area under the curve; CART: Classification and regression tree; CHAID: Chi-square automated interaction detection.



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Figure 7 Model output value. The model output value in the eXtreme Gradient Boosting model. These parameters make a direct impact on the output generated by the eXtreme Gradient Boosting model. tot_proteins: Total protein; tot_bilirubin: Total bilirubin; ag_ratio: Ratio of compound albumin to globulin; alkphos: Alkaline phosphatase; sgpt: Serum glutamic pyruvic transaminase; sgot: Serum glutamic-oxaloacetic transaminase.

were 0.724 and 0.445, respectively (Figure 8B). The Gini coefficient showed that the ability of the model to categorize was not good.

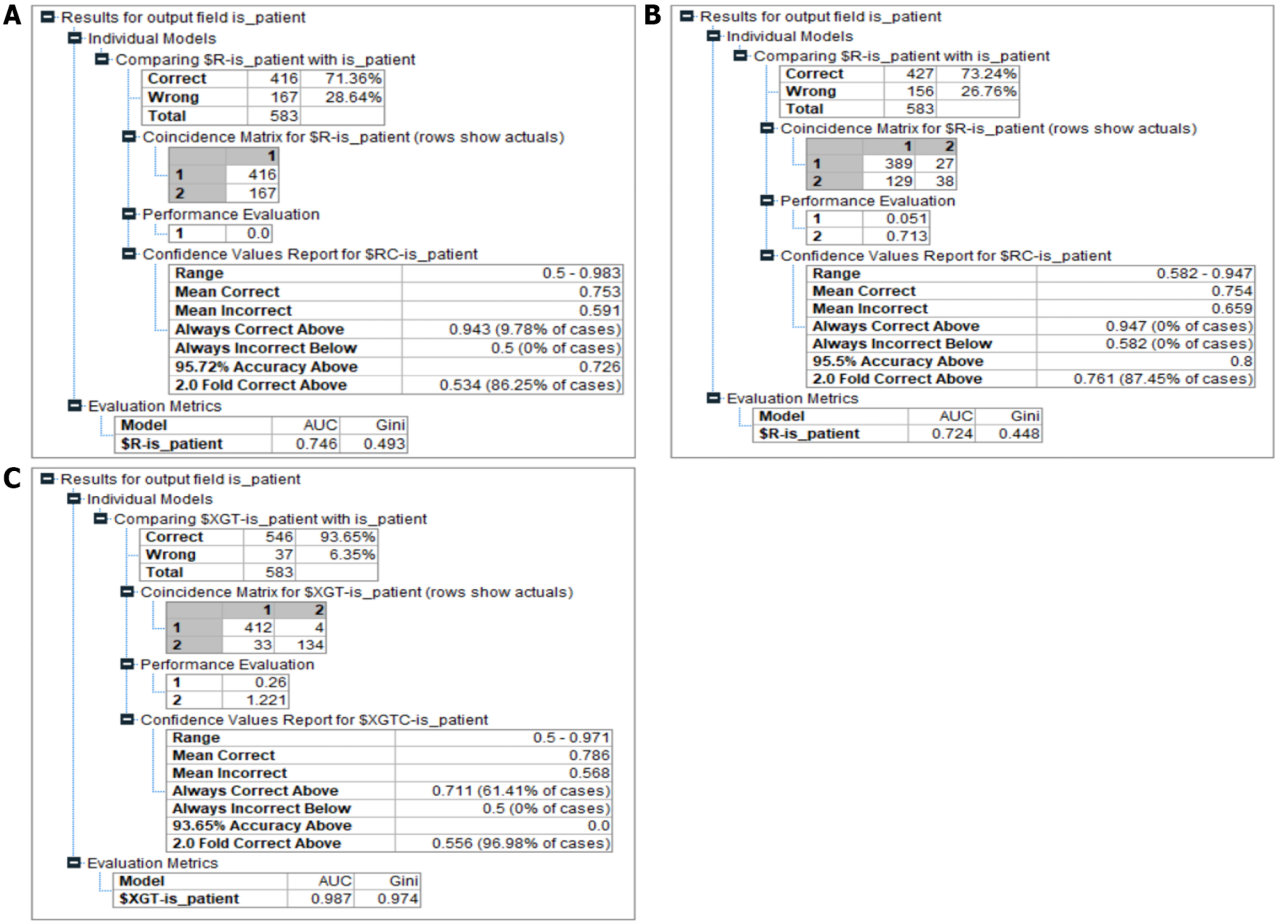
Our model had an accuracy of 93.65% in predicting liver disease, outperforming the other models significantly (Figure 8C). It also showed a Gini index of 0.97, categorizing it as a highly efficient model in making the distinction between a patient who has liver disease and a patient who is healthy in the given context (Table 2).

DISCUSSION

Artificial intelligence plays a very important role in predicting liver disease. It consists of different machine learning algorithms that may be applied in liver disease prediction. In this study, multiple machine learning algorithms were implemented on the abovementioned dataset of Indian liver patients. This work used the CART and CHAID algorithms to predict liver disease. If bilirubin, total protein, alkaline phosphatase, and albumin were present and tests such as serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) indicated that a person needs to be diagnosed, the results were stated through the various machine learning algorithms. The proposed algorithm was efficient in handling the missing values and overfitting problem. It was capable of handling high variance problems normally faced in execution of the machine learning algorithms.

CONCLUSION

The decision tree algorithms, *i.e.* CART and CHAID, were not extremely accurate for the prediction of liver disease in our Indian patient cohort. The proposed machine learning model (hybrid XGBoost model) designed in this study showed an accuracy level of 93.65%. The proposed model faced the



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Figure 8 Model results. A: Chi-square automated interaction detection (CHAID) results. The CHAID model had 71.36% accuracy in predicting liver disease. The area under the curve (AUC) and Gini values of this model were 0.746 and 0.493, respectively; B: Classification and regression tree results. The result for the classification and regression tree model showed a better accuracy at predicting the liver disease compared to the CHAID model at 73.24%. The AUC and Gini values of this model were 0.724 and 0.4448, respectively; C: Proposed model results. The model produced an accuracy of 93.65% in predicting liver disease, outperforming the other models significantly. It also recorded a Gini index of 0.97, categorizing it as a highly efficient model in making the distinction between a patient who has liver disease and a patient who is healthy in the given context.

overfitting issue during its execution phase. In future studies, the image dataset of liver disease should be added to the algorithm to avoid other tests such as SGOT and SGPT. Such approaches are very useful for minimizing the workload of clinicians.

ARTICLE HIGHLIGHTS

Research background

Liver disease is a leading cause of mortality in the United States and is regarded as a life-threatening condition across the world. It is possible for people to develop liver disease at a young age.

Research motivation

Predicting liver disease with precision, accuracy, and reliability can be accomplished through the use of a modified eXtreme Gradient Boosting model with hyperparameter tuning in comparison to the chi-square automated interaction detection (CHAID) and classification and regression tree models.

Research objectives

This study was conducted with the aim of fulfilling various objectives. The first objective was identifying the symptoms of liver disease and their impact on the patient. The authors studied various machine learning approaches for predicting liver disease and evaluated the performance of decision tree algorithms in prediction of liver disease. The next objective was to propose a modified eXtreme Gradient Boosting model with a hyperparameter tuning mechanism. Finally, the performance of the

proposed model was validated with the existing models.

Research methods

Hybrid eXtreme Gradient Boosting model with hyperparameter tuning was designed using data from patients who had liver disease and patients who were healthy.

Research results

The experimental results demonstrated that the accuracy level in the CHAID and classification and regression tree models were 71.36% and 73.24%, respectively. The proposed model was designed with the aim of gaining a sufficient level of accuracy. Hence, 93.65% accuracy was achieved in our proposed model.

Research conclusions

The existing machine learning models, *i.e.* the CHAID model and the classification and regression tree model, do not achieve a high enough accuracy level. The proposed model predicted liver disease with 93.65% accuracy. This model has real-time adaptability and cost-effectiveness in liver disease prediction.

Research perspectives

The proposed model can better predict liver-related disease by identifying the disease causes and suggesting better treatment options.

FOOTNOTES

Author contributions: Onyema EM contributed to the introduction, background, results, and analysis; Dalal S contributed to the design, methods, conclusion, and background; Malik A contributed to the discussion, data collection, and review of the final draft.

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Clinical trial registration statement: This letter is to confirm that the results are being generated on open access data for this study and does not involve any clinical trial.

Informed consent statement: The patients were not required to obtain informed consent for this study as the dataset is available on the open access Kaggle website.

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ORIGINAL ARTICLE

Clinical Trials Study

Diagnostic evaluation of endoscopic ultrasonography with submucosal saline injection for differentiating between T1a and T1b early gastric cancer

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Abstract

BACKGROUND

Endoscopic ultrasonography (EUS) has become a reliable method for predicting the invasion depth of early gastric cancer (EGC). However, diagnostic accuracy of EUS is affected by several factors. In particular, it is difficult to differentiate between T1a and T1b EGC through EUS.

AIM

To confirm whether submucosal saline injection (SSI) could improve the accuracy of EUS in distinguishing T1a and T1b lesions in EGC.

METHODS

Twenty-four patients with EGC were examined by EUS and subsequently by SSI combined EUS to compare the degree of tumor invasion. Then, they underwent endoscopic or surgical resection within 7 d. The diagnostic accuracy of EUS and SSI combined EUS was evaluated based on the final pathological findings postoperatively. Saline injected into the submucosa acted as an echoic contrast enhancing agent and had the effect of distinguishing the mucosal and submucosal layers clearly.

RESULTS

Of total 24 patients, 23 were diagnosed with EGC (T1 cancer: 13 as T1a, and 10 as T1b). Standard EUS identified 6 of 13 T1a cancer patients and 3 of 10 T1b cancer patients. Whereas, EUS-SSI identified 12 of 13 T1a cancer patients and 6 of 10 T1b cancer patients. In this study, SSI combined EUS was more accurate than EUS alone in diagnosing T1a and T1b lesions of EGC (75.0% and 37.5%, respectively).

CONCLUSION
SSI improved the diagnostic accuracy of EUS in distinguishing between the T1a and T1b stages in EGC.

Key Words: Endoscopic ultrasonography; Gastric cancer; Endoscopy; Surgery

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Core Tip: Submucosal saline injection improved the diagnostic accuracy of endoscopic ultrasonography in distinguishing between the T1a and T1b stages in early gastric cancer.

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INTRODUCTION

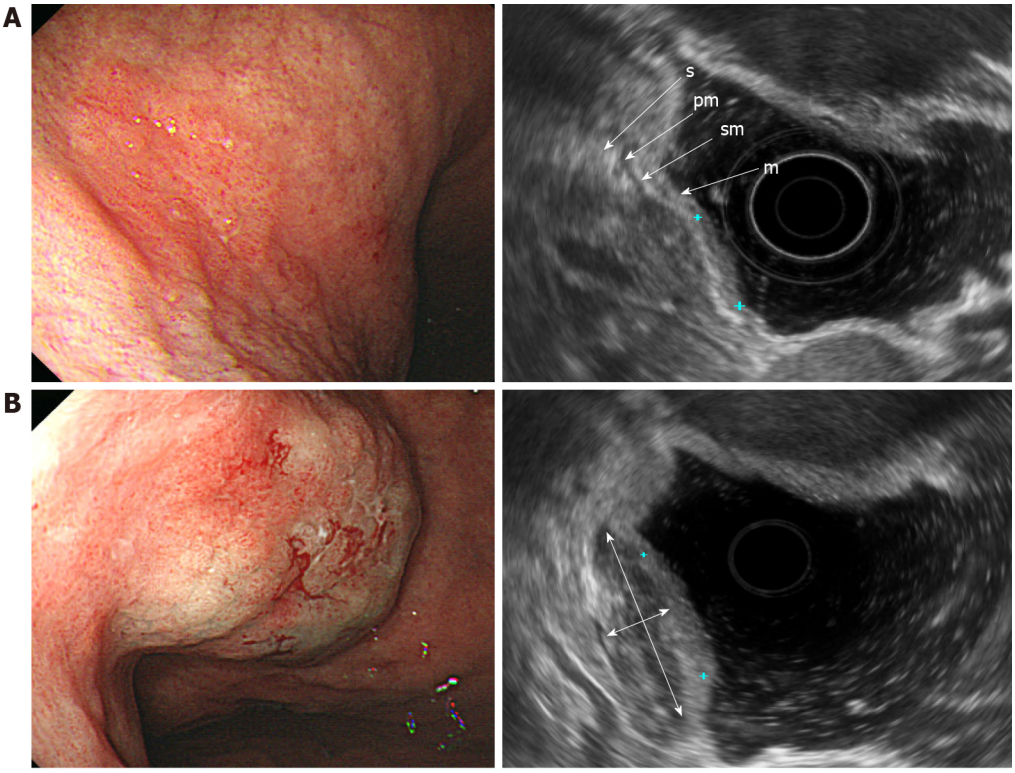
Early gastric cancer (EGC) is a malignant lesion confined to the mucosa or submucosa (SM), regardless of lymph-node metastasis[1,2]. Endoscopic submucosal dissection (ESD) is widely used to treat EGC, and the indications for ESD are expanded in the cases assumed to have a low risk of lymph-node metastasis[3-5]. Even if the pathological depth of invasion is T1b (tumor invading the SM), ESD can be performed if the invasion is confined to SM1 (submucosal invasion to < 500 μm from the muscularis mucosae)[6,7]. However, an additional surgery is recommended for EGC when deep submucosal invasion is identified by pathological evaluation after ESD (more than SM2; depth of submucosal invasion, ≥ 500 μm) owing to the risk of lymph-node metastasis[8]. Therefore, the depth of invasion (T-stage) of gastric cancer is vital for determining the treatment strategy[3-7]. Endoscopic ultrasonography (EUS) has been used for T-staging of gastric cancer[9,10]. Although previous studies showed the clinical efficacy of EUS in T-staging of gastric cancer, the results have revealed a wide level of variability[1,2,11]. The diagnostic accuracy may be affected by endoscopic findings, lesion location, tumor size, and the skill of the examiner[1,12]. Specifically, EUS is difficult to distinguish between T1a (tumor invading the lamina propria and muscularis mucosae) and T1b lesions because the boundary between the mucosa and submucosa is thin and the difference in echogenicity is unclear[1,10].

Submucosal saline injection (SSI) is routinely administered prior to ESD to prevent damage to the surrounding tissue of the gastric wall and to avoid perforation during ESD[13]. SSI creates a cushion within the loose connective tissues of the submucosa, which has been reported as an effective medium and echoic contrast-enhancing agent for ultrasound transmission, enabling good distinction between the mucosal and submucosal layers[13-15]. Moreover, saline can increase the thickness of the submucosa [13-15]. According to previous studies, SSI improved the performance of EUS in characterizing the invasion depth of esophageal and colorectal cancers[13-15]. Therefore, this study was conducted to confirm whether SSI could be a method to improve the accuracy of EUS in distinguishing T1a and T1b lesions even in EGC and determine the feasibility of EUS for beginners.

MATERIALS AND METHODS

Case series

Methods: During March–April 2019, 24 endoscopically diagnosed EGC lesions in 24 patients were examined by EUS. The macroscopic tumor classification was as follows: type I (protruded), type IIa (superficial elevated), type IIb (flat), type IIc (superficial depressed), and type III (excavated). Types I and IIa were classified as the elevated type, and IIb IIc, and III as the depressed type. All patients underwent standard EUS followed by EUS with SSI (EUS-SSI). EUS findings of T1a gastric cancers were defined as low-echoic lines of muscularis mucosae that were clearly demarcated from the submucosa, and T1b gastric cancers on EUS were defined as low-echoic line lesions that were not clearly distinguished from the boundary of submucosal layer. Subsequently, they underwent endoscopic or surgical resection within 7 d. Definitive classification was determined based on the postoperative pathology. All recruited patients agreed to be enrolled as participants in this clinical trial and were provided informed consent. This study was approved by the Institutional Review Board of the Inje University Sanggye Paik Hospital (SGPAIK2021-10-019).



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Figure 1 Endoscopic and ultrasonographic images and associated schematic diagrams of T1a early gastric cancer. A: Standard endoscopic ultrasonography (EUS) showing that it is difficult to differentiate the extent of invasion from the mucosal layer to the submucosal layer; B: EUS after submucosal saline injection showing clearly the boundary between the mucosa and the submucosa, meaning that the T1a stage can be easily identified. m: Mucosa; sm: Submucosa; pm: Proper muscle; s: Serosa; double arrow, saline layer.

EUS examination and staging were simultaneously conducted by one endoscopist with only 6 mo' experience with EUS. The examiner performed EUS with a 12-MHz ultrasonic probe (Olympus GF-UE260-AL5 Endoscopic System, Olympus Co., Tokyo, Japan). SSI was thereafter conducted as follows: after the lesion was confirmed by conventional endoscopy and subsequently by iodine dye-enhanced endoscopy, the examiner injected 3–5 mL saline slowly into the submucosa using a single-use 22G mucosal needle (Endo-Flex Co., Voerde, Germany). The puncture points were located 0.5 cm beyond the edge of the lesion, and saline injection was stopped once the gastric mucosa had been elevated by approximately 1 cm. After SSI, the examiner determined the depth of the lesion using EUS.

RESULTS

All patients showed good tolerance of EUS-SSI without severe adverse events, such as significant bleeding, asphyxia, perforation, or problems related to anesthetics.

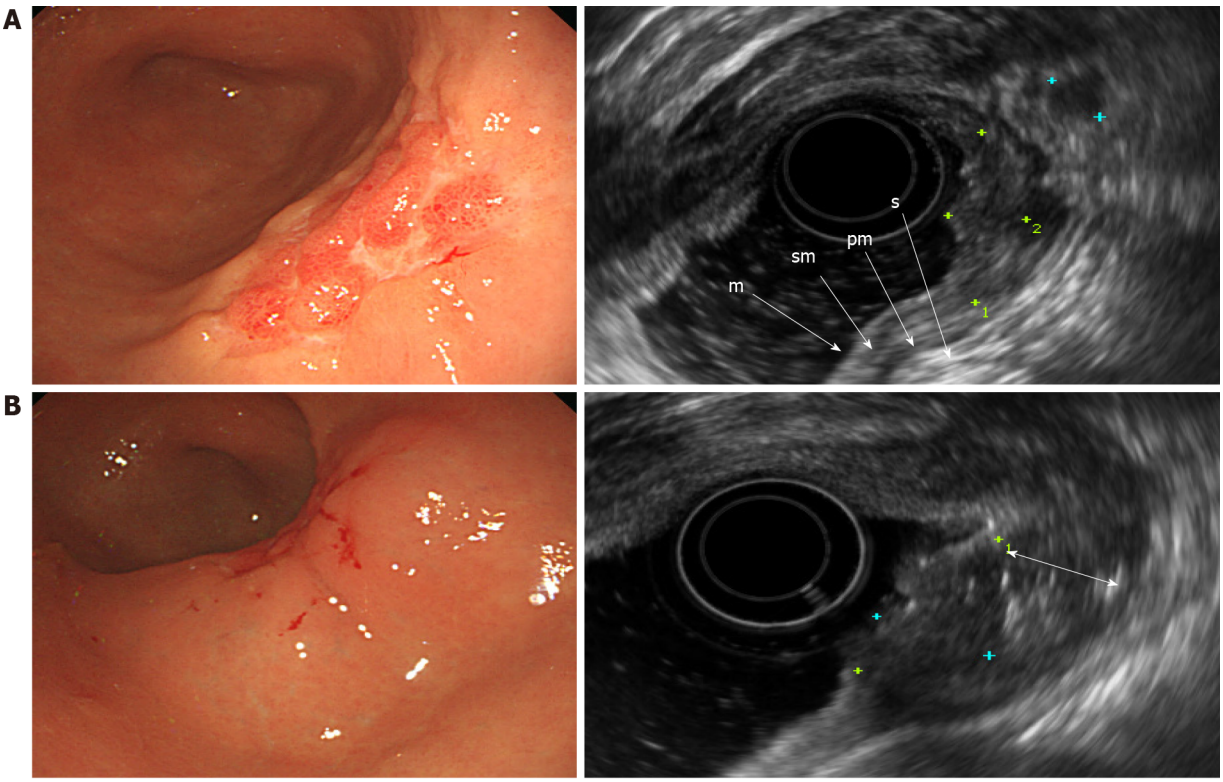
Of total 24 patients, 23 were diagnosed with EGC (T1 cancer: 13 as T1a, and 10 as T1b), except for one who was diagnosed with T2 cancer after the surgery. According to the macroscopic classification of tumors, there were 4 patients with elevated type lesions and 20 with depressed-type lesions. In 12 of the patients, the pathological T-stage was different between the standard EUS and EUS-SSI. Among them, EUS-SSI findings were consistent with the final pathological findings in 10 patients and standard EUS findings were consistent in one patient. The other patient was diagnosed with EGC stage-T2, which differed before and after the surgery (Table 1).

EGC was observed by using standard EUS as a localized thickening of the gastric mucosa or depression of the mucosal wall with a relatively low echogenicity. In patients with stage T1a disease, the muscularis mucosae was displayed as a low-echoic line between the mucosa and submucosa (Figure 1A). On the other hand, in patients with stage T1b, the muscularis mucosae was not clearly distinguished, and the boundary between the submucosal layer and the lower margin of the lesion was blurred, making it difficult to determine the degree of invasion of the submucosal layer on standard EUS (Figure 2A). After SSI, the mucosa had relatively enhanced echogenicity compared to the submucosa that was filled with saline. The boundary between the edge of the lesion and submucosa was apparent after SSI due to the saline-formed cushion in the submucosa (Figure 1B and 2B). Since the echoic difference between the lesion and the surrounding normal tissue became clear in EUS-SSI, the

Table 1 Clinical features, endoscopic ultrasonography findings before and after submucosal saline injection, and pathological results of 24 patients with early gastric cancer

Patient No.	Age	Sex	Location	Size (max, mm)	Endoscopic morphology (EGC type)	Ulcer	EUS-assessed preoperative stage		Final pathology	Type of resection	Differentiation	Regional LN invasion	Vascular invasion
							Before SSI (EUS-only)	After SSI (EUS-SSI)					
1	72	M	Lower third	20	0-III	Y	T1a	T1b	T1b (sm3)	Surgery	Mod		
2	64	M	Lower third	20	0-IIc		T1b	T1b	T1b (sm1)	Surgery	Poor (signet ring)	Y	
3	59	M	Lower third	10	0-IIc		T1b	T1b	T1b (sm1)	Surgery	Poor		
4	53	M	Upper third	27	0-IIa		T1a	T1a	T1a	Surgery	Mod		
5	56	F	Lower third	38	0-IIc		T1b	T1a	T1a	Surgery	Poor		
6	73	M	Upper third	22	0-IIc		T1b	T1a	T1a	Surgery	Mod		
7	62	M	Mid third	65	0-IIc		T1b	T1a	T1a	Surgery	Mod		
8	68	M	Upper third	8	0-IIb		T1a	T1a	T1a	ESD	Well		
9	69	M	Lower third	15	0-IIa		T1a	T1a	T1a	ESD	Well		
10	71	M	Lower third	17	0-IIb		T1b	T1a	T1a	ESD	Poor		
11	54	F	Lower third	25	0-IIc		T1b	T1a	T1a	Surgery	Poor (signet ring)		
12	82	M	Upper third	15	0-IIb		T1a	T1a	T1b (sm1)	ESD	Poor		
13	71	F	Lower third	25	0-IIc		T1b	T1b	T1b (sm3)	Surgery	Poor	Y	Y
14	36	F	Lower third	20	0-IIc		T1a	T1b	T1b (sm3)	Surgery	Poor	Y	
15	60	M	Lower third	10	0-IIc		T1a	T1a	T1a	ESD	Mod		
16	62	F	Mid third	50	0-IIc		T2	T1b	T1b (sm3)	Surgery	Poor		
17	74	F	Upper third	25	0-IIb		T1a	T1a	T1b (sm1)	Surgery	Poor		
18	60	F	Upper third	15	0-IIa		T1a	T1b	T2	Surgery	Poor (signet ring)		
19	80	F	Lower third	15	0-IIb		T1a	T1b	T1a	ESD	Poor		
20	48	F	Lower third	45	0-IIc		T1a	T1a	T1b (sm3)	Surgery	Poor		
21	72	F	Lower third	10	0-IIc		T1a	T1a	T1b (sm1)	ESD	Mod		
22	50	M	Lower third	27	0-IIb		T1a	T1a	T1a	ESD	Well		
23	74	M	Upper third	15	0-IIc		T1b	T1a	T1a	Surgery	Mod		
24	76	M	Upper third	23	0-Is		T1b	T1a	T1a	Surgery	Well		

EGC: Early gastric cancer; EUS: Endoscopic ultrasonography; SSI: Submucosal saline injection.



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Figure 2 Endoscopic and ultrasonographic images and associated schematic diagrams of T1b early gastric cancer. A: Standard endoscopic ultrasonography (EUS) showing that it is difficult to differentiate the extent of invasion from the mucosal layer to the submucosal layer; B: EUS after submucosal saline injection (SSI) showing clearly the boundary between the mucosa and the submucosa, meaning that the lesion, its infiltration depth into the mucosa, and the submucosa can be easily identified. m: Mucosa; sm: Submucosa; pm: Proper muscle; s: Serosa; double arrow, saline layer.

extent of tumor invasion was more distinct than that demonstrated by standard EUS (Figure 3).

Standard EUS identified 6 of 13 T1a cancer patients and 3 of 10 T1b cancer patients. Whereas, EUS-SSI identified 12 of 13 T1a cancer patients and 6 of 10 T1b cancer patients. The diagnostic accuracies of the standard EUS and EUS-SSI are shown in Table 2 (37.5% and 75.0%, respectively).

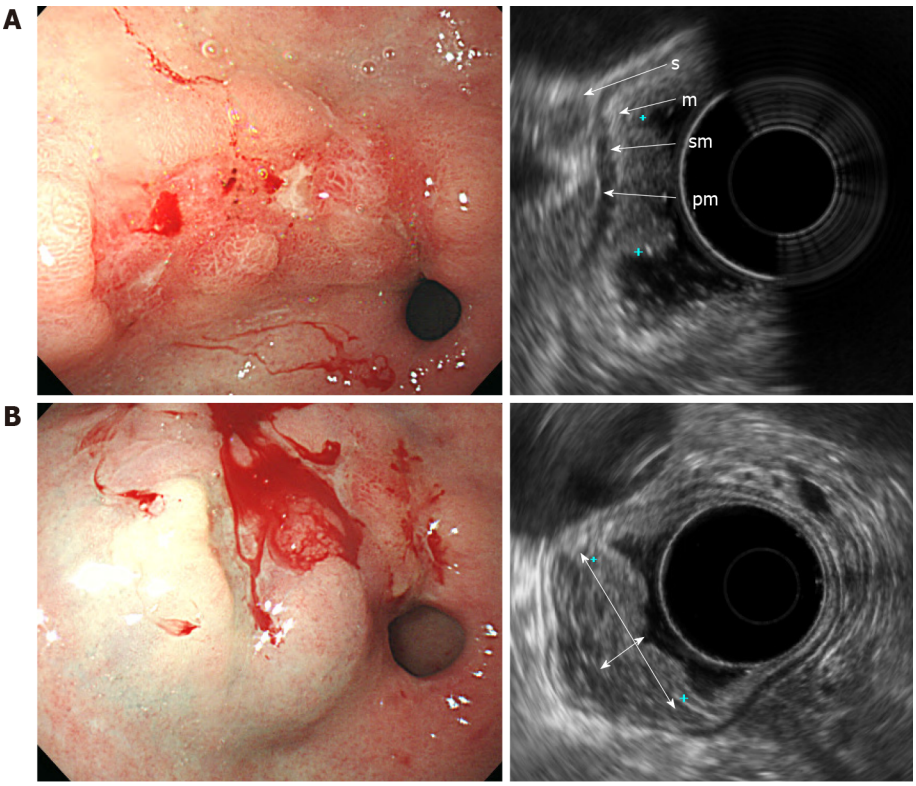
DISCUSSION

EUS accurately characterizes the locoregional stage of gastric cancer and although the diagnostic accuracy of EUS in evaluating the invasion of depth of EGC has been reported, the results lack a consensus and have varying accuracy rates of 64.8%-92%[9-11]. Several studies also concluded that EUS has no significant advantage over conventional endoscopy in predicting the invasion depth[16]. Hence, it has been clarified that the accuracy of EUS can vary greatly depending on the experience of the endoscopist, macroscopic type of tumor, presence of ulceration, tumor located in the stomach, tumor size, and differentiation type[1,9,10,12]. Regarding ulcerative lesions, submucosal fibrosis occurs, which is observed on EUS as a hypoechoic lesion, similar to tumor invasion[2,10,16]. For lesions in the upper third of the stomach, the accuracy of EUS may decrease because of the different thicknesses of the stomach layer and presence of fibrosis or blood vessels surrounding the tumor[10,16]. In addition, it is difficult to fill the deaerated water and locate the EUS probe near the lesion because of the angulation of the EUS scope[10,16]. Previous studies have reported that a large tumor size is a risk factor for misdiagnosing the depth of invasion[10]. This is probably because the lesions might not extend even if the deaerated water is stored in cases of large tumors[11]. Undifferentiated-type tumors might have a diffuse or vesicular invasion of tumor cells to the submucosal layer of the gastric wall compared to differentiated-type tumors[11]. Thus, EUS cannot visualize these microinvasions and might underestimate the depth of invasion[11]. In our study, reviewing 15 patients with different results between final pathology and EUS-only findings, all patients had tumors located in the upper third of the stomach, sized ≥ 2 cm, ulcerative lesions, or undifferentiated type.

Table 2 Preoperative and postoperative stages for early gastric cancer in the endoscopic ultrasonography after submucosal saline injection and endoscopic ultrasonography -only examinations

Preoperative EUS reported stage	Postoperative pathologic stage	
	T1a	T1b
EUS-SSI, *n* (%)		
T1a	12 (92.3)	4 (40)
T1b	1 (7.7)	6 (60)
EUS-only, *n* (%)		
T1a	6 (46.2)	6 (66.7)
T1b	7 (53.8)	3 (33.3)

EUS: Endoscopic ultrasonography; SSI: Submucosal saline injection.



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Figure 3 Endoscopic and ultrasonographic images and associated schematic diagrams of T1a early gastric cancer. A: With the use of standard endoscopic ultrasonography (EUS), the boundary between the lesion and the submucosal layer was unclear. The distance between the mucosa and the submucosa was short. This lesion was diagnosed as T1b as it appeared to partially invade the submucosa when observed with standard EUS; B: With the use of endoscopic ultrasonography after submucosal saline injection (EUS-SSI), the boundary between the lesion and the submucosal layer was apparent. It was much easier to determine whether the lesion had invaded the submucosal layer due to the increased thickness of the gastric wall and an effect of increasing echoic contrast by saline cushion. This lesion was diagnosed as T1a as the submucosal layer was intact when observed with EUS-SSI. m: Mucosa; sm: Submucosa; pm: Proper muscle; s: Serosa; double arrow, saline layer.

Regardless of the tumor characteristics, the diagnostic accuracy of EUS in predicting the T-stage of EGC in this study was 37.5%, which is low compared to that reported in previous studies. This study was conducted by a beginner endoscopist with approximately 6 mo' experience. To increase the diagnostic accuracy of EUS for staging of gastric cancer, an endoscopist with a high experience and proficiency is required, but some techniques are also required for the classification of EGC. EUS may overestimate the depth of invasion due to underlying inflammation or fibrosis[10,11,16]. EUS-SSI showed improved results in reducing the overestimation and overall diagnostic accuracy (Table 3). By reducing over-staging, an unnecessary surgery can be avoided, surgery-related adverse events can be prevented, the recovery period can be further shortened, and the patient's quality of life can be

Table 3 The misdiagnosis rate for T staging of early gastric cancer in the endoscopic ultrasonography after submucosal saline injection and endoscopic ultrasonography -only examinations

	EUS-only	EUS-SSI
Overstaging, *n* (%)	8 (33.3)	1 (4.2)
Understaging, *n* (%)	7 (29.2)	5 (20.8)

EUS: Endoscopic ultrasonography; SSI: Submucosal saline injection.

improved.

As limitations, we noted that EUS-SSI required a longer examination time than EUS-only, which may cause more patient discomfort. However, the patients in this study did not complain of discomfort and did not develop any adverse events related to SSI.

In our study, SSI improved the diagnostic accuracy of EUS in distinguishing between the T1a and T1b stages in EGC. This is probably because the saline injected into the submucosa serves as an echoic contrast-enhancing agent for the clear visualization of the boundary between the mucosa and the submucosa. However, our study is a clinical study conducted at a single institution, and the sample size is small, so there is a limit to interpreting the results. Therefore, a large-scale, prospective, randomized clinical trials for this are needed in the future. In particular, we suggest that beginners who are beginning EUS should try the EUS-SSI method when evaluating the depth of invasion of gastric cancer.

CONCLUSION

SSI improved the diagnostic accuracy of EUS in distinguishing between the T1a and T1b stages in EGC in this study. However, this needs to be confirmed in large-scale, prospective, randomized clinical trials in the future.

ARTICLE HIGHLIGHTS

Research background

Although endoscopic ultrasound (EUS) is a method to predict the depth of invasion in early gastric cancer (EGC), it is still difficult to differentiate between T1a and T1b EGCs *via* EUS.

Research motivation

In particular, we considered a method to increase the accuracy of diagnosis for endoscopists who are beginning to perform EUS. It was thought that submucosal saline injection (SSI) during endoscopic mucosal resection may be helpful for examination because it can expand the submucosal layer.

Research objectives

The objectives of this study was to confirm whether SSI could be a method to improve the accuracy of EUS in distinguishing T1a and T1b lesions even in EGC and determine the feasibility of EUS for beginners.

Research methods

During March-April 2019, 24 endoscopically diagnosed EGC lesions in 24 patients were examined by EUS. All patients underwent standard EUS followed by EUS with SSI (EUS-SSI). Thereafter, endoscopic or surgical resection was performed within 7 days. T1a and T1b lesions were diagnosed based on the final pathology results after treatment. The diagnostic accuracy of EUS and EUS-SSI for T stage was compared.

Research results

Standard EUS identified 6 of 13 T1a cancer patients and 3 of 10 T1b cancer patients. Whereas, EUS-SSI identified 12 of 13 T1a cancer patients and 6 of 10 T1b cancer patients. In this study, SSI combined EUS was more accurate than EUS alone in diagnosing T1a and T1b lesions of EGC (75.0% and 37.5%, respectively).

Research conclusions

SSI improved the diagnostic accuracy of EUS in distinguishing between the T1a and T1b stages in EGC

in this study. However, this needs to be confirmed in large-scale, prospective, randomized clinical trials in the future.

Research perspectives

In our study, SSI improved the diagnostic accuracy of EUS in distinguishing between the T1a and T1b stages in EGC. In particular, we suggest that beginners who are beginning EUS should try the EUS-SSI method when evaluating the depth of invasion of gastric cancer. However, our study is a clinical study conducted at a single institution, and the sample size is small. Therefore, a large-scale, prospective, randomized clinical trials for this are needed in the future.

FOOTNOTES

Author contributions: Jeon TJ and Park JY contributed to conception and design; Park JY contributed to acquisition of data (acquired and managed patients); Jeon TJ contributed to development of methodology; Jeon TJ and Park JY contributed to analysis and interpretation of data; Park JY contributed to writing and review of manuscript; Park JY and Jeon TJ contributed to study supervision.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the Inje University Sanggye Paik Hospital (SGPAIK2021-10-019).

Clinical trial registration statement: This study is registered at "Clinical Research Information Service (https://cris.nih.go.kr)". The registration identification number is KCT0007919.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: Dr Ji Young Park and Tae Joo Jeon declare that they have no conflicts of interest.

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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ORIGINAL ARTICLE

Randomized Controlled Trial

Safety and efficacy of purified clinoptilolite-tuff treatment in patients with irritable bowel syndrome with diarrhea: Randomized controlled trial

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Abstract

BACKGROUND

Irritable bowel syndrome (IBS) is a highly prevalent gastrointestinal disorder with poor response to treatment. IBS with predominant diarrhea (IBS-D) is accompanied by abdominal pain as well as high stool frequency and urgency. Purified clinoptilolite-tuff (PCT), which is approved by the Food and Drug Administration for use as a dietary supplement with the brand name G-PUR®, has previously shown therapeutic potential in other indications based on its physical adsorption capacity.

AIM

To assess whether symptoms of IBS-D can be ameliorated by oral treatment with PCT.

METHODS

In this randomized, placebo-controlled, double-blind pilot study, 30 patients with

IBS-D diagnosis based on Rome IV criteria were enrolled. Following a 4-wk run-in phase, 14 patients were randomized to receive a 12-wk treatment with G-PUR® (2 g three times daily), and 16 patients received placebo. The relief from IBS-D symptoms as measured by the proportion of responders according to the Subject's Global Assessment (SGA) of Relief was assessed as the primary outcome. For the secondary outcomes, validated IBS-D associated symptom questionnaires, exploratory biomarkers and microbiome data were collected.

RESULTS

The proportions of SGA of Relief responders after 12 wk were comparable in both groups, namely 21% in the G-PUR® group and 25% in the placebo group. After 4 wk of treatment, 36% of patients in the G-PUR® group *vs* 0% in the placebo group reported complete or considerable relief. An improvement in daily abdominal pain was noted in 94% *vs* 83% (P = 0.0353), and the median number of days with diarrhea *per* week decreased by 2.4 d *vs* 0.3 d in the G-PUR® and placebo groups, respectively. Positive trends were observed for 50% of responders in the Bristol Stool Form Scale. Positive trends were also noted for combined abdominal pain and stool consistency response and the Perceived Stress Questionnaire score. Only 64% in the G-PUR® group compared to 86% in the placebo group required rescue medication intake during the study. Stool microbiome studies showed a minor increase in diversity in the G-PUR® group but not in the placebo group. No PCT-related serious adverse events were reported.

CONCLUSION

In this randomized, double-blind, placebo-controlled study, the PCT product, G-PUR®, demonstrated safety and clinical benefit towards some symptoms of IBS-D, representing a promising novel treatment option for these patients.

Key Words: Irritable bowel syndrome; Diarrhea; Functional gastrointestinal disorder; Clinoptilolite; Zeolite; Treatment

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Core Tip: The purified clinoptilolite-tuff (PCT) product, G-PUR®, provided improvement in abdominal symptoms and stool abnormalities in patients with irritable bowel syndrome (IBS) with predominant diarrhea. Additionally, it reduced the use of rescue medication and tended to enrich gut microbiome diversity compared to placebo, while showing no safety concerns. Hence, the PCT product, G-PUR®, represents a promising novel treatment option for patients with IBS with predominant diarrhea.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic gastrointestinal disorder of high global prevalence with a substantial impact on quality of life and is associated with abdominal pain and altered bowel habits. According to the Rome IV criteria, IBS is characterized by recurrent abdominalgia occurring at least once a week over a period of 3 mo, together with at least two of the following criteria: Pain related to defecation; change in stool frequency; or change in form/appearance of stool, with the onset of symptoms at least 6 mo prior to diagnosis. IBS can be divided into IBS subgroups with predominant constipation, diarrhea (IBS-D), mixed bowel habits, or unclassified[1,2]. The prevalence of IBS reported in the literature ranges between 1%-45%, reflecting the geographical and methodological variety. IBS affects at least twice as many women as men, occurring in women primarily in the late teenage years to the mid-40s[3].

While no underlying morphologic correlation has yet been identified in IBS, the multifactorial biopsychosocial model postulates a contribution of genetic predisposition, history of gastrointestinal infections with possible increase in gut permeability and activation of the immune system, malabsorption of bile acids, neuropsychological disorders and the bidirectional brain-gut axis[4-6]. The therapeutic approach is empirical and aims to reduce symptoms of IBS.

Several natural mineral adsorbents, *e.g.*, dioctahedral smectite, spherical carbon, bentonite or zeolites, have been investigated in patients with IBS and have shown variable degrees of effectiveness in alleviating symptoms[7-11]. Clinoptilolite, a commonly found mineral from the group of natural zeolites, is characterized by high ad/absorptive capacity, the ability to support ion exchange and to act as a molecular sieve, owing to its microporous crystalline structure with multiple microcavities[12,13]. A number of clinical studies have evaluated the potential of clinoptilolite-based products in relieving symptoms of gastric discomfort, such as diarrhea[14], gastric hyperacidity[15,16], or veisalgia[17]. Furthermore, clinoptilolite has shown benefits in enhancing the intestinal wall integrity as indicated by decreased concentrations of the tight-junction modulator zonulin, reduction of inflammation-associated markers, such as α1-antitrypsin and high-sensitivity C-reactive protein and a trend towards beneficial microbiome changes[10,18].

In a pilot study with clinoptilolite in IBS patients, significant decreases in clinical parameters such as pain, distension or bowel habits were reported. Despite the pronounced placebo effect seen in this study, clinoptilolite showed a trend towards an augmented clinical benefit[19]. Based on these encouraging results, we aimed to determine the clinical effectiveness and safety of purified clinoptilolite-tuff (PCT) product G-PUR® in patients with IBS-D symptoms in a randomized controlled trial.

MATERIALS AND METHODS

Trial design

We conducted a randomized, placebo-controlled, double-blind, parallel-arm study to evaluate the safety and clinical efficacy of a 12-wk oral treatment with G-PUR® (2 g three times daily) or matching placebo in a cohort of 30 patients with IBS-D diagnosis based on Rome IV criteria[2]. Glock Health, Science and Research GmbH acted as sponsor of this multicenter study and was responsible for the design and report of this study. The study protocol was approved by the Ethics Committee of the Medical University of Vienna (No. 1295/2019), Ethics Committee of Upper Austria (No. 1208/2019) and the Austrian Federal Office for Safety in Health Care (BASG) and is registered at clinicaltrials.gov (NCT04138186). This study was conducted at the Department of Clinical Pharmacology at the Medical University of Vienna and Klinikum Wels-Grieskirchen, Department of Internal Medicine in accordance with the Declaration of Helsinki and the ISO 14155 guideline. All study participants provided written informed consent before any study-specific procedures were performed. The clinical investigation was initiated in September 2019 and completed in February 2021.

Patient population

Eligible patients had to be between 18-75 years of age and fulfill the Rome IV criteria for IBS-D, *i.e.* more than 25% of the bowel movements graded as the Bristol Stool Form Scale (BSFS) type 6 or 7 (equivalent to mild/severe diarrhea) and less than 25% of bowel movements graded as type 1 or 2 (equivalent to mild/severe constipation)[20]. Moreover, patients had to have moderate to severe abdominal pain as defined by an IBS Symptoms Severity Scale (IBS-SSS) score > 175 and stable eating habits 1 mo prior to randomization. Patients > 50 years of age had to have an unremarkable colonoscopy within the preceding 5 years. Patients were excluded if they failed to record > 50% of daily diary entries during the screening/run-in phase, had rectal bleeding in the absence of documented bleeding hemorrhoids or anal fissures, had a history of major gastrointestinal surgery, had been or were being treated with antibiotic medicines, including rifaximin, or used pro- or antikinetic agents (except as a rescue medication, see below), tricyclic antidepressants or immunosuppressive therapy. Patients with a history of positive tests for ova, parasites, or *Clostridioides difficile* infection had to undergo repeated testing with negative results during the screening/run-in phase.

Investigational medical device

G-PUR® is a PCT, prepared from a high-grade raw material with low heavy metal content, sourced from a mine in the eastern Slovak Republic[21,22] and has been marketed in the United States as a dietary supplement since 2016. G-PUR® has been successfully applied in different therapeutic indications[23,24]. The patented purification process is technically based on ion exchange mechanisms of the clinoptilolite mineral, micronization and terminal heating, which results in the removal of all natural impurities and a homogeneous, very fine-grained particle size[25]. The production process is thoroughly quality-assured, meeting all required regulatory standards. The purified product has been evaluated by independent laboratories and conforms with the safety requirements for human consumption. Besides the very high clinoptilolite content in the purified product (> 75%), other mineral phases like cristobalite, feldspars, accessory biotite and quartz are contents of G-PUR®. The almost completely inert product is characterized by a high adsorption capacity for a variety of toxins, heavy metals and other undesirable substances[26-28]. G-PUR® is not absorbed or metabolized in the gastrointestinal tract and therefore excreted unchanged *via* stool. The therapeutic potential is based on the physical adsorption capacity of G-PUR®[23,24].

For this clinical investigation, G-PUR® was provided in hard-gelatin Capsugel® AAA capsules, each containing 400 mg of PCT. Colloidal silicon dioxide (Aerosil®) was added as an excipient at a concentration of 0.5% to enhance flowability and enable accurate filling of the capsules. Placebo was provided in identical-looking capsules, each containing 400 mg maltodextrin and 0.5% magnesium stearate as excipient.

Randomization and blinding

Randomization was performed by Assign Data Management and Biostatistics GmbH (Innsbruck, Austria). Based on the randomization list, blinding was performed centrally by NUVISAN Pharma Services. The randomization list was prepared by computer software (random permuted blocks with confidential block size) using a 1:1 ratio. One sealed randomization list was stored at Assign Data Management and Biostatistics and was not to be opened until after database lock. Patients fulfilling all eligibility criteria as confirmed at visit 3 were enrolled into the clinical investigation and randomized. Enrolled patients were allocated to the next highest randomization number (medication kit identification number). Investigational site staff, including the investigator, and patients were blinded to treatment allocation.

Trial procedures

Eligible patients underwent a 4-wk screening/run-in phase, followed by a 12-wk double-blind treatment phase and a 2-wk withdrawal phase. The total study duration *per* patient was 18–21 wk and included seven study visits. Patients also had to complete at least 14 d of baseline diary entries over the 28-d screening/run-in phase. Throughout the double-blind 12-wk treatment phase and the 2-wk withdrawal phase, a limited use of loperamide and hyoscine butylbromide was allowed as rescue medication. Following the run-in phase, patients were randomized to receive five G-PUR® capsules or identical-looking placebo capsules, three times a day before meals with a minimum of 200 mL tap water. The total daily dose of PCT in the treatment arm was 6 g. Due to the high adsorption capacity and the possible risk of interaction between the PCT product G-PUR® and other medicines, participants were instructed to allow for a minimum 2-h window between G-PUR® and other oral medication intake. Throughout the entire study duration, patients were supported by health care professionals and psychological therapists.

According to the guidelines by the European Medicines Agency (EMA)[29] and the Food and Drug Administration (FDA)[30], abdominal pain based on an 11-point numerical rating scale as well as stool consistency according to the BSFS should be assessed daily to determine the effect of medicine treatment in patients with IBS-D. Accordingly, patients kept daily diaries of abdominal pain, bloating, urgency, stress, stool frequency, stool consistency, treatment adherence and use of any concomitant or rescue medications by using an electronic patient-reported outcome (ePRO) system throughout the study. At the end of the treatment phase, *i.e.* at visit 6, ePRO diary usability was evaluated.

Outcomes

The primary objective of this clinical investigation was to assess the relief from IBS-D symptoms after a 12-wk treatment with G-PUR® (2 g three times daily) *vs* placebo as measured by the Subject's Global Assessment (SGA) of Relief questionnaire[31]. The secondary objectives were to assess the safety and tolerability of treatment with G-PUR®, impact of treatment on additional IBS-related symptoms, bowel habits, health-related quality of life, psychological status (*i.e.* anxiety, depression, and stress), patient satisfaction with the ePRO diary and additional exploratory parameters including microbiome analysis.

The primary endpoint of this clinical investigation was the proportion of responders according to the SGA of Relief[31] using the last four post-randomization assessments in the treatment period, or if fewer than four post-randomization SGAs were available, then on all post-randomization SGA of Relief questionnaires. Patients were considered responders if they answered "considerably relieved" or "completely relieved" at least 50% of the time or at least "somewhat relieved" 100% of the time.

The following variables were assessed as secondary endpoints: (1) SGA of Relief estimated by time point, absolute change in SGA of Relief compared to baseline and relationship between SGA of Relief by treatment groups in a logistic regression model with IBS-SSS, Hospital Anxiety and Depression Scale (HADS), pain intensity, stool frequency and stool consistency at visit 3 as independent variables; (2) Incidence of (serious) adverse events (AEs); (3) Daily intensity of abdominal pain using an 11-point numerical rating scale (NRS), including the proportion of abdominal pain responders, with abdominal pain response defined as an at least 30% improvement in abdominal pain on at least 50% of days with available ePRO diary entries compared to the patient's worst abdominal pain reported in the screening/run-in phase (baseline abdominal pain), additional analyses of abdominal pain response, defined as an at least (a) 40%, 50% or 60% improvement in abdominal pain on at least 50% of days; (b) 30%, 40%, 50% or 60% improvement in abdominal pain on at least 40% of days; or (c) 30%, 40%, 50% or 60% improvement in abdominal pain on at least 30% of days, with available ePRO entries compared to the patient's worst baseline abdominal pain or proportion of days with an at least 30% improvement in abdominal pain compared to the patient's worst baseline abdominal pain; (4) Daily intensity of bloating using an 11-point NRS; (5) Daily intensity of urgency using an 11-point NRS; (6) Daily stress level using

an 11-point NRS; (7) Daily stool frequency; (8) Daily stool consistency using the BSFS[32], including the proportion of BSFS responders, with BSFS response defined as an at least 50% reduction in the number of days *per* week with at least one BSFS type 6 or 7 stool ('diarrhea') compared to the screening/run-in phase ('baseline diarrhea'), daily proportion of patients with BSFS type 6 or 7 (*i.e.* diarrhea) and number of days *per* week with diarrhea; (9) Proportion of patients with a combined abdominal pain and BSFS response; (10) Patient compliance with daily ePRO diary reporting; (11) ePRO diary usability; (12) Gastrointestinal symptoms using the IBS-SSS[33] during each study visit assessing the proportion of patients with a ≥ 50% reduction in the IBS-SSS score and number of pain-free days using the IBS-SSS; (13) Quality of life using the 12-item Short Form Survey (commonly referred to as SF-12)[34]; (14) Anxiety and depression using the HADS[35]; (15) Stress response using the Perceived Stress Questionnaire (PSQ)[36]; (16) Use of rescue medication; (17) Visual analog scale of Treatment Expectation and Relief[37]; (18) Freiburger Ernährungsfragebogen[38]; and (19) Global Physical Activity Questionnaire[39].

The following variables were assessed as exploratory endpoints before and after 12 wk of treatment: (1) Indoleamine-2,3-dioxygenase (IDO)[40] and zonulin[41] in capillary blood; (2) Bile acid[42], human beta defensin 2 (HBD2)[43], gluten[44] and zonulin[45] in stool; and (3) Microbiome in stool (next-generation sequencing; myBioma GmbH, Vienna, Austria).

Safety analysis included AEs and adverse device effects (ADEs), device deficiency, laboratory results and aluminum levels.

Statistical analysis

No formal sample size calculation was performed owing to the exploratory pilot study design. Overall, 30 patients were randomized to treatment with either G-PUR® or placebo. Analyses were performed using Statistical Analysis Software 9.3 or higher (SAS Inc., Cary, NC, United States). The Full Analysis Set analysis was provided in this publication including data from all patients who were randomized. Patients were analyzed in the treatment group they were randomized to, regardless of the actual treatment received. For the inferential analysis of the primary endpoint, the rate of responders according to SGA was compared in an intention-to-treat analysis between the two treatment groups using Fisher's exact test. A two-sided significance level of 5% was applied. For all secondary efficacy endpoints, categorical variables were compared between treatment groups using Fisher's exact test. Continuous variables were compared using a Wilcoxon rank-sum test (Mann-Whitney *U* test). In general, the last available post-randomization assessment during the treatment period was used to compare secondary efficacy parameters between treatment groups. Data were summarized by treatment group and, where appropriate, by visit. Descriptive statistics (number of observations, mean, standard deviation, minimum, median and maximum) were provided for continuous variables. Frequency counts and percentages were presented for categorical variables. Five patients who were withdrawn from the study prematurely were not included in this assessment of adherence and efficacy. Logistic regression analysis for parameters SGA of Relief and IBS-SSS, HADS, NRS and stool frequency and consistency included only patients with available results for all relevant independent variables (n = 27).

RESULTS

Patient population

We screened 44 patients in order to include 30 trial participants, of whom 14 were randomized into the G-PUR® arm and 16 into the placebo arm (Figure 1). The two study groups were similar regarding their demographic and baseline characteristics, but patients in the G-PUR® group had a slightly longer disease duration. The median age of the study population was 34 years (range: 20–73), and 21 of the 30 patients (70%) were women. Baseline patient demographics and concomitant medications are summarized in Table 1 and medical history in Table 2. At baseline, analgesic and antipyretic medications were used by 2 patients (14%) in the G-PUR® arm and 8 patients (50%) in the placebo arm; the use of medicines for functional gastrointestinal disorder was comparable in both groups.

Primary outcome

After 12 wk of treatment the proportion of responders according to the SGA of Relief was 21% (n = 3) in the G-PUR® group and 25% (n = 4) in the placebo group (P = 1.0; Table 3).

Secondary outcomes

A between-group difference in SGA of Relief was obvious after 4 wk of treatment, with 36% of patients in the G-PUR® group reporting complete or considerable relief, compared to 0% in the placebo group (Figure 2). Consistent with this observation, the median proportion of days with an at least 30% improvement in abdominal pain compared to the patient's worst abdominal pain at baseline was higher in the G-PUR® group (94%) than in the placebo group (83%, P = 0.0353). This was also seen for abdominal pain response, where 93% of patients (n = 13) in the G-PUR® group were classified as

Table 1 Demographic characteristics and concomitant medications at baseline

Characteristics and medications		G-PUR®, *n* = 14	Placebo, *n* = 16	Total, *n* = 30
Age, yr		34 (range: 24–61)	35 (range: 20–73)	34 (range: 20–73)
Sex	Male	5 (35%)	4 (25%)	9 (30%)
	Female	9 (64%)	12 (75%)	21 (70%)
Duration of IBS symptoms, yr		12 (IQR: 6–19)	9 (IQR: 6–21)	11 (IQR: 6–20)
Duration of IBS diagnosis, yr		8 (IQR: 3–11)	2 (IQR: 1–11)	4 (IQR: 1–11)
BMI, kg/m^2		21 (IQR: 20–25)	24 (IQR: 21–26)	23 (IQR: 20–26)
Smoking status	Non-smoker	9 (64%)	11 (69%)	20 (67%)
	Ex-smoker	3 (21%)	4 (25%)	7 (23%)
	Smoker	2 (14%)	1 (6%)	3 (10%)
Concomitant medications				
Any concomitant medication		12 (86%)	15 (94%)	27 (90%)
Analgesics		2 (14%)	8 (50%)	10 (33%)
Drugs for functional gastrointestinal disorders		4 (29%)	5 (31%)	9 (30%)
Selective serotonin reuptake inhibitors		4 (29%)	4 (25%)	8 (27%)
Systemic antihistamines		5 (36%)	1 (6%)	6 (20%)
Loperamide PRN		2 (14%)	2 (13%)	4 (13%)
Antacids		1 (7%)	1 (6%)	2 (7%)
Hypnotics, sedatives, antipsychotics		0 (0%)	2 (13%)	2 (7%)
Tonics		1 (7%)	1 (6%)	2 (7%)
Systemic antibiotics[1]		0 (0%)	1 (6%)	1 (3%)
Digestives, incl. enzymes		1 (7%)	0 (0%)	1 (3%)
Drugs for constipation		1 (7%)	0 (0%)	1 (3%)

[1]Macrolides, lincosamides and streptogramins.

BMI: Body mass index; IBS: Irritable bowel syndrome; IQR: Interquartile range; PRN: Pro re nata (as necessary).

responders compared with 81% (n = 13) in the placebo group (P = 0.6015). Although not reaching the threshold for statistical significance, the proportion of BSFS responders was more pronounced in the G-PUR® group. However, the median number of days with diarrhea *per* week decreased by 2.4 d in the G-PUR® group and by only 0.3 d in the placebo group.

Quality of life as assessed using the SF-12, anxiety and depression evaluated using the HADS, and IBS-SSS scores showed no absolute differences between groups. The significant difference between groups at the end of the study was likely related to the less favorable baseline PCS-12 in the placebo group. Overall, 7/11 patients (64%) in the G-PUR® group and 12/14 patients (86%) in the placebo group reported intake of rescue medication during the trial. In the analysis of perceived stress level, the change in total PSQ score was not different between groups. When score items were analyzed separately, 'tension' was significantly relieved in patients receiving G-PUR® (P = 0.0399 *vs* placebo). The main secondary outcomes are summarized in Table 3.

In the exploratory biomarker analysis, IDO and zonulin levels in capillary blood and levels of bile acid, HBD2 and zonulin in stool were not altered during the observation period. Microbiome studies in stool showed a mild increase in diversity in the G-PUR® group but not in the placebo group. Although these effects were minor, this finding was further supported by the spatial changes of beta diversity seen in the G-PUR® group but not in the placebo group (Figures 3-5).

Safety analysis

G-PUR® was well tolerated throughout the study. Only 2 of the 69 AEs in the G-PUR® group, *i.e.* mild mucosal dryness and mild abdominal distension, were considered treatment-related ADEs. With an overall exposure of 805 treatment days in the G-PUR® group, this corresponds to an ADE rate *per* patient year of 0.9, which was similar to placebo (0.7 *per* patient year). The AE rate *per* patient year differed between groups, with 31 events *per* patient year in the G-PUR® group and 90 in the placebo group. With

Table 2 Medical history at baseline

System organ class MedDRA term	G-PUR®, *n* = 14	Placebo, *n* = 16	Total, *n* = 30
Immune system disorders	10 (71%)	6 (38%)	16 (53%)
Seasonal allergy	7 (50%)	3 (19%)	10 (33%)
Drug hypersensitivity	2 (14%)	2 (13%)	4 (13%)
Allergy to animal	1 (7%)	1 (6%)	2 (7%)
Allergy to plants	2 (14%)	0 (0%)	2 (7%)
Food allergy	1 (7%)	1 (6%)	2 (7%)
Mite allergy	1 (7%)	1 (6%)	2 (7%)
Perfume sensitivity	1 (7%)	0 (0%)	1 (3%)
Metabolism and nutrition disorders	3 (21%)	7 (44%)	10 (33%)
Vitamin D deficiency	1 (7%)	5 (31%)	6 (20%)
Lactose intolerance	2 (14%)	1 (6%)	3 (10%)
Fructose intolerance	1 (7%)	1 (6%)	2 (7%)
Folate deficiency	0 (0%)	1 (6%)	1 (3%)
Food intolerance	0 (0%)	1 (6%)	1 (3%)
Hypercholesterolemia	0 (0%)	1 (6%)	1 (3%)
Psychiatric disorders	3 (21%)	5 (31%)	8 (27%)
Depression	2 (14%)	2 (13%)	4 (13%)
Anxiety disorder	0 (0%)	3 (19%)	3 (10%)
Burnout syndrome	1 (7%)	0 (0%)	1 (3%)
Sleep disorder	1 (7%)	0 (0%)	1 (3%)
Gastrointestinal disorders	1 (7%)	5 (31%)	6 (20%)
Gastritis	0 (0%)	3 (19%)	3 (10%)
Gastroesophageal reflux disease	1 (7%)	1 (6%)	2 (7%)
Hiatus hernia	0 (0%)	1 (6%)	1 (3%)
Nausea	0 (0%)	1 (6%)	1 (3%)

Data are presented for patients randomized to receive G-PUR® or placebo, given as absolute numbers and percent of group. Headings represent the standard nomenclature and structure from the Medical Dictionary for Regulatory Activities. MedDRA: Medical Dictionary for Regulatory Activities.

many of the reported AEs related to the patients' IBS symptoms, this large between-group difference in AE frequency may be at least in part related to the effects of treatment. Because G-PUR® contains alumino-silicates, the release of aluminum has been a potential source of concern. In this study, an elevation in aluminum levels above normal limits was not seen in any of the patients of the entire cohort. In the G-PUR® group, the proportion of patients with aluminum levels below the reference range increased from 14% (n = 2) at baseline to 40% (n = 4) at the end of treatment. In the placebo group, this proportion was 13% (n = 2) at baseline and 0% (n = 0) at the end of treatment.

DISCUSSION

This study provided evidence for the effectiveness and safety of the PCT product G-PUR® in the treatment of IBS-D. While no consistent change in SGA of Relief in response to G-PUR® was demonstrable, the favorable results of G-PUR® were seen in the reduction of cardinal symptoms of IBS-D, *i.e.* abdominal pain and improvement in stool consistency, compared to placebo. This is further supported by symptom improvements over time and lower use of rescue medications in the G-PUR® group than in the placebo group. Importantly, the proportion of days with an at least 30% improvement in abdominal pain was significantly higher in the G-PUR® group compared to placebo. The particular

Table 3 Study outcome variables at week 12 of treatment with G-PUR® or placebo

Outcome variable		G-PUR®, *n* = 14	Placebo, *n* = 16	RD/MD (95%CI)	Odds ratio (95%CI)	*P* value
SGA of Relief responders, end of study[1]		3 (21%)	4 (25%)	0.04 (-0.28-0.38)	0.82 (0.18-3.73)	1.0000
Abdominal pain responders[2]		13 (93%)	13 (81%)	0.12 (-0.20-0.40)	3 (0.39-41.55)	0.6015
Daily abdominal pain reduction[3]		94 (IQR: 81-100)	83 (IQR: 65-92)	-14.9 (-31.87-2.07)	N/A	0.0353
Proportion of BSFS 50% responders		7 (50%)	5 (31%)	0.19 (-0.19-0.51)	2.2 (0.55-11.05)	0.4572
Proportion of BSFS 30% responders		10 (71%)	7 (44%)	0.28 (-0.11-0.58)	3.21 (0.76-12.03)	0.1590
Combined abdominal pain and BSFS 50% response		6 (43%)	4 (25%)	0.18 (-0.19-0.50)	2.25 (0.49-8.68)	0.4421
Decrease in d with diarrhea *per* week		2.4	0.3	N/A	N/A	0.4176
IBS-SSS		-90 (IQR: -170 to -40)	-55 (IQR: -100 to -10)	31.6 (-42.25-105.5)	N/A	0.3950
SF-12		53 (IQR: 50-55)	49 (IQR: 42-51)	-5.40 (-9.15 to -1.65)	N/A	0.0127
PSQ absolute change	Total	-7 (IQR: -28-0)	5 (IQR: -5-12)	11.0 (-2.65-24.65)	N/A	0.0843
	Tension	-20 (IQR: -40-0)	-3 (IQR: -13-13)	16.2 (0.7-31.7)	N/A	0.0399
	Joy	13 (IQR: -7-20)	-10 (IQR: -20-0)	-10.4 (-25.3-4.5)	N/A	0.1176
	Demands	-7 (IQR: -33-13)	10 (IQR: -7-20)	14.3 (-5.35-33.95)	N/A	0.1795
	Worries	0 (IQR: -13-0)	0 (IQR: -7-13)	3.2 (-11.56-17.96)	N/A	0.5587

[1]Subject's Global Assessment (SGA) of Relief response assessment was based on the last four post-randomization SGAs, or if fewer than four post-randomization SGAs were available, then all post-randomization SGAs were used. Patients were considered responders if they answered 'considerably relieved' or 'completely relieved' at least 50% of the time or at least 'somewhat relieved' 100% of the time.

[2]Abdominal pain response was defined as an at least 30% improvement in abdominal pain on at least 50% of days with available electronic patient-reported outcome diary entries as compared to the patient's worst abdominal pain reported in the screening/run-in phase ('baseline abdominal pain').

[3]The median proportion of days with an at least 30% improvement in abdominal pain compared to the patient's worst abdominal pain documented in the screening/run-in phase.

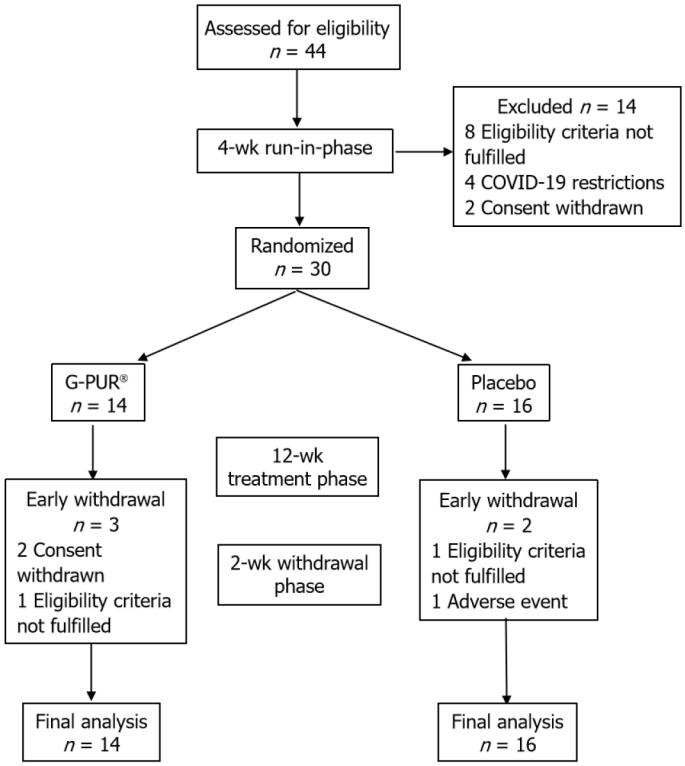
Data are presented as absolute numbers and percent of group or median with interquartile range. Risk difference for proportional variables and mean differences for normally distributed variables, with 95% confidence intervals are given. Odds ratio is provided for proportional variables. BSFS: Bristol Stool Form Scale; N/A: Not available; PSQ: Perceived Stress Questionnaire; SF-12: 12-item Short Form survey; IBS-SSS: Irritable Bowel Syndrome-Symptoms Severity Scale; IQR: Interquartile range; RD: Risk difference; MD: Mean differences; CI: Confidence intervals.

attention and patient support in this study by health care professionals and psychological therapists might have contributed to the strong placebo effect and reduced disease-associated anxiety in both groups. While the PSQ total score improved in the G-PUR® group, it deteriorated in the placebo group, with the between-group difference in change over time trending towards significance (P = 0.0843). This advantage of G-PUR® was driven mainly by the 'tension' subscore, whose absolute change differed significantly between groups (P = 0.0399) and to a lesser extent by the 'joy' and 'demands' subscores.

The comparison of IBS treatment efficacy is generally difficult because there have been only a few head-to-head RCTs. The fluctuations of IBS symptoms over time and the scale of the placebo effect complicates endpoint assessment at scheduled time points. Hence, our primary endpoint of symptom relief at week 12 does not necessarily cover the wide range of symptoms associated with IBS-D, and it cannot be used to describe a continuous clinical improvement over the treatment period.

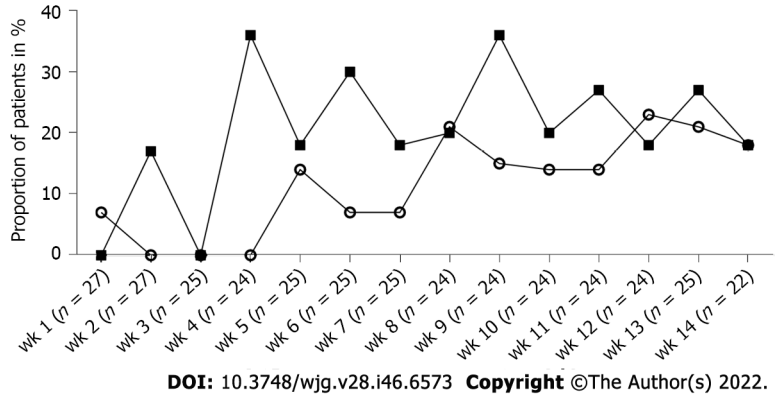
In a clinical trial, micro-activation zeolite improved IBS-associated symptoms of abdominal discomfort with stool frequency and stool consistency to the same extent as placebo, indicating a pronounced placebo effect[10]. A recent randomized trial in 190 IBS patients[46] found some improvement for abdominal pain, discomfort and IBS severity after an 8-wk treatment with small-intestinal-release peppermint oil. However, the main outcomes including abdominal pain response or overall symptom relief were not significant when using endpoints recommended by the FDA and the EMA. Similarly, another study assessing peppermint oil treatment in IBS patients based on an IBS-SSS endpoint did not lead to a significant result[47].

In this trial, G-PUR® was administered over a period of 12 wk. The daily dose of 6 g G-PUR® was well tolerated, and there were no clinical or laboratory safety signals throughout the study. The overall rate of AEs was lower in the G-PUR® group than in the placebo group, and the low ADE rate *per* patient year was the same as the placebo. Although G-PUR® is an aluminum-containing substance, no elevation in serum aluminum levels were observed in patients in this trial. This is related to the fact that within the product's mineral phases, aluminum is not mobile but tightly bound, building up the silicate mineral



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Figure 1 Patient flow diagram. COVID-19: Coronavirus disease 2019.

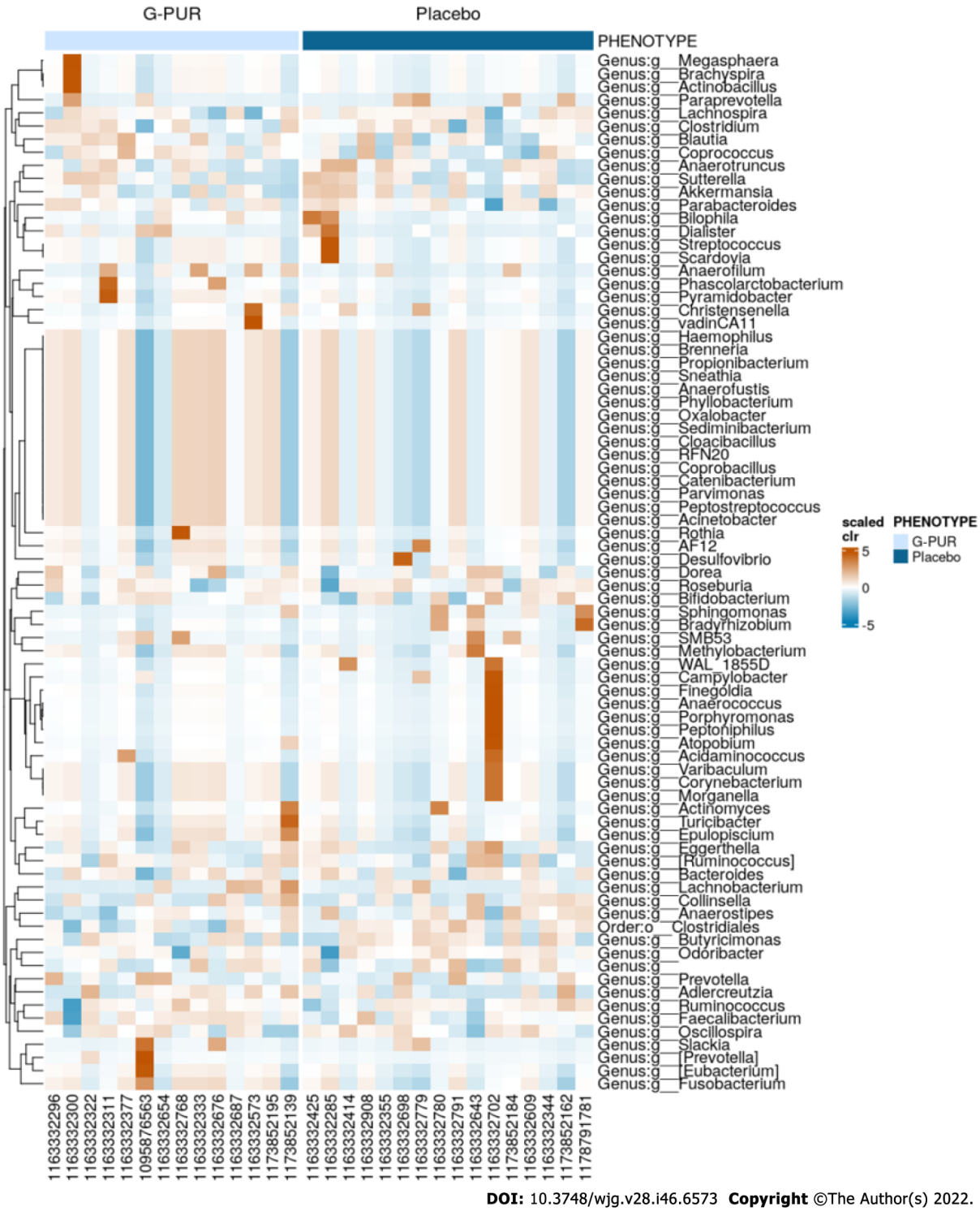


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Figure 2 Subject's Global Assessment of Relief. The proportion of patients (%) reporting 'completely relieved' or 'considerably relieved' by time point during the 12-wk treatment and 2-wk withdrawal period with G-PUR® (solid squares) or placebo (open circles). "*n*" represents the number of patients with available data at the respective time point, while patients with missing data were considered as non-responders in the intention-to-treat-analysis.

crystal lattice[21]. Interestingly, the proportion of patients in whom aluminum was below the reference range at the end of the study increased in the G-PUR® group. This may also be related to the ability of clinoptilolite to adsorb metal ions in the gastrointestinal tract.

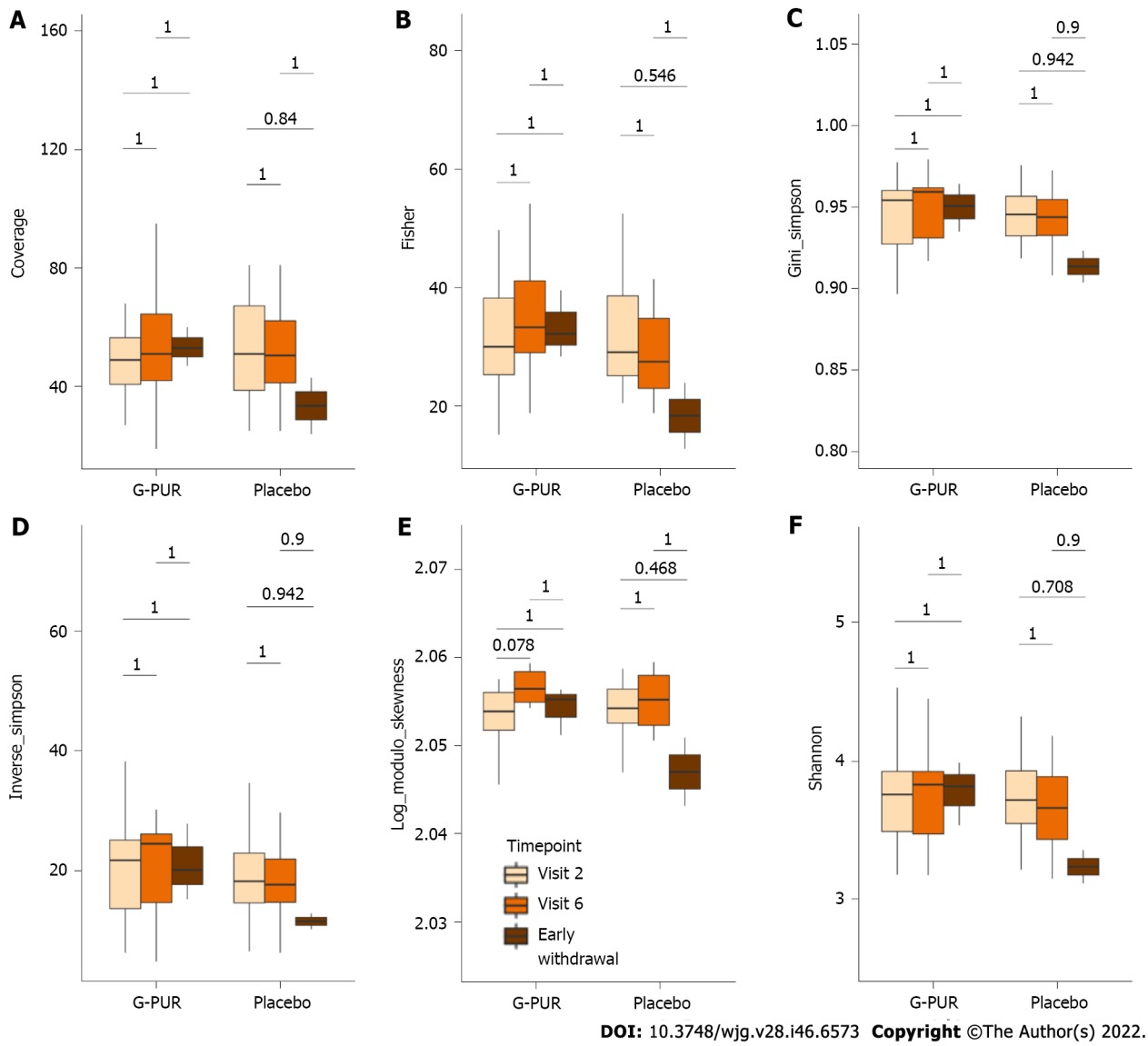
Currently, various pharmacological and non-pharmacological therapeutic interventions are available for the management of IBS-D. The treatment is individualized for the patients' needs and predominant symptoms by their physician, considering the risk-benefit ratio for each strategy. Loperamide, which was used as rescue therapy in the present trial, is often used as a first-line agent to treat diarrhea in IBS-D. However, despite its widespread clinical use, it is worth noting that loperamide is not effective against IBS-associated abdominal pain and bloating[48-50]. Similarly, eluxadoline, a mixed opioid receptor drug, can reduce visceral hypersensitivity and prolong the gastrointestinal transit time, but the effects on abdominal pain relief are modest[51,52]. In a recent meta-analysis of established traditional therapies in IBS, tricyclic antidepressants are recommended for treatment of abdominal pain, but careful dosing is warranted based on the side-effect profile[53,54].



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Figure 3 Microbiome heatmap. Overall visualization of the change in the gut microbiome genera in comparison to baseline by treatment group. Microbiome studies in stool across multiple calculation methods showed an increase in diversity in the G-PUR® group but not in the placebo group.

Dietary and lifestyle changes constitute an important non-pharmacological approach in treating IBS symptoms. A recent meta-analysis showed that patients receiving a diet low in fermentable oligosaccharides, disaccharides, monosaccharides and polyols in RCTs experienced a statistically significant reduction in pain and bloating compared to patients receiving a traditional diet[55]. Overall, the exclusion of foods high in fermentable oligosaccharides, disaccharides, monosaccharides and polyols may reduce IBS symptoms and can be recommended to affected patients, but there is still a need for higher-quality evidence to guide management[56]. Similarly, supplements such as vitamin D have shown modest effects compared to placebo[57-59]. PCT has been shown to be an effective sorbent for gluten derived from food sources[27], which could also contribute to its supposed benefit.

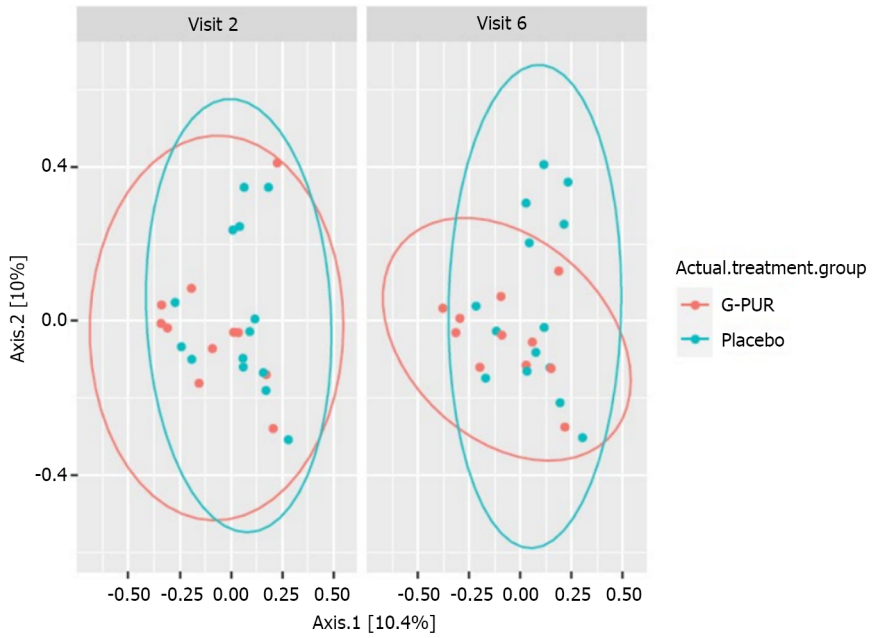


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Figure 4 Alpha diversity indices in phenotypes between time point by treatment group. A: Coverage; B: Fisher; C: Gini_simpson; D: Inverse_simpson; E: Log_modulo_skewness; F: Shannon.

In research concerning functional disorders, treatment effects are generally assessed *via* patient-reported outcomes, which renders the objective evaluation difficult. A strength of this study is that it was designed to meet all of the essential methodological quality criteria for functional gastrointestinal research[60], including a randomized, placebo-controlled, double-blind design, the use of the Rome IV criteria for enrollment, the selection of patients with moderate to severe IBS based on the IBS-SSS, the prohibition of a wide range of concomitant medications, a treatment duration of 12 wk, the use of validated symptom scores and the formal documentation of safety data. Despite the limitation of small sample size, the clinical benefit of PCT could be demonstrated in various clinically meaningful endpoints.

The gut microbiome analysis of the patients revealed a trend towards greater alpha and beta microbial diversity in the treatment group, which has been associated with a healthy gut microbiome and improved symptoms. This change has not been observed in the placebo group. Larger cohorts would be needed to identify a causal relationship to the change in abundance of a single or several bacteria species. A pilot study with 41 patients who received 3 g of zeolite or microcrystalline cellulose twice daily showed that zeolite may lower intestinal inflammation of IBS patients. A positive effect on the gut microbiome was in line with our results[10]. Alterations in gut microbiome may impact the intestinal immune, barrier and neuromuscular junction functions and cause imbalance in the bidirectionally communicating gut-brain axis. However, the exact composition of a healthy microbiome remains unclear[61,62].



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Figure 5 Beta diversity of the gut microbiome by treatment group and visit (longitudinal analysis, statistical methods LefSe and Maaslin).

CONCLUSION

In conclusion, this randomized placebo-controlled, double-blind pilot study provided evidence that the PCT product G-PUR® can be used safely over a prolonged period of 12 wk for the treatment of patients with IBS-D. A favorable result of G-PUR® was detectable for some symptoms of IBS, *i.e.* abdominal pain and stool consistency. Further, a lower use of rescue medication in the G-PUR® group than in the placebo group and a trend towards greater microbial diversity generally associated with a healthy gut microbiome was observed.

ARTICLE HIGHLIGHTS

Research background

Irritable bowel syndrome with diarrhea (IBS-D) is a highly prevalent chronic gastrointestinal disorder with a substantial impact on quality of life. Despite the advancements in the available treatment, there is a need for effective therapy options with a favorable safety profile.

Research motivation

Previous studies have shown positive effects of clinoptilolite-tuff G-PUR® in multiple indications, especially for its adsorption capacity for a variety of toxins, heavy metals and other undesirable substances. Thus, clinoptilolite-tuff might be an effective therapy in IBS-D.

Research objectives

The primary objective of this clinical investigation was to assess the relief from IBS-D symptoms after a 12-wk treatment with G-PUR®. The main secondary objectives were to assess the safety and tolerability of treatment with G-PUR®, the impact of treatment on IBS-related symptoms, quality of life and additional exploratory parameters including microbiome analysis.

Research methods

We performed a randomized, placebo-controlled, double-blind pilot study on 30 patients with IBS-D. Over a treatment period of 12 wk, 14 patients received 2 g of G-PUR® three times daily, and 16 patients received placebo. The response was assessed with validated IBS-D associated symptom questionnaires. Exploratory biomarkers and microbiome data were collected and analyzed.

Research results

After 12 wk of treatment, the proportions of Subject's Guide of Assessment of Relief responders were comparable in both groups, while after 4 wk of treatment significantly more patients in the G-PUR®

group *vs* placebo group reported complete or considerable relief. An improvement in daily abdominal pain, diarrhea-free days, abdominal pain and stool consistency response was seen in the G-PUR® group compared to the placebo group.

Research conclusions

In this randomized, double-blind, placebo-controlled study, the purified clinoptilolite-tuff product G-PUR® demonstrated safety and clinical benefit towards some symptoms of IBS-D, representing a promising novel treatment option for these patients.

Research perspectives

Further research is needed to evaluate clinical efficacy of clinoptilolite-tuff product G-PUR® in larger cohorts.

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FOOTNOTES

Author contributions: Wolzt M, Moser G, Meisslitzer C, Gouya G and Tschegg C conceptualized the study; Wolzt M, Meisslitzer C, Gouya G, Freissmuth M and Tschegg C designed the methodology; Anderle K, Wolzt M, Moser G, Keip B and Peter J performed the investigation; Anderle K, Wolzt M, Moser G, Keip B, Peter J, Meisslitzer C, Gouya G, Freissmuth M and Tschegg C performed the data curation and reviewed/edited the manuscript for important intellectual content; Gouya G performed the formal analysis of data; Moser G and Freissmuth M performed the data validation; Anderle K wrote the original draft of the manuscript; Wolzt M and Tschegg C provided resources; Keip B and Peter J provided software; Anderle K, Meisslitzer C, Gouya G and Tschegg C performed project administration; Tschegg C and Meisslitzer C were responsible for funding acquisition; Wolzt M and Gouya G performed study supervision; All authors had access to the study data and reviewed and approved the final manuscript.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Medical University of Vienna (No. 1295/2019), Ethics Committee of Upper Austria (No. 1208/2019) and the Austrian Federal Office for Safety in Health Care (BASG).

Clinical trial registration statement: This study was registered at ClinicalTrials.gov registry (Identifier NCT04138186).

Informed consent statement: All study participants provided verbal and written informed consent prior to study inclusion.

Conflict-of-interest statement: The Medical University of Vienna and Klinikum Wels-Grieskirchen were reimbursed for all trial-related work hours, material and effort spent by the study sponsor. Investigators or study staff did not receive any payment. Michael Freissmuth is the recipient of an unrestricted research endowment by G.H. Glock.

Data sharing statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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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ORIGINAL ARTICLE

Randomized Clinical Trial

Prevalence of functional gastrointestinal disorders in children with celiac disease on different types of gluten-free diets

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Abstract

BACKGROUND

Functional gastrointestinal disorders (FGIDs) are common during the pediatric age. FGIDs are not related to biochemical or structural abnormalities. However, since they have a high prevalence, several studies have evaluated an overlap between FGIDs and organic diseases. Individuals with celiac disease (CD) have been shown to be at an increased risk for functional abdominal pain, even if they adhere well to a gluten-free diet (GFD). Little information is available for the pediatric age group. The aims of our study were to evaluate the prevalence of FGIDS in CD children 1 year after diagnosis and to compare the prevalence of FGIDs in CD children on a GFD with processed foods compared with those on a GFD with natural products.

AIM

To assess the prevalence of FGIDs in children with CD after 1 year of follow-up and to compare the prevalence of FGIDs in children with CD on a GFD with processed foods and in children on a GFD with natural products.

METHODS

We recruited pediatric patients aged 1-18 years with a new CD diagnosis.

Participants were randomized to two groups: Group A on a GFD with processed foods (diet 1); and group B on a GFD with natural products (diet 2). Clinical monitoring, diet assessment and the questionnaire on pediatric gastrointestinal symptoms-Rome IV version were performed at diagnosis (T0) and after 12 mo of follow-up (T1). Dietary intake was assessed using a 3-d food diary record. Data from the diaries were evaluated using WinFood nutrient analysis software. We assessed the prevalence of FGIDs at T1 and the correlation with the type of GFD.

RESULTS

We registered 104 CD children, with 55 patients in group A (53.0%) and 49 patients in group B (47.0%). Initially, 30 of the 55 (54.5%) CD children were symptomatic in group A, while 25 of 49 (51.0%) were symptomatic in group B. At T1, in spite of a low or negative serology for CD, FGIDs prevalence was 10/55 (18.0%) in group A and 8/49 (16.3%) in group B, with no statistically significant difference between the two groups (P = 0.780). At T1 the macro- and micronutrient intake was similar across the two groups with no significant differences in nutrient analysis. However, in both groups at T1 we found that a lower prevalence of FGIDs (P = 0.055) was associated with an inferior caloric (odds ratio = 0.99, 95% confidence interval: 0.99-1.00) and fat (odds ratio = 0.33, 95% confidence interval: 0.65-0.95) intake.

CONCLUSION

Our results showed that CD children on a GFD have gastrointestinal symptoms with an elevated prevalence of FGIDs. Our study suggests that developing FGIDs may be linked to caloric intake and percentage of food fat, but it does not change between a GFD with processed foods or a GFD with natural products. However, long-term monitoring is required to evaluate a correlation between FGIDs and various types of GFDs.

Key Words: Functional gastrointestinal disorders; Celiac disease; Gluten free diet; Gastrointestinal symptoms; Children

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Core Tip: In spite of a strict adhesion to a classic gluten-free diet, patients with celiac disease are more likely to suffer from functional abdominal pain disorders. Our findings suggest that the prevalence of functional gastrointestinal disorders may be linked to the caloric intake and fat content in the diet. However, it does not differ between a processed gluten-free diet with commercial products or a gluten free diet with natural products.

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INTRODUCTION

Celiac disease (CD) is a chronic immune-mediated enteropathy characterized by mucosal inflammation and villous atrophy[1] and triggered by the ingestion of gluten in genetically predisposed individuals. CD is now recognized as a global disease with a prevalence of about 1% of the world's population[2]. The clinical presentation ranges from features of malabsorption such as abdominal pain, diarrhea, steatorrhea and weight loss or growth failure to atypical forms of CD with more subtle gastrointestinal (GI) manifestations similar to functional GI disorders (FGIDs) or asymptomatic individuals diagnosed by screening high-risk groups[3].

The only available treatment for CD is lifetime adherence to a gluten-free diet (GFD)[4]. Just over a decade ago, Turco *et al*[5] showed that subjects with CD have a higher risk of developing FGIDs, fulfilling the Rome III diagnostic criteria, despite strict adherence to a classic GFD. However, concordant data has not been produced in this area in recent years[6,7]. When it is present an organic abnormality FGID cannot be diagnosed. However, these disorders have a high prevalence. Therefore, numerous studies have assessed the potential for overlap between FGIDs and organic diseases[8]. A great innovation introduced in the new Rome IV criteria is the specification that the diagnosis can only be made 'after appropriate medical evaluation, the symptoms cannot be attributed to another medical

condition'. This wording replaces the previous statement that there should be an 'absence of inflammatory, anatomic, metabolic, or neoplastic process that explains the subject's symptoms'. This change makes it possible for a patient suffering from another organic disease, like CD or inflammatory bowel disease, to also have a functional disorder (common event) and should reduce the amount of tests necessary to diagnose FGIDs[9].

However, only little and contrasting data are available in children regarding the prevalence of symptoms of FGID in children with CD or the effects of a GFD on those symptoms. The objectives of this study were to evaluate the prevalence of FGIDs among children with CD at the moment of diagnosis and after 1 year of follow-up comparing a conventional GFD with commercially available products and a GFD based mainly on natural products.

MATERIALS AND METHODS

Patient selection

We prospectively followed up for 1 year two groups of CD children who received two different GFDs to evaluate if specific dietary factors were involved in the development of FGIDs at 1 year of follow-up. This group consisted of 104 consecutive children [34 male children, 69 female children; mean age 7.2 (4.1) years; range: 4-17 years] who received a diagnosis of CD from December 2017 to January 2019 at the Department of Pediatrics, University 'Federico II' in Naples, Italy and the Department of Pediatrics, University of Campania, "Luigi Vanvitelli" in Naples, Italy. All children received a GFD and were followed up for 1 year. CD diagnosis, according to the ESPGHAN criteria[10], was based on the serum concentration of anti-transglutaminase-immunoglobulin A (IgA) determined by an indirect solid-phase enzyme immunoassay test and serum concentration of endomysial antibodies determined by indirect immunofluorescence using monkey's esophagus sections as substrate. To exclude the presence of selective IgA deficiency, serum IgA levels were assayed by nephelometry. According to the ESPGHAN guidelines[11], in children and adolescents with signs or symptoms suggestive of CD, high anti-transglutaminase titers (> 10 times), positivity of endomysial antibodies and human leukocyte antigen DQ2/8, biopsies were omitted. In all other cases, patients underwent upper endoscopy with multiple duodenal biopsies. Class II antigen human leukocyte antigen typing was also performed by polymerase chain reaction sequence-specific oligonucleotide using DQ-CD Typing Plus[12]. We excluded from the study patients with systemic or GI infection, patients with other known GI, renal, cardiac, pulmonary, hematological, neurological and cerebral pathologies and patients with an inability or unwillingness to give informed consent.

Study design

The patients were randomly divided into two groups: Group A (n = 55) received a controlled GFD with processed foods (diet 1); and group B (n = 49) received a controlled GFD with > 60% natural products (diet 2). All registered patients and/or their parents underwent validated questionnaires for GI symptoms according to the Rome IV criteria. The pediatric GI symptoms Questionnaire-Rome IV version was used to diagnose FGIDs. Subjects were classified as having an FGIDs through their questionnaire responses and were able to meet criteria for multiple disorders.

A clinical follow-up and symptom questionnaire based on the Rome IV criteria were carried out for each child at two different times: At diagnosis (T0) and at the 12-mo (T2) follow-up. At 12 mo of follow-up, children were considered in clinical remission from CD if they reverted positive serological tests to negative following treatment with a strict GFD, despite having GI symptoms defined as functional according to the Rome IV criteria. The potential consumption of gluten-containing products was assessed using a combination of CD serology, self-reported adherence questions and an interview with an experienced dietician. Dietary intake was assessed on the basis of compiling a 3-d food diary and a dietary interview by an expert nutritionist. The nutrients analysis was conducted using the WinFood software.

Statistical analysis

Data were presented as frequency (percent) for categorical variables and as mean (standard deviation) for continuous variables. Differences in nutrient intake between patients in group A and group B were computed with the student's t test or Mann-Whitney U as appropriate. Associations between nutrient intake and FGIDs prevalence for the two groups were computed with logistic regression. Comparison between the prevalence of FGIDs at T0 or T1 in patients were computed with the McNemar test. For all analyses, a P value of 0.05 was considered significant. All statistical analyses were performed using the R statistical environment, version 4.0.3.

RESULTS

The symptomatic children at enrollment were 30 of 55 (54.5%) in group A and 25 of 49 (51.0%) in group B. Constipation (28.8%) was the most prevalent symptom, followed by abdominal pain (24.0%) and vomiting (4.8%). As expected after 1 year of GFD the frequency of GI symptoms significantly decreased in both groups (Table 1). At 1 year from CD diagnosis children were investigated for repeated serology for CD (endomysial and anti-transglutaminase antibodies), of which 99/104 (95.2%) were negative, 2 (1.9%) were positive, and 3 (2.9%) were borderline.

Despite negative serology for CD, the prevalence of FGIDs, classified according to the Rome IV criteria, was 10/55 (18.0%) in group A and 8/49 (16.3%) in group B. There was no statistically significant difference between the two groups at T1 (P = 0.780) (Figure 1). Among CD children with FGIDs, functional constipation (FC) (12.5%) was the most prevalent disorder (7.7% in group A and 6.7% in group B) followed by postprandial distress syndrome (1.9% in group A and 1.0% in group B) at T1.

We observed that children who presented with symptoms at diagnosis, more frequently developed a FGID at 1 year than patients who were asymptomatic at the time of diagnosis. The individual analysis of the intake of macro- and micronutrients at T1 showed that there were no important differences in nutrient intake between group A and group B (Table 2). However, in both groups, we found a relationship between reduced caloric and fat intake (odds ratio = 0.99, 95% confidence interval: 0.99-1.00 and odds ratio = 0.33, 95% confidence interval: 0.65-0.95, respectively) and a decreased prevalence of FGIDs after 1 year of a GFD (P = 0.05) (Figure 2). No statistically significant difference was found in the subcategories of fat analyzed (saturated, polyunsaturated and monounsaturated fatty acids).

DISCUSSION

To the best of our knowledge this is the first pediatric study that investigated the prevalence of FGIDs in children with CD on a GFD with processed products *vs* a GFD with natural products. We found that there was no difference in the prevalence of FGIDs according to the type of diet (18.4% *vs* 16.3%), and the most frequent FGID was FC in both groups. Interestingly, in both groups we found an association between lower caloric and fat intake and lower prevalence of FGIDs after 1 year of a GFD.

In a previous work we found a higher prevalence of CD patients who continued having GI symptoms and fulfilled Rome III criteria for FGIDs despite the GFD compared to control (28.0% *vs* 8.9%, respectively). FC was the most frequent disorder. The likely cause for the difference in the current study is related to the application of the Rome IV criteria, which resulted in a lower prevalence of FGIDs as previously described[13,14]. Indeed, our findings are in accordance with the results of a recent large study conducted in Italy from Cristofori *et al*[6] that found a prevalence of functional abdominal pain disorders of 11.5% according to Rome IV criteria among patients with celiac disease compared to 6.7% of the control. FC and irritable bowel syndrome (IBS) were the most frequent disorders found in CD patients. We also found that FC was the most common disorder. However, we noted an increase of functional dyspepsia and a decrease of IBS compared to Cristofori *et al*[6]. Two large American reports and our recent European study conducted on healthy subjects also reported an increased level of functional dyspepsia (3.0%-7.6%), with postprandial distress syndrome being the most common subtype (2.7%-7.2%), as in our population[13-15]. It is interesting to note that in these studies, the prevalence of functional dyspepsia exceeded that of IBS, which was previously the most prevalent functional abdominal pain disorder[16].

Patients with CD and FGIDs can be categorized as "atypical forms" of CD. Although, the term "atypical" CD is primarily used for patients who present with extraintestinal symptoms like IgA-nephropathy, hemosiderosis of the lungs and a variety of neurological diseases. Moreover, another difference is that the atypical forms generally respond to the GFD with a disappearance of symptoms [17].

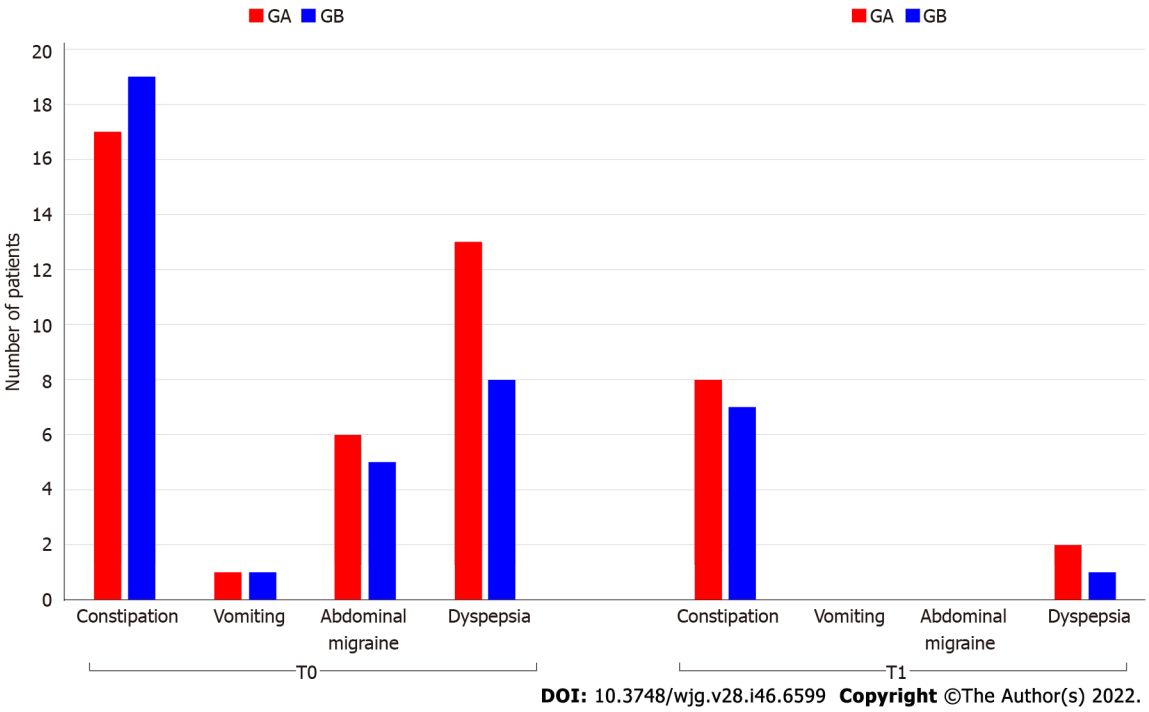
Although numerous studies have been carried out to study the pathogenesis of both FGIDs and CD, there are still many unanswered questions. Possible explanations behind the persistence of GI symptoms could be the presence of another unrecognized GI disease, altered bowel motility due to the persistence of low-grade inflammation despite a GFD, microbiota alteration or a continuous intentional or inadvertent intake of gluten[6]. Only a few studies have been reported on the overlap between CD and functional abdominal pain disorders in children, but it seems likely that intestinal inflammation (infectious and non-infectious) predisposes children to develop visceral hypersensitivity that can manifest as functional abdominal pain disorders[18].

O'Leary *et al*[19] hypothesized that despite the GFD, particularly in treated, occasionally noncompliant celiac patients, low-grade of inflammation that induced sensory or motor dysfunction and IBS-type symptoms persisted and precipitated a motility disturbance in many patients. Another theory is persistent intestinal inflammation could be due to a short follow-up on a GFD. Moreover, gut microbiota of CD patients is characterized by increased *Bacteroides spp*, *Escherichia coli*, *Proteobacteria* and *Staphylococcus* and decreased *Bifidobacterium spp* and *Lactobacillus*[20]. Multiple studies reported similar changes in the microbiota of IBS patients[21,22]. According to the most recent data, intestinal dysbiosis

Table 1 Distribution of gastrointestinal symptoms in the celiac disease patients at T0 and T1 in both groups

All disorders	Group	T1 absence	T1 presence	*P* value
T0 absence	Group A	19	6	< 0.001[1]
	Group B	20	4	0.001[1]
T0 presence	Group A	26	4	< 0.001[1]
	Group B	21	4	0.001[1]

[1]*P* values were computed with the McNemar test. T0 was the time at the initial diagnosis of celiac disease. T1 was the time at the 1-year follow-up after diagnosis of celiac disease. In group A, patients consumed a gluten-free diet with processed foods. In group B, patients consumed a gluten-free diet with natural foods.



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Figure 1 Prevalence of functional gastrointestinal disorders in children affected by celiac disease at diagnosis and after 1 year of follow-up in group A (consumed a gluten-free diet with processed foods) and group B (consumed a gluten-free diet with natural foods). T0 was the time at the initial diagnosis of celiac disease. T1 was the time at the 1-year follow-up after diagnosis of celiac disease. GA: Group A; GB: Group B.

might be responsible for the persistence of symptoms, even in patients on a GFD. In fact, a GFD, though capable of improving the nutritional status of CD patients without causing nutritional problems, is only partially effective in restoring microbiota and may be partly responsible for intestinal dysbiosis due to the reduction in the intake of polysaccharides (fructans), which has a prebiotic action on *Bifidobacterium spp*.

Moreover, the reduced amount of fiber in a GFD may be considered as one of the reasons why CD patients often suffer from FC[5]. The new appearance of constipation after the introduction of a GFD likely reflects a decrease in fiber intake, and many of these patients may react to the addition of dietary fiber. Constipation can also reflect a return to a predisposition for constipation after resolution of malabsorption. Another possible explanation could be that functional abdominal pain can be triggered not only by gluten but also by other components of wheat including α-amylase/trypsin inhibitors, wheat, lectin, agglutinin and fructans as described in the study by Llanos-Chea and Fasano[23]. They showed that the consumption of wheat and other cereal grains can contribute to the manifestation of chronic inflammation and autoimmune diseases by increasing intestinal permeability and initiating a proinflammatory immune response.

Chronically increased intestinal permeability allows for the increased translocation of both microbial and dietary antigens to the periphery, which can then interact with cells of the immune system and stimulate pathways of innate immunity. According to Barone *et al*[24], CD patients compared to healthy individuals eat significantly higher amounts of fat and sugar and small amounts of fiber on a GFD[20-23]. Numerous studies have demonstrated that the total fat content of gluten-free foods is at least double

Table 2 Comparison of macro- and micronutrient daily intake in group A and group B at the 1-year follow-up

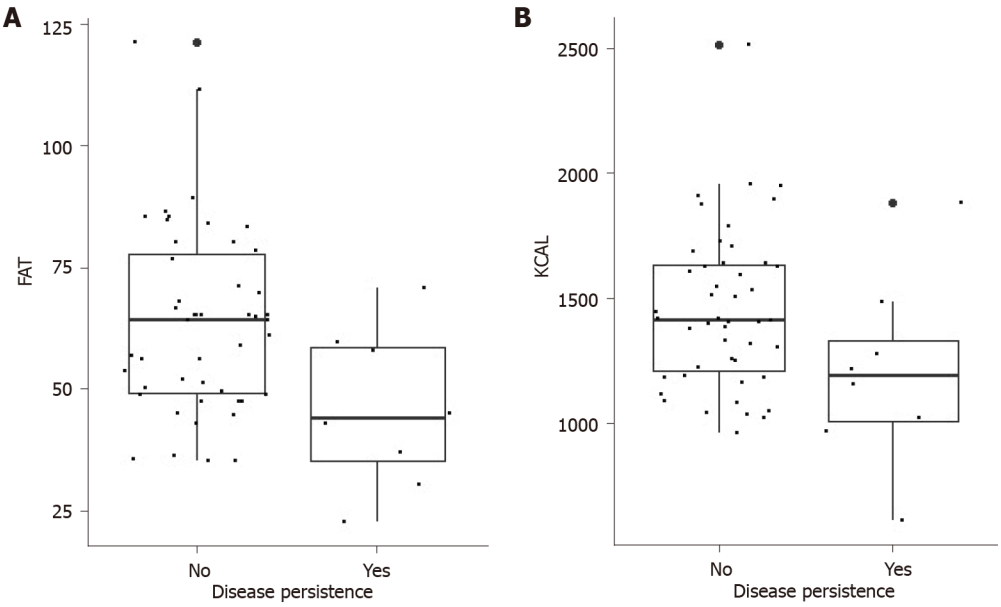
Variable, mean (SD)	Group A, *n* = 55	Group B, *n* = 49	*P* value
Energy intake, kcal/d	1417 (338)	1376 (367)	0.558
Total protein, g/d	52 (14)	50 (15)	0.509
Lipids, g/d	63 (19)	59 (20)	0.324
Carbohydrates, g/d	189 (137)	167 (51)	0.277
Oligosaccharides, g/d	40 (20)	34 (17)	0.119
Starch, g/d	41 (33)	33 (33)	0.250
Cholesterol, mg/d	135 (60)	155 (109)	0.251
Saturated fatty acids, g/d	15.1 (7.0)	13.7 (6.0)	0.277
Poly-unsaturated fatty acids, g/d	4.99 (1.83)	4.02 (2.02)	0.012
Mono-unsaturated fatty acids, g/d			
Fiber, g/die	25 (10)	22 (11)	0.089
Calcium, mg/d			
Sodium, mg/d	8.1 (5.5)	7.1 (5.2)	0.382
Potassium, mg/d	412 (190)	421 (219)	0.819
Phosphorus, mg/d	881 (565)	780 (669)	0.410
Iron, mg/d	1.386 (592)	1.300 (550)	0.433
Zinc, mg/d	672 (229)	653 (259)	0.685
Folic acid, mcg/d	4.68 (2.70)	4.47 (2.29)	0.644
Niacin, mg/d	5.36 (2.38)	5.04 (2.16)	0.482
Riboflavin, mg/d	100 (74)	104 (75)	0.793
Thiamine, mg/d	8.2 (4.4)	7.5 (4.2)	0.433
Vitamin A, mcg/d	0.82 (0.33)	0.90 (0.64)	0.466
Vitamin B6, mg/d	0.53 (0.23)	0.49 (0.21)	0.373
Vitamin C, mg/d	333 (201)	382 (214)	0.234
Vitamin D, mcg/d	0.87 (0.51)	0.82 (0.48)	0.577
Vitamin E, mg/d	71 (87)	57 (49)	0.341
	1.53 (1.73)	1.47 (1.43)	0.826
	7.1 (3.2)	6.4 (3.8)	0.308

In group A, patients consumed a gluten-free diet with processed foods. In group B, patients consumed a gluten-free diet with natural foods. SD: Standard deviation.

that of gluten-containing foods, which helps to improve the taste of these products.

In our study we found that there was no significant difference in the prevalence of GFIDs between children on an industrial manufactured GFD and children who followed a GFD using natural products. However, in both groups we found an association between lower calorie and fat intake and a lower prevalence of FGIDs after 1 year of a GFD.

Dietary fat has been associated with the onset of symptoms after a meal challenge or reported as inducing symptoms of dyspepsia in some studies[25-27], specifically dyspeptic symptoms of nausea[25,26], bloating[25,26], post-prandial fullness/discomfort[25,27] and epigastric pain[26,27]. It is not entirely clear how food factors cause dyspeptic symptoms. Studies have shown that sensitivity to stomach distention and chemical stimuli like nutrients (fat), acid and GI hormones (CCK) and interactions between them have a central role inducing symptomatic response[28]. Zito *et al*[29] showed that some alimentary regimens or even some meals are able to trigger GI symptoms in predisposed individuals. Fats, in particular, seem to influence gastric activity by delaying gastric emptying and promoting relaxation of the fundus. These mechanisms are closely related to the onset of GI symptoms. Functional dyspeptic patients, for example, usually report that meal size, eating patterns, caloric intake as well as



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Figure 2 Decreased fat and calorie intake was correlated with disease persistence of functional gastrointestinal disorders after 1 year of follow-up.

nutrient composition (lipid content in particular) strongly influences the onset of dyspeptic symptoms.

Our study has several strengths including a large sample size representative of the various age groups and well-defined CD and FGIDs diagnoses. The main limitations of our study was the short follow-up associated with the absence of a follow-up endoscopy, which could underestimate the persistence of a slight degree of inflammation. The self-compilation of food diaries associated with an autonomous choice of foods to be consumed during the duration of the study was another limitation. This may have led to bias in the comparison of the macro- and micronutrients of the two groups, possibly leading to a difference in the persistence of symptoms. However, the presence of an experienced dietician who performed a diet check with the patient at each visit coupled with a negative serological test ensured us that the patient was strictly adhering to the GFD. Therefore, accidental and/or occasional consumption of gluten is unlikely to explain GI symptoms. However, this approach limits our ability to evaluate whether the lack of compliance could be responsible for the appearance of symptoms.

CONCLUSION

In conclusion, this is the first study to show that the presence of functional GI symptoms in children with CD on a GFD are possibly related to higher caloric and fat intake. It remains to be determined whether the risk is due to the persistence of a chronic inflammatory process or to nutritional factors. Long-term monitoring studies will assist in determining the natural history of these functional symptoms.

ARTICLE HIGHLIGHTS

Research background

When an organic disorder, like celiac disease (CD), is present, functional gastrointestinal disorders (FGIDs) cannot be diagnosed. However, these disorders have a high prevalence. Therefore, a number of studies have assessed the possibility of overlap between FGIDs with organic disorders.

Research motivation

Few data are available regarding the risk of FGIDs in children strictly adhering to a conventional gluten free diet (GFD).

Research objectives

The objectives of this study were to estimate the prevalence of FGIDs in patients affected by CD at the moment of the diagnosis (T0) and after 1 year of follow-up (T1) comparing two different types of GFD.

Research methods

This study involved 104 celiac pediatric patients (aged 1 year to 18 years) randomized to: Group A, on a GFD with processed foods; and group B, on a GFD with natural products. Clinical follow-up, a 3-d dietary diary evaluation and a questionnaire based on the Rome IV criteria were completed for each child at T0 and T1. We examined the FGIDs after 12 mo and the relationship to the type of GFD.

Research results

At the time of enrollment, 54.5% of CD children had symptoms in group A, and 51.0% of CD children had symptoms in group B. At T1, in spite of low or negative CD serology, the prevalence of FGIDs was 18.0% in group A and 16.3% in group B (P = 0.780). In both groups after 12 mo of a GFD an intraindividual analysis showed a significantly lower prevalence of FGIDs (P = 0.055) in patients lower calorie and fat intake.

Research conclusions

Many children still have gastrointestinal symptoms and FGIDs despite a strict GFD, and it could be linked to the caloric intake and the amount of fat in the diet. However, it does not seem affected by a GFD with commercial or natural products.

Research perspectives

To evaluate the correlation between FGIDs and different types of GFDs, long-term monitoring is necessary.

FOOTNOTES

Author contributions: Strisciuglio C, Fiori Nastro F, Serra MR, Pacella D and Cenni S were responsible for the study conception and design, data analysis and interpretation and manuscript drafting; Martinelli M, Miele E, Staiano A, Tolone C and Auricchio R critically revised the article for important intellectual content; and all authors reviewed and approved the final version to be published.

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SYSTEMATIC REVIEWS

Correlation between COVID-19 and hepatitis B: A systematic review

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Abstract

BACKGROUND

There is growing evidence that patients with coronavirus disease 2019 (COVID-19) frequently present with liver impairment. Hepatitis B virus (HBV) remains a major public health threat in current society. Both severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and HBV can cause liver damage, and current findings on whether HBV infection increases disease severity in COVID-19 patients are inconsistent, and whether SARS-CoV-2 infection accelerates hepatitis B progression or leads to a worse prognosis in hepatitis B patients has not been adequately elucidated.

AIM

To explore the complex relationship between COVID-19 and hepatitis B in order to inform the research and management of patients co-infected with SARS-CoV-2 and HBV.

METHODS

An experienced information specialist searched the literature in the following online databases: PubMed, China National Knowledge Infrastructure, Google Scholar, Scopus, Wiley, Web of Science, Cochrane, and ScienceDirect. The literature published from December 2019 to September 1, 2022 was included in the search. We also searched medRxiv and bioRxiv for gray literature and manually scanned references of included articles. Articles reporting studies conducted in humans discussing hepatitis B and COVID-19 were included. We excluded duplicate publications. News reports, reports, and other gray literature were included if they contained quantifiable evidence (case reports, findings, and qualitative analysis). Some topics that included HBV or COVID-19 samples but did not have quantitative evidence were excluded from the review.

RESULTS

A total of 57 studies were eligible and included in this review. They were from 11 countries, of which 33 (57.9%) were from China. Forty-two of the 57 studies reported abnormalities in liver enzymes, three mainly reported abnormalities in blood parameters, four indicated no significant liver function alterations, and another eight studies did not provide data on changes in liver function. Fifty-seven studies were retrospective and the total number of co-infections was 1932, the largest sample size was 7723, and the largest number of co-infections was 353. Most of the studies suggested an interaction between hepatitis B and COVID-19, while 12 studies clearly indicated no interaction between hepatitis B and COVID-19. Six of the 57 studies clearly reported HBV activation. Six studies were related to liver transplant patients.

CONCLUSION

There is some association between COVID-19 and hepatitis B. Future high-quality randomized trials are needed to further elucidate the interaction between COVID-19 and hepatitis B.

Key Words: COVID-19; SARS-CoV-2; Hepatitis B virus; Chronic hepatitis B; Liver injury; Co-infection

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Core Tip: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and hepatitis B virus (HBV) infections are two major current global public health crises. Both infection with SARS-CoV-2 and infection with HBV can cause liver damage. There are conflicting views on whether HBV infection aggravates the prognosis of patients with coronavirus disease 2019 (COVID-19). There is a potential association between COVID-19 and hepatitis B. Clarification of this association could benefit these special patients with SARS-CoV-2 and HBV co-infection.

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INTRODUCTION

Since the outbreak of coronavirus disease 2019(COVID-19), there has been increasing evidence that patients with COVID-19 frequently present with hepatic impairment[1-3]. This may be caused by pre-existing liver disease, viral infection of hepatocytes, and certain medications. Hepatitis B virus (HBV) remains a major current public health threat to society, with approximately 300 million people chronically infected worldwide[4]. Since both severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and HBV can cause liver damage, current findings on whether HBV infection increases disease severity in patients with COVID-19 are inconsistent, with some reports suggesting that co-infection with SARS-CoV-2 and HBV has no effect on the course and prognosis of COVID-19[5,6], while several studies have shown that patients with SARS-CoV-2 and HBV co-infection are more likely to have serious outcomes[7-9]. Does pre-existing HBV infection increase susceptibility to SARS-CoV-2 infection, leading to more severe disease and a worse prognosis? Conversely, does SARS-CoV-2 infection accelerate the progression of hepatitis B or lead to worse outcomes? The potential association between hepatitis B and COVID-19 has not been fully elucidated, so it is important and interesting to understand how COVID-19 and hepatitis B interact with each other. In order to elucidate this complexity, we summarize almost all current clinical studies to provide a detailed description of the interaction between COVID-19 and hepatitis B, possible mechanisms, and clinical interventions to provide a reference for the clinical management of these special patients.

MATERIALS AND METHODS

Identification of research questions

Does SARS-CoV-2 and HBV co-infection have any effect on the course and prognosis of COVID-19? And *vice versa*, does COVID-19 accelerate the progression of hepatitis B and lead to a severe prognosis?

Identification of relevant types of evidence

The literature published from December 2019 to September 1, 2022 was included in the search. To avoid missing any relevant and important literature, we used an inclusive search strategy. An experienced information specialist searched the literature in the following online databases: PubMed, China National Knowledge Infrastructure, Google Scholar, Scopus, Wiley, Web of Science, Cochrane, and ScienceDirect. We also searched medRxiv and bioRxiv for gray literature and manually searched the references of the included studies to further ensure the comprehensiveness of the search. Articles that met the criteria in the *Reference Citation Analysis* (https://www.referencecitationanalysis.com) were also included in this review. Articles performed in humans and discussing SARS-CoV-2 in patients with hepatitis B and HBV in COVID-19 patients were included. Detailed search strategies are shown in Supplementary material.

Study selection

Several types of articles were included after our initial search, including case reports, case series, qualitative studies, and systematic and scoping reviews. We excluded repetitive publications. Articles containing more complete descriptions of the data were used for data charting. News articles, case reports, survey results, qualitative analyses, and other gray literature were also included in our review if they contained quantifiable evidence. We read the full text, synthesized the relevant evidence, and organized the literature thematically. All five authors participated in the discussion and decided on a theme. Some topics that included HBV or COVID-19 samples but did not have quantitative evidence were also excluded from the review. Figure 1 shows a visual representation of inclusion workflow.

Data charting

As of September 1, 2022, we retrieved 1136 publications, adding six manually retrieved papers for a total of 1142. We tested 139 full-text articles for eligibility after being screened by the above inclusion criteria, and 57 were finally included in this review (Figure 1). Two independent reviewers extracted data from the full-text papers of eligible studies, including the name of the first author, publication month and year, country of the study, number of included patients, number of SARS-CoV-2 and HBV co-infections, main biochemical characteristics of co-infected patients, their clinical outcome (death or survival), and main conclusion of each study. A summary of information on the included studies is presented in Table 1[9-60], and details of important parameters are presented in Supplementary Table 1. This systematic review has been registered on the PROSPERO platform: https://www.crd.york.ac.uk/prospero/#recordDetails.

RESULTS

A total of 57 studies were eligible and included in this review. They were from 11 countries, of which 33 (57.9%) were from China (Figure 2). Forty-two of the 57 studies reported abnormalities in liver enzymes [2,5,7-46], three mainly reported abnormalities in blood parameters[47-49], four indicated no significant liver function alterations[6,50-52], and another eight studies did not provide data on changes in liver function[53-60]. Fifty-seven studies were retrospective, and the total number of co-infections was 1932, the largest sample size was 7723[54], and the largest number of co-infections was 353[16]. Most of the studies suggested an interaction between hepatitis B and COVID-19, while 12 studies clearly indicated no interaction between hepatitis B and COVID-19[5,11,16,18,21,25,28,50,51,54-56]. Six of the 57 studies clearly reported HBV activation[23,29,41,43,46,52]. Six studies were related to liver transplant patients [36,38,39,49,57,58].

Interpretation of 'no effect' results

As previously mentioned, the possible reasons why HBV infection was not associated with clinical outcomes of COVID-19, despite the fact that some patients had high liver enzyme levels, are as follows: (1) The infection rate is not affected. The main consideration is the positive effect of nucleoside analogues on resistance to SARS-CoV-2[59]. In the included studies, some hepatitis B patients received long-term treatment with nucleoside analogues, such as tenofovir, which binds tightly to RNA-dependent RNA polymerase and terminates SARS-CoV-2 RNA synthesis[61,62], resulting in a low rate of SARS-CoV-2 infection in chronic hepatitis B (CHB) patients treated with anti-HBV drugs; (2) The severity of the disease is not affected. The main consideration is the key role played by immune dysfunction in SARS-CoV-2 and HBV co-infected patients. The cytokine storm caused by SARS-CoV-2 infection leads to overproduction of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-2, while HBV infection with persistent viral antigens leads to virus-specific $CD4^+$ and $CD8^+$ T cell depletion, resulting in impaired cytokine secretion, especially IL-2 and TNF-α, in which the depleted and immunosuppressed state of HBV-specific T lymphocytes may attenuate or avoid the excessive host immune response to SARS-CoV-2 and reduce the cytokine storm, resulting in a less severe disease[63-65]; and (3) Prognosis is not affected. Previous studies have demonstrated that hepatitis C virus infection can limit the replication of HBV[66], a phenomenon called viral interference, in which one virus in the host prevents the replication of another co-infected virus by

Table 1 Characteristics of included studies

Ref.	Site	Sample size (*n*)	No. of patients with HBV infection, *n* (%)	Major serum biochemical characteristics of co-infected patients	Outcomes	
					Death, *n* (%)	Survival, *n* (%)
Guan *et al*[53]	China	1590	28 (1.8)	NR	1 (3.6)	27 (96.4)
Zou *et al*[2]	China	93	93 (100.0)	Abnormal liver function	7 (7.5)	86 (92.5)
Song *et al*[47]	China	4	2 (50.0)	Abnormal blood parameters	0 (0.0)	2 (100.0)
Qi *et al*[10]	China	3	1 (33.3)	Abnormal liver enzyme	1 (100)	0 (0.0)
Hambali *et al*[11]	Malaysia	1	1 (100.0)	Abnormal liver enzyme	0 (0.0)	1 (100.0)
Ali *et al*[12]	Qatar	1	1 (100.0)	Elevated liver enzymes	1 (100)	0 (0.0)
Zha *et al*[13]	China	31	2 (6.5)	Elevated liver enzymes	0 (0.0)	2 (100.0)
Cai *et al*[14]	China	298	5 (1.7)	Elevated liver enzymes	NR	NR
Naderi *et al*[15]	Iran	93[1]	13 (13.8)[2]	Elevated liver enzymes	0 (0.0)	13 (100.0)
Yip *et al*[16]	Hong Kong SAR, China	5639	353 (6.3)[3]	ALT abnormality	8 (2.3)	345 (97.7)
Richardson *et al*[17]	United States	5700	8 (0.1)	Elevated liver enzymes	NA	NA
Kang *et al*[54]	Korea	7723	267 (3.5)	NR	12 (5.1)	255 (94.9)
Li *et al*[18]	China	7	7 (100.0)	Elevated liver enzymes	0 (0.0)	7 (100.0)
Chen *et al*[9]	China	123	15 (12.2)	Abnormal liver enzyme	2 (13.3)	13 (86.7)
Bongiovanni *et al*[19]	Italy	1	1 (100.0)	Liver enzymes increased	0 (0.0)	1 (100.0)
He *et al*[20]	China	571	15 (2.6)	Liver enzymes increased	0 (0.0)	15 (100.0)
Wen *et al*[21]	China	110	5 (4.5)	Abnormal liver enzyme	NA	NA
Zou *et al*[7]	China	105	105 (100.0)	Liver enzymes increased	98 (93.3)	7 (6.7)
Wang *et al*[8]	China	436	109 (25.0)	Abnormal liver enzyme system	13 (11.93)	96 (88.1)
Chen *et al*[5]	China	326	20 (6.1)	Lower level of prealbumin	0 (0.0)	20 (100.0)
Zhang *et al*[22]	China	23	23 (100.0)	Liver enzymes increased	0 (0.0)	23 (100.0)
Liu *et al*[48]	China	220	50 (22.7)	Abnormal blood parameters	4 (8)	46 (92.0)
Yu *et al*[6]	China	67	7 (10.4)	No significant change	0 (0.0)	7 (100.0)
Liu *et al*[23]	China	71[4]	20 (28.2)[4]	Abnormal liver enzyme system	0 (0.0)	20 (100.0)
Lin *et al*[24]	China	133	17 (12.8)	Liver enzymes increased	NR	NR
Bekçibaşı and Arslan[25]	Turkey	156	20 (12.8)	Liver enzymes increased	0 (0.0)	20 (100.0)
Colaneri *et al*[26]	Italy	1	1 (100.0)	Liver enzymes increased	0 (0.0)	1 (100.0)
Ma *et al*[50]	China	109	1 (0.9)	Normal liver enzymes	NA	NA
Chen *et al*[27]	China	274	11 (4.0)	Abnormal liver function	5 (45.5)	6 (54.5)
Ding *et al*[28]	China	2073	134 (6.5)	Liver enzymes increased	8 (6.0)	126 (94.0)
Rodríguez-Tajes *et al*[29]	Spain	484	72 (14.9)	Liver enzymes increased	8 (11.1)	64 (88.9)
Parlar *et al*[51]	Turkey	479[5]	43 (9.0)[2]	No significant change	0 (0.0)	43 (100.0)
Yang *et al*[30]	China	2899	105 (3.6)	Liver enzymes increased	18 (17.1)	87 (82.9)
Yigit *et al*[31]	Qatar	1	1 (100.0)	Liver enzymes increased	1 (100)	0 (0.0)
Aldhaleei *et al*[32]	United Arab Emirates	1	1 (100.0)	Liver enzymes increased	0 (0.0)	1 (100.0)
Ji *et al*[33]	China	140	7 (5.0)	Liver enzymes increased	0 (0.0)	7 (100.0)

Kim *et al*[34]	United States	867	62 (7.2)	Liver enzymes increased	5 (8.1)	57 (91.9)
Wang *et al*[35]	China	1	1 (100.0)	Abnormal liver enzyme system	0 (0.0)	1 (100.0)
Zhong *et al*[36]	China	2	1 (50.0)	Liver enzymes increased	0 (0.0)	1 (100.0)
Fernández-Ruiz *et al*[49]	Spain	18	2 (11.1)	Decreased white blood cells	1 (50.0)	1 (50.0)
Huang *et al*[37]	China	1	1 (100.0)	Elevated total bilirubin	1 (100)	0 (0.0)
Patrono *et al*[57]	Italy	10	2 (20.0)	NR	0 (0.0)	2 (100.0)
Qin *et al*[38]	China	1	1 (100.0)	Abnormal liver enzyme system	0 (0.0)	1 (100.0)
Liu *et al*[58]	China	1	1 (100.0)	NR	0 (0.0)	1 (100.0)
Loinaz *et al*[39]	Spain	19	4 (21.1)	Abnormal liver enzyme system	1 (25.0)	3 (75.0)
Adali *et al*[40]	Turkey	231	77 (33.3)	Abnormal liver enzyme system	6 (7.8)	71 (92.2)
Oruç *et al*[55]	Turkey	92	4 (4.3)	NR	0 (0.0)	4 (100.0)
Guardigni *et al*[56]	Italy	606	12 (2.0)	NR	NA	NA
Sagnelli *et al*[41]	Italy	1	1 (100.0)	Liver enzymes increased	1 (100)	0 (0.0)
Lens *et al*[59]	Spain	1764[5]	9 (0.5)[2]	NR	0 (0.0)	9 (100.0)
Phipps *et al*[42]	United States	2273	15 (0.7)	Abnormal liver enzyme system	NA	NA
Li *et al*[52]	China	85	2 (2.4)	Normal liver enzymes	0 (0.0)	2 (100.0)
Wu *et al*[43]	China	1	1 (100.0)	Elevated liver enzyme	0 (0.0)	1 (100.0)
Wu *et al*[44]	China	620	70 (11.3)	Elevated liver enzymes	0 (0.0)	70 (100.0)
Iavarone *et al*[45]	Italy	50	5 (10.0)	Elevated liver enzymes	NA	NA
Marjot *et al*[60]	United Kingdom	745	96 (12.9)	NR	23 (24.0)	73 (76.0)
Huang *et al*[46]	China	1	1 (100.0)	Elevated liver enzymes	0 (0.0)	1 (100.0)
Total	57	37375	1932			

[1]93 cases were the sample size of the experimental group and that of the other healthy control group was 62 cases. 93 cases were all chronic hepatitis B patients.
[2]Patients with co-infection with hepatitis B virus and severe acute respiratory syndrome coronavirus 2.
[3]The 353 cases were patients with current co-infection with hepatitis B virus (HBV), and another 359 patients with past infection with HBV were not included.
[4]Sample size after propensity score matching.
[5]All were chronic hepatitis B patients.
No.: Number; NA: Data not available; NR: Data not reported; HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

competitive inhibition rather than by the action of immune antibodies. Impaired type I interferon activity has been found to be a major feature of patients with severe COVID-19[67-69]. When co-infected, HBV can inhibit the replication and proliferation of co-infected SARS-CoV-2 by enhancing type I interferon signaling, thereby reducing viral load and prolonging survival. In addition to this, the small number of HBV co-infected patients in the 57 studies that we included may have influenced the results.

Interpretation of altered liver enzymes and other biochemical abnormalities

Explanation of enzyme system abnormalities: Many studies have suggested that elevated liver enzyme systems are associated with drug therapy (Supplementary Table 1). Corticosteroids have been widely used to treat and benefit COVID-19[70]. Systemic corticosteroids are also associated with an increased risk of aspartate aminotransferase (AST) elevation[71]. Several studies have reported that elevated liver enzyme systems are associated with antiviral therapy medication[72-74]. The results of these studies showed that liver enzyme levels in patients dropped to near normal within a few days after discontinuation of medication. In addition, a study by Lei *et al*[71] showed that elevated AST and alkaline phosphatase levels were associated with the use of antifungal drugs. Another explanation is that some enzymes reflecting liver function, such as glutamic aminotransferase, are nonspecific and are expressed in a wide range of tissues, including heart, skeletal muscle, red blood cells, and liver, and can originate from other sites[75,76].

Explanation for the decrease in albumin: Patients with SARS-CoV-2 and HBV co-infection show a decrease in albumin, especially in heavy and critically ill patients, which may be due to the high

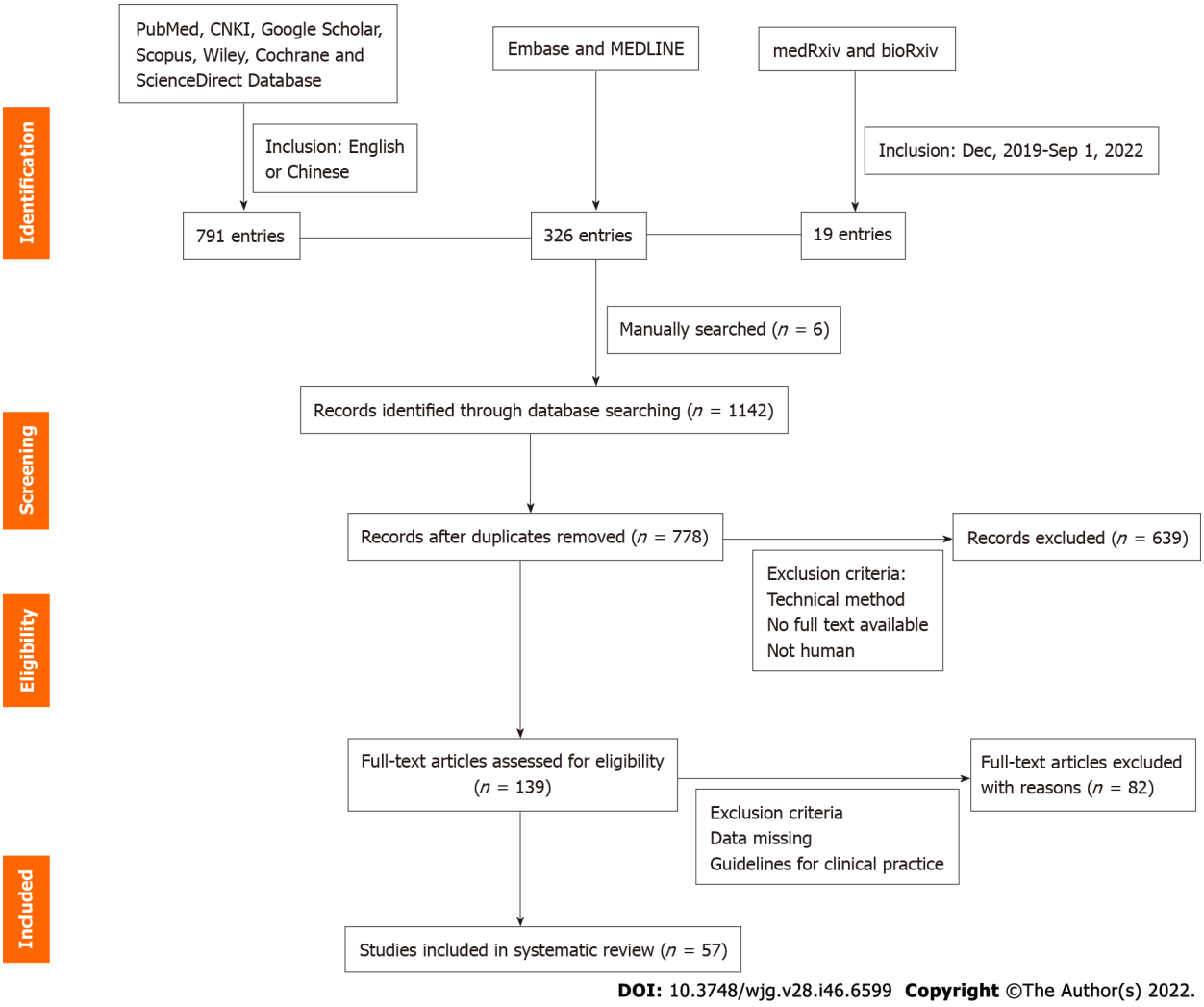
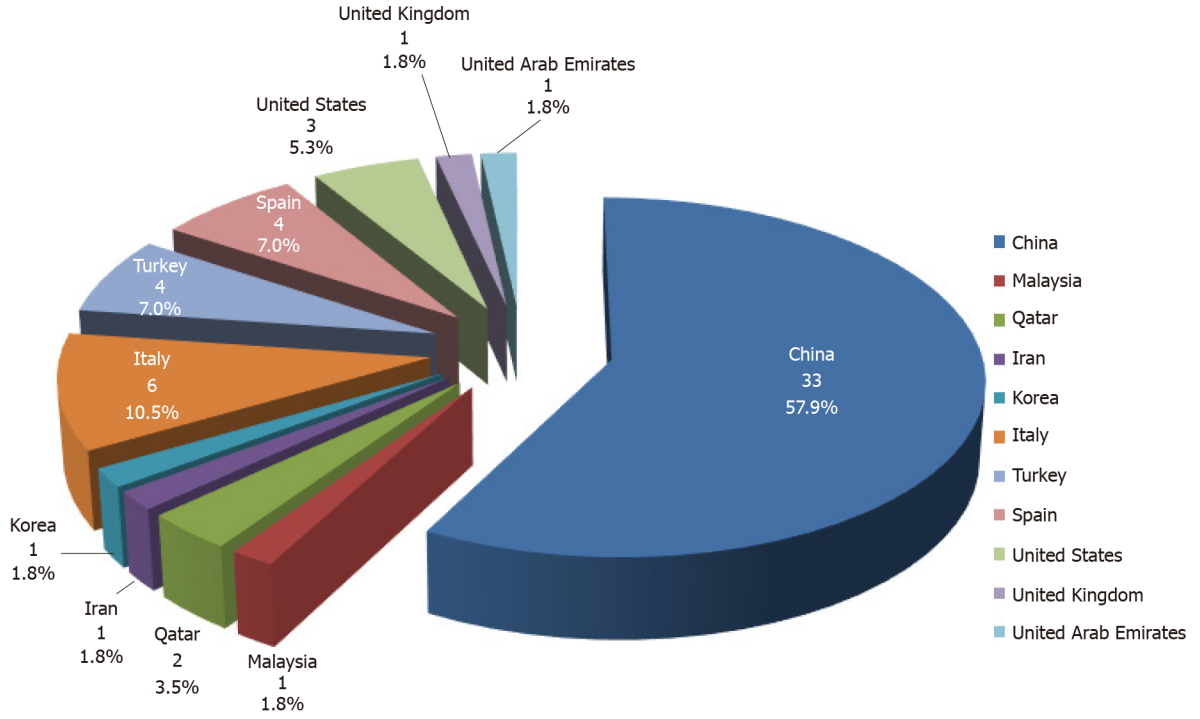


Figure 1 Flow diagram of literature search.

catabolic state of the patient's body, poor nutritional status, negative nitrogen balance, and insufficient raw materials for synthesizing albumin, as well as impaired hepatic synthesis of albumin due to hepatocyte damage. Corticosteroid treatment can also cause a decrease in albumin during the treatment process[21]. COVID-19 patients co-infected with HBV have lower prealbumin levels due to weaker hepatic reserve capacity, which also contributes to the decrease in albumin. With the resolution of the disease, most of the patients' liver function will return to normal.

Explanation for lymphocytopenia and thrombocytopenia: Patients with SARS-CoV-2 and HBV co-infection have significantly lower white blood cell counts, mainly due to reduced lymphocyte counts and significantly lower monocyte levels. The mechanism leading to lymphocytopenia remains unclear. Since low lymphocyte counts are associated with increased disease severity and mortality in COVID-19 [77,78], patients with SARS-CoV-2 and HBV co-infection may be in greater need of increased clinical surveillance. Thrombocytopenia is common in liver disease caused by HBV infection. The mechanism of thrombocytopenia in COVID-19 may be that SARS-CoV-2 reduces platelet production, increases platelet destruction, or increases platelet consumption[79]. Specifically, the lung is one of the organs where megakaryocytes dynamically release platelets[80], and SARS-CoV-2 damages the lungs of COVID-19 patients through angiotensin-converting enzyme 2 (ACE2), leading to increased destruction of megakaryocytes in the lung and resulting in decreased platelet production. In addition, SARS-CoV-2 may directly invade hematopoietic cells or infect bone marrow stromal cells by binding to CD13 or CD66a receptors, *etc.*, damaging megakaryocytes and platelets and exacerbating apoptosis[81]. Cytokine storm leads to immune hyperactivation, causing cellular damage and increased platelet destruction through autoantibody or immune complex activation of complement; at the same time, immune hyperactivation results in the release of large amounts of inflammatory factors that promote excessive platelet activation and platelet-monocyte aggregation formation[82], forming thrombi at the site of injury and further leading to increased platelet depletion and destruction.



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Figure 2 Pie-chart distribution by country of the 57 included studies.

DISCUSSION

Impact of COVID-19 on hepatitis B

We mainly describe the effects of COVID-19 on hepatitis B from the aspects of HBV reactivation, abnormal liver enzyme system, mortality, and liver histopathological changes.

HBV reactivation: Six of the 57 included studies reported COVID-19-induced HBV reactivation (Supplementary Table 1), which primarily refers to the re-detection of HBV DNA in patients who previously had HBV DNA below the lower limit of detection or a sudden and substantial increase in HBV DNA levels in individuals with detectable HBV DNA[83]. In some cases, it may also evolve into fulminant liver failure. The mechanism of HBV reactivation after SARS-CoV-2 infection is mainly due to a disruption of the balance between the immune status of the host and viral replication. The intensity of glucocorticoid or immunosuppressive therapy is a major risk factor for HBV reactivation during COVID-19 treatment[29,41,84]. Age, male sex, and severe comorbidities (such as hypertension, diabetes, hypercholesterolemia, and chronic kidney disease) are considered risk factors for HBV reactivation[29]. In addition, SARS-CoV-2 infection causes significant lymphopenia, which may increase the likelihood of HBV reactivation.

Although there is a risk of HBV reactivation with SARS-CoV-2 infection, studies have shown that the overall risk is low. In a study by Rodríguez-Tajes *et al*[29], 61 patients with severe COVID-19 on immunosuppressive therapy were followed up for at least one month and no cases of hepatitis B surface antigen (HBsAg) reversal were found, with only two (3%) patients having detectable serum HBV-DNA.

Considering the risk of reactivation, it is recommended that HBV patients should start or continue anti-HBV therapy once COVID-19 is diagnosed[85], and some scholars recommend treatment with the nucleoside analogs entecavir and tenofovir[83], and closely monitoring HBV virological indicators and indicators related to liver injury. It has been suggested that the use of IL-6 receptor antagonists can reduce the risk of HBV reactivation, and in Watanabe *et al*[86]'s study, 152 patients who recovered from HBV infection treated with disease-modifying antirheumatic drugs had a very low risk of HBV reactivation (< 5%).

Abnormalities in liver enzymes: Most studies have evaluated the effects of COVID-19 in HBV infected patients mainly manifesting as abnormalities in liver enzymes, dominated by elevated levels of alanine aminotransferase (ALT), AST, and total bilirubin (TBIL), especially in severely ill patients[2,5,7-46] (Supplementary Table 1). Several studies have shown a predominance of AST elevations in COVID-19 patients[14,17,71,87], reflecting the severity of the disease. A large study including 5700 subjects showed elevated levels of AST (58%) and ALT (39%) in hospitalized COVID-19 patients[17]. Sixty-two percent of critically ill patients had elevated AST levels[88]. Abnormal liver function tests ranged from 37% to 69%, ALT and AST increased by 18.2% to 31.6% and 14.8% to 35.4%, respectively[14,52,89,90], and several

studies have reported elevated TBIL elevation (5.1% to 11.5%)[89,91]. It is worth noting that AST alone does not prove with certainty that more liver damage occurred, as other tissues, including heart or muscle tissue, can also release glutathione[75,76].

Zhao *et al*[92] also noted that COVID-19 patients had significantly higher levels of γ-glutamyl transpeptidase, lactate dehydrogenase (LDH), and α-hydroxybutyric dehydrogenase than non-COVID-19 patients. A study by Chen *et al*[27] including 113 deceased patients (five of whom were co-infected with SARS-CoV-2 and HBV) showed that the deceased patients had creatinine, creatine kinase (CK), LDH, cardiac troponin I, N-terminal pro-brain natriuretic peptide (NT-proBNP), D-dimer, IL-2 receptor (IL2R), IL-6, IL-8, IL-10, and TNF-α concentrations that were significantly higher compared to those in recovered patients. Among these, several studies have confirmed that NT-proBNP is strongly and independently associated with mortality in COVID-19 patients[93-95]. In addition, serum albumin was below the normal range, and prothrombin time was prolonged due to the compromised synthetic function of the liver.

Mortality: Several reports have suggested that pre-existing liver disease may be associated with high mortality in patients with COVID-19[96-99]. Studies have noted that the risk of mortality is associated with pre-existing liver disease status and not with age, race, body mass index, hypertension, or diabetes [96]. Several studies have evaluated the relationship between the severity of abnormal liver tests and mortality in patients with COVID-19[1,14,96,97]. A study from the United States showed that among 2780 patients with COVID-19, the mortality rate was 12% in patients with chronic liver disease (10 out of 250 patients with chronic liver disease have CHB), compared to 4% in patients without liver disease[99]. Another study of 152 consecutively submitted COVID-19 cases in patients with chronic liver disease found a higher mortality rate in COVID-19 patients with combined chronic liver disease, 11.8% of whom were hepatitis B patients[96]. These studies support that SARS-CoV-2 affects the immune response, exacerbating HBV replication during the acute and chronic phases of hepatitis B infection[12]. Iavarone *et al*[45] also concluded that COVID-19 is associated with increased mortality in patients with deteriorating liver function. The cause of death may be related to the failure of patients to receive timely specialist care, inadequate hepatoprotective therapy, and hepatitis B infection complicated by SARS-CoV-2 leading to fulminant hepatitis and multi-organ failure.

Histopathological changes of the liver: Histopathology-specific manifestations of the liver in patients with COVID-19 include binucleated or multinucleated hepatocytes, swollen mitochondria, and reduced glycogen granules[47]. Macrovesicular steatosis is one of the common nonspecific manifestations[72], in addition to mild portal lymphocytic infiltration, mild sinusoidal dilatation, inflammatory cells in the sinusoids, and portal vein inflammation, with electron microscopy also showing the presence of viral particles[100-103].

Effect of hepatitis B on COVID-19

We will describe the effect of hepatitis B on COVID-19 in terms of the rate of infection in patients with hepatitis B, changes in biochemical indicators and cytokines, and the prognostic effect of hepatitis B on COVID-19 (including mortality, complications, and severe COVID-19).

Rate of infection: An important indicator of the impact of hepatitis B on COVID-19 is the rate of infection. It has been shown that hepatitis virus infection reduces the chance of COVID-19 infection to some extent in people with hepatitis B[15]. Antiviral drugs also reduce susceptibility to COVID-19 infection. Data from Naderi *et al*[15] showed that the risk of COVID-19 in healthy individuals was 2.3 times higher than that in patients with CHB. Antiviral therapy in patients with CHB, including tenofovir and entecavir, resulted in a decreased incidence of SARS-CoV-2 positivity. Kang *et al*[54] reported that the occurrence of CHB resulted in a lower rate of SARS-CoV-2 positivity. A recently published survey of a larger cohort in Spain showed a reduced incidence of COVID-19 infection in CHB patients given tenofovir, suggesting a positive effect of tenofovir on SARS-CoV-2[59]. A large study in the United States including 5700 inpatients with COVID-19 showed that 14% (13/93) of CHB patients and 32.25% (20/62) of controls were infected with SARS-CoV-2, respectively, and the risk of developing COVID-19 was 2.3 times higher in healthy controls than in CHB patients, suggesting that antiviral drugs also reduced the susceptibility to SARS-CoV-2 infection[17].

Cytokines and biochemical indicators: As with the effects of COVID-19 on hepatitis B, SARS-CoV-2 and HBV co-infection aggravates liver injury in COVID-19 patients[5,24], leading to abnormalities in the liver enzyme system. In addition to ALT, AST, alkaline phosphatase, and TBIL described previously[30,32,37], IL-6, LDH, D-dimer TNF-α, CK, and other inflammatory factors levels are elevated[14,27,104,105].

Among the cytokines in co-infected patients, IL-6 was most significantly elevated. IL-6 is the result of SARS-CoV-2 activation of the immune system leading to massive cytokine release[106]. IL-6 plays a role as a pro-inflammatory factor in the regulation of vascular leakage, complement activation, and coagulation pathways. The coagulation biomarker D-dimer is derived from the formation and cleavage of cross-linked fibrin, and the systemic pro-inflammatory cytokine response following viral infection

may induce endothelial cell dysfunction, leading to overproduction of thrombin, which activates platelets and stimulates fibrinolysis, resulting in elevated D-dimer[107,108]. D-dimer has also been found in COVID-19 patients to be associated with poor outcome and abnormally dysregulated in CHB patients[109]. LDH not only plays an important role in glucose metabolism by catalyzing the conversion of pyruvate to lactate, but also regulates the immune response by inducing T cell activation and enhancing immunosuppressive cells through lactate production[110]. Serum CK levels are the most sensitive indicator of muscle damage[111]. Hypovolemia occurring in dehydrated patients with severe COVID-19 may lead to renal failure, which may increase CK levels[25]. Two recent studies suggest that high CK, IL-6, and D-dimer levels may be early warning indicators of serious disease and monitoring these indicators may help in the early detection of adverse events[25,112].

Mortality, complications, or serious prognosis: Patients with SARS-CoV-2 and HBV co-infection are more likely to have severe disease and a worse prognosis, including higher mortality and complications [44,55,60]. Two recent studies have shown that patients with COVID-19 who have pre-existing liver disease have a higher mortality rate compared to patients without liver disease[96,97]. Mirzaie *et al*[113] found that of the reported SARS-CoV-2-HBV co-infection cases, the mortality rate was 4.7%, 14.1% were transferred to the intensive care unit (ICU), and 38.8% reported severe COVID-19. In a study by Chen *et al*[9], seven of 15 HBV-infected patients (46.7%) developed severe disease, compared with 24.1% of severe cases in COVID-19 patients without HBV infection. In a study of 436 patients with COVID-19, more patients with CHB developed a severe status compared to non-CHB patients [30/109 (27.52% *vs* 5.20%) 17/327], with mortality rates of 13/109 (11.93%) and 8/327 (2.45%), respectively[8]. Another recent study by Wu *et al*[44] involving 70 co-infections showed a higher proportion of severe patients and critical patients in the COVID-19 and HBV co-infected group than in the non-HBV infected group (32.86% *vs* 15.27%). Data from a study by Yang *et al*[30] showed that patients with COVID-19 combined with CHB had a higher risk of death and ICU admission than patients without CHB. These data support the hypothesis that patients with liver disease may be associated with serious outcomes in patients with COVID-19. However, it is unclear whether these results reflect the severity of the patient's pre-existing liver disease or the severe prognosis caused by SARS-CoV-2 itself. However, it has also been reported to the contrary, that is, the incidence of ICU admission or death may be lower in patients with observed pre-existing HBV infection [none (0%) *vs* 36 (6.47%)] compared to 556 of 571 COVID-19 cases without HBV infection[20]. These findings suggest that the immune status of the host is influenced to some extent by CHB, which may affect the consequences of infection by SARS-CoV-2.

COVID-19 has no effect on hepatitis B

Yu *et al*[6] included 7 HBsAg(+) and 60 HBsAg(-) (a total of 67) COVID-19 patients and found no extensive fluctuations in markers of HBV replication during the acute course of SARS-CoV-2 infection, indicating that SARS-CoV-2 had no effect on HBV kinetics, and that co-infection did not prolong viral shedding or latency cycles in COVID-19 patients, and it did not trigger reactivation or seroconversion of CHB. Therefore, they concluded that SARS-CoV-2 infection would not be a source of HBV reactivation. Ding *et al*[28] conducted a study in which 204 of 2073 patients with COVID-19 had pre-existing liver disease, including 134 patients with hepatitis B and five patients with CHB cirrhosis, and after adjusting for baseline characteristics by matching propensity scores between groups, the in-hospital mortality rate in patients with hepatitis B was similar to that in patients without liver disease. These results suggest that SARS-CoV-2 infection may not increase the risk of severe liver injury in patients with hepatitis B and in patients with compensated liver function.

Hepatitis B has no effect on COVID-19

In a study by Wen *et al*[21], patients with COVID-19 combined with hepatitis B had no significant increase in the probability of abnormal ALT, AST, and albumin compared to patients without combined hepatitis B. No differences were found in various adverse clinical outcomes in patients with CHB compared to HBsAg negative patients. Chen *et al*[5] divided 326 COVID-19 confirmed cases into 20 patients with co-infection with HBV and 306 patients without HBV infection for observation and found no significant differences in the levels of liver function parameters, discharge rates, length of stay, or mortality between the two groups. Similarly, a study by Yu *et al*[6] showed that co-infection with HBV did not increase the severity of COVID-19 or prolong the length of hospital stay. There was also no significant difference between the two groups in terms of disease recovery at discharge. Zou *et al*[7] and Zhang *et al*[22] showed that the proportion of organ damage in patients co-infected with HBV was not significantly different from that in patients infected with SARS-CoV-2 alone. Liu *et al*[23] matched 21 patients with COVID-19 and HBV co-infection to 51 patients with COVID-19 without HBV, and found that HBV did not prolong the shedding of SARS-CoV-2, nor did it add to the progression of COVID-19 or increase the risk of poor prognosis. Similarly, Li *et al*[18] and He *et al*[20] had similar conclusions. Hepatic biochemistry (ALT, AST, and TBIL) was similarly dynamic and insignificantly different between the two groups of patients at each time point. In addition, HBV antiviral therapy has been reported to have a limited impact on the incidence and outcome of COVID-19[114]. The analysis by Zhu *et al*[115] also supports the view that HBV is not a significant risk factor for serious adverse outcomes among hospitalized patients with COVID-19.

Data from several studies have shown that co-infection did not negatively affect the course of COVID-19, although the SARS-CoV-2/HBV co-infection group had higher results in terms of liver function index levels[16,25,28]. Guardigni *et al*[56] in a study of 606 subjects found no statistical difference in mortality between the two groups, despite the higher rate of admission to the ICU in HBV-positive individuals. Pre-existing viral liver infections had no impact on the clinical and virological evolution of COVID-19. A study by Liu *et al*[48] found that although patients co-infected with SARS-CoV-2 and HBV exhibited more severe monocytopenia, thrombocytopenia, and hepatic dysfunction in albumin production and lipid metabolism, no differences in inflammatory cytokine levels were observed between patients co-infected with SARS-CoV-2 and HBV and those infected with SARS-CoV-2 alone. Most of the disturbances could be reversed with recovery from COVID-19, and HBV co-infection had no significant effect on the outcome of COVID-19.

Possible mechanisms of interaction between COVID-19 and liver injury

The mechanisms underlying liver dysfunction in patients with SARS-CoV-2 and HBV co-infection are not fully understood and may include direct injury, immune injury, drug injury, ischemia and hypoxia, and reactivation of HBV with immunosuppressive drugs[8-11], or may be the result of a combination of factors.

Direct injury: SARS-CoV-2 replication directly causes liver damage. There is increasing evidence that SARS-CoV-2 can directly cause liver damage in patients with COVID-19. ACE2 is widely present in human organ tissues, with the highest expression in the small intestine and also in the spleen, brain, muscle, heart, and liver[116]. It has been shown that SARS-CoV-2 can use ACE2 as a receptor for cell entry[117,118]. Dipeptidyl peptidase 4 and transmembrane serine protease 2 can also act as receptors to mediate SARS-CoV-2 virus entry into host cells[119,120]. Pre-existing liver disease is associated with increased expression of ACE2 in hepatocytes, thereby increasing the likelihood of SARS-CoV-2 entry into hepatocytes[121,122]. Recent data have shown higher ACE2 expression in bile duct cells than in hepatocytes, suggesting that the virus may bind directly to ACE2 expressed on bile duct cells, leading to hepatic impairment[117,123]. These findings suggest that abnormal liver function may be caused by the preferential binding of SARS-CoV-2 to bile duct cells[124]. SARS-CoV-2 granules in the cytoplasm of COVID-19 patients' hepatocytes, mitochondrial swelling and structural damage, and massive apoptosis of hepatocytes were shown by electron microscopy, strongly suggesting direct cytopathy of SARS-CoV-2 in hepatocytes[125,126].

Immune impairment: Currently, the most discussed immune injury is the 'cytokine storm'. During the immune activation phase, the replication and proliferation of SARS-CoV-2 lead to cellular inflammatory necrosis and release of pro-inflammatory cytokines[127], activating T and B cells and recruiting macrophages[128-130], which in turn produce additional inflammatory factors, such as C-reactive protein, IL-6, IL-1, IL-8, and IL2R, leading to a cytokine storm that causes severe immune damage to the lung as well as the liver[131,132]. IL-6 is a potential risk factor for severe liver injury in patients with COVID-19[133,134]. IL-6 is produced *via* classical CIS-signaling or TRANS-signaling, two different pathways that lead to a systemic cytokine storm[135].

Drug-induced liver injury: The liver is an important organ for the metabolism of drugs, and antivirals are one of the main measures for the treatment of COVID-19. The drugs currently used for treatment, including antibacterial drugs, nonsteroidal anti-inflammatory drugs, traditional Chinese medicine, antiviral drugs, and other drugs used in clinical trials, such as chloroquine, hydroxychloroquine, and tocilizumab, may produce hepatotoxicity[72,96]. Cai *et al*[72] observed that lopinavir and ritonavir contributed significantly to liver injury in patients with COVID-19. Antifungal drugs and systemic corticosteroids have also shown a positive association with liver injury[71]. Traditional Chinese medicine plays a crucial role in the treatment of COVID-19, but it can also contribute to liver injury [136]. The mechanisms of liver injury are variable and it is generally believed that the pathogenesis of liver injury with antiviral drugs is related to mitochondrial toxicity, hypersensitivity/induced autoimmune hepatitis, and secondary bacterial infections[124,137]. In addition to this, HBV reactivation is also considered to be one of the manifestations of drug-induced liver injury, as HBV reactivation occurs at an increased rate in patients treated with high doses of long-term corticosteroids or immunomodulators[43].

Ischemia and hypoxia of the liver

Ischemia and hypoxia in the tissues and organs of COVID-19 patients after SARS-CoV-2 infection are common pathophysiological phenomena[78]. The consequences of progressive hypoxia may include viral proliferation, cytokine release, inflammation, and intravascular coagulation[78], leading to hypoxic injury in several organs, including the liver, and in severe cases, hypoxic hepatitis[138]. Cellular damage caused by hepatic ischemia and inflammatory response caused by reperfusion both lead to hepatocyte apoptosis and elevation of liver enzymes[139,140]. In addition, patients' prior underlying diseases, such as chronic lung disease[91], diabetes[105], and gastrointestinal disease[141], have been predicted as possible mechanisms of liver injury in the context of viral infection. Interestingly, being male has also

been reported to be associated with a high risk of liver injury[71,142].

Drug treatment and alternative therapies

COVID-19 combined with chronic liver disease has become a very prominent clinical problem, and the treatment and prevention of COVID-19 combined with liver injury are particularly important. In the treatment of COVID-19 combined with hepatitis B, the first priority is to treat the cause, followed by a comprehensive treatment plan of antiviral therapy, use of antibiotics, liver protection, and nutritional support, as well as aggressive treatment of comorbidities, such as hypertension and diabetes. Corticosteroids are now widely used to treat COVID-19, antiviral therapy is a basic treatment, and in China, traditional Chinese medicine is also used to treat SARS-CoV-2 infection[143]. Nucleoside (acid) analogues are used in the treatment of CHB. These drugs are effective in inhibiting HBV replication, reducing viral load, and improving liver histological lesions. Some studies have shown that in patients with SARS-CoV-2 and HBV co-infection, the use of nucleoside analogs is safe and does not lead to exacerbation of the disease due to drug administration. However, antiviral drugs may also cause liver damage, and corticosteroids may increase the risk of hepatitis exacerbation in patients with chronic HBV infection, especially when combined with other immunosuppressive drugs[144]. Therefore, the use of hepatoprotective drugs and close monitoring of changes in liver function are recommended in clinical work to minimize the risk of liver injury and HBV reactivation.

Interestingly, some authors have suggested that hemoperfusion can delay the progression of the hyperinflammatory process of COVID-19 and remove the toxins involved in acute liver failure, and could be an alternative option to mitigate disease progression in patients with COVID-19[26]. In addition, plasma therapy in recovering patients has been reported to be very effective in patients who still have the virus in their system[145,146].

Care monitoring and social management

During the COVID-19 pandemic, delays in the care of patients other than those with COVID-19 may result in negative outcomes for CHB patients due to discontinuation of antiviral therapy. Patients with hepatitis B, especially those in the decompensated phase of cirrhosis, many of whom have a decline in blood cells associated with hypersplenism, will frequently present with pulmonary and abdominal infections due to their reduced immune function, and this is a particularly important situation to screen and identify. For patients with HBV combined with SARS-CoV-2 infection, it is recommended not to discontinue anti-HBV drugs and to strengthen the dynamic monitoring of the patient's liver function. The diagnosis and treatment of chronic diseases are long-term, and a variety of problems may still be faced in the post-pandemic era. Therefore, it is important to emphasize the importance of network hospitals, strengthen the use of network cloud platforms, increase network consultation and follow-up, provide online assistance to these patients, and reduce unnecessary hospital visits. It is also important to avoid large gatherings during a pandemic, and be careful to wear masks and wash your hands more often in public places.

Vaccination

The already licensed COVID-19 vaccine is immunogenic and has a good short-term safety record[147-151]. Given the potential for SARS-CoV-2 infection to have serious health consequences in patients with hepatitis B, patients with liver disease should be considered a priority population for receiving the vaccine. The potential interactions between the COVID-19 vaccine and other vaccines have not been well studied, and the few data available is limited to the concurrent use of the COVID-19 vaccine and influenza vaccine, with studies showing that antibody responses to both vaccines were maintained, and no safety issues were reported[137]. Future clinical studies on the interaction between the COVID-19 vaccine and hepatitis B vaccine are urgently needed.

Limitations

The present review has several limitations. First, most of the current research papers on SARS-CoV-2 and HBV co-infection were retrospectively designed, and no randomized controlled studies were found, which means that a selection bias must be acknowledged. Second, some studies do not distinguish the clinical stage of HBV infection and do not clarify the immune stage of the patient. Clinical outcomes may be different in patients with chronic HBV infection or acute HBV infection, and the prognosis of COVID-19 may be influenced by the status of the patient's immune system, which may affect the interpretation of the results. Third, the results related to hepatitis B were derived from a limited sample size of observational and case studies, some of which were not classical studies on hepatitis B. The participation of patients with hepatitis B may have partially contributed to these studies, and thus the overall analysis may have overstated its role in COVID-19. Finally, because different countries have different institutions, policies, and economic conditions regarding COVID-19, and the 57 studies extracted for our review were distributed across 11 countries, it is necessary to carefully interpret whether there is regional or population bias in these results (Figure 2) and whether they are representative of the entire population.

CONCLUSION

In conclusion, both SARS-CoV-2 and HBV are globally pathogenic viruses, and there are currently few and conflicting data on the potential association between COVID-19 and hepatitis B. In the future, high-quality randomized trials are needed to further elucidate the interaction between COVID-19 and hepatitis B. To our knowledge, our review is the most comprehensive report to date describing the association between COVID-19 and hepatitis B. By reviewing these studies, we provide direct evidence of some association between COVID-19 and hepatitis B, explain the clinical phenomena that they exhibit, explore the possible mechanisms of the interaction between COVID-19 and hepatitis B, and provide management measures for co-infected patients, and therefore, our review can provide a reference for future scientific studies and clinical management.

ARTICLE HIGHLIGHTS

Research background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and hepatitis B virus (HBV) infections are two major current global public health crises. Both SARS-CoV-2 and HBV infections can cause liver damage. It is unclear whether HBV itself makes patients more susceptible to coronavirus disease 2019 (COVID-19), or whether COVID-19 leads to worse outcomes in patients with HBV infection. There are few and conflicting data on the association between COVID-19 and hepatitis B.

Research motivation

All current studies on patients with both COVID-19 and hepatitis B will be searched to explore the complex relationship between COVID-19 and hepatitis B in order to inform the research and management of patients co-infected with SARS-CoV-2 and HBV.

Research objectives

We searched almost all current clinical studies on COVID-19 combined with hepatitis B to describe their interaction, possible mechanisms, and clinical interventions to inform the clinical management of this special population with SARS-CoV-2 and HBV co-infection.

Research methods

We used an inclusive search strategy and searched the literature in the following online databases: PubMed, China National Knowledge Infrastructure, Google Scholar, Scopus, Wiley, Web of Science, Cochrane, and ScienceDirect, as well as medRxiv and bioRxiv, and also manually searched references of the included studies. Articles reporting studies conducted in humans and discussing hepatitis B and COVID-19 were included. We extracted the relevant data from the full text of eligible studies for the table after excluding duplicative publications. Some topics that included HBV or COVID-19 samples but did not have quantitative evidence were excluded from the review.

Research results

After excluding duplications and publications without quantitative evidence, a total of 57 studies were eligible and included in this review; all were retrospective, and they were from 11 countries. Most of the studies suggested an interaction between hepatitis B and COVID-19, mainly in the form of abnormal liver enzymes, abnormal blood parameters, and HBV reactivation; however, 12 of these studies clearly indicated no effect between hepatitis B and COVID-19.

Research conclusions

Both SARS-CoV-2 and HBV are both globally pathogenic viruses, and there are few and conflicting data on the potential association between COVID-19 and hepatitis B. Our review provides direct evidence for some associations between COVID-19 and hepatitis B, explains the clinical phenomena that they exhibit, explores the possible mechanisms of the interaction between COVID-19 and hepatitis B, and provides management measures for co-infected patients.

Research perspectives

To our knowledge, our review is the most comprehensive report to date describing the association between COVID-19 and hepatitis B. In the future, high-quality randomized trials are needed to further elucidate the interaction between COVID-19 and hepatitis B.

FOOTNOTES

Author contributions: He YF designed the study, reviewed the literature, and drafted the manuscript; Jiang ZG and Wu N retrieved and summarized the literature; Ren JL and Bian N advised on the review and reviewed the final manuscript; and all authors have read and approved the final manuscript.

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OPINION REVIEW

How to avoid overtreatment of benign colorectal lesions: Rationale for an evidence-based management

Marco Bustamante-Balén

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Abstract

Implementing population-based screening programs for colorectal cancer has led to an increase in the detection of large but benign histological lesions. Currently, endoscopic mucosal resection can be considered the standard technique for the removal of benign lesions of the colon due to its excellent safety profile and good clinical results. However, several studies from different geographic areas agree that many benign colon lesions are still referred for surgery. Moreover, the referral rate to surgery is not decreasing over the years, despite the theoretical improvement of endoscopic resection techniques. This article will review the leading causes for benign colorectal lesions to be referred for surgery and the influence of the endoscopist experience on the referral rate. It will also describe how to categorize a polyp as complex for resection and consider an endoscopist as an expert in endoscopic resection. And finally, we will propose a framework for the accurate and evidence-based treatment of complex benign colorectal lesions.

Key Words: Colorectal polyps; Endoscopic mucosal resection; Colorectal surgery

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Core Tip: Despite endoscopic treatment being of choice for the treatment of large benign colorectal neoplasms, many lesions are still being referred to surgery. Problems in identifying a polyp as complex to resect, too much self-confidence of the endoscopists, and the lack of a referral pathway may be causes underlying this situation. The organization of a structured referral network may be the main step to reducing the overtreatment of benign lesions. Decisive support from Medical Societies and Public Administration is warranted to set up this paradigm change.

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INTRODUCTION

The implementation of population-based screening programs for colorectal cancer (CRC) has led to an increase in the detection of large lesions with benign histology. For example, in a French study of a population screening program based on fecal occult blood testing, 5% of the polyps found were larger than 3 cm[1]. Many of these patients are asymptomatic and of intermediate age, and removal of the lesions should be performed with the goal of maximum efficacy (complete resections, few recurrences) and maximum safety (few adverse effects). Traditionally, most polyps considered "large" were biopsied and then referred to surgery for segmental resection of the colon. However, in recent years, with the improvement of endoscopes and the development of new techniques, endoscopic treatment can be considered the treatment of choice. However, too many benign lesions are still being referred to surgery, with associated morbidity and increased costs. In this review, we will justify the selection of endoscopic therapy as the treatment of choice, dig into the main causes for referring benign lesions to surgery, and propose an organizational solution for this situation. Most of the evidence that will be reviewed here focuses on endoscopic mucosal resection (EMR) because is the endoscopic technique of choice in most instances, while other endoscopic techniques [*e.g.*, endoscopic submucosal dissection (ESD) or full-thickness resection] are indicated for a more specific type of lesions.

WHY SHOULD ENDOSCOPIC RESECTION AND NOT SURGERY BE THE THERAPY OF CHOICE FOR THE TREATMENT OF BENIGN COLONIC LESIONS?

EMR can currently be considered the standard technique for the removal of benign lesions of the colon due to its excellent safety profile and good clinical results. Large series of patients, especially from the Australian endoscopic resection group, support this claim. Moss *et al*[2] performed a prospective evaluation of all patients referred for EMR of polyps ≥ 20 mm. In this cohort of particularly complex polyps, complete resection was achieved in a single session in 89.2% of patients with a recurrence rate of 20.4%. This recurrence was mostly minute and easily treated endoscopically. As for adverse effects, the same group reported a clinically significant bleeding frequency of 6.0%, of which only 44% required endoscopy and only one case required embolization[3]. The proportion of deep mural damage or perforation was only 3% and 0.6%, treated in all cases by endoscopic methods[4].

At least two meta-analyses confirm these results. The first, which evaluated the endoscopic management of lateral spreading tumors, and which analyzed separately EMR and DSE, reported a proportion of complete resections for the former of 99.5%, and a proportion of recurrences of 12.6%, most of which were manageable endoscopically. In terms of adverse effects, there was an aggregate proportion of perforation of 1.2% and bleeding of 9.6%[5]. In the second meta-analysis, the proportion of surgeries attributable to complications of EMR was less than 1%[6]. Therefore, we have an effective and safe endoscopic technique for the treatment of benign colon lesions.

The therapeutic alternative to endoscopic resection is surgery, which has classically been the treatment of choice. However, even with current techniques, surgery is not free of complications. The overall rate of adverse effects at 30 days is between 14% and 25% in the most modern series (Table 1)[7-10]. In general, the proportion of adverse effects is higher in open surgery, and as the age of the patient increases[7,8,10]. Surgery is also associated with a non-negligible risk of mortality which, although it is usually somewhat less than 1% (Table 1), in patients > 80 years of age can reach almost 3%[7].

No randomized studies are comparing both therapeutic strategies and such a study is unlikely to be performed due to ethical problems. One way to overcome this is the use of propensity score matching. Wickham *et al*[11] evaluated 95 patients referred because of endoscopically unresectable colorectal lesions and compared them to 190 propensity score-matched controls. Endoscopic resection was achieved in 66 (70%) of patients with a reduced hospital stay, a lower unplanned 30-day readmission rate, and fewer postoperative complications (4.2% *vs* 33.9%; $P < 0.001$) compared to surgery. Another attempt to make this comparison has been made using theoretical models. Ahlenstiel *et al*[12] compared the theoretical mortality of colon surgery, calculated using a proprietary Association of Surgeons of Great Britain and Ireland score, with the actual mortality from EMR of benign lesions in a cohort of 1,061 patients. While the theoretical mortality from surgery was 3.3%, there were no deaths in the first 30 days after EMR. The NNT to prevent one death was only 30. A recent Dutch study, using a microsimulation system and taking into account fatal complications of surgery, compared expert endoscopic resection of benign lesions *vs* laparoscopic surgery. Referral to an expert reduced from 2.1 surgeries for

Table 1 Main recent series on the morbidity and mortality of surgery for benign colorectal lesions

Ref.	Year	Country	Data source	N	Mortality (%)	Colostomy/ Ileostomy (%)	Major adverse event (%)	Readmission (%)	Surgical re-intervention (%)
Peery *et al* [7]	2018	USA	National Inpatient Sample[1]	12.732	0.7	2.2	14.0	7.8	3.6
Zogg *et al* [8]	2016	USA	National Inpatient Sample[1]	68.462[2]	-	-	14.7	-	1.0
de Neree *et al*[9]	2019	Netherlands	Systematic Review	139.897	0.7	-	24.0	-	0-8.9
Ma *et al* [10]	2019	USA	National Inpatient Sample[1]	262.843	0.8		25.3	-	-

[1]All-payer inpatient healthcare database.

[2]Overall colon surgery (not only colorrectal epithelial lesions): % of adverse events are specific for surgery of benign colorectal lesions.

benign polyps/1000 individuals to 0.2/1000 reducing also the number of deaths[13]. Some observational studies performed in Eastern countries and focused on ESD confirm that the latter has a shorter hospital length stay, an inferior 30-day readmission rate, and a lower complication rate[14,15].

Furthermore, endoscopic treatment is cost-effective compared to surgery. At least four studies in different countries and contexts have compared endoscopic resection with surgery in terms of cost-effectiveness (Table 2). All agree that endoscopic resection (EMR or ESD) is cost-effective compared to surgery, and this difference widens if the complications of surgery are taken into account[13,16-19]. The development of adverse effects after surgery has been associated with a 106% increase in the average length of stay and a 91% increase in the average cost of hospitalization[10].

Therefore, and this is reflected in the clinical practice guidelines[20,21], it seems clear that endoscopic treatment should be the first-choice treatment for benign colonic lesions, provided that quality standards are maintained[21].

WHAT IS THE SITUATION IN ACTUAL CLINICAL PRACTICE?

Several studies from different geographic areas agree that many benign colon lesions are still referred to surgery. In a French study conducted in a population-based screening program with fecal occult blood test, out of 4,251 patients with at least one polyp, 4.1% were referred to surgery[1]. In a study conducted in the USA, 47% of colorectal lesions sent for surgery were benign polyps[22]. In the aforementioned study by Zogg *et al*[8] the mean number of annual colectomies performed for benign pathology in the United States was around 22,000. A study of the British CRC screening program showed that, in the period from 2006 to 2009, 21.7% of the polyps sent for surgery were directly operated on, without a prior endoscopic attempt. Depending on the centers, the use of surgery as the first therapeutic option varied between 7% and 36%[23]. Finally, a recent Australian survey study showed that 16.7% of respondents would send directly to surgery a 45 mm benign polyp[24]. Saade *et al*[25], in a retrospective review of 144 patients with surgical resection for benign colorectal polyps found that 82% were referred for surgery without attempting an endoscopic resection. Of those, 22% had polyps < 2 cm, a size that should be resected en bloc by an average endoscopist[21].

But in addition, the rate of referral to surgery is not decreasing over the years, despite the theoretical improvement in endoscopic resection techniques. In a retrospective review of a national surgical database also in the United States, it was found that the incidence rate of surgery for benign polyps increased significantly over time, from 5.9 *per* 100,000 patients in 2000 to 9.4 *per* 100,000 patients in 2014, while during this same period the rate of surgery for CRC decreased. This increase was significantly greater in urban academic hospitals, which is just where one would expect it to decrease[26]. A Dutch study reviewed a national database of anatomic pathology reports looking for all cases of benign colon lesions removed by surgery. They showed that the ratio of the number of resections for benign lesions to the total number of colonoscopies performed was significant and remained constant over the last decade (2005-2015), ranging from 0.37 to 0.26[27].

Table 2 Cost-effectiveness studies, endoscopic therapy *vs* surgery

Ref.	Year	Country	Endoscopic technique	Design	Comparison	Costs analyzed	Results
Swan *et al*[19]	2009	Australia	EMR	Observational monocentric	Endoscopy *vs* surgery, Considering surgery without major complications	Direct costs including a 1-day hospital stay for EMR, Loss of utility not considered	EAC: $2051 pp, SAC: $9041
Jayanna *et al*[16]	2016	Australia	EMR	Observational multicentric	Endoscopy *vs* surgery, Considering surgery with and without complications	Direct costs including hospital stay and adverse events, 1st surveillance endoscopy	EAC: $4668 pp, SAC: $12720, If surgery 7.5% complications -> SAC: $45530
Law *et al*[17]	2016	USA	EMR	Decision analysis tree (hybrid Markov model)	Endoscopy (resection + surveillance, surgery if recurrence at 12 mo) *vs* laparoscopic surgery, Considering complications in both arms	Direct costs, Loss of utility considered, QALY, Sensitivity analysis	EAC: $5570 ppEndoscopy QALY: 9.64, SAC: $18717 pp, Surgery QALY: 9.58, Laparoscopy is cost-effective if complete EMR < 75.8%, EMR adverse events rate > 12% and laparoscopy cost < $14.000
Dahan *et al*[18]	2019	France	ESD	Observational monocentric	Endoscopy *vs* surgery, Considering complications in both arms	Direct costs including hospital stay and endoscopy costs	EAC: €3190, SAC: €8490
Buskermolen *et al*[13]	2022	Netherlands	Non-specified	Microsimulation screening analysis (MISCAN-colon)	Surgery *vs* attempted removal by an expert endoscopist, Considering complications in both arms	Direct costs, Loss of utility considered, QALY, Sensitivity analysis	EAC: €60.200, SAC: €72.700, Endoscopy QALY: 33.1/1000 individuals, Surgery QALY: 32.9/1000 individuals

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosa dissection; QUALY: Quality adjusted life years; EAC: Endoscopy average cost; SAC: Surgical average cost; pp: Per patient.

WHAT IS THE MAIN REASON THAT BENIGN LESIONS ARE STILL BEING SENT DIRECTLY FOR SURGERY? THE ENDOSCOPIST FACTOR.

Le Roy *et al*[1] evaluated different variables related to referral to surgery. Size, location, and histology (villous component and high-grade dysplasia) were influential. The most relevant factor was size > 20 mm. These data have been confirmed in a recent meta-analysis, in which the most frequent causes of referral to surgery were polyp location (right colon), size (median size = 4 cm), and sessile morphology [9].

However, the assessment of the relative importance of these factors when deciding whether to perform endoscopic or surgical resection is highly subjective and dependent on the endoscopist. In fact, in the study by Le Roy *et al*[1] great variability was found among endoscopists when referring the patient to surgery, a difference that was maximal for polyps > 20 mm (0 to 46.6%). These results were confirmed in a study in which 154 endoscopists of different expertise (residents, gastroenterologists, surgeons, and experts in polyp resection) responded to a survey on how to treat 6 polyps presented in video format. Endoscopists specializing in complex resections referred the patient to surgery at a significantly lower percentage than other specialists (3.1% *vs* 13.3% non-specialists endoscopists *vs* 17.2% surgeons). In the univariate analysis that took into account the size of the polyp, its location, the patient's ASA, and the type of specialist, the fact that the endoscopist was not a specialist in the resection of complex polyps was the only variable related to the probability of referring the patient to surgery [odds ratio (OR) 4.93, 95%CI (1.5-16.26)][28].

This variability in clinical practice has direct practical consequences. A retrospective review of the Dutch pathology registry reviewed the medical reports of patients referred for surgery for benign polyps over 9 years. Three expert endoscopists reviewed the records to assess whether the patient could have benefited from endoscopic treatment. The conclusion was that 73% of the cases could have been treated endoscopically. This referral to surgery as a primary elective treatment was more frequent in county hospitals than in tertiary referral centers[29]. In other words, the endoscopist is perhaps the most influential factor in whether a patient is referred for surgery, and experience in complex resections seems to be the fundamental characteristic.

IS IT EFFECTIVE TO REFER THE PATIENT TO AN ENDOSCOPIST WITH EXPERTISE IN COMPLEX ENDOSCOPIC RESECTIONS?

The possibility of reducing surgeries if the patient was referred to an expert endoscopist has also been demonstrated in practice. In one study, 58 patients referred to surgery for colorectal polyps were collected. An expert endoscopist re-evaluated these lesions in a new colonoscopy to decide whether endoscopic resection was possible. Of these 48 could be resected endoscopically although 5 of them underwent surgery later either because of malignancy in the specimen (4 cases) or recurrence (1 case). In any case, surgery could be avoided in 43 (74.1%)[30]. Other studies of similar design agree that surgery is avoidable in 30-70% of cases when the polyp is reviewed by an expert endoscopist, including up to 26% of lesions with previously attempted resection[31-33].

WHY IS AN EXPERT ENDOSCOPIST MORE EFFECTIVE?

The main advantage of an experienced endoscopist when removing complex lesions is that he or she will have a higher proportion of complex resections with a lower frequency of adverse effects.

Few studies are comparing the results of EMR in terms of efficacy according to the experience of the endoscopist. The St. Marks group evaluated the proportion of successful resection between a group of expert and non-expert endoscopists. Experts were successful in 76% of cases while non-experts were successful in only 40%[34]. A retrospective study showed that the performance of resection by an expert endoscopist was protective against incomplete resection in the presence of other risk factors for incomplete resection [adjusted OR 0.13, 95%CI (0.04-0.41)][35]. The CARE study demonstrated that the rate of incomplete resection in polyps that, in the judgment of the endoscopist, were assumed to be completely removed was high (10.1%), increased with polyp size, and was highly dependent on the endoscopist. These findings suggest that technical skill in complex resections is not universal[36].

The experience of the endoscopist is also a key factor for adverse effects. In a study of 97,091 colonoscopies performed on an outpatient basis, the OR for bleeding or perforation increased significantly when the endoscopist performed fewer than 300 colonoscopies per year[37]. In a similar study of 24,509 endoscopies, the complication rate was significantly higher for endoscopists performing fewer than 200 procedures per year [RR 2 95%CI (1.1-3.7)][38]. Finally, a study of 2,315,126 colonoscopies confirmed that endoscopists performing fewer than 300 colonoscopies per year had a higher rate of bleeding and perforation[39].

HOW DO WE KNOW THAT AN ENDOSCOPIST IS AN EXPERT? THE EGO OF ENDOSCOPISTS.

Some of the studies evaluating the rate of surgery for benign polyps have found that this rate is higher in urban teaching hospitals, hospitals that often have experts or units specialized in endoscopic resection[7]. There may be too much self-confidence in the endoscopist (*e.g.*, "if I cannot resect this lesion, nobody can") or there may be some feeling of shame in referring a lesion to a colleague.

The endoscopists' perception of their expertise is often not supported by objective criteria. This fact was elegantly highlighted in a study in which 268 surveys were conducted among endoscopists asking them, among other things, about their experience in resection and their surgical referral practices. Eighty-one (30%) of them considered themselves capable of performing complex resections on lesions that could perfectly well have been referred to surgery. However, of this group of "experts" 17% had never removed a polyp > 5 cm and 32% did not perform more than 20 EMRs per year. In other words, a significant number of endoscopists considered themselves experts in resection when there was no objective evidence of this. And this had consequences for patient management because endoscopists who considered themselves non-experts tended to send patients to another colleague, while "experts" more frequently sent them to surgery (26% *vs* 68%)[40].

Inappropriately mischaracterizing oneself as an expert endoscopist directly affects patient management in three key ways: (1) The endoscopist will initiate a resection that he or she cannot complete, and complications are possible; (2) If resection is not attempted the patient is more likely to be sent to surgery than a more expert colleague; and (3) As a consequence of the previous two, the patient is more likely to be incorrectly sent to surgery.

The definition of some objective criteria to classify an endoscopist as an expert in resections could help in this situation, reassuring the less-experienced endoscopist to refer the lesion to a better-prepared endoscopist. However, there are no established criteria to identify the expert endoscopist in performing EMR, perhaps due to the lack of structured training for this technique.

WHAT LESIONS SHOULD BE REFERRED AND TO WHOM? HOW TO TRANSFORM SUBJECTIVITY INTO OBJECTIVITY

As we have seen, the assessment of the difficulty of resection of a particular polyp depends primarily on polyp factors (size, morphology, location, suspicion of submucosal invasion, *etc.*) that may seem rather subjective in their evaluation. It seems logical, therefore, to develop systems that are as objective as possible to define which polyp should be sent to an expert endoscopist, in such a way as to help endoscopists of varying degrees of experience to make the decision. On the other hand, it seems necessary to have criteria for evaluating endoscopists to define, as objectively as possible, what is an expert endoscopist, aimed to easily identify referral specialists. Finally, the expertise of the individual endoscopist is not enough. His or her work environment must allow for comprehensive treatment of benign colon lesions, with the use of different resection techniques depending on the case, and must have sufficient casuistry to maintain the skills acquired. In this section, we will review these three sides of the management of large colorectal lesions: The complex polyp, the expert endoscopists, and the reference endoscopy unit.

The "complex" polyp

To avoid or at least reduce individual subjectivity in the assessment of the difficulty of resection, objective evaluation criteria are necessary. A group of experts, following the Delphi methodology, defined a score to classify the theoretical difficulty in the resection of colon polyps. This score ("SMSA" scoring system) has four parameters (Site, Morphology, Site, and endoscopic Access), and assigns different scores to the values adopted by each one. Thus, a polyp > 4 cm, with a flat morphology, located in the right colon, and with difficult access obtains the highest score (17 points). All polyps scoring > 12 points are considered level 4, and appropriate for truly expert endoscopists[41].

The British Society of Gastroenterology (BSG) suggests other lesion´s objective features that anticipate a complex resection, grouped into three areas: Increased risk of malignancy evidenced by optical diagnosis, increased risk of incomplete resection, and increased risk of adverse effects (Table 3). Notably, the experience of the endoscopist is included as a criterium for defining a complex polyp because of an increased risk of adverse events[42]. The definition of a complex polyp, therefore, involves a judicious and sensible evaluation of the endoscopist´s expertise.

The categorization of a polyp as complex involves more than the difficulty in its removal. The management of a complex polyp may also need, to some extent, and depending on the lesion´s characteristics, the need for expert, interdisciplinary management. In this sense, the European Society of Gastrointestinal Endoscopy (ESGE) recommends sending the lesion to be evaluated in an expert center, besides the aforementioned criteria, when superficial submucosal invasion is suspected[21].

The "expert" endoscopist

The level of experience required of endoscopists to resect polyps is not objectively defined. The ASGE guidelines indicate that all endoscopists should be able to resect pedunculated or sessile polyps < 2 cm [43], but resection of complex polyps requires special skill, specific learning, and experience, and it seems unreasonable to expect this from all centers[44].

The most objective criterion could be the number of resections performed. However, this particular number has not been defined yet. Several studies place the experience necessary to perform EMR with adequate quality standards between 100 and 125 resections[45,46]. Other authors, based on a retrospective study of a new EMR unit for 4 years, suggest a number of 30 EMRs per year, but the SMSA level of their lesions was not described [47]. This figure seems a bit low when dealing with SMSA level 4 Lesions. The BSG broadly suggests that the number of resections per year should be enough to maintain acceptable quality and safety standards, but also indicates that there is no evidence to recommend a specific figure[42]. Regarding ESD, there is also a high variability in the reported number of cases needed to achieve proficiency ranging from 20 to 250 cases[48]. To maintain proficiency, the ESGE curriculum recommends performing at least 25 cases per year[49].

Some more objective methods to evaluate polypectomy competency have been developed, like the Direct Observation of Competence Skills (DOPyS). This instrument assesses several items, like optimal polyp position, determining the full extent of the lesion, polypectomy technique, *etc.* Using this tool Duloy *et al*[50] described significant variation in polypectomy competency rates (30% to 90%) with rates decreasing for larger polyps. However, it has not been designed specifically for EMR. The BSG has proposed auditable indicators to assess the ability of endoscopists to perform EMR, focusing on efficacy (% recurrences), safety (% complications), and annual case volume (Table 3)[42].

The implementation of structured learning tools or courses could help to evaluate who may be competent in endoscopic resection techniques. *In vivo* and virtual tools have been described for EMR and ESD[51,52], and a formal curriculum for ESD has been developed by the ESGE[49]. However, there is not a similar curriculum for EMR training, which has essentially been limited to that obtained during residency and has repeatedly proven to be insufficient[53].

Table 3 Objective parameters for assessing lesions, endoscopists, and units

Complex polyp	Expert endoscopist	Reference endoscopy unit
SMSA score ≥ 12 (Level 4)[41]	BSG criteria[42]	BSG criteria[42]
BSG criteria[42]	500 independent colonoscopies	Ensure that endoscopists undertake a sufficient number of procedures a year to maintain acceptable standards[4]
Increased risk of malignancy	100-125 EMR to obtain competence	
Kudo´s pit pattern V	A non-defined number[1] of EMR procedures to maintain competence	
Paris 0-IIc/0-IIa+IIc		Time from referral to definitive management: < 8 wk
LST-NG/LST-Gm (dominant nodule)	Fulfilling key performance indicators	
NICE 3/Sano III	Presence of recurrence/residual polyp at 12 mo < 10%	Geraghty *et al*[40]
Increased risk of incomplete resection/recurrence		Provided endoscopy list time for the additional workload with a dedicated list
Size ≥ 40 mm	EMR perforation rate: < 2%	
Difficult location (ileocecal valve, appendix, diverticulum, dentate line)	Post-polypectomy bleeding rate: < 5%	Staff to include at least two endoscopists that can cover each other and endoscopy nurses with training in complex polypectomy
Within an inflamed segment of the colon	DOPyS[2]	
Prior failed resection attempt	ESGE[3] curriculum for optical diagnosis[59]	
Non-lifting sign	Assessing competence: ≥ 80 % accuracy for identifying submucosal invasion in large (≥ 20 mm lesions), Maintaining competence: *in vivo* audit and review of at least 10 large (≥ 20 mm) lesions within a year	Equipment: including necessary snares and hemostatic devices
Increased risk of adverse events		Surgeons for discussion in the MDT and case of operative treatment of adverse events
Cecum		
Endoscopist´s expertise		Robust referral system including administrative staff support and tools for virtual MDT
ESGE criteria[21]		
Difficult location or poor access (ileocecal valve, periapendicular, anorectal junction)		
Prior failed resection attempts		
Non-lifting sign		
SMSA level 4		

[1]Enough to maintain quality standards.
[2]Direct Observation of Competence Skills (not specific for EMR).
[3]For achieving competence in optical diagnosis of early colorectal cancer.
[4]Review in conjunction with other key performance indicators.
BSG: British Society of Gastroenterology; EMR: Endoscopic mucosal resection; LST-NG: Non-granular lateral spreading tumor; LST-Gm: Granular mixed lateral spreading tumor; NICE: NBI International Colorectal Endoscopic classification; ESGE: European Society of Gastrointestinal Endoscopy; MDT: Multi-disciplinary team; DOPyS: Direct Observation of Competence Skills; SMSA: Site, Morphology, Size, Access.

Moreover, for increasingly larger polyps, with flat morphology, in difficult locations, or patients with previous colon pathology such as inflammatory bowel disease, the endoscopist must also master alternative mucosal resection techniques such as the underwater technique, or fragmented cold loop resection, and know how to choose between them by changing the initial resection plan. Strategies to decrease the recurrence rate, like margin ablation, margin marking, or hybrid argon plasma coagulation [54-56] should be mastered as well. Finally, an endoscopist specializing in endoscopic resection of larger lesions must also know and apply optical diagnosis (use of NICE and JNET classifications, use of dyes, use of magnification, *etc.*) to identify those that, due to a higher probability of superficial submucosal invasion, require an en bloc resection, using ESD or full-thickness resection[20,57]. And also, those that, due to a high probability of deep submucosal invasion, must be surgically removed[58]. Following, for instance, the BSG guidelines, these skills are needed to correctly classify a polyp as complex (Table 3) [42]. Mastering optical diagnosis also needs proper training and practice to obtain and maintain competence[59].

The "reference" endoscopy unit

It seems that the number of procedures is the single most important factor influencing on efficacy and safety results of an endoscopy unit specialized in complex resections. In the aforementioned Australian study on risk factors for post-polypectomy bleeding, the unit (one that had performed fewer than 75 procedures) was directly related to the likelihood of immediate post-polypectomy bleeding [adjusted OR 3.78 (2.35-6.10)] and to bleeding occurring beyond the first 48 h[60]. And immediate post-polypectomy bleeding was related to the probability of recurrence at the first endoscopic control. Other studies describe a lower rate of complications in colonoscopies performed in a hospital center than in an outpatient clinic[39]. It thus appears that units that accumulate a larger number of cases are more effective in the treatment of complex colon lesions. This has also been shown in studies on newly developing EMR units, in which the rate of complications decreased as experience time was gained[47]. The frequency of SMSA level 4 Lesions in a single institution is unlikely to be enough to maintain competency, therefore a centralized referral system seems advisable[40].

But in addition to the experience and casuistry of the units, the adequate management of complex colon lesions requires adequate infrastructure. High-definition endoscopes that allow precise optical diagnosis, electrosurgical units with automatic microprocessors, CO_2 insufflation, specific pumps for lavage channels, *etc.*[21]. They also need the availability of a variety of resection devices (snares, knives, injection substances, hemostatic, *etc.*) allowing switching resection techniques and dealing with complications. Resections of complex polyps lengthen the procedure time beyond that required for a conventional colonoscopy[61], prolongation which is closely related to the size of the polyp in question. Therefore, the unit will have to have the facility to adjust the citation slots to the performance of longer and more complex procedures. Finally, the work of this kind of unit must be integrated into a background with experienced surgeons, a multidisciplinary team for the management of complex lesions[40], and all the infrastructure (computed tomography scanning, *etc.*) to handle possible adverse events.

The BSG has also proposed measurable domains for accrediting Endoscopy Units for performing EMR (Table 3). Regarding ESD, the American Society of Gastrointestinal Endoscopy recommends setting up an "ESD cart" with the necessary equipment for the procedure and the management of adverse events. The presence of experienced nurses and technicians is also addressed[48].

IS IT TRULY EFFICIENT IN REAL PRACTICE TO REFER COMPLEX POLYPS TO EXPERIENCED UNITS?

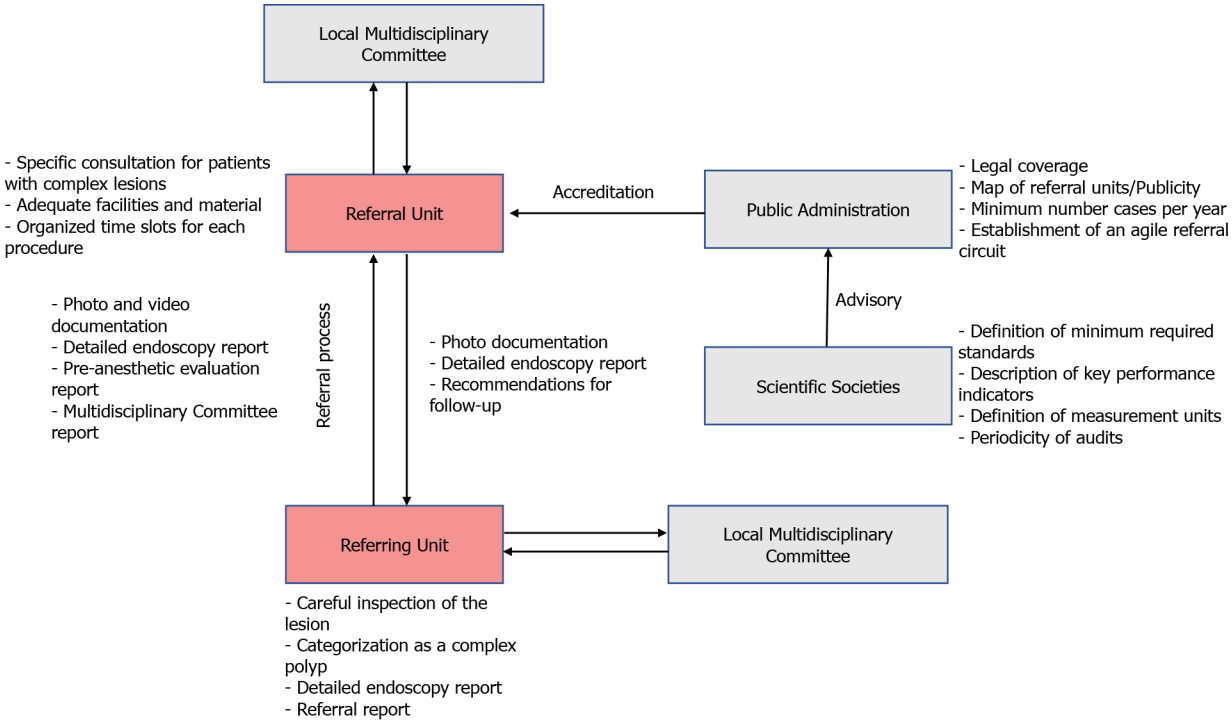
We have learned that an appropriate referral of complex polyps to an expert endoscopist increases the rate of successful endoscopic treatment and reduces adverse events compared to surgery. We have also learned how a referral endoscopy unit should be to ensure efficacy. How has this been translated onto clinical practice?

The Australian group was the first to demonstrate the efficacy of a referral unit for the treatment of large colorectal lesions. Out of 174 patients referred for 193 complex polyps, 90% avoided surgery with a procedural success of 95% excluding those patients with invasive cancer[19]. Another Australian retrospective study comparing the surgical rate of benign colorectal lesions before and after the introduction of a specific EMR service in a tertiary referral center showed a 56% reduction in the number of patients referred to surgery[62]. More recently, in France, a study evaluated the evolution of surgical management of benign polyps > 2 cm after the implementation of a regional referral network for the management of these lesions. This regional care network included two specialized endoscopists in the referral center with direct access by e-mail or by phone to all general gastroenterologists in the region and with twice-a-year regular meetings with general gastroenterologists. The surgical management rate of benign lesions decreased significantly after the implementation of the referral network from 14.6% in 2012 to 5% in 2017[63]. Similarly, in the Northwest of the Netherlands, a reference panel of expert endoscopists for the general endoscopist to consult was organized. Eleven centers participated and 88 patients were evaluated by the panel. Overall, 43.2% of consulting endoscopist changed their initial management strategy after consultation, and in 56 cases (63.3%), the patient was referred to another endoscopy center[64].

In conclusion, setting up a referral system for the management of complex polyps is efficient and translates into immediate clinical advantages.

HOW TO SET UP A REFERRAL ENDOSCOPY UNIT FOR THE MANAGEMENT OF COMPLEX POLYPS: PRACTICAL TIPS AND AN ORGANIZATION PROPOSAL

To achieve the objective of an adequate and comprehensive treatment of large benign colon lesions, several actors must be involved: The Administration, the Scientific Societies, the Units themselves, and



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Figure 1 Organizational proposal for the management of complex benign colorectal lesions.

finally the referring endoscopists (Figure 1).

One side of the referral network is the referral endoscopy unit. Having established the main characteristics that a referral unit must have to be considered as such, a certification system should be put in place. For instance, using criteria similar to that of the BSG plus others adapted to the specific background, periodical audits of the organization and key performance indicators should be performed. The local Endoscopy Societies should collaborate in the design of the certification protocol (definition of key indicators, measurement units, audit´s periodicity, *etc.*). An example of this kind of collaborative effort, although not applied to complex resections, is the Qualiscopia initiative in Spain, which aimed to monitor and certify endoscopy units and endoscopists in quality in colorectal screening colonoscopy [65]. Ideally, the endoscopy unit has to establish an internal Quality Management Program, including the definition of a system for recording staff´s initial competency and continued competency on an annual basis. Working together with the referral unit there should be a multidisciplinary team in place, made up of surgeons, oncologists, radiologists, and every specialist that could be involved in the management of complex polyps, especially when facing a deep submucosal invasion or dealing with adverse events. Finally, the referral unit should have enough administrative staff to handle the communication and documentation workload from and to the referring units. Clean communication systems should be established (e-mail, telephone, videoconference, *etc.*).

The Administration should, in agreement with the Societies, should give legal cover to the concept of Referral Unit, should establish a map of referral units according to population needs, ensure a minimum number of cases per unit to maintain competence, and should participate in the establishment of an agile regional referral circuit[21,42]. It is the Administration that should make available for all possible referring physicians a list with the accredited Units.

The other face of the referral network is the referring endoscopist. These endoscopists have to carefully evaluate and characterize the lesion using the usual classifications (NICE, Paris, *etc.*), categorize it as a "complex lesion" and decide if he/she can resect it. If not, the lesion should not be biopsied (unless an invasive carcinoma is suspected), and a tattoo should be placed not too close to the lesion[66]. Several pictures and videos should be taken. All this information should be provided in a detailed and structured endoscopy report. Ideally, a Multidisciplinary Committee (including at least an endoscopist and surgeons) should evaluate the patient and make a report that should be incorporated into the referral report.

The referral process should be detailed including clinical and administrative data from the patient, photo and video documentation of the lesion, a detailed endoscopic report including size, location, and morphology of the lesion, if biopsies were taken or if there was any resection attempt. The reason for referring the lesion should also be described. In correspondence, once the lesion has been treated in the referral unit, a thorough report of the applied treatment should be done, again with photo documentation. The technical result of this treatment (success/failure) should be provided. Finally,

recommendations for patient follow-up should be attached to the report. It is mandatory to maintain fluid communication between referring and referral units during the therapeutic process. Al the steps back and forth in the referral process should be subjected to the Quality Management Program and should be auditable.

CONCLUSION

Endoscopic resection is the treatment of choice for large colorectal lesions. However, overtreatment is still an important issue in many countries. Organizing a network of specialized endoscopy units in complex resections seems to be the main approach to tackling this situation. This development should be accompanied by the organization of an accreditation system and a Quality Management Program, a process in which endoscopy units, endoscopists, Scientific Societies, and the Public Administration should be involved.

FOOTNOTES

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REVIEW

Mucosal imaging in colon polyps: New advances and what the future may hold

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Abstract

An expanding range of advanced mucosal imaging technologies have been developed with the goal of improving the detection and characterization of lesions in the gastrointestinal tract. Many technologies have targeted colorectal neoplasia given the potential for intervention prior to the development of invasive cancer in the setting of widespread surveillance programs. Improvement in adenoma detection reduces miss rates and prevents interval cancer development. Advanced imaging technologies aim to enhance detection without significantly increasing procedural time. Accurate polyp characterisation guides resection techniques for larger polyps, as well as providing the platform for the "resect and discard" and "do not resect" strategies for small and diminutive polyps. This review aims to collate and summarise the evidence regarding these technologies to guide colonoscopic practice in both interventional and non-interventional endoscopists.

Key Words: Colonoscopy; Colorectal cancer; Mucosal imaging; Chromoendoscopy; Polyp surveillance; Polyp characterization

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Core Tip: Advanced mucosal imaging enhances polyp detection and characterization. This detailed review summarises existing advanced mucosal imaging technologies to guide everyday colonoscopic practice for interventional and non-interventional endoscopists.

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INTRODUCTION

Colorectal cancer (CRC) accounts for 10% of cancer incidence and is the third leading cause of cancer-related death worldwide[1]. Whilst CRC incidence and mortality are increasing globally, there is now tangible evidence of the evolving efficacy of screening programs in developed countries including Australia, the United States, Iceland, New Zealand and Japan, where there have been improvements in both CRC incidence and mortality[2,3]. While these decreases are multifactorial and partly a result of lifestyle modification (reduction in smoking, weight loss, dietary changes), the implementation of population CRC screening programs has been integral to the prevention and early detection of CRC[4,5].

CRC develops through a well-documented adenoma-carcinoma cascade consisting of multiple differing pathways. Although underlying genetic mutations are diverse and heterogenous, most CRCs arise as either traditional tubular adenomas or serrated adenomas. Eventually these adenomas acquire additional carcinogenic mutations sufficient to develop invasive potential[6]. This sequence forms the basis of colonoscopic screening and surveillance programs. Not only can cancers be detected at an early stage where curative and non-invasive treatment is possible, but in many cases these pre-cancerous adenomas can be resected prior to their differentiation into carcinomas with invasive potential.

Unfortunately, interval CRCs still develop in patients who have undergone appropriate colonoscopic screening, accounting for 4.8%-7.9% of all CRCs[7-11]. Given that most adenomas take an estimated 5-15 years to develop into CRC, these interval cancers likely represent adenomas missed at the time of colonoscopy[12]. In fact, a 2019 meta-analysis found miss rates for adenomas to be as high as 26%[13]. Studies have consistently demonstrated that location in the proximal colon leads to an increased chance of missed adenomas, with interval cancers more than twice as likely to be proximally located[11]. Multiple factors contribute to this risk, as proximally located polyps are more likely to be flat, more likely to be sessile serrated polyps, more dysplastic whilst smaller and less likely to be hyperplastic polyps without malignant potential[14-16].

While certain polyp-related factors contribute to the likelihood of missed adenomas, overall adenoma detection rates (ADRs) are also highly operator-dependent. For example, a retrospective propensity-score matched study demonstrated an ADR of 44% for "high-ADR endoscopists" *vs* 26.9% for "low-ADR endoscopists" in the same Japanese screening population[17]. In this study, "high-ADR endoscopists" were more likely to detect proximal, non-protruding and high-risk adenomas. It is therefore not surprising that studies have demonstrated an inverse correlation between endoscopists" ADR and interval cancer development, with each 1% increase in ADR resulting in a 3% reduction in interval cancer risk[18,19]. Kaminski *et al*[19] also demonstrated an increase in interval cancer development in endoscopists with an ADR < 20%. Accordingly, societal guidelines recommend a minimum ADR of 25% (20% in women, 30% in men) as a means of ensuring quality control among colonoscopists[20]. More recently, the mean number of adenomas detected during colonoscopy has been raised as a possible alternative quality indicator, as the number of adenomas detected directly impacts surveillance intervals. Denis *et al*[21] found that even endoscopists with an ADR of more than 35% had considerable variation in mean adenoma detection over 42817 surveillance colonoscopies, from 0.36 to 0.98. The adenoma miss rate has also been demonstrated to vary considerably between high ADR endoscopists, instead correlating strongly with adenomas detected per colonoscopy[22].

Given the heterogeneity among proceduralists and the ongoing prevalence of interval CRCs, multiple add-on devices and techniques have been developed to increase mucosal visualisation and reduce adenoma miss rates. A 2020 network meta-analysis demonstrated that add-on devices such as "Endocuff vision" and techniques such as water-immersion colonoscopy do improve adenoma detection [relative risk (RR) 1.53 and 1.41 respectively] however they require additional equipment and cost while often increasing procedure times[23]. The addition of a transparent cap attached to the tip of the colonoscope has been demonstrated to improve adenoma detection while also reducing caecal intubation time[24-26]. However, a 2012 meta-analysis found the impact of these measures to be small, with a RR of 1.08 for adenoma detection and a mean 0.64 min reduction in caecal intubation time[27]. In the context of expansive population screening programs, small changes in equipment costs and procedure times have a considerable impact on a larger scale.

Advanced mucosal imaging techniques function by either improving image definition, application of dyes/altering the light source to enhance certain tissue features, digitally enhancing images in real time, or by providing "alerts" to the proceduralist for abnormal findings detected by artificial intelligence (AI). In doing so, these technologies aim to improve detection and characterisation of polyps without increasing equipment costs. This review aims to consider and summarise the numerous available advanced imaging technologies and examine their efficacy in both polyp detection and polyp characterisation. Whilst this is not a formal systematic review, it has been based largely on a structured interrogation of existing literature using Pubmed and Embase, with abstracts screened for relevance and reference lists searched for additional pertinent studies.

POLYP DETECTION

Standard and high-definition white light imaging

White light imaging (WLI) is the original unenhanced form of endoscopic imaging. Standard definition (SD-WLI) endoscopes produce a signal of up to 100000 to 400000 pixels, compared to high-definition (HD-WLI) endoscopes which produce from 850000 to more than 1 million pixels[28]. Despite this considerable improvement in image quality, studies comparing HD-WLI to SD-WLI have found an only marginal benefit in adenoma detection, with a 2020 meta-analysis of 6 randomised-controlled trials (RCTs) involving 4594 patients finding an ADR of 40% for HD-WLI *vs* 35% for SD-WLI (RR 1.13, P = 0.001)[29-31]. However, various studies have demonstrated a more significant increase in detection of flat adenomas (8.2%-9.5% *vs* 2.4%-3.8%), right sided adenomas (34% *vs* 19%) and sessile serrated polyps (RR 1.55, P = 0.03) with HD-WLI[29,31,32]. In the context of inflammatory bowel disease (IBD) where dysplasia detection is notoriously difficult, HD-WLI leads to increased likelihood of dysplasia on targeted biopsies, with an adjusted prevalence ratio of 2.99 (CI 1.16-7.79) in one 2013 study[33]. In fact, Krugliak *et al*[34] described 36 patients who underwent colectomy for dysplasia in IBD found using HD-WLI colonoscopy, in which no metachronous lesions were discovered that had not been detected endoscopically. While the overall benefit in adenoma detection may be marginal, the improved detection of high-risk, flat, right sided lesions, along with the fact that HD-WLI is now widely available, has led to almost universal uptake of HD-WLI in screening colonoscopy.

Chromoendoscopy

Chromoendoscopy involves topical application of dyes to enhance mucosal characterisation and improve detection of pathologic lesions. For adenoma detection during colonoscopy, the most commonly used dye is methylene blue, which is rapidly absorbed into healthy colonic mucosa and more slowly absorbed in dysplastic tissue[35]. More recently, chromoendoscopy using acetic acid has been described, acting as a mucolytic agent as well as increasing mucosal surface opacity[36].

Multiple studies have demonstrated the efficacy of chromoendoscopy for neoplasia detection (particularly proximal serrated lesions) during screening and surveillance colonoscopy, with a 2016 Cochrane review (7 studies, 2727 participants) finding an odds ratio (OR) of 1.53 for detection of at least one neoplastic lesion[37,38]. However, the incremental benefit in many of these studies has been marginal and not associated with any increase in detection of advanced adenomas or larger polyps[39,40]. The strongest evidence for the benefit of chromoendoscopy has been for detection of dysplasia in the IBD population. Compared to SD-WLI, multiple meta-analyses have demonstrated the superiority of chromoendoscopy, with a RR of up to 2.05 for dysplasia detection[41,42]. However, the utility of chromoendoscopy in IBD has become more controversial as more recent studies have not demonstrated a difference between chromoendoscopy and HD-WLI[41,43,44].

Chromoendoscopy has been shown to improve dysplasia detection in other high-risk populations, particularly in those with an increased risk of flat, right-sided lesions. A 2019 tandem study comparing HD-WLI and chromoendoscopy with indigo carmine in patients with serrated polyposis syndrome found a higher additional ADR (39% *vs* 22%, P < 0.001) in the chromoendoscopy group[45]. In hereditary non-polyposis colon cancer (HNPCC), a 2019 meta-analysis demonstrated improved adenoma detection with a relative risk (RR) of 1.53 (CI 1.07-2.17)[46]. However, again recent evidence has found the benefit of chromoendoscopy over HD-WLI to be marginal in this setting, with a 2021 meta-analysis of three RCTs not reaching statistical significance (OR 1.17, CI 1.81-1.70)[47-49].

Irrespective, widespread uptake of chromoendoscopy has been limited by the increase in procedure time required for dye application. A 2019 meta-analysis in IBD surveillance found the total procedure time to be a mean of 21.69 min (CI 9.01-34.38) longer for chromoendoscopy[50]. One method to counter this was described by Repici *et al*[51], using oral dye (methylene blue) ingested at the time of bowel preparation. Promisingly, this led to an 8.5% increase in ADR without increasing procedure times, although there was no difference in detection of larger or more advanced polyps.

Virtual chromoendoscopy

Virtual, or electronic chromoendoscopy have been developed in attempt to digitally recreate the enhanced mucosal visualisation of chromoendoscopy without increasing procedure time. However, no

form of virtual chromoendoscopy has been able to conclusively demonstrate a benefit with respect to polyp detection at colonoscopy.

Narrow-band imaging

Narrow-band imaging (NBI) uses optical filters to produce two narrow bands of light centred at wavelengths of 415 nm and 540 nm, corresponding to the primary and secondary light absorption peaks of haemoglobin. Superficial capillaries appear brown, highlighted by the 415 nm wavelength, while deeper vessels in the mucosa and submucosa are cyan due to the deeper penetration of the 540 nm wavelength[52].

The role of NBI in adenoma detection during routine colonoscopy in the general population has been extensively studied. Studies that have found a benefit for NBI in this setting have demonstrated an improvement particularly in the detection of flat or depressed lesions (Figure 1), with a pooled RR of 1.96 in a 2012 meta-analysis[53-56]. However, the majority of studies, including a 2012 Cochrane review by Nagorni *et al*[57], have shown no difference in overall adenoma detection[32,57-61]. In fact, one 2017 RCT demonstrated a reduction in ADR with NBI when adjusted for increased withdrawal time[62].

Multiple possible factors may contribute to the limitations of NBI in screening colonoscopy. Earlier-generation NBI resulted in a reduction in overall brightness due to the narrow bandwidths, which may limit overall visualisation in the wide colorectal lumen. The second-generation bright NBI has been developed to counter this, although recent studies have again demonstrated no difference in overall adenoma detection[58,63]. NBI also appears to be disproportionately affected by poor bowel preparation (which may also be in part due to reduced brightness), with a 2019 meta-analysis finding superior adenoma detection with second-generation NBI only in patients with maximal bowel preparation scores[64]. In addition, the colour spectrum of NBI is different to WLI and therefore may require experience and familiarity with the technology in order to be effective. This was demonstrated by Minamide *et al*[63] who retrospectively reviewed 1831 patients that underwent colonoscopy using second-generation bright NBI or WLI and found a higher polyp detection rate (PDR) with NBI (80.9% *vs* 71.4%, $P = 0.02$) in academic centres familiar with its use, while in community centres, there was actually a trend towards a higher PDR with WLI (51.1% *vs* 47.7%). Additionally, in the NBI group, the ADR for NBI-experienced proceduralists was 63.2% *vs* 39.2% for NBI-inexperienced proceduralists ($P < 0.001$).

i-SCAN

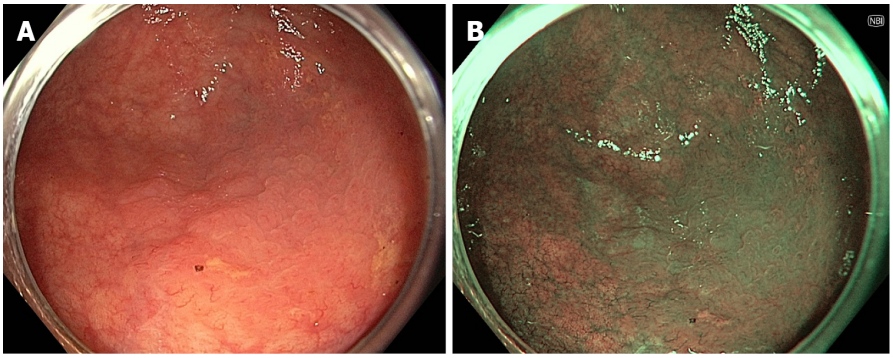
i-SCAN is a software-based post-processing technology, which digitally enhances WLI output through surface and contrast enhancement (i-SCAN mode 1) as well as tone enhancement (i-SCAN modes 2 and 3)[52]. Evidence has again been inconsistent regarding its efficacy for adenoma detection. Multiple studies have found an improvement in polyp and adenoma detection, the largest of which demonstrated a non-statistically significant improvement in ADR from 27% to 33% ($P = 0.33$)[65-68]. As demonstrated by Kidambi *et al*[69] in 2019, this effect has mainly been due to improved detection of diminutive, flat, right-sided adenomas[68,69]. In terms of high-risk populations, Bisschops *et al*[70] found a reduction in adenoma miss rates from 62% to 12% using i-SCAN in 61 patients with HNPCC. On the contrary, a 2012 prospective back-to-back study comparing HD-WLI with i-SCAN modes 1 and 2 in 389 screening colonoscopies showed no difference in ADR or adenoma miss rates, while a 2014 meta-analysis also demonstrated no difference in ADR[71,72]. There is therefore insufficient evidence to recommend routine use of i-SCAN in screening colonoscopy at this stage.

Flexible spectral imaging colour enhancement

Flexible spectral imaging colour enhancement (FICE) also involves digital enhancement of WLI images from the video processor, emphasising certain wavelengths which can be determined by the proceduralists according to 10 factory-determined pre-set modes[52]. FICE was developed with the goal of providing mucosal enhancement without compromising the familiarity of colour patterns from WLI. While one early back-to-back colonoscopy study in 2012 demonstrated reduced adenoma miss rate using FICE[73], multiple studies have demonstrated no significant impact, with the largest RCT in 2010 by Aminalai *et al*[74] finding no difference in ADR between FICE and HD-WLI over 1318 colonoscopies.

Linked colour imaging

Linked colour imaging (LCI) uses both pre- and post-processing technology with narrow wavelength light to separate colours, increasing the vividity of the red and white colour spectrums and enhancing the contrast of mucosal surface patterns and superficial capillaries (Figure 2). It was developed with the aim of enhancing lesion visibility and surface characterisation without compromising brightness or familiarity of colour spectrums, offering perhaps the most promising early evidence for improved adenoma detection[75-77]. It has been demonstrated to improve lesion visibility in both video- and image-based studies when compared to HD-WLI, particularly for nongranular, flat lesions[75,78,79]. While evidence varies with regard to overall ADR, studies have found improvements in proximal adenoma detection and miss rates[80-84]. In addition, a 2020 meta-analysis of 7 studies including 3097 patients demonstrated improved adenoma detection (RR 1.26, $P < 0.001$), particularly in the right colon



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Figure 1 Sessile serrated adenoma/polyp seen on high-definition white light imaging and narrow-band imaging. A: High-definition white light imaging; B: Narrow-band imaging.



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Figure 2 Sessile serrated adenoma seen on white light imaging, linked colour imaging, and blue light imaging. A: White light imaging; B: Linked colour imaging; C: Blue light imaging.

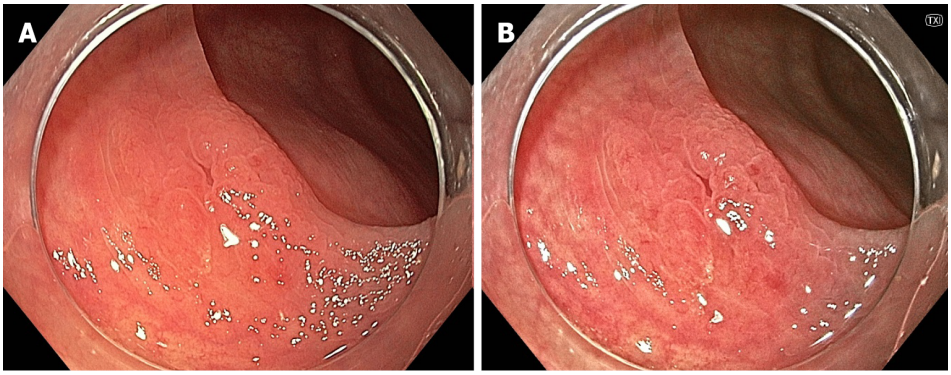
(RR 2.68, $P < 0.001$) and a mean of 0.27 additional adenomas detected per colonoscopy[85]. In a high-risk population of patients with HNPCC, LCI was found to improve ADR compared to HD-WLI (36.3% *vs* 25.6%, $P = 0.04$)[86]. Interestingly, while advanced imaging such as NBI appears to have a greater impact when used by experienced endoscopists, a 2021 study by Hasegawa *et al*[87] found a strong negative correlation between the baseline ADR with HD-WLI and the improvement ratio, indicating that perhaps the familiar colour pattern allows effective use by non-expert proceduralists.

Blue light imaging

Blue light imaging (BLI) is form of digitally enhanced imaging which concentrates and enhances a specific wavelength of light between 410-450 nm, increasing the contrast of superficial micro-vessels and mucosal surface structures (Figure 2). BLI uses four independent light-emitting diodes rather than the xenon light used in NBI, which is postulated to improve brightness[88]. This new technology has not been as extensively studied, however a video-based 2015 study demonstrated improved visibility scores with BLI bright mode compared to WLI according to both expert and non-expert proceduralists[89]. On a smaller scale this translated into improved adenoma detection, with two studies (including 182 and 127 patients respectively), finding an improvement in ADR from 27.8% to 46.2% ($P = 0.01$) and a reduction in adenoma miss rate from 10% to 1.6% ($P = 0.001$) compared to HD-WLI[90,91]. In contrast, the largest prospective study to date, including 963 patients, did not find a difference in ADR, though did find a non-statistically significant increase in mean adenomas per patient (APP) (1.27 *vs* 1.01, $P = 0.08$)[92].

Texture and colour enhancement imaging

Texture and colour enhancement imaging (TXI) is a recently developed technology, where the HD-WLI image is split into two layers, each individually undergoing brightness enhancement, tone mapping and texture enhancement before the images are stacked (TXI mode 1) and undergo further colour enhancement (TXI mode 2)[93]. Similarly to LCI, this aims to enhance mucosal visualisation without compromising familiarity of colour patterns or brightness (Figure 3). As an only recently developed technology, clinical studies examining adenoma detection are not yet available, however preliminary studies have demonstrated improved visibility of adenomas and sessile serrated polyps using TXI compared to HD-WLI[94,95].



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Figure 3 Sessile serrated adenoma seen on white light imaging and texture and colour enhancement imaging. A: White light imaging; B: Texture and colour enhancement imaging.

Virtual chromoendoscopy summary

While virtual chromoendoscopy theoretically offers enhanced mucosa visualisation without the increase in procedure time required for dye-based chromoendoscopy, none of the currently available technologies have conclusively demonstrated a meaningful improvement in ADR compared to HD-WLI. These technologies may all have a role particularly in improving detection of flat, right sided adenomas and may be used as additional tools for examination during screening colonoscopy, but evidence is not yet sufficient for recommendation in societal guidelines. Data appear most promising for newer forms of post-processing technology where brightness and familiarity of color patterns are preserved, however additional research is required to confirm this efficacy.

Autofluorescence imaging

Light of a specific wavelength induces cell autofluorescence produced by endogenous fluorophores, with varied characteristics between normal (green), inflamed (dark green) and neoplastic (magenta) tissue. Autofluorescence imaging (AFI) relies on the detection and delineation of this natural fluorescence after stimulating the mucosal cells with short wavelength light[96,97]. In doing so, AFI aims to detect neoplastic or dysplastic tissue even before it manifests as an anatomically distinguishable discrete lesion. McCallum *et al*[98] demonstrated that colonic adenomas have a significantly higher autofluorescence intensity than non-neoplastic polyps. It is therefore unsurprising that the greatest impact of AFI across multiple studies has been improved detection of flat, right sided polyps rather than elevated polypoid adenomas, with one RCT reporting an ADR for flat neoplasms of 42.5% *vs* 29.2% ($P < 0.001$)[99,100]. However, a 2015 meta-analysis found that while the adenoma and polyp miss rates were lower with AFI, there was no difference in overall ADR despite an average of 8 min longer procedural time for the AFI group[101].

Artificial intelligence

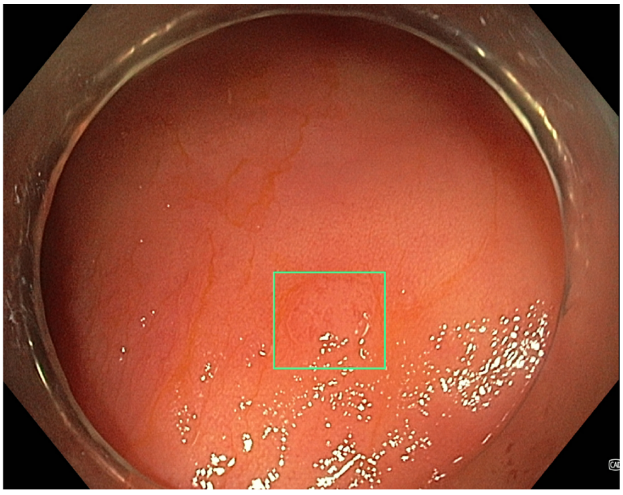
Multiple AI systems have been developed for polyp detection during surveillance colonoscopy, referred to as computer aided detection (CADe). These systems use convulational neural networks (CNNs) which are trained using still images and videos of polyps[102]. The most recent systems then output a real-time alert to the proceduralist to the presence of the polyp, most commonly with a square around the perimeters of the image output or around the polyp itself (Figure 4). CADe systems were initially analysed in still image- and video-based studies, demonstrating a sensitivity of 95%-99% and accuracy of 96%[103-107]. Subsequently, large studies by Repici *et al*[102] (2020) and Wang *et al*[108,109] (2019 and 2020) in real-time AI-assisted colonoscopy have demonstrated an increase in ADR (RR 1.61 *vs* 1.30), as well as a 1.46- to 1.72-fold increase in total adenomas detected. The adenoma miss rate in tandem colonoscopy studies has also been demonstrated to be lower with CADe-assisted colonoscopy (14%-20%) compared to WLI (31%-40%)[110,111]. Subsequently, multiple meta-analyses have consistently demonstrated improved ADR and adenomas detected per colonoscopy with CADe systems (Table 1)[112-115].

An alternative role for AI-assistance in screening colonoscopy is based on quality assurance, employing AI to monitor withdrawal speed, endoscope slipping and blind spots to ensure consistency in colonoscopic practice. Gong *et al*[116] studied ENDOANGEL for this purpose and in a 2020 RCT involving 704 patients demonstrated an odds ratio of 2.3 for adenoma detection. Similar results were demonstrated by Su *et al*[117] using their Automatic Quality Control System (AQCS). Although not yet explored in studies, it may be that the combination of these AI systems using quality control and CADe may facilitate optimal adenoma detection. This is an area for further study as these systems become more widely available.

Table 1 Meta-analyses on efficacy of real-time computer aided detection

Ref.	Studies, patients	ADR			Adenoma per patient			Withdrawal time	False positives
		AI	WLI	RR	AI	WLI	Mean difference	Mean difference CADe *vs* control	
Aziz *et al*[112], 2020	3 studies, 2815 patients	32.9%	20.8%	1.58	0.47	0.26	0.20	0.9 min (P = 0.03)	4.87% (n = 137)
Hassan *et al*[113], 2021	5 studies, 4354 patients	36.6%	25.2%	1.44	0.58	0.36	0.22	0.34 min (P = 0.13)	-
Spadaccini *et al*[114], 2021	6 studies, 5178 patients	34.0%	26.6%	1.78	-	-	-	No significant difference	-
Barua *et al*[115], 2021	5 studies, 4311 patients	29.6%	19.3%	1.52	0.41	0.23	0.18	0.5 min	11.2%

ADR: Adenoma detection rate; AI: Artificial intelligence; WLI: White light imaging; RR: Relative risk; CADe: Computer aided detection.



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Figure 4 Computer aided detection detection system with a real-time alert seen around a flat tubular adenoma.

Fluorescence molecular endomicroscopy

Fluorescence molecular endomicroscopy (FME) involves targeted fluorescent agents that bind to specific cellular components of dysplastic cells, allowing detection using a specialised near infra-red FME (NIR-FME) probe[118]. For example, Hartmans *et al*[119] developed a fluorescently-labelled antibody against vascular endothelial growth factor A (which is upregulated in colonic adenomas) and injected this intravenously 3 d prior to colonoscopy. In their pilot study, all 39 adenomas from 15 patients were detected using the NIR-FME probe, demonstrating the feasibility of this technique[119]. Alternatively, Joshi *et al*[120] identified a peptide sequence that binds specifically to sessile serrated adenomas/polyps (SSA/Ps) which was administered topically using a spray catheter to 38 subjects undergoing routine outpatient colonoscopy, distinguishing SSA/Ps from normal colonic mucosa with 89% sensitivity and 92% specificity[120].

Problem: Over surveillance?

As a result of the expanding range of advanced imaging technologies (Table 2) and improved adenoma detection, patients will increasingly meet societal guidelines for more frequent surveillance colonoscopy. To counter this, guidelines may eventually need to be adjusted to reduce the frequency of colonoscopy based on diminishing adenoma miss-rates. However, a 2014 study by Gómez *et al*[121] demonstrated no difference in adenoma detection at follow-up colonoscopy after prior procedures completed by higher ADR endoscopists using HD-WLI. Currently, the duration of use of these advanced technologies has been insufficient to analyse polyp detection at future surveillance, hence further research is required as experience grows.

Table 2 Summary of strengths and weakness of advanced imaging technologies in adenoma detection

Modality		Strengths	Weaknesses
HD-WLI		Widely available	Marginal incremental benefit over SD-WLI
		Increased detection of flat, right-sided adenomas and SSAs	
Chromoendoscopy		Increased detection of small and flat adenomas	No significant increase in detection of advanced adenomas
		Increased dysplasia detection in IBD (compared to SD-WLI)	Increased procedural time
		May increase polyp detection in high-risk syndromes (serrated polyposis syndrome, HNPCC)	
Virtual chromoendoscopy	NBI	May improve flat lesion detection	Loss of brightness and familiarity of colour patterns
		Effective in those with experience using NBI	No evidence of increased total adenoma detection
			Less effective when used by proceduralists inexperienced in NBI
	i-SCAN	May reduce miss-rates in high-risk populations	Not widely available
			No difference in adenoma detection in larger studies
			Insufficient evidence to recommend use
	FICE	Retains familiar colour patterns	Not widely available
			No difference in ADR
	LCI	Retains familiar colour patterns	Not widely available
		Effective when used by non-LCI experienced proceduralists	Variable evidence regarding overall adenoma detection
		Improve adenoma detection, particularly right sided and flat lesions	
	BLI	Improved adenoma detection and miss rate in smaller studies	Not widely available
			No difference in ADR in largest study to date
	TXI	Retains familiar colour patterns	Not widely available
			New technology therefore insufficient evidence
AFI		Improved detection of flat/right sided polyps	Not widely available
			Increased procedure time
			No difference in overall ADR
AI		Improves ADR	Expensive currently
		Improves consistency between proceduralists	Not widely available
		Quality assurance	Some increase in procedure time
FME		In theory may improve detection of flat/poorly visible polyps	Insufficient evidence
			Requires injection/ingestion of tracer

HD-WLI: High-definition white light imaging; SD-WLI: Standard definition white light imaging; HNPCC: Hereditary non-polyposis colon cancer; NBI: Narrow-band imaging; IBD: Inflammatory bowel disease; BLI: Blue light imaging; TXI: Texture and colour enhancement imaging; FME: Fluorescence molecular endomicroscopy; AI: Artificial intelligence; AFI: Autofluorescence imaging; SSA/Ps: Sessile serrated adenomas/polyps; ADR: Adenoma detection rate.

POLYP CHARACTERISATION

Importance of polyp characterisation

Polyp characterisation is critically important for both small and larger polyps. In the context of diminutive (< 5 mm) and small (< 10 mm) polyps, accurate characterisation has facilitated the "resect and discard" and "do not resect" strategies. For larger polyps, accurate endoscopic characterisation guides the selection of suitable polyps for endoscopic resection as well as the most appropriate resection technique.

Diminutive and small polyps

Traditionally, all polyps identified during colonoscopy have been resected and examined histologically. However, as the accuracy of endoscopic identification of polyps has improved, the "resect and discard" or even "do not resect" strategies have been developed to minimise the resource consumption of routine histological analysis. These strategies were developed after large studies found that advanced histology (at least high-grade dysplasia) is present in as few as 1.7% of diminutive (≤ 5 mm) polyps, and only 6.6%-10.0% of small (< 10 mm) polyps[122,123]. In fact, a 2013 meta-analysis including 6280 polyps found only 56.7% of diminutive polyps are even neoplastic[124]. On this basis, the American Society of Gastrointestinal Endoscopy (ASGE) published the Preservation and Incorporation of Valuable endoscopic Innovations (PIVI) thresholds for adopting real-time endoscopic assessment of polyps for "resect and discard" and "do not resect"[125]. For diminutive polyps to be discarded without pathological assessment, endoscopic imaging should provide a ≥ 90% agreement in assignment of post-polypectomy surveillance. Polyps > 5 mm in size should be sent for histological assessment given the up to 10% frequency of more advanced histology which would alter surveillance intervals[123]. For diminutive rectosigmoid hyperplastic polyps, imaging should provide ≥ 90% negative predictive value for adenomatous histology. Even hyperplastic-appearing diminutive polyps proximal to the sigmoid colon should be resected as these polyps have a more than 10% chance of being SSA/Ps histologically [126]. These strategies would result in significant cost-savings to the healthcare sector. For example, Solon *et al*[127] examined the potential financial impact of this strategy for the National Health Service (NHS) in England in 2016, demonstrating potential annual cost savings of £141192057.

Larger polyps

For larger polyps, endoscopic characterisation is critical to guiding suitability for endoscopic resection as well as appropriate resection techniques. Even for non-interventionalists who are not proceeding with immediate resection, accurate characterisation without the need for biopsy may be ideal to guide appropriate referral. Kuroha *et al*[128] highlighted this in a 2021 study examining predictors of success in 369 colorectal ESDs. Severe fibrosis was associated with increased mean procedure time, as well as lower en bloc and complete resection rates, with the greatest predictors of severe fibrosis on multivariate analysis being prior resection attempt (OR 175.4) and pre-treatment biopsy (OR 8.3)[128]. In addition, pre-resection biopsies can be inaccurate in large lesions, with false negative rates as high as 86% for adenocarcinoma, therefore characterisation with advanced imaging and upfront endoscopic resection may be more appropriate[129].

Training in polyp characterisation

Accurate polyp characterisation using advanced mucosal imaging is impacted to some extent by proceduralist experience. A 2014 video-based study demonstrated that interventional endoscopists specialising in complex polypectomy were more accurate in identifying malignant polyps when compared to other endoscopists[130]. However, multiple studies support the efficacy of specialised training in advanced mucosal imaging for polyp characterisation, irrespective of endoscopist experience. Both Bae *et al*[131] and Patel *et al*[132] have studied the accuracy of endoscopists before and after a training module on identification diminutive rectal polyps, in whom the negative predictive value (NPV) for diminutive neoplastic polyps improved from 82.1% to 92.5%-94.7%, thus meeting the PIVI threshold. In addition, studies have demonstrated accurate characterisation after training even in medical residents with no endoscopy experience, while Basford *et al*[133] found no difference in the accuracy of interpretation of HD-WLI and i-SCAN images prior to specific training between consultant gastroenterologists, trainees and medical students[133-136]. Proceduralists should therefore engage in specific training in advanced mucosal imaging rather than relying on experience alone, in order to improve accuracy of polyp characterisation.

Classifications systems

Multiple polyp classification systems have been developed to improve polyp characterisation (Table 3). While not reliant on advanced imaging, the Paris classification aids in risk stratification for larger polyps prior to consideration of endoscopic resection (Figure 5)[137]. A large multicentre 2017 study found that the presence of any 0-IIc ("depressed") component predicted submucosal invasive cancer in almost 30% of patients. In laterally spreading tumours (LSTs), the presence of an elevated component (0-IIa + Is) predicted submucosal invasion in over 10% over patients *vs* 4.9% for those with flat lesions alone (0-IIa)

Table 3 Summary of existing classification systems using advanced mucosal imaging

System	Imaging modality	Polyp features	Accuracy	Complexity	TA/TVAs included	SSAs included
Kudo	Any	Pits	AUC 0.94[143]	Complex	Yes	No
NICE	NBI	Vessels and pits	Sensitivity 98%, NPV 97.8%[145]	Moderate	Yes	No
JNET	NBI	Vessels and pits	AUC 0.97 for JNET 1, 0.84 for JNET 2A, 0.9 for JNET 3 but less accurate for JNET 2B (AUC 0.72)[152]	Moderate	Yes	No
BASIC	BLI	Vessels, pits and surface	Accurate surveillance prediction in 90%, NPV for rectosigmoid polyps 91%[160]	Moderate	Yes	No
WASP	Any	Pits, surface, shape	May improve SSA detection[162]	Simple	No	Yes
mSano	NBI	Vessels, pits and surface	AUC 0.92[169]	Simple	Yes	Yes

NBI: Narrow-band imaging; WASP: Workgroup serrAted polypS & polyposis; TVA: Tubulo-villous adenomas; AUC: Area under the receiver operating characteristic curve; NPV: Negative predictive value; BASIC: Blue light imaging adenomas serrated international classification; JNET: Japan NBI Expert Team developed the Japan NBI expert team; NICE: NBI International Colorectal Endoscopic; LCI: Linked colour imaging; SSA: Sessile serrated adenomas.

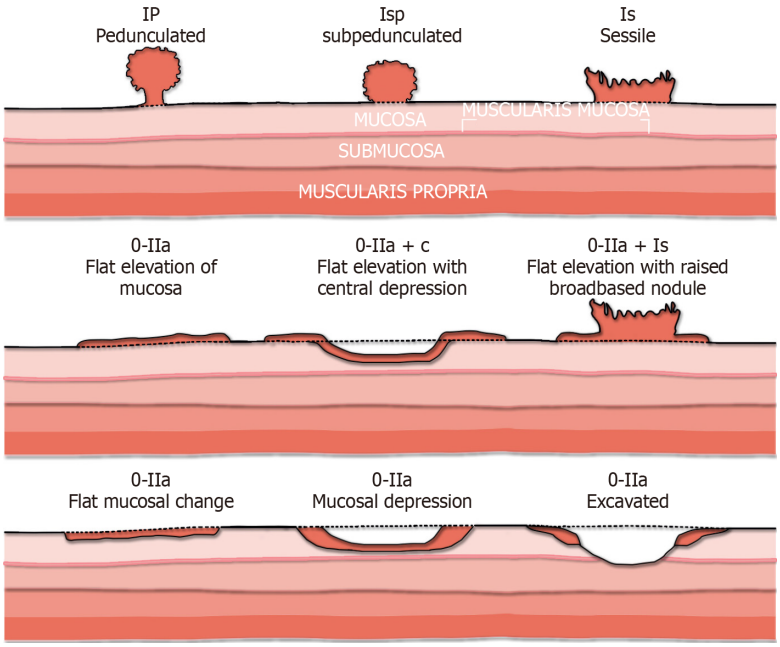


Figure 5 Paris classification. Citation: Mathews AA, Draganov PV, Yang D. Endoscopic management of colorectal polyps: From benign to malignant polyps. *World J Gastrointest Endosc* 2021; 13: 356-370[141].

(P < 0.001)[138]. However there is considerable inter-observer variability, particularly with regard to classification of lesions as flat *vs* sessile, with one study finding a kappa statistic of 0.42[139]. Van Doorn *et al*[139] proposed a simplified classification system of "pedunculated", "elevated" (including flat and sessile) and "depressed" in order to address this, which resulted in improved interobserver agreement and 91.6% accuracy for prediction of invasive cancer[140].

Kudo classification

The Kudo classification (Figure 6) was developed in 1996 to classify polyps according to their "pit patterns" on magnifying endoscopy[141]. Type I pits appear round, while type II appear stellate or papillary, both representing benign changes (normal, hyperplastic or inflammatory). Type III-s pits are smaller, round, tubular pits while type III-L are larger tubular pits, representing tubular adenomas (TA). Type IV pits are branch-like or gyrus-like and represent tubulo-villous adenomas (TVA), while type V pits are non-structured representing HGD or cancer[142]. Multiple studies have assessed the accuracy of the Kudo classification, summarised by a 2014 meta-analysis of 20 studies, including 5,111 colorectal

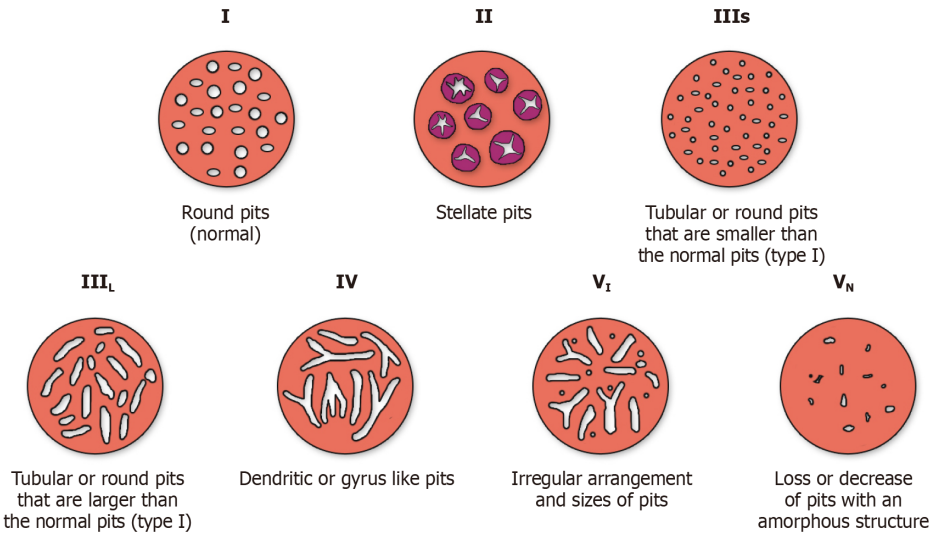


Figure 6 Kudo's classification. Citation: Mathews AA, Draganov PV, Yang D. Endoscopic management of colorectal polyps: From benign to malignant polyps. *World J Gastrointest Endosc* 2021; 13: 356-370[141].

lesions[143]. Pit pattern classification differentiated neoplastic from non-neoplastic polyps with a pooled sensitivity of 89.00%, specificity of 85.78% and area under the receiver operating characteristic curve (AUC) of 0.94[144].

NBI International Colorectal Endoscopic classification

The NBI International Colorectal Endoscopic (NICE) classification was developed in 2012 with the goal of developing an international consensus for classification using NBI[145]. This classification takes into account the polyp colour, vessel pattern and surface pattern to characterise polyps into NICE type 1 (hyperplastic) type 2 (adenoma) and type 3 (invasive cancer) (Figure 7). Using this simplified classification results in highly accurate differentiation of neoplastic from non-neoplastic polyps, with sensitivity of 97%-99%, specificity of 85%-95% and accuracy of 89%-98% across 3 Large prospective studies[145-147]. In the 2012 validation study, 471 predominantly diminutive and small polyps were predicted with high-confidence with sensitivity of 98% and NPV of 97.7%, while 119 low-confidence predictions resulted in a sensitivity of 94.2% and NPV of 94.4%, both easily exceeding PIVI thresholds [145]. However, in a study of 2123 larger lesions, the NICE classification predicted deep invasive cancer with a sensitivity of just 58.4%. Nevertheless, due to low rates of deep invasion this was still associated with an NPV of 96.4% and specificity of 98.1%, therefore the authors suggested that even large NICE 1 and 2 Lesions should be considered for endoscopic resection[148]. The NICE classification has also been validated in a smaller cohort using i-SCAN rather than NBI, with similar results[149].

Japan NBI expert team classification

More recently, the Japan NBI Expert Team developed the Japan NBI expert team (JNET) classification specifically for the classification of colorectal polyps based on their appearance on magnification NBI using a combination of vessel and surface pattern analysis (Figure 8)[150,151]. The JNET classification is highly accurate for differentiating neoplastic *vs* non-neoplastic polyps, with an AUC of 0.97 for JNET 1 (hyperplastic/SSA/Ps) and 0.84 for JNET 2A (adenoma with LGD) in a 2020 meta-analysis[152]. In a retrospective 2020 study, this resulted in an increase in the number of adenomas resected per colonoscopy (1.7 *vs* 1.2, $P < 0.01$) and a reduction in resection of non-neoplastic lesions (8.9% *vs* 17.0%, $P < 0.01$)[153]. It is also highly specific in predicting deep invasive cancer in JNET 3 Lesions, with specificity of 100% and an AUC of 0.9[152]. In addition, unlike other systems, the JNET classification has been validated for characterisation of dysplasia within SSA/Ps, with Murakami *et al*[154] finding that the presence of JNET 2A/B/3 foci within a JNET 1 Lesion is 83.9% sensitive, 95.5% specific and 94.5% accuracy for detection of dysplasia within sessile serrated lesions. However, the main limitation of the JNET classification is in the interpretation of JNET 2B lesions, with studies demonstrating a wide range of advanced pathology, from HGD to superficial invasive cancer and even deep invasive cancer in JNET 2B polyps, with an AUC of 0.72[152,155,156]. This was highlighted in a recent study that retrospectively reviewed 297 colorectal adenocarcinomas, in which the probability of deep invasion was only 1.8% for JNET 2A, 30.1% for JNET 2B and 96.6% for JNET 3[157,158]. In this study, JNET 2B lesions were then further analysed using chromoendoscopy and Kudo"s classification of pit patterns. In Kudo non-V lesions, the risk of deep invasion was only 4.3%. Overall, JNET differentiates accurately for JNET 1, 2A, and 3 lesions, however proceduralists should consider further examination with magnified chromoendoscopy for JNET 2B lesions to improve accuracy of histology prediction.

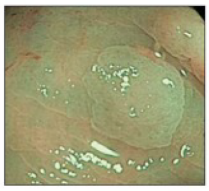
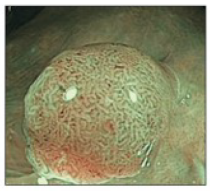
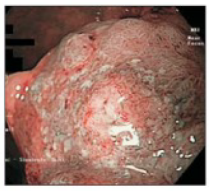
	Type 1	Type 2	Type 3
Color	Same or lighter than background	Browner relative to background (verify that color arises from vessels)	Brown to dark brown relative to background, sometimes patchy whiter areas
Vessels	None or isolated lacy vessels coursing across the lesion	Brown vessels surrounding white structures	Has area(s) with markedly distorted or missing vessels
Surface pattern	Dark or white spots of uniform size, or homogeneous absence of pattern	Oval, tubular, or branched white structures surrounded by brown vessels	Areas with distortion or absence of pattern
Most likely pathology	Hyperplastic	Adenoma	Deep submucosally invasive cancer
			

Figure 7 Narrow-band Imaging International Colorectal Endoscopic classification. Citation: Puig I, Kaltenbach T. Optical Diagnosis for Colorectal Polyps: A Useful Technique Now or in the Future? *Gut Liver* 2018; 12: 385-392[150]. Copyright© The Author(s) 2018. Published by The Korean Society of Gastroenterology, the Korean Society of Gastrointestinal Endoscopy, the Korean Society of Neurogastroenterology and Motility, Korean College of Helicobacter and Upper Gastrointestinal Research, Korean Association the Study of Intestinal Diseases, the Korean Association for the Study of the Liver, Korean Pancreatobiliary Association, and Korean Society of Gastrointestinal Cancer (Supplementary material).

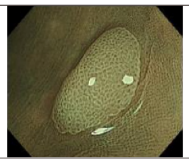
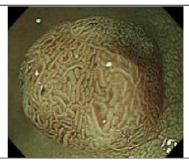
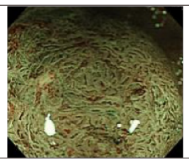
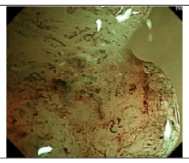
	Type 1	Type 2A	Type 2B	Type 3
Vessel pattern	•Invisible	•Regular caliber •Regular distribution (meshed/spiral pattern)	•Variable caliber •Irregular distribution	•Loose vessel areas •Interruption of thick vessels
Surface pattern	•Regular dark or white spots •Similar to surrounding normal mucosa	•Regular (tubular/branched/papillary)	•Irregular or obscure	•Amorphous areas
Most likely histology	Hyperplastic polyp/ Sessile serrated polyp	Low grade intramucosal neoplasia	High grade intramucosal neoplasia/ Shallow submucosal invasive cancer	Deep submucosal invasive cancer
Endoscopic image				

Figure 8 Japan Narrow-band Imaging Expert Team developed the Japan Narrow-band Imaging expert team classification. Citation: Hirata D, Kashida H, Iwatate M, Tochio T, Teramoto A, Sano Y, Kudo M. Effective use of the Japan Narrow Band Imaging Expert Team classification based on diagnostic performance and confidence level. *World J Clin Cases* 2019; 7: 2658-2665[158]. Copyright© The Author(s) 2019. Published by Baishideng Publishing Group Inc (Supplementary material).

BLI adenomas serrated international classification

The BLI adenomas serrated international classification (BASIC) classification was developed in 2018 for classification of polyps using BLI, based on assessment of surface, pit patterns and vessels, classifying polyps as either hyperplastic, traditional adenomatous, sessile serrated or cancer[159]. In the largest prospective validation study of 748 diminutive polyps this classification reached PIVI thresholds with accurate surveillance prediction in 90% and an NPV for rectosigmoid polyps of 91%[160].

Dutch Workgroup serrAted polypS & polyposis classification

The dutch workgroup serrAted polypS & polyposis (WASP) classification was developed in 2016 to facilitate accurate differentiation of SSA/Ps from hyperplastic and traditional adenomatous polyps as many existing classification systems did not allow for inclusion of SSA/Ps[161]. It's accuracy has been validated by Lee *et al*[162] who demonstrated that the implementation of a specific training program in the WASP classification led to a statistically significant increase in SSA/P resection over the 6-mo

training period, from 4.5% to 8% (P = 0.003).

Sano and mSano classification

The Sano classification (Figure 9) characterises polyps according to their capillary pattern, with barely visible honeycomb pattern capillaries in type I (normal or hyperplastic), larger elongated capillaries in type II [adenoma with low-grade dysplasia (LGD)] and irregular branching vessels in type III [high-grade dysplasia (HGD)] or adenocarcinoma)[163,164]. In a validation study, 97% of Sano II lesions were diagnosed as LGD while 87% of Sano III lesions were HGD or invasive cancer[165]. In 2013, this system was modified by Singh *et al*[166] (mSano classification) to include type IIo lesions in order to distinguish hyperplastic from sessile serrated polyps (Figure 7). Across multiple studies, the overall accuracy of the mSano classification has been between 90%-97%, with near-perfect interobserver agreement (k 0.89)[166,167]. The NPV for diminutive rectosigmoid polyps is as high as 100% and the accuracy for post-polypectomy surveillance 97%, exceeding the PIVI thresholds described above[167]. mSano as a standalone classification system was compared to the combination of the WASP and JNET classification in 2020, with superior high-confidence predictions (85% *vs* 69%, P < 0.05) and equivalent interobserver reliability[168]. It was also compared to the NICE classification in a 2018 RCT including 348 colonoscopies, with an AUC of 0.92 for prediction of neoplasia by mSano *vs* 0.78 for NICE (P = 0.02) and an AUC of 0.92 for prediction of suitability for endoscopic resection *vs* 0.83 for NICE (P = 0.04)[169]. The mSano is therefore a highly accurate standalone criteria for characterisation of colonic polyps including differentiation of neoplasia (including SSA/Ps) as well as invasive cancer.

HD-WLI

There appears to be some incremental benefit from examination with HD-WLI alone *vs* SD-WLI for polyp characterisation, although this may be smaller than expected. In the largest direct comparison from Rastogi *et al*[31] in 2011, HD-WLI improved sensitivity for characterisation of small adenomas from 51.7% to 66.8% (P < 0.001) however the overall accuracy did not change. Minimal evidence exists comparing the accuracy of HD-WLI to SD-WLI for characterisation and prediction of invasion in larger polyps, however with the vast expansion of advanced imaging technologies, evidence increasingly supports the use of ancillary technology over HD-WLI in this context[170].

Chromoendoscopy

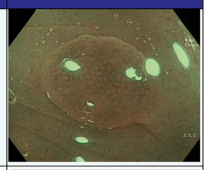
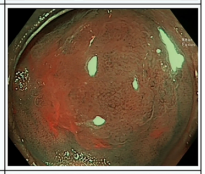
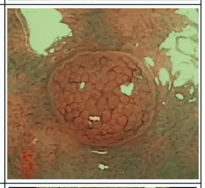
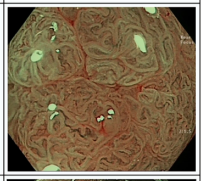
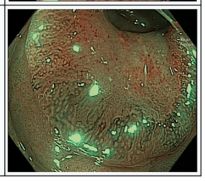
Chromoendoscopy has been demonstrated to be highly effective in differentiating neoplastic from non-neoplastic small colonic polyps, with overall diagnostic accuracy of greater than 99%[171-173]. However, with increasingly accurate forms of virtual chromoendoscopy for assessment of these diminutive and small polyps, the procedure time required for chromoendoscopy is likely to limit its ongoing use. Instead, the main ongoing role for chromoendoscopy may be in the prediction of invasion depth in larger lesions to guide resection techniques[174]. For example, the European Society of Gastrointestinal Endoscopy (ESGE) guidelines recommend the use of chromoendoscopy for pit pattern analysis in JNET 2B lesions (where NBI lacks accuracy), in order to further qualify the risk of deep invasion according to Kudo's classification as described above[175]. This recommendation has been supported by Hosotani *et al*[157] in their 2021 study which demonstrated a PPV of 76% for invasive cancer in the presence of a "VH" pit pattern and a NPV of 96% for non-V pit patterns. Even in this context however, a recent prospective study including 400 patients found that there was no overall incremental benefit for the use of chromoendoscopy in addition to HD-WLI and NBI for the characterisation of large nonpedunculated polyps[176]. Novel indications for chromoendoscopy include the use of acetic acid chromoendoscopy or submucosal methylene blue injection (Figure 10) to clearly delineate polyp margins prior to resection[177-179].

Virtual chromoendoscopy

While virtual chromoendoscopy has not been conclusively demonstrated to improve polyp detection, an expanding body of evidence supports its use for polyp characterisation to guide endoscopic resection strategies, as well as the "resect and discard" and "do not resect" strategies in diminutive polyps. For classification of highly prevalent small and diminutive polyps where dye-based chromoendoscopy may no longer be efficient on a population level, virtual chromoendoscopy has been demonstrated to have equivalent accuracy with a reduction in median procedural and interpretation time[180].

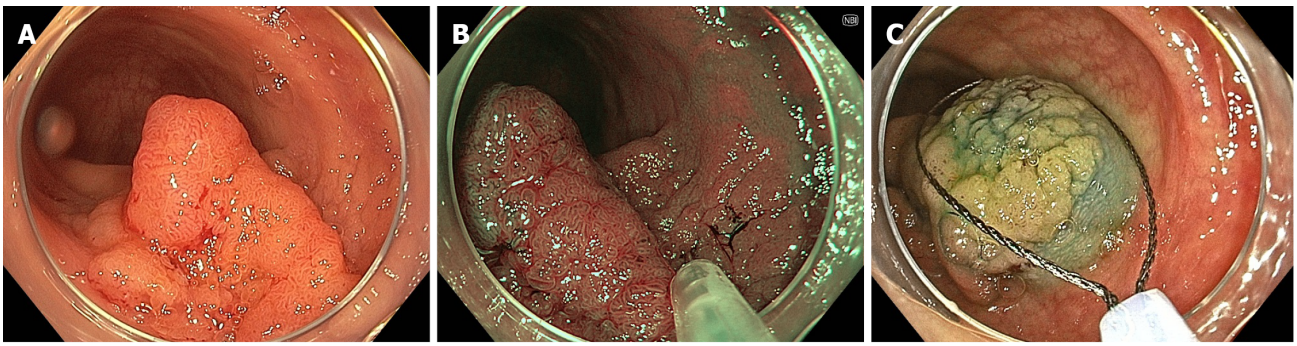
NBI

For diminutive colorectal polyps, multiple studies have shown that characterisation using NBI is able to easily exceed PIVI thresholds, with correct surveillance interval prediction in 92%-99% of cases and an NPV for diminutive rectosigmoid polyps of 91%-92%[62,124,181,182]. While many of these studies have been performed by expert endoscopists with experience in NBI, it has also been demonstrated that non-interventional endoscopists are able to achieve significant improvement following specific training, with Higashi *et al*[133] reporting an overall accuracy of 90% for non-interventionalists following a single training module[133,183].

MS classification (predicted histology)	Description	Example
MS I (HP - hyperplastic polyp)	Pale colour ± round pits with central brown star-like dots or bland appearance ± minute capillaries that may meander across polyp	
MS IIo (SSA/P - sessile serrated adenoma/polyp)	Pale or light dark colour ± open pits ± 3 out of 5: cloud-like surface, inconspicuous margins, mucous cap, irregular shape and varicose microvascular vessels	
MS II (tubular adenoma with low grade dysplasia)	Light dark or dark colour ± white linear or oval pits ± linear or oval regular capillary network surrounding pits	
MS IIIa (high grade dysplasia‡/ villous or tubulovillous adenoma/superficial cancer)	Light dark or dark colour ± white villous/cerebriform pits ± tortuous/branched mildly regular capillary network surrounding pits§	
MS IIIb (invasive cancer)	Dark surroundings with pale central area ± loss of pit and vascular pattern	

‡High-grade dysplastic SSA/Ps are included in this category
§May have slight loss of pit pattern and vascularity when leaning towards superficial cancer

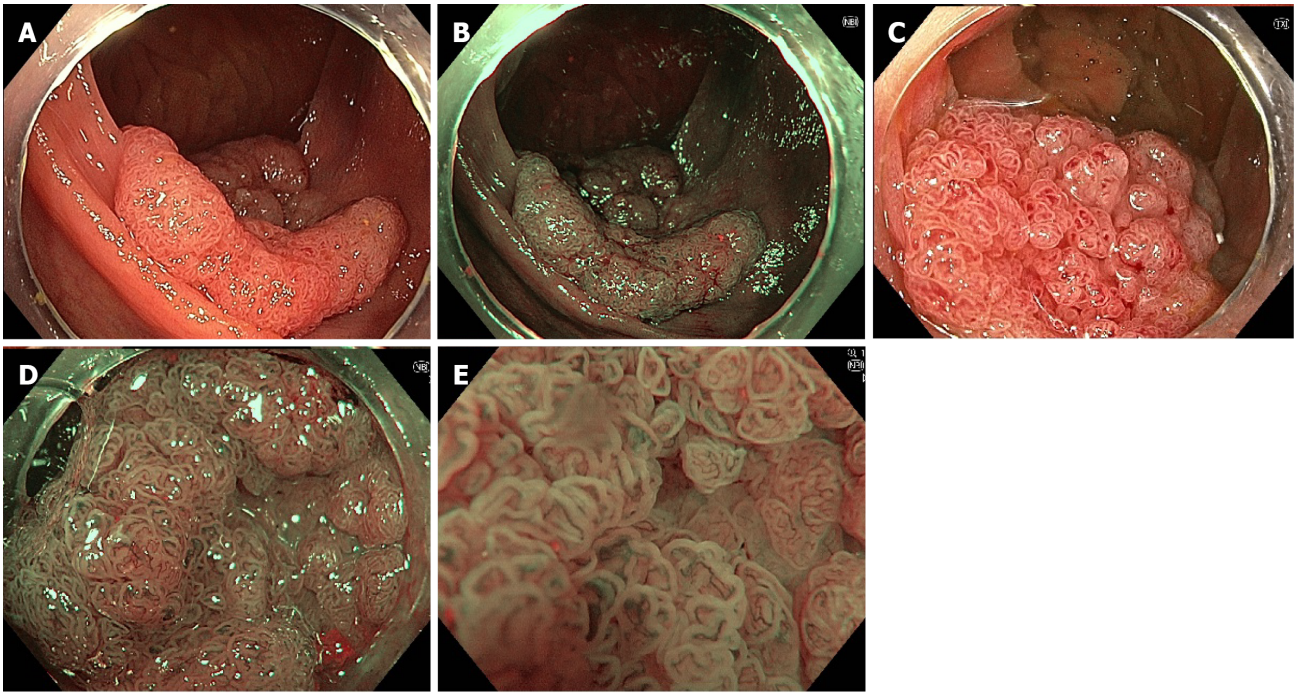
Figure 9 mSano classification demonstrating delineation between sessile serrated adenomas/polyps and hyperplastic polyps. Citation: Zorron Cheng Tao Pu L, Yamamura T, Nakamura M, Koay DSC, Ovenden A, Edwards S, Burt AD, Hirooka Y, Fujishiro M, Singh R. Comparison of different virtual chromoendoscopy classification systems for the characterization of colorectal lesions. *JGH Open* 2020; 4: 818-826[168]. Copyright© The Author(s) 2019. Published by Journal of Gastroenterology and Hepatology Foundation and John Wiley & Sons Australia, Ltd (Supplementary material).



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Figure 10 Large colonic laterally spreading tumour. A: White light imaging with poor differentiation between polyp and normal tissue; B: Flat extension seen more clearly on NBI; C: Submucosal methylene blue injection prior to resection clearly delineating the margins of the flat spreading component.

Additionally, NBI has been used for the characterisation and prediction of invasion depth within larger colonic polyps (Figure 11). As early as 2008, Katagiri *et al*[165] demonstrated that an irregular capillary pattern (designated CP III) on NBI predicted a 65.6% (21/31) rate of invasive adenocarcinoma. Subsequently, Ikematsu *et al*[184] differentiated CP III into IIIA (characterised by high microvessel density with a lack of uniformity, blind ending, branching and curtailed irregularly) and IIIB (characterised by the presence of a nearly avascular or loose microvascular area). They found that IIIA lesions defined adenomas, intramucosal cancers and superficial submucosal invasive cancer, while IIIB lesions defined deep submucosal invasive cancers, with a sensitivity of 84.8%, specificity of 88.7% and overall accuracy of 87.7%. NBI has also been examined for detection of dysplasia and cancer within SSA/Ps. Tate *et al*[185] found that the presence of an adenomatous (NICE II) pattern within an SSA had 95%



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Figure 11 Laterally spreading tubulo-villous adenoma with high-grade dysplasia. A: White light imaging; B: Narrow-band imaging (NBI); C: Texture and colour enhancement imaging; D: NBI with magnification; E: NBI with high-magnification using the underwater technique.

accuracy and a 98.1% NPV for detection of dysplasia within SSA/Ps, while Chino *et al*[186] demonstrated 100% sensitivity and 99% specificity with NBI for detection of cancers within SSA/Ps.

FICE

FICE has also been demonstrated to be highly accurate for the characterisation of colorectal polyps, with sensitivity of 89.4%-94.7%, specificity of 81.0%-89.2% and accuracy of 87.0%-89.4%[172,187-190]. However, Yoshida *et al*[187] did demonstrate its accuracy to be inferior to that of chromoendoscopy (89.4% *vs* 94.7%, $P < 0.05$). While minimal direct comparative data exists between modalities of virtual chromoendoscopy, Akarsu *et al*[191] found the NPV of FICE (80%) to be inferior to that of NBI (96.3%, $P < 0.001$), although there was no difference in overall accuracy.

i-SCAN

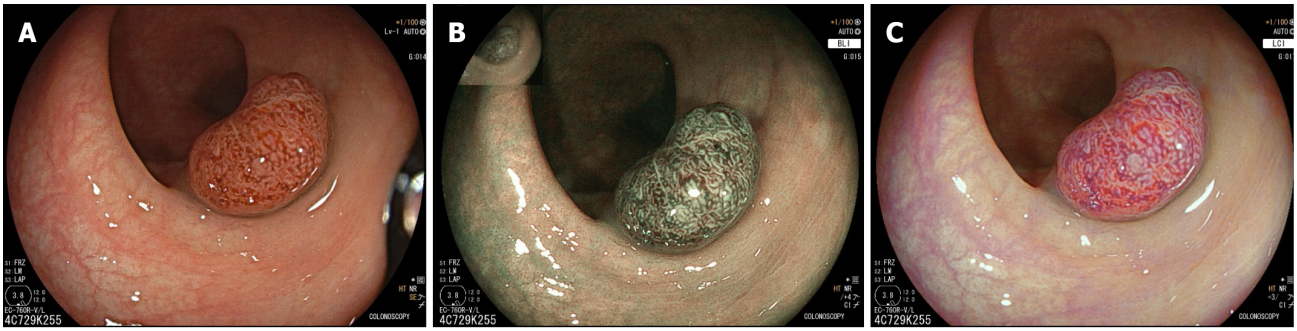
i-SCAN has achieved similar results with respect to diminutive and small colorectal polyp categorisation, with sensitivity and specificity consistently above 90% across multiple studies[149,192-194]. It also appears to be an accessible form of advanced imaging for non-experts, with junior residents achieving similar accuracy to experts in one study after a 30-min training session[149]. There have been two RCTs directly comparing the accuracy of NBI and i-SCAN for polyp characterisation, both of which have found no difference in accuracy between these modalities but did demonstrate superiority for both NBI and i-SCAN when compared to HD-WLI[195,196].

LCI

LCI was developed in conjunction with BLI, aiming to improve polyp detection while BLI aimed to improve characterisation (Figure 12). Accordingly, minimal evidence exists regarding the accuracy of LCI for polyp characterisation. However, in 2017 Wu *et al*[197] employed the NICE classification using LCI, and reported a sensitivity of 96.5%, specificity of 83.8% and NPV of 93.9% for neoplastic lesion prediction.

BLI

The accuracy of BLI for polyp characterisation has been more extensively studied. Both retrospective and prospective studies have demonstrated the superiority of BLI over WLI for the characterisation of < 10 mm colonic polyps, with the largest 2019 prospective randomised study by Rondonotti *et al*[198] finding the overall accuracy for BLI to be 92% *vs* 84% for WLI ($P = 0.01$)[198-200]. BLI has also been compared to NBI using the JNET classification in a retrospective study where there was no significant difference in accuracy (92.1% for BLI *vs* 91.7% for NBI)[201].



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Figure 12 Tubulo-villous adenoma. A: White light imaging; B: Blue light imaging; C: Linked colour imaging.

AFI

While studies on AFI have been promising regarding polyp detection, its role in polyp characterisation appears limited. A 2011 RCT comparing HD-WLI, AFI and NBI did find that the overall accuracy of AFI is equivocal to that of NBI for distinguishing adenoma from hyperplastic polyps (84.9% *vs* 88.4%)[202]. However, the interobserver agreement for NBI with magnification is superior to that of AFI, while a 2017 meta-analysis demonstrated inferior specificity using AFI (44%) compared to NBI (69%, $P = 0.031$) [203,204].

TXI

As the most recently developed form of advanced mucosal imaging, TXI has yet to be studied in the context of polyp characterisation. Given the familiarity of color patterns, it may have some role for differentiation of neoplastic from non-neoplastic diminutive polyps which may increase its uptake during population screening. In addition, this familiarity may benefit proceduralists during resection by more clearly delineating polyp margins without compromising visualisation.

Artificial intelligence

Extensive research has been undertaken in recent times into the development of AI systems for characterisation of colonic polyps, designated computer aided diagnosis (CADx) (Table 4). These systems have proven to be highly accurate in assessment of diminutive polyps, with a 2020 meta-analysis demonstrating a pooled AUC of 0.96 (CI 0.95-0.98) and a pooled NPV of 95.1%[104]. Interestingly, across multiple studies, CADx systems have not proven to be superior to expert endoscopists regarding histology prediction, although they have consistently led to improved histology prediction in non-expert endoscopists, nearing that of experts[205-208]. In these studies, the NPV for diminutive polyps has been 90%-97%, with an accurate surveillance interval in 93-94%, well surpassing PIVI thresholds [205,206,208-220].

There are fewer studies examining the efficacy of AI for delineation of submucosal invasive adenocarcinoma to guide resection strategies. Lu *et al*[221] found the accuracy of their AI model "Endo-CRC" to be 93.78% for polyps with and 91.71% for polyps without advanced CRC. Lui *et al*[222] developed an AI model to classify polyps more than 2 cm in size as being endoscopically resectable (less than 1 mm submucosal invasion, no lymphovascular invasion and no more than well-differentiated adenocarcinoma) or non-resectable. The overall accuracy was 85.5% for prediction of endoscopically resectable lesions, but improved to 94.3% when the AI system was interpreting NBI images. However, while AI models have been more effective than non-expert interventionalists for detection of invasive carcinoma, in each of these studies AI was not superior to expert endoscopists, suggesting the main role of AI for larger polyps may be in improving inter-endoscopist consistency as well as perhaps aiding in selection of suitable referrals to interventionalists by non-expert endoscopists[221,222].

In vivo histologic diagnosis

Emerging technologies have been developed with the goal of achieving *in vivo* histological diagnosis, termed "optical biopsy". Accurate optical biopsies would allow endoscopists to not only surpass PIVI thresholds for small and diminutive polyps but would also allow accurate endoscopic diagnosis for larger polyps and LSTs where existing mucosal imaging technology may have deficiencies.

Endocytoscopy

Endocytoscopy is a novel technology that allows *in vivo* visualisation of tissue at the cellular level in real-time[223]. The device can either be incorporated into the endoscope or comes as a probe-based system, utilising a high-power fixed-focus objective lens to achieve ultra-high magnification in excess of 450 ×, generally following methylene blue staining[224]. Studies have demonstrated superior accuracy compared to advanced mucosal imaging and chromoendoscopy, with accuracy as high as 93.3%-96.8%

Table 4 Studies on the accuracy of AI for polyp histology prediction

Ref.	Study type	Imaging modality	Number of patients/polyps	Sensitivity	Specificity	NPV	Accurate surveillance interval
Kominami *et al* [209], 2016	Retrospective	NBI	41 patients, 118 polyps	93%	95%	93%	92.7%
Chen *et al*[210], 2018	Retrospective	NBI	284 polyps	96%	78%	90%	-
Mori *et al*[211], 2018	Prospective	NBI	325 patients, 466 polyps	93%	90%	95%	-
Renner *et al*[212], 2018	Retrospective	WLI, NBI	100 polyps	92%	63%	90%	-
Byrne *et al*[213], 2019	Retrospective	NBI	125 polyps	98%	83%	97%	-
Min *et al*[214], 2019	Prospective	LCI	91 patients, 217 polyps	83%	70%	71%	-
Sánchez-Montes *et al*[206], 2019	Retrospective	WLI	225 polyps	92%	89%	87%	-
Horiuchi *et al*[215], 2019	Prospective	AFI	95 patients, 258 polyps	80%	95%	93%	-
Ozawa *et al*[216], 2020	Retrospective	WLI, NBI	309 polyps	97% for NBI, 90% for WLI	-	91% for NBI, 85% for WLI	-
Jin *et al*[205], 2020	Retrospective	NBI	300 polyps	83%	90%	94%	-
Zacharia *et al*[208], 2020	Retrospective	WLI, NBI	524 polyps	96%	90%	93%	94%
Rodriguez-Diaz *et al*[217], 2021	Retrospective	NBI	119 patients, 280 polyps	96%	84%	91%	94%
Van der Zander *et al*[218], 2021	Retrospective	WLI, BLI	54 patients, 60 polyps	96%	93%	88%	-
Yoshida *et al*[220], 2021	Retrospective	BLI	25 patients, 100 polyps	91%	85%	92%	-
Sakamoto *et al* [219], 2022	Retrospective	WLI, BLI	604 polyps	96% for WLI, 96% for BLI	84% for WLI, 89% for BLI	-	-

NBI: Narrow-band imaging; NPV: Negative predictive value; BLI: Blue light imaging; WLI: White light imaging; LCI: Linked colour imaging.

for distinction of neoplastic *vs* non-neoplastic diminutive polyps[225]. Endocytoscopy has been shown to be similarly highly accurate for larger polyps in detection of submucosal invasion, with an overall accuracy of 85.8%-97.0%[226-229]. The main limiting factors for this technology are the requirement for specific equipment, as well as the time and training required to facilitate accurate interpretation of the images. However, its uptake may evolve with the development of AI technologies which could allow effective use by inexperienced proceduralists. Misawa *et al*[230] developed and published a new AI system for interpretation of endocytoscopy images (using NBI rather than methylene blue staining) named "EndoBRAIN" in 2016. Their study demonstrated overall sensitivity, specificity, and accuracy for high-confidence predictions of 97.6%, 95.8%, and 96.9% respectively. In 2020, Kudo *et al*[231] compared "EndoBRAIN" to trainee and expert endoscopists using both dye-based and virtual chromoendoscopy and found the AI system to be superior to both groups, with sensitivity of 96.9%, specificity of 100% and overall accuracy of 98%.

Multiphoton microscopy

Multiphoton microscopy is based on the detection of signals at specific emission wavelengths after laser excitation, offering real-time high-resolution visualisation. The use of longer photons allows deeper tissue penetration and visualisation up to a depth of several hundred microns[232]. Recently, Terradillos *et al*[233] developed an AI system for interpretation of multiphoton microscopy images of colorectal polyps, with a specificity of 91% and sensitivity of 82% for malignant colorectal lesions. Further study is clearly required into the application of this technology, however the greater depth of visualisation may allow *in vivo* assessment of invasion depth for submucosal invasive adenocarcinoma.

CONCLUSION

New and existing advanced mucosal imaging technologies facilitate improved adenoma detection and characterisation in both expert and non-expert endoscopists (Table 5). The use of virtual chromoendoscopy for polyp detection has been limited by reduced brightness and loss of familiarity of color patterns, however new technologies such as LCI and TXI enhance visualisation without significantly altering color patterns and may lead to more consistent improvement in polyp detection. Additionally, the availability of AI systems is increasing and may improve consistency between expert and non-expert endoscopists. Advanced mucosal imaging also allows accurate *in vivo* assessment of polyps to guide resection techniques, while clearly exceeding PIVI thresholds for the "resect and discard" and "do not resect" strategies. NBI has been at the forefront of polyp characterisation, improving delineation of neoplastic from non-neoplastic diminutive and small polyps, while improving prediction of invasion depth in larger polyps. AI technologies are yet to surpass expert endoscopists for histology prediction but facilitate accurate prediction by non-experts to rival that of expert endoscopists. Effective use of these advanced mucosal imaging technologies is not out of reach of any endoscopist following brief but dedicated training programs, thereby maximising the efficacy of everyday colonoscopy and improving patient outcomes.

Table 5 Summary and conclusions for each form of advanced mucosal imaging discussed

Modality		Detection	Characterisation	Comment
HD-WLI		Advantages: Marginal benefit in overall adenoma detection; and improved detection of right-sided, flat polyps, and SSAs[1]	Advantage: Marginal benefit for small adenomas; disadvantage: Insufficient evidence for large polyps	Advantage: Widely available
Chromo-endoscopy		Advantage: Increases polyp detection; disadvantage: Increases withdrawal time	Advantages: Highly effective for small polyps (although inefficient); and useful in prediction of invasion depth for large polyps[1]	Disadvantage: Increases procedural time
Virtual chromo-endoscopy	NBI	Disadvantage: No significant difference in ADR	Advantages: Accurate for distinguishing neoplastic from non-neoplastic small and diminutive polyps; and accurate for prediction of invasion depth[1]	Disadvantage: Loss of brightness; neutral: Greater efficacy when used by expert proceduralists
	i-SCAN	Neutral: Variable results, increased detection of flat and right-sided polyps	Advantage: Effective for diminutive and small polyps	
	FICE	Disadvantage: No significant difference in ADR	Disadvantage: Inferior to NBI	Advantage: Familiar colour spectrum
	LCI	Advantages: Improves adenoma detection; and effective for non-expert proceduralists[1]	Disadvantage: Insufficient evidence	Advantage: Familiar colour spectrum
	BLI	Disadvantage: No significant difference in ADR	Advantage: Similar to NBI in terms of colour spectrum and accuracy[1]	Advantage: Similar colour spectrum to NBI
	TXI	Advantage: Increases polyp visibility in image-based studies	Disadvantage: Insufficient evidence	Disadvantage: Insufficient evidence; advantage: Familiar colour spectrum
AFI		Disadvantage: Insufficient evidence; advantage: Improves detection of flat, right-sided polyps and reduces miss rates	Disadvantage: Inferior to NBI	Disadvantage: Not widely available
AI		Advantage: Increases adenoma detection; no significant difference in withdrawal time[1]	Advantages: Highly accurate; superior to non-expert endoscopists for histology prediction; not superior to experts using NBI[1]	Disadvantage: Not yet widely available
FME		Disadvantages: Expensive; insufficient evidence		Disadvantage: Not widely available
Endo-cystoscopy			Neutral: Accurate but requires expertise for interpretation; advantages: Uptake may increase with incorporation of AI	Disadvantages: Requires additional equipment; and not widely available
Multiphoton microscopy			Disadvantage: Insufficient evidence	Disadvantage: Requires additional equipment

[1]The most promising technologies.

HD-WLI: High-definition white light imaging; NBI: Narrow-band imaging; BLI: Blue light imaging; TXI: Texture and colour enhancement imaging; FME:

Fluorescence molecular endomicroscopy; AFI: Autofluorescence imaging; ADR: Adenoma detection rate; AI: Artificial intelligence; LCI: Linked colour imaging; SSA: Sessile serrated adenomas.

FOOTNOTES

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REVIEW

Acute liver injury in COVID-19 patients hospitalized in the intensive care unit: Narrative review

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Abstract

In recent years, humanity has been confronted with a global pandemic due to coronavirus disease 2019 (COVID-19), which has caused an unprecedented health and economic crisis worldwide. Apart from the respiratory symptoms, which are considered the principal manifestations of COVID-19, it has been recognized that COVID-19 constitutes a systemic inflammatory process affecting multiple organ systems. Across the spectrum of organ involvement in COVID-19, acute liver injury (ALI) has been gradually gaining increasing attention by the international scientific community. COVID-19 associated liver impairment can affect a considerable proportion of COVID-19 patients and seems to correlate with the severity of the disease course. Indeed, COVID-19 patients hospitalized in the intensive care unit (ICU) run a greater risk of developing ALI due to the severity of their clinical condition and in the context of multi-organ failure. The putative pathophysiological mechanisms of COVID-19 induced ALI in ICU patients remain poorly understood and appear to be multifactorial in nature. Several theories have been proposed to explain the occurrence of ALI in the ICU setting, such as hypoperfusion and ischemia due to hemodynamic instability, passive liver congestion as a result of congestive heart failure, ischemia-reperfusion injury, hypoxia due to respiratory failure, mechanical ventilation itself, sepsis and septic shock, cytokine storm, endotheliitis with concomitant coagulopathy, drug-induced liver injury, parenteral nutrition and direct cytopathic viral effect. It should be noted that no specific therapy for COVID-19 induced ALI exists. Therefore, the therapeutic approach lies in preventive measures and is exclusively

supportive once ALI ensues. The aim of the current review is to scrutinize the existing evidence on COVID-19 associated ALI in ICU patients, explore its clinical implications, shed light on the underlying pathophysiological mechanisms and propose potential therapeutic approaches. Ongoing research on the particular scientific field will further elucidate the pathophysiology behind ALI and address unresolved issues, in the hope of mitigating the tremendous health consequences imposed by COVID-19 on ICU patients.

Key Words: Liver injury; COVID-19; Intensive care; Pathophysiological mechanisms; Cytokine storm; Multi-organ failure

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Core Tip: In recent times, coronavirus disease 2019 (COVID-19) pandemic has substantially altered the hitherto existing medical landscape, causing tremendous perturbations among the global scientific community and imposing a disproportionate burden on healthcare systems worldwide. It soon became apparent that COVID-19 affects multiple organ systems, including the liver. Acute liver injury has been progressively identified as a common, yet often under-recognized, complication of COVID-19, especially in the intensive care unit (ICU) setting, resulting in higher mortality rates. This review attempts to elucidate the underlying pathophysiological mechanisms that contribute to the development of acute liver injury in ICU patients with COVID-19, summarize emergent data and propose therapeutic strategies.

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INTRODUCTION

In recent years, humanity has been confronted with a global pandemic due to coronavirus disease 2019 (COVID-19), which has caused an unprecedented health and economic crisis worldwide. The initial isolation of the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and the subsequent first outbreak of COVID-19 in Wuhan, China, marked a grim milestone for mankind, substantially altering the existing scientific, social and economic landscape[1-3]. Since then, COVID-19 has rapidly spread throughout the world across all nations and ages and has resulted in the emergence of numerous mutant SARS-CoV-2 variants, triggering recurrent COVID-19 surges that continue to drive the ongoing pandemic[4-6]. Due to its dynamic and ever-changing epidemiological course, COVID-19 still remains an international public health emergency and accounts for considerable morbidity and mortality, inflicting an overwhelming death toll and a disproportionate burden on healthcare systems worldwide, thus raising major concerns among the global scientific community[7,8].

The clinical spectrum of COVID-19 is highly variable with a wide range of clinical manifestations. In most cases, the course of the disease is either asymptomatic or presents as a mild self-limited infection; however, in some patients, especially those with underlying comorbidities, COVID-19 evolves to a severe or even a critical life-threatening disease, culminating in the development of acute respiratory distress syndrome (ARDS) which requires intensive care support and may eventually progress to death [9,10]. Undoubtedly, SARS-CoV-2 infection demonstrates an inherent propensity for the respiratory system; hence, lung involvement is the predominant feature in patients hospitalized with COVID-19. Nonetheless, as the complete magnitude of COVID-19 sequelae continues to unravel, it has been realized that this new nosological entity constitutes a diverse, complex and multifaceted syndrome that extends beyond the respiratory system and affects multiple organs, including the liver[11,12]. Bearing this in mind, specific attention has been gradually drawn towards the pathogenesis of liver injury in the setting of COVID-19, since acute liver injury (ALI) has been progressively identified as a common, yet often under-recognized, complication of COVID-19. As a matter of fact, there have been numerous literature reports that a considerable proportion of COVID-19 patients develop liver injury characterized by abnormalities in liver chemistry levels. Notably, ALI seems to correlate with the severity of the disease course[13-16]. Indeed, COVID-19 patients hospitalized in the intensive care unit (ICU) run a greater risk of developing ALI, mainly due to the severity of their clinical condition and in the context of multi-organ failure, thus resulting in higher mortality rates[17-20].

Studies dealing with COVID-19 induced ALI in the ICU setting are rather limited. Accordingly, the present review aims to summarize relevant existing evidence regarding ALI in COVID-19 patients

hospitalized in the ICU and explore its clinical implications, while at the same time an attempt is made to elucidate the underlying pathophysiological mechanisms that contribute to the development of ALI in ICU patients, and to propose therapeutic approaches.

DEFINING ACUTE LIVER INJURY

A universal definition of ALI is still lacking. This poses significant challenges and oftentimes generates confusion among clinicians when referring to liver injury and trying to accurately interpret abnormal liver tests[21]. Based on the latest American College of Gastroenterology clinical guidelines for the evaluation of abnormal liver chemistries[22], markers of liver injury comprise routinely measured liver chemistries, namely aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and bilirubin, whereas markers of hepatocellular function include albumin, bilirubin and prothrombin time. Sometimes, elevation of gamma-glutamyl transferase (GGT) can be used as a supplement to confirm the hepatic origin of an elevated ALP level. According to the same guidelines [22], AST and/or ALT elevation is defined as borderline when values are less than 2-fold the upper limit of normal (ULN); mild when they are between 2 and 5 times the ULN; moderate when they are between 5 and 15 times the ULN; severe if they exceed 15 times the ULN; and massive in case they are above 10000 IU/L. Furthermore, in order to characterize the type of liver injury, the following categories have been proposed based on the proportion of elevated AST and ALT values as compared to ALP values: Hepatocellular injury (disproportionate elevation of AST/ALT), cholestatic injury (predominant elevation of ALP) and mixed pattern of injury (elevation of both AST/ALT and ALP levels). For this purpose, the R ratio has been used, which is derived from the formula R = (ALT value:ALT ULN)/(ALP value:ALP ULN), whereby an R ratio > 5 indicates hepatocellular injury, an R value < 2 suggests cholestatic injury and an R value 2-5 is consistent with a mixed pattern of liver injury[22].

The lack of a uniform definition of ALI has led to the arbitrary use of different criteria for identifying ALI, which has resulted in great heterogeneity of reported research results. The adoption of diverse criteria by researchers, either for defining ALI or characterizing its pattern and severity, is also evident in the current literature regarding COVID-19 induced ALI. For instance, in a large retrospective United States cohort of COVID-19 patients, ALI was defined as any elevation of ALT and AST above normal and was subsequently classified, according to the degree of ALT elevation, as none/mild, moderate or severe when ALT values were less than 2-fold the ULN, between 2 and 5 times the ULN and more than 5-fold the ULN respectively[23]. On the other hand, Yip *et al*[24] defined ALI as an elevation of ALT and/or AST ≥ 2 times the ULN, with a concomitant increase of total bilirubin by more than 2-fold the ULN and/or international normalized ratio (INR) ≥ 1.7[24], whereas Cai *et al*[25] defined liver injury either as an increase of ALT and/or AST ≥ 3 times the ULN or as a more than 2-fold increase of ALP, GGT and/or total bilirubin above the ULN[25]. Meanwhile, other researchers avoided the use of the term ALI and employed other general terminology instead, such as ''liver test abnormalities'' or ''abnormal liver function tests'', to include any increase of at least one of AST, ALT, ALP, bilirubin and GGT above the ULN (as *per* local laboratory reference range standards) or even a reduction of albumin levels below normal cut-off values[26,27].

Based on the above, it is more than obvious that there is a wide discrepancy in the current COVID-19 literature with regard to ALI definition, thus rendering interpretation and comparison of results rather burdensome and obscure. On this account, it would be desirable for researchers to reach a consensus on a unifying definition in order to eliminate existing disparities.

EXISTING EVIDENCE

In the current COVID-19 literature there is a dearth of published clinical studies with an exclusive focus on COVID-19 induced ALI in ICU patients. Moreover, most of the available data on the topic are rather diverse owing to the lack of a uniform definition for liver injury, disparate thresholds applied for ALI, divergent study designs and different endpoints of each study.

The allegedly first study to explore the incidence, clinical characteristics and outcomes of ALI exclusively in ICU patients with COVID-19 was conducted in Germany and included 72 critically ill patients between March and July 2020[28]. The investigators used the term severe liver dysfunction and defined it as the occurrence of hypoxic liver injury (manifested by elevated aminotransferase levels > 20-fold the ULN in the setting of cardiac, circulatory or respiratory failure and after exclusion of other possible causes) and/or jaundice (total bilirubin ≥ 2 mg/dL). They found that 31% of the ICU patients developed severe liver dysfunction during their ICU stay approximately a week after ICU admission, predominantly in the form of cholestatic liver injury (45%) followed by equally contributing hepatocellular (27%) and mixed pattern injury (27%). Patients with severe liver dysfunction had a higher simplified acute physiology score II on admission, indicating a more severe clinical condition, as well as higher rates of viremia during their ICU stay. Severe liver dysfunction was associated with more severe respiratory failure, as manifested by more frequent development of ARDS and lower values of

Horowitz index, namely lower ratio of partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2 ratio). Furthermore, patients with severe liver dysfunction were more likely to require mechanical ventilation (MV), circulatory support with vasopressors, renal replacement therapy and rescue therapy by means of veno-venous extracorporeal membrane oxygenation (ECMO). Accordingly, patients with severe liver dysfunction experienced higher mortality rates and, more importantly, severe liver dysfunction was found to be an independent predictor of mortality[28].

Along the same lines, Huang *et al*[29] conducted a retrospective study with the aim to investigate the prevalence of hypoxic hepatitis in 51 COVID-19 patients hospitalized in a Chinese ICU from December 2019 to March 2020[29]. They used the same definition of hypoxic hepatitis; that is, a massive but transient elevation of ALT levels > 20-fold the ULN in the setting of cardiac, circulatory or respiratory failure, after excluding other putative causes of ALI. Based on this terminology, hypoxic hepatitis was evident in 3 male patients, corresponding to 5.88% of all ICU patients, with peak ALT values being 1665, 1412 and 1140 U/L respectively. All 3 patients progressed to respiratory failure and eventually died due to multiple organ failure. When looking at the dynamic changes of liver enzymes, it was observed that ALT and AST elevations occurred at an earlier stage, while bilirubin levels increased sharply at a later stage when transaminase values had already started to show a downward trend after peaking in the third week. The authors came to the conclusion that hypoxic hepatitis was not an infrequent condition in ICU patients with severe COVID-19 and was accompanied by high mortality[29].

Meanwhile, Salik *et al*[30] conducted a study which included 533 COVID-19 patients admitted to the ICU with the aim to determine the impact of observed liver test abnormalities on mortality[30]. Liver injury was defined as ALT and/or AST levels above 3 times the ULN, and/or total bilirubin levels greater than 2-fold the ULN. Patients were divided into three groups: Group 1 consisted of patients with normal liver chemistries; Group 2 included patients with abnormal liver test values not falling into the category of liver injury; Group 3 comprised patients with liver injury. Any kind of liver test abnormality, reflected by groups 2 and 3, was observed in 52% of the total cohort and was more frequent in males. However, as *per* study's definition, only 8.6% of all ICU patients developed ALI, represented by group 3 alone. Paradoxically, the authors reported that patients with ALI and milder liver test abnormalities (groups 2 and 3) had shorter ICU lengths of stay compared with group 1, but this result is most likely attributed to the unacceptably high total mortality rates of patients belonging to these groups (71.4% and 78.3% for groups 2 and 3 respectively), which erroneously were not taken into account as confounding factors during the analysis. The authors concluded that liver test abnormalities were predictive of higher mortality, given that groups 2 and 3 experienced higher than expected 7-d, 28-d and total mortality rates[30].

A similar definition of ALI was employed by Arentz *et al*[31] who published the first case series of ICU patients with COVID-19 in the United States, dating back to February-March 2020[31]. A total of 21 patients were included. On ICU admission, abnormal liver tests were reported in 8 patients (38%) with one patient displaying very high levels of AST (4432 U/L) and ALT (1414 U/L). During the course of the disease, among patients who were mechanically ventilated, 3 (14.3%) developed ALI, defined as a more than 3-fold rise above the ULN in AST or ALT levels. No further details about ALI were provided by this study, since its focus was not on liver injury in the ICU, but rather its aim was to describe the clinical characteristics and outcomes in the initial ICU cases of COVID-19 patients in the United States [31].

Likewise, in a retrospective observational cohort study from France with 600 COVID-19 patients, 153 of whom required ICU hospitalization, it was reported that 9.8% of the ICU patients developed ALI, defined as AST levels higher than 3-fold the ULN[32]. ALI occurred more frequently in the ICU compared to common ward patients, as did most of the other extrapulmonary complications, like acute kidney injury, cardiovascular events and thromboembolic events. Data analysis demonstrated that factors associated with higher risk for ALI included age > 75 years, concomitant cancer, chronic cardiac disease, as well as higher levels of C-reactive protein (CRP), serum creatinine and hemoglobin[32].

Another retrospective observational study from China reported an 18.1% incidence of ALI in 83 ICU patients with COVID-19[33]. In this case, ALI was defined as an elevation of hepatic biomarkers more than 2-fold the ULN or as disproportionately elevated AST and ALT levels compared to ALP levels. However, the focus of this study was on acute gastrointestinal injury (defined as various grades of malfunction of the gastrointestinal tract) in critically ill patients with COVID-19 and not specifically on ALI; hence, no additional data on ALI were available. Nevertheless, since ALI could be considered one of the components of acute gastrointestinal injury, it could be assumed that the main study conclusions may also be applicable to ALI. The major findings of this study were that patients with worse grades of acute gastrointestinal injury had worse clinical severity features, while sequential organ failure assessment score, MV duration and white blood cell count arose as independent risk factors for the development of more severe acute gastrointestinal injury. What is more, these patients exhibited higher rates of both septic shock and 28-d in-hospital mortality[33].

In a case series examining the temporal evolution of blood liver tests in 20 consecutive ICU patients with COVID-19, liver injury was reported to be frequent, albeit transient and non-severe[34]. However, liver injury was ill-defined as any elevation of at least one liver test (AST, ALT, ALP, GGT, total bilirubin) above the ULN. Consequently, all 20 patients exhibited some form of liver injury during the first 10 d after ICU admission. Interestingly, only median levels of AST, ALT and GGT (but not bilirubin

or ALP) increased above the ULN, while only GGT showed pronounced elevations (≥ 3-fold the ULN) with a peak on day 8 after ICU admission. Based on these findings, the researchers commented that late cholestasis was frequently observed[34].

In addition, Shousha *et al*[35] conducted a prospective cohort study in 547 Egyptian patients with COVID-19 in order to investigate the underlying prevalence and severity of liver and gastrointestinal disturbances, as well as their effect on disease outcomes[35]. Among patients who required ICU admission (122/547), 48.50% had elevated AST and 35.60% had elevated ALT. Elevated AST, but not ALT, levels were associated with increased mortality in univariate analysis. Moreover, patients admitted to the ICU displayed significantly higher hospital admission levels of fibrosis-4 index (FIB-4), which incorporates 4 variables (age, AST, ALT and platelet count) and is considered a predictive marker for significant liver fibrosis. Similarly high levels of FIB-4 were observed in severe COVID-19 cases and in non-survivors. In the multivariate analysis, FIB-4 score > 3.25 was a significant predictor of mortality. Unfortunately, no other specific ICU details could be extrapolated from this study, owing to the trial design and aim, which was not focused on ICU patients[35].

Based on the observation that black population in the United States had been disproportionately affected by COVID-19, Currier *et al*[36] investigated potential differences in outcomes between black and non-black patients with COVID-19 and elevated liver enzymes[36]. They included a total number of 8028 patients, out of whom 3937 patients had available liver test data for interpretation. The analysis demonstrated that 45% of both black and non-black patients exhibited elevations in their liver chemistries. Among black patients with liver test elevations, 46% were intubated compared to 34.8% of non-black patients with elevated liver enzymes. This study highlighted that black patients who had liver test abnormalities were more prone to ICU admission and intubation than non-black counterparts, who albeit run a significantly higher risk of death[36].

Besides, ALI has also been appreciated in the context of specific patient categories with a prior history of liver disease. In this perspective, a multicenter observational cohort study from the United States aimed to explore the prevalence and impact of COVID-19 induced ALI in liver transplant recipients of various races and ethnicities[37]. 112 adult liver transplant recipients with COVID-19 were included, 81 of whom required hospitalization. Among hospitalized patients, 30 (37%) were admitted to the ICU with the majority of them requiring MV and circulatory support with vasopressors. ICU patients were more likely to develop ALI, which was determined according to ALT values at the peak of COVID-19 and was defined as ALT levels greater than 2-fold the ULN. On multivariate analysis, use of vasopressors in the ICU was found to be an independent predictor of liver injury. Furthermore, ICU patients had a higher likelihood of having their immunosuppression therapy modified. However, reduction in immunosuppression was not associated either with ALI or with risk of mortality or graft rejection, in contrast to antibiotic administration which was related to increased risk for ALI. Overall, the presence of ALI independently predicted risk for ICU admission and mortality in liver transplant recipients with COVID-19[37].

Other than cohort studies, there have also been case reports regarding COVID-19 induced ALI in ICU patients. Of note, a case report from Italy highlighted a rare case of ALI progressing to acute liver failure and eventually death[38]. It involved 2 critically ill COVID-19 patients hospitalized in the ICU due to severe ARDS. The patients were treated with tocilizumab, as part of the anti-cytokine storm regimen, which however seemed to have aggravated the underlying COVID-19 immunosuppression, thus facilitating the development of opportunistic infections in the already immunocompromised patients. Indeed, a few days prior to their death, lab tests revealed Herpes simplex virus 1 viremia leading to fulminant hepatitis with dramatic increases in ALT, AST, bilirubin and INR levels and resulting in fatal outcomes for both patients[38].

Furthermore, one should bear in mind that within the spectrum of liver injury in COVID-19 patients lies the cholestatic pattern of liver injury, along with its late sequelae, such as the development of secondary sclerosing cholangitis in critically ill patients. In this regard, Bütikofer *et al*[39] described the incidence and severity of cholestatic liver injury in 34 ICU patients with COVID-19[39]. Cholestatic liver injury was termed mild if ALP and GGT levels were higher than 1.5-fold and 3-fold the ULN respectively, whereas it was considered severe in case the above abnormalities were accompanied by a concomitant elevation of total bilirubin levels more than 2-fold the ULN. The investigators reported that 59% of the ICU patients developed some degree of cholestasis (32% mild and 27% severe). Patients with severe cholestatic injury displayed a more complicated clinical course, required more intensified supportive treatment (in terms of vasopressor support, renal replacement therapy and ECMO) and had a more extended length of ICU stay. Moreover, 4 out of 9 patients with severe cholestatic injury developed secondary sclerosing cholangitis, eventually resulting in 2 deaths and 1 candidacy for liver transplantation. By the same token, irrespective of the degree of cholestasis, the vast majority of patients with pronounced elevations of ALT levels (> 10-fold the ULN) developed untoward outcomes, progressing either to secondary sclerosing cholangitis or death[39].

From a similar perspective, on the grounds of a higher than expected incidence of cholangiopathies in critically ill patients with COVID-19, Wendel-Garcia *et al*[40] addressed the issue of drug-induced liver injury (DILI) in a prospective observational cohort of patients with COVID-19 associated ARDS[40]. They performed a post hoc analysis on 243 ICU patients who were on invasive MV, with the aim to investigate whether a causal relationship between the prolonged infusion of high-dose ketamine and the

occurrence of cholestatic liver injury existed. Acute cholestatic DILI was defined as ALP levels greater than 1.5-fold and GGT levels greater than 3-fold the ULN, whereas patients were deemed to have severe cholestatic liver injury in case a concurrent increase in bilirubin levels more than 2-fold the ULN was present. During their ICU stay, 114 patients developed cholestatic liver injury, 100 of whom had received long-term ketamine infusion, while severe cholestatic liver injury occurred in 33% of the latter. The analysis revealed a duration-effect and dose-effect relationship between ketamine infusion and bilirubin and ALP levels. In other words, prolonged duration of infusion and higher doses of ketamine were positively correlated with rising bilirubin and ALP levels. Interestingly, no such effect was observed with long-term infusion of propofol and sufentanil, even at high doses. The study clearly demonstrated an increased hazard of developing cholestatic liver injury in ICU patients who had received long-term ketamine infusion as a co-sedative agent; yet, no association between ketamine infusion and increased in-hospital mortality was depicted[40].

The aforementioned studies regarding the prevalence and clinical implications of COVID-19 induced ALI in ICU patients are summarized in Table 1.

PATHOPHYSIOLOGY

The pathophysiology of ALI in COVID-19 patients hospitalized in the ICU has not been fully elucidated and still awaits to be unraveled, since the underlying mechanisms have not been sufficiently decoded and appear to be multifactorial in nature. So far, the scientific research conducted in the field has proposed several mechanisms that associate SARS-CoV-2 infection with ALI. These mechanisms may have direct or indirect impact on liver function. In fact, there seems to be a complex interplay among several distinct pathophysiological pathways implicated in the course of critically ill patients with COVID-19 (Figure 1). Presumably, these diverse pathophysiological mechanisms act in synergy and exert cumulative effects, since no single mechanism can completely explain the vast spectrum of liver involvement in ICU patients with COVID-19. It is worth noting that COVID-19 induced ALI can occur either in the context of an underlying liver disease, thus leading to decompensation of the preexisting state of equilibrium, or can manifest as a de novo nosological entity in ''naïve'' patients with no previous history of documented hepatic dysfunction[41-44].

Hypoperfusion

ICU patients represent a specific subgroup of patients with distinctive features. Hemodynamic instability is frequently encountered among patients hospitalized in the ICU setting, who therefore require administration of vasopressors or even inotropes, sometimes in particularly high doses. There are miscellaneous factors contributing to the observed hemodynamic instability of ICU patients, including hypovolemic, distributive, cardiogenic or obstructive shock, MV, sedation and drugs[45,46]. It is worth mentioning that in the COVID-19 era a specific cause leading to cardiogenic shock may be related to myocardial injury caused by SARS-CoV-2. Cardiovascular involvement in COVID-19 may manifest in the form of myocarditis and pericarditis, but also as arrhythmias, acute coronary syndromes and stress-induced cardiomyopathy. When severe, all of these cardiovascular manifestations may potentially lead to acute heart failure and shock, especially in vulnerable patients with pre-existing cardiovascular disease[47-49]. Regardless of the cause, hemodynamic instability results in decreased splanchnic blood flow, leading to inadequate blood supply to the liver and subsequent liver ischemia. The resultant decreased end-organ perfusion is exaggerated in cases of hypovolemia[50].

Moreover, in the setting of passive liver congestion due to right heart failure of various etiologies, the superimposed elevated hepatic venous pressures further impair hepatic circulation by reducing the gradient between portal and hepatic venous pressures, which primarily drives the flow within the portal venous system. On top of that, the liver autoregulatory mechanisms are disrupted and become maladaptive, while the hepatic arterial buffer system fails to sustain hepatic blood flow under conditions of low mesenteric perfusion and cannot compensate for the changes in liver blood supply caused by low blood pressure, thus placing the liver at risk for further ischemic injury[51,52].

It needs to be emphasized that, although vasopressors can have beneficial hemodynamic effects in terms of restoring and maintaining hemodynamic stability and supporting vital functions, they are not void of adverse effects, which can often be detrimental. Indeed, they can cause excessive vasoconstriction and hence impair tissue perfusion through reduction of blood flow in vasoconstricted vascular beds, while at the same time they may exert deleterious effects on cardiac, metabolic, microbiome and immune function[53-55].

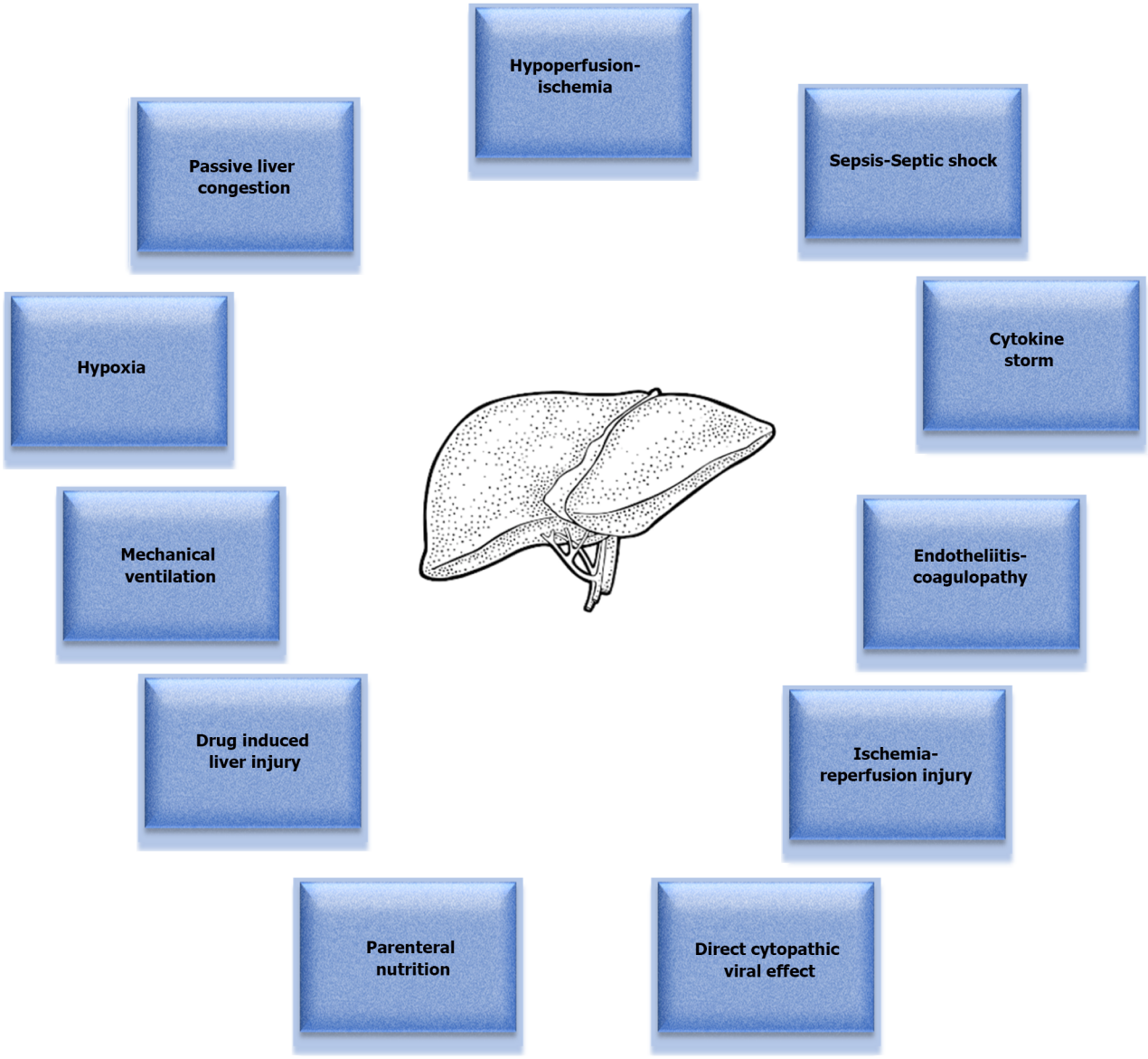
From a cellular standpoint, the sudden and profound reduction in systemic blood pressure, in conjunction with increased hepatic venous pressures, establish a low-flow state, which can result in the so-called hypoxic hepatocellular injury characterized by prominent centrilobular hepatocellular necrosis, since the central areas of the liver (commonly referred to as zone 3) are more susceptible to ischemic insults[51]. Mitochondrial damage and DNA fragmentation are the principal mechanisms implicated in the process of injury during hypoxic ALI, as evidenced by elevated plasma levels of glutamate dehydrogenase and cytochrome c oxidase mitochondrial DNA on the one hand and elevated

Table 1 Studies examining coronavirus disease 2019 induced acute liver injury in intensive care unit patients

Ref.	ALI definition	Number of ICU patients	ALI incidence	Major findings/outcomes
Roedl *et al*[28], 2021, Germany, Single-center, retrospective	↑ Transaminases > 20 × ULN or BIL ≥ 2 mg/dL	72	31%	ALI pts experienced higher mortality (83% for hepatocellular, 66% for mixed and 60% for cholestatic ALI), developed respiratory failure more frequently and had higher need for MV, ECMO, vasopressors and RRT
Huang *et al*[29], 2020, China, Single-center, retrospective	↑ALT > 20 × ULN	51	5.88%	ALI was not rare and was associated with high mortality due to MOF
Salik *et al*[30], 2021, Turkey, Single-center, retrospective	↑ALT/AST > 3 × ULN and/or ↑TBIL > 2 × ULN`	533	8.6%	ALI pts had higher total, 7-d and 28-d mortality, as well as higher SOFA score. ALI was more frequent in males
Arentz *et al*[31], 2020, United States, Single-center, case series	ALT or AST levels > 3 × ULN	21	14.3%	67% mortality for the overall cohort, no mortality data for ALI pts. All ALI pts (3) were on MV. High rate of cardiomyopathy in the overall cohort
Martinot *et al*[32], 2021, France, Single-center, retrospective observational cohort	AST ≥ 3 × ULN	153	9.8%	Factors associated with higher risk for ALI: Age > 75, cancer, cardiac disease, higher levels of CRP, serum Cr and Hb
Sun *et al*[33], 2020, China, Single-center, retrospective observational	↑Serum levels of liver biomarkers (*e.g.* ALT) > 2 × ULN or disproportionate ↑ ALT/AST *vs* ALP	83	18.1%	The study focused on AGI, defined as various grades of malfunction of the GI tract, and not on ALI. Pts with worse AGI grades had higher 28-d mortality, higher incidence of septic shock and worse clinical variables
Cardoso *et al*[34], 2020, Portugal, Single-center, case series	Any elevation of at least one liver test (AST, ALT, ALP, GGT, TBIL) above ULN	20	100%	Liver injury was frequent, but transient and non-severe. Late cholestasis was mainly observed
Shousha *et al*[35], 2021, Egypt, Multi-center, prospective cohort	↑Transaminases > 3 × ULN	122 ICU pts. Overall cohort consisted of 547 pts	4.91% or 3.70% (based on AST or ALT levels respectively). Data represent overall (not ICU) pts cohort	FIB-4 on admission was significantly higher in pts admitted to the ICU, those with more severe COVID-19 and non-survivors. FIB-4 score > 3.25 and ICU admission were significant predictors of mortality
Currier *et al*[36], 2021, United States, Single-center, retrospective	ALT/AST > 60, ALP > 150, or BIL > 1.5	No data available for ICU pts. Overall cohort consisted of 8028 pts	45%. Data represent overall (not ICU) pts cohort	Black COVID-19 pts with liver test abnormalities were at greater risk for ICU admission and intubation compared to other races, but non-Black pts with liver test abnormalities were at increased risk of death
Rabiee *et al*[37], 2020, United States, Multi-center, observational cohort	ALT ≥ 2 × ULN (moderate: ALT 2-5 × ULN, severe: ALT > 5 × ULN)	30 ICU pts. Overall cohort consisted of 81 hospitalized LT recipients	34.6%. Data represent overall (not ICU) pts cohort	ALI was associated with higher risk for ICU admission and higher mortality. Hispanic ethnicity, metabolic syndrome, use of vasopressors, antibiotic use and younger age were independent risk factors for ALI. ICU pts were more likely to have their immunosuppression therapy modified
Roncati *et al*[38], 2022, Italy, Case report	N/A	2	100%	Fulminant herpetic hepatitis developed as a superimposed opportunistic infection due to tocilizumab-induced immunosuppression. Both pts died due to acute liver failure
Bütikofer *et al*[39], 2021, Switzerland, Single-center, retrospective cohort	Cholestatic ALI: ALP ≥ 1.5 × ULN and GGT ≥ 3 × ULN (termed severe if additionally TBIL ≥ 2 × ULN)	34	59% (27% severe)	Pts with severe ALI had higher mortality, significantly longer ICU stay with a more complicated course and required higher levels of support
Wendel-Garcia *et al*[40], 2022, Switzerland, Single-center, prospective observational cohort (post-hoc analysis)	Acute cholestatic liver injury: ALP > 1.5 × ULN and GGT > 3 × ULN (severe if additionally BIL > 2 × ULN)	243	47%	Pts who received ketamine had an increased risk of developing cholestatic liver injury than pts who didn't. Ketamine infusion demonstrated a dose-dependency and duration-dependency association with increasing BIL and ALP levels, but it was not associated with increased hospital mortality

AGI: Acute gastrointestinal injury; ALI: Acute liver injury; ALT: Alanine transaminase; ALP: Alkaline phosphatase; AST: Aspartate transaminase; BIL: Bilirubin; COVID-19: Coronavirus disease 2019; Cr: Creatinine; CRP: C-reactive protein; ECMO: Extracorporeal membrane oxygenation; FIB-4: Fibrosis-4 index; GI: Gastrointestinal; GGT: Gamma-glutamyl transferase; Hb: Hemoglobin; ICU: Intensive care unit; LT: Liver transplant; MOF: Multiple organ failure; MV: Mechanical ventilation; Pts: Patients; RRT: Renal replacement therapy; SOFA: Sequential organ failure assessment; TBIL: Total bilirubin; ULN:

Upper limit of normal.



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Figure 1 Pathophysiological mechanisms implicated in the development of coronavirus disease 2019 acute liver injury in the intensive care unit setting. Figure 1 depicts the various pathophysiological mechanisms that come into play in the setting of coronavirus disease 2019 (COVID-19) infection, which eventually culminate in the development of acute liver injury (ALI) in intensive care unit (ICU) patients. Hypoperfusion and ischemia due to the frequently observed hemodynamic instability in the ICU patients play a major role in the process. Passive liver congestion as a result of congestive heart failure of various etiologies is another key determinant. The situation can be further aggravated by ischemia-reperfusion injury. Sepsis can also cause hepatic dysfunction, which is usually cholestatic in nature and reversible, but, when it progresses to septic shock, it results in multiple organ dysfunction syndrome and liver hypoperfusion, manifesting as shock liver. Besides, hypoxia due to respiratory failure and acute respiratory distress syndrome may also lead to ALI. Paradocixally, mechanical ventilation *per se* can exert deleterious effects on the liver through positive pressure ventilation, especially when accompanied by the application of high positive end-expiratory pressure and large tidal volumes. Furthermore, ALI can stem from the inflammatory cascade generated by the cytokine storm, while it can be further complicated by impaired microcirculation and thrombosis as a result of endotheliitis with concomitant coagulopathy. Drug-induced liver injury, as well as parenteral nutrition, must also not be overlooked as potential causative factors. Finally, liver injury could also be attributed to a direct cytopathic viral effect. There seems to be a complex interplay among these numerous underlying mechanisms, which may act either independently and cause COVID-19 induced ALI in the ICU setting, or more frequently synergistically and generate a relentless ALI vicious cycle.

circulating levels of nuclear DNA fragments on the other[56].

Even after restoring hemodynamic stability with the use of vasoactive drugs, it should be kept in mind that ischemia reperfusion injury may ensue, whereby reperfusion after prolonged ischemia can trigger a cascade of molecular mechanisms, paradoxically begetting further liver injury. This cascade of events involves the recruitment and activation of cellular mediators, the engagement of the complement

system, the generation of reactive oxygen species and the release of a wide variety of various cytokines, chemokines, adhesion molecules and other chemical mediators, which lead to microvascular alterations, acute inflammatory responses, derangements in microcirculation and increased hepatocellular apoptosis and necrosis[57].

Sepsis-Septic shock

In the ICU environment, sepsis represents a relentless plague with grave consequences for the critically ill patients, accounting for a significant proportion of the observed high morbidity and mortality rates. This becomes even more relevant for COVID-19 patients hospitalized in the ICU, given the fact that they require extended ICU stay with prolonged periods of invasive MV and vasopressor support, in the setting of a severely compromised immune system with a markedly dysregulated host response to infection. Sepsis may stem from multiple sources of infection and invariably results in organ dysfunction[58]. With regard to the liver, sepsis can cause hepatic dysfunction, which primarily manifests as sepsis-associated cholestatic dysfunction and to a lesser extent as hypoxic hepatitis. Sepsis, especially when caused by infections from gram-negative bacteria, increases intestinal permeability and results in endotoxin translocation from the intestinal lumen into the portal circulation. In the liver, endotoxins mount an inflammatory response through activation of Kupffer cells and macrophages, which results in secretion of proinflammatory cytokines[21,59]. This inflammatory reaction induces changes in the architecture and function of hepatocytes and cholangiocytes, leading to dysregulation of the liver metabolic signaling pathways, repression of the hepatobiliary transporter systems and decrease in canalicular contractility. All these result in bile acid retention with defective bile acid uptake, impaired bile production and secretion, inhibition of bile flow and formation of biliary sludge. The condition is further exacerbated by the impaired hepatic microcirculation due to sepsis-induced microvascular endothelial injury resulting in coagulopathy and microthrombi formation[21,60]. In the majority of critically ill patients, this type of cholestasis is reversible, but, in rare cases, destruction of the biliary epithelium due to ischemia and inflammation may cause irreversible biliary damage, progressing to secondary sclerosing cholangitis with formation of biliary casts and scarring of the bile ducts[60,61].

When sepsis culminates in septic shock, hemodynamic instability prevails, necessitating administration of vasopressors or inotropes[62]. Under these circumstances, the imminent end-organ hypoperfusion may eventually lead to shock liver in the context of multiple organ dysfunction syndrome. Indeed, the impaired hepatic perfusion leads to hypoxic hepatitis by causing direct hepatocellular injury. Despite the associated hyperdynamic state with increased cardiac output, septic shock is characterized by ongoing liver ischemia owing to impaired tissue oxygen extraction or, in other words, inability of hepatocytes to utilize oxygen. Once hepatocytes, especially those residing in the centrilobular regions of the liver, are deprived of oxygen, they start malfunctioning[21,63]. In fact, progressive tissue hypoxia promotes a catabolic state of anaerobic metabolism, suppresses mitochondrial energy production, compromises cellular membrane integrity due to loss of energy-dependent ion pumps and causes significant structural and functional abnormalities to hepatocytes[63]. The final result is direct hepatocellular damage, which is clinically described as hypoxic or ischemic liver injury and characterized by a massive, acute and transient rise in serum levels of aminotransferases[64].

Hypoxia

One of the cardinal features of COVID-19 critical illness is severe systemic hypoxia due to respiratory failure or ARDS[65]. Regardless of hemodynamic status, severe hypoxia *per se* can lead to ALI[66]. As a matter of fact, a pronounced and prolonged imbalance between oxygen supply and expenditure, secondary to profound hypoxia, can result in oxygen deprivation to the liver and thus trigger hypoxic hepatitis[64]. Hypoxia has long been implicated in the pathogenesis of liver diseases[67]. It has been shown that hypoxia induces alterations in gene expression by effectuating the activation of hypoxia-inducible factor 1α and nuclear factor kappa B, which in turn stimulate angiogenesis, chronic inflammation and epithelial-mesenchymal transition. Accordingly, the activation of these transcriptional pathways promotes liver fibrosis and increases hepatic vascular resistance, thereby diminishing liver blood flow and further aggravating liver hypoxia[68].

In most ICU cases, hypoxia, the predominant characteristic of ARDS, mandates the use of invasive MV. During ARDS management, protective ventilation strategies are employed, which incorporate the use of low tidal volumes with permissive hypercapnia[69]. However, both hypoxia and hypercapnia have been known to induce pulmonary vasoconstriction, which in turn increases pulmonary vascular resistance and right ventricular afterload[70,71]. This may cause derangements in the subtle balance between the respiratory and cardiovascular system, eventually provoking cardiocirculatory instability, which sequentially establishes an even harsher hypoxic environment[71].

In view of the fact that severely hypoxemic patients require invasive MV, often with the application of high positive end-expiratory pressure (PEEP) levels, the strain imposed on the right ventricle by MV may elicit right ventricular failure and reduce venous return and right ventricular preload, thus further affecting hemodynamic stability[72,73]. These mechanical effects are aggravated in patients with ARDS or in mechanically ventilated patients with intrinsic PEEP due to dynamic hyperinflation. As a result, the clinical course of the patients may be complicated by acute cor pulmonale. Factors associated with a

higher risk for developing acute cor pulmonale include worse oxygenation, hypercapnia, high ventilator pressures and pneumonia-related ARDS[72].

MV

Owing to the fact that respiratory failure is the most frequent indication for ICU admission, the majority of ICU patients are mechanically ventilated. Although life-saving, MV may elicit untoward effects on the function of extrapulmonary organs, including the liver, *via* multiple interactions.

It has long been recognized that positive pressure ventilation can induce a decrease in cardiac output accompanied by a reduction in hepatic arterial blood flow, while high intrathoracic pressures can jointly incur increases in hepatic venous and inferior vena caval pressures. In parallel, liver compression by the descent of the diaphragm increases hepatic venous resistance and causes a rise in intravascular portal pressure. The constellation of these MV-triggered events can cause congestive hepatomegaly and induce hepatocellular dysfunction[74].

The aforementioned effects on liver hemodynamics can be further aggravated by the application of PEEP. Indeed, it is widely known from the literature that PEEP may increase the backpressure to liver venous outflow and cause an elevation in liver venous resistance, thus decreasing total venous return and resulting in hepatic blood pooling and liver congestion[75,76]. This decrease in venous return is further amplified in hypovolemic states[71]. Besides, in a study assessing risk factors for liver injury in critically ill patients, high levels of PEEP were found to promote hepatic dysfunction[77].

Furthermore, in a study by Schricker *et al*[78], it was found that PEEP affected liver metabolism by promoting hepatic gluconeogenesis and enhancing oxidative hepatic lipid utilization for energy coverage in the liver[78]. A parallel increase in the splanchnic oxygen extraction rate was reported in response to a decline in hepatic oxygen delivery caused by a PEEP-dependent decrease in cardiac output[78]. This mechanism has been corroborated by various human studies which have shown that increasing levels of PEEP reduced splanchnic blood flow, thereby jeopardizing oxygen delivery to the abdominal viscera; yet, splanchnic oxygen consumption was usually maintained through a compensatory increase in splanchnic oxygen extraction[79].

Moreover, the application of large tidal volumes during MV markedly increases pulmonary vascular resistance. In turn, the increased pulmonary vascular resistance may compromise the systolic performance of the right ventricle, since ejection will have to take place against an increased right ventricular afterload[71]. The ensuing right ventricular dysfunction will result in high right atrial pressures, which will then be transmitted backwards to the liver, causing hepatic congestion due to a retrograde increase in the pressures of the inferior vena cava and the hepatic veins[80].

Additionally, the use of high PEEP, high inspiratory pressures and high tidal volumes during MV may alter hepatosplanchnic perfusion by precipitating varying degrees of elevation in the intra-abdominal pressure[81-83]. As a matter of fact, MV, especially with high levels of PEEP, induces changes in pressure in the intra-abdominal compartment through transmission of increased intrathoracic pressures to the abdomen and downward displacement of the diaphragm. Should a sustained rise in intra-abdominal pressure occur, this can lead to an abdominal compartment-like syndrome, thus affecting abdominal venous return and causing a decline in cardiac output as a result of a marked decrease in right ventricular preload[84]. Concurrently, the resultant intra-abdominal hypertension may compromise liver perfusion and jeopardize the physiologic hepatic function by compressing the portal vein and causing intestinal congestion[85]. At the same time, the increased abdominal pressure is transmitted to the thoracic cavity, affecting lung volumes and respiratory mechanics and generating increases in peak inspiratory, plateau and mean airway pressures of the mechanically ventilated patients[86]. Accordingly, this may negatively affect cardiac and respiratory performance, potentially leading to cardiovascular collapse, which will further exacerbate the vicious cycle of poor tissue perfusion and organ dysfunction[84]. The clinical importance of this phenomenon may be further magnified by the presence of causal factors which predispose to additional elevations of intra-abdominal pressure, such as acidosis, coagulopathy, sepsis, MV, use of PEEP or presence of auto-PEEP, pneumonia, prone positioning, aggressive fluid resuscitation or increased severity scores[87].

Finally, MV *per se* can induce an early systemic inflammatory reaction, governed by an increased expression of soluble adhesive molecules and cytokines, along with a concomitant activation of the neurohumoral axis[88].

Collectively, it becomes evident from the above that MV may exert negative effects on liver function in terms of both mechanics and hemodynamics, as well as from a neurohumoral and metabolic perspective[89].

Cytokine storm

SARS-CoV-2 activates the host immune system at different levels and in varying degrees. In critically ill COVID-19 patients, the virus triggers an uncontrolled immune response, which results in a state of generalized overt inflammation. This overwhelming inflammatory reaction is termed cytokine storm and is heralded by the excessive production of proinflammatory cytokines on the grounds of a dysregulated immune system. During this chain of events, cytokines play a pivotal role in the inflammatory cascade triggered by SARS-CoV-2. Principal cytokines involved in the process include interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). The excessive expression of proinflammatory cytokines,

chemokines and adhesion molecules attracts neutrophils, monocytes, macrophages and platelets, which further potentiate cytokine production. The resultant overflooding of cytokines and chemokines poses a systemic stress on multiple organs, leading to tissue injury through activation of numerous signaling pathways. Ultimately, the severe systemic inflammatory response syndrome (SIRS) results in multi-organ failure. Of course, the liver is not spared throughout this process and sustains immune-mediated direct and indirect injury of varying magnitude[90-92].

It has been demonstrated that COVID-19 patients exhibit elevated levels of various inflammatory cytokines, such as IL-1β, IL-2, IL-6, IL-10, TNF-α, interferon-gamma (IFN-γ), IFN-γ-inducible protein 10, granulocyte macrophage-colony stimulating factor and monocyte chemoattractant protein-1. What is more, some of these cytokines have been shown to correlate with the severity of COVID-19 disease course. Moreover, inflammatory infiltrates, indicative of immune-mediated injury, have been found to be present in numerous tissue samples of COVID-19 patients[93]. In addition, elevated levels of IL-6 and IL-10 and low counts of CD4+ T cells have been reported to be independent risk factors for development of ALI in COVID-19 patients[94]. All the above point towards the fact that an exaggerated immune-mediated response, generating an inflammatory cascade of events known as cytokine storm, plays a key role in the development of COVID-19 induced ALI.

Endothiliitis-coagulopathy

COVID-19 is considered a thrombotic disease which affects the vessel endothelium *via* inflammation and promotes endotheliitis, coagulopathy and thrombosis[95,96]. Indeed, endotheliitis seems to be the aftermath of the complex interplay between SARS-CoV-2 and the host immune response. SARS-CoV-2 initiates an inappropriate inflammatory response that triggers the overproduction of chemical mediators and mounts cell-mediated interactions with a subsequent burst of a cytokine storm. Activated immune cells bind to the adhesion molecules expressed on the surface of endothelial cells and generate an inflammatory state. The complex and sustained activation of cellular downstream signaling pathways mediated by pro-inflammatory cytokines drives SIRS, which affects the endothelium. The integrity of the endothelial barrier is compromised, thus promoting endothelial capillary leak and interstitial oedema. Normal endothelial function is also disrupted due to the production of free radicals, the reduction of nitric oxide (NO) levels and the decrease of the endothelial NO synthase activity. Through impairment of endothelial function, NO dysregulation and increased oxidative stress lead to maldistribution of microvascular blood flow, eventually resulting in abnormal vascular tone, attenuated endothelium-dependent vasodilation, compromised tissue oxygen delivery and tissue hypoxia[97,98]. In addition, dysfunctional endothelial cells may create a procoagulant milieu, by triggering fibrin formation as well as platelet adhesion and aggregation. These derangements alter local hemorheological conditions and promote microcirculatory stasis, microthrombi formation and capillary plugging. The generalized hypercoagulable state leads to maldistribution of tissue perfusion and microcirculatory ischemia, further aggravating tissue hypoxia[97]. The reduced peripheral perfusion due to the aforementioned endothelial dysfunction affects multiple systems and may result in multi-organ failure. The liver is not left intact throughout this process and subsequently ALI emerges[98,99].

DILI

ICU patients present several unique characteristics which render them much more susceptible to the development of DILI, compared to common ward patients. First of all, ICU patients typically have multiple co-morbidities and require treatment with numerous pharmacologic agents. Oftentimes, complex drug-combination strategies are employed in an attempt to cope with infections due to multi-drug resistant microorganisms[100]. However, polypharmacy exposes them to the risk of many potential drug-drug interactions[101]. Second, their ICU stay is usually prolonged, thus mandating long-course treatment[102]. Furthermore, due to their underlying critical condition, drug pharmacokinetics are substantially modified and are therefore unpredictable, increasing DILI risk[103]. Additionally, when considering the co-existence of other causes of liver injury that are almost invariably present in ICU patients with COVID-19, it becomes evident that the risk of DILI is further accentuated.

A variety of drugs used in the ICU for the treatment of COVID-19 patients could potentially lead to liver injury. Acetaminophen is a drug widely used in the ICU with well-documented hepatotoxic properties, which may confer liver injury even at lower than maximum daily recommended doses[104]. Besides, ICU patients with COVID-19, especially those with ARDS and on invasive MV, are particularly prone to bacterial infections and other opportunistic super-infections and are therefore treated with several antibiotics and antifungals. However, many of the antibiotics prescribed in the ICU carry an inherent risk of DILI, such as penicillins, cephalosporins, fluoroquinolones, macrolides and tetracyclines [105,106]. The same holds true for antifungal agents with triazoles demonstrating the highest potential for hepatotoxicity[106,107].

Furthermore, antiviral drugs used in the treatment of COVID-19 have been implicated in the development of ALI. The effect of antiviral treatment on liver function was studied in critically ill patients with COVID-19 in a retrospective cohort study and it was concluded that the overall use of antivirals was associated with increased risk of ALI[108]. Remdesivir is the sole antiviral drug currently approved for COVID-19 treatment, although its use and benefits are still under debate[109]. Early reports from studies examining compassionate use of remdesivir indicated that hepatotoxicity was

among one of the most frequent adverse events observed during remdesivir treatment. It manifested as an increase in transaminase levels, which resulted in discontinuation of the drug in some cases[110,111]. A randomized, double-blind, placebo-controlled multicenter trial from China also reported increases in aminotransferase or bilirubin levels with remdesivir, sometimes leading to premature discontinuation of therapy[112]. However, the authors concluded that, overall, remdesivir was adequately tolerated and the serious adverse events tended to be lower in the remdesivir group than in the placebo group[112]. According to a systematic review and meta-analysis, the use of remdesivir frequently resulted in elevated transaminases with the incidence of DILI being 15.2% among COVID-19 patients receiving remdesivir[113]. Remdesivir was also found to confer an increased risk of ALI in a study analyzing data derived from VigiBase, a pharmacovigilance global database system available from the World Health Organization[114]. Indeed, the most frequently reported adverse effects related to remdesivir were increased liver enzymes, mainly transaminases and to a lesser extent bilirubin. Of note, most cases were deemed serious by virtue of requiring hospitalization or resulting in prolonged hospital stay[114]. Other studies have also shown that remdesivir can cause hepatocellular injury, but it seems that in most cases ALI is mild, asymptomatic and not clinically apparent; it is not associated with jaundice and does not progress to severe liver damage or failure, while it is characterized by low discontinuation rates and reversibility after discontinuation[115-119]. Nevertheless, remdesivir's use in the ICU is strictly limited in patients with severe COVID-19 who are not on MV[120].

Apart from antiviral drugs, immunomodulatory therapies have been put forward as therapeutic options for COVID-19. Tocilizumab, which is an anti-IL-6 receptor monoclonal antibody, is among the most utilized ones. It has been used in patients with respiratory deterioration and high oxygen requirements and has been associated with a reduction in mortality and need for intubation, when combined with corticosteroid use and administered early in the disease course[121-123]. However, its use has been associated with the development of a hepatocellular pattern of DILI, characterized by a mild to moderate elevation of transaminases in most cases[124-126]. The observed hepatotoxicity has been reported to be dose-dependent and generally transient[119]. Moreover, owing to the fact that tocilizumab induces immunosuppression, it should be used with caution, since it can increase the risk of infectious complications and may cause reactivation of hepatitis B virus (HBV) in patients with latent infections[127,128]. Thus, clinicians should always keep in mind that tocilizumab-induced ALI could also potentially arise from HBV reactivation[14].

Furthermore, low molecular weight heparins are among the most frequently used regimens in the ICU. Heparin-induced hepatotoxicity has been described in the literature, but is generally mild, transient and self-limited and does not warrant discontinuation of heparin therapy, which is an essential component of COVID-19 treatment[118,119,129-131]. Likewise, the use of systemic corticosteroids, which are routinely recommended in mechanically ventilated patients with COVID-19 and ARDS, has been rarely associated with DILI[119,120,132]. Moreover, amiodarone, which is frequently used in the ICU setting as an antiarrhythmic drug to terminate supraventricular and ventricular tachyarrhythmias, is well-known for its hepatotoxic effects and could thus contribute to the development of DILI[133].

Lastly, the use of anesthetic drugs deserves specific mention, since COVID-19 pandemic has posed unique challenges to intensivists with regard to sedative strategies. Oftentimes, achieving satisfactory and deep sedation in invasively mechanically ventilated patients has been proven problematic, since an inordinate resistance to standard sedative regimens has been typically observed. In order to overcome this impediment, ICU physicians have been compelled to resort to alternative methods of sedation[134]. As a result, ketamine has been increasingly used as a second-line anesthetic agent for long-term sedation, in combination with the standard course of sedative and analgesic treatment. However, there have been several reports associating the prolonged infusion of ketamine in high doses with cases of hepatotoxicity[40,135-138]. The resulting ALI manifests in the form of cholestatic liver injury, which may further progress to the development of secondary sclerosing cholangitis. In general, ketamine infusion requires prolonged period of administration and high total cumulative doses in order to exert its cholangiotoxic effect[40,135]. The postulated mechanism through which ketamine induces or exacerbates cholestatic liver injury includes bile stasis, which promotes the precipitation of the water-insoluble norketamine (the main active metabolite of ketamine) within the biliary tree, ultimately resulting in biliary tract dysfunction and leading to biliary strictures, biliary obstruction, cholangitis or even secondary biliary cirrhosis[40,136]. In brief, the blockade of the N-methyl-D-aspartate (NMDA) receptors in smooth muscle cells by ketamine favours bile stasis and bile duct dilation, while the ketamine-induced contraction of the sphincter of Oddi increases flow resistance within the biliary tree, thus further aggravating bile accumulation. Meanwhile, bile stasis is additionally exacerbated by gall bladder dyskinesia, which is caused by the ketamine-mediated blockade of NMDA receptors in the dorsal motor nucleus of the vagal nerve[40]. These phenomena collectively establish the ideal predisposing conditions for the precipitation of norketamine and the resultant biliary tract injury. Notably, the biliary system sustains multiple assaults, since the aforementioned sequential events act in concert with other COVID-19 related direct and indirect insults to the biliary tract, such as hypoxia, ischemia, hypoperfusion, hemodynamic instability, MV, SIRS or the virus SARS-CoV-2 itself[135,139,140].

Parenteral nutrition

Parenteral nutrition is often initiated in ICU patients in order to cover their metabolic demands in case of intolerance or contraindications to enteral feeding or whenever caloric targets are not met by enteral nutrition alone[141]. However, it has been demonstrated that ICU patients receiving total parenteral nutrition run a significantly greater risk of developing ALI than those receiving enteral nutrition[142]. Indeed, parenteral nutrition has been associated with liver injury, resulting in elevations of all liver enzymes, namely transaminases, ALP, GGT and bilirubin. Since parenteral nutrition by-passes the gut and results in decreased luminal content, it eliminates the hepatoprotective gut-derived signals, alters the enterohepatic circulation of bile acids and disrupts the normal crosstalk between the gut and the liver. Through various complex signaling pathways, parenteral nutrition causes cholestasis, steatosis, altered glucose and fat metabolism and hepatic fibrosis. Moreover, it interrupts gut mucosal integrity, causes derangements in the gut microbiota, promotes bacterial translocation and induces gut inflammation as well as increased cytokine release, all of which may contribute to further liver injury[143,144].

In addition, the lack of enteral feeding suppresses the secretion of cholecystokinin, gastrin and peptide YY. This leads to reduced intestinal motility, attenuated gallbladder contraction and decreased stimulation of bile flow, thus establishing the ideal environment for bacterial overgrowth, bile stasis, biliary sludging and subsequent bile duct obstruction. These conditions render the hepatocytes more susceptible to both direct and indirect toxic effects[144,145].

Besides, parenteral nutrition *per se* can be hepatotoxic through its components, mainly soy-derived phytosterols, manganese, aluminium and copper. Soybean oil-based lipid emulsions contain predominantly ω-6 polyunsaturated fatty acids which possess proinflammatory properties and lead to Kupffer cell activation, while phytosterols impede bile acid transport to the liver by antagonizing bile nuclear receptors. Furthermore, lipid emulsion infusion may be rarely complicated by fat overload syndrome with deleterious effects to the liver and other systemic organs[144,145].

Another potential risk of parenteral nutrition is energetic overfeeding, which increases the hazard for hepatobiliary complications[146]. Similarly, the strategy of combining parenteral with enteral nutrition, that may sometimes be employed to optimize nutritional intake in ICU patients, carries the risk of overfeeding, which predisposes to hepatic steatosis and hepatitis[141,145].

On the other side of the spectrum lies underfeeding of ICU patients, which may be related to various factors, such as feeding intolerance, hemodynamic instability, underestimation of caloric needs, frequent feeding interruptions due to diagnostic procedures or therapeutic interventions[147]. Underfeeding may contribute to a decrease in serum albumin concentration, which is a frequent underlying finding in ICU patients. Albumin levels reflect the liver's synthetic function and hypoalbuminaemia is associated with worse outcomes in critically ill patients[148].

Direct cytopathic viral effect

It has been postulated that ALI may be caused by direct viral invasion, infection and damage of hepatocytes. On this account, SARS-CoV-2 may exert direct cytopathic effects on hepatic cells, by causing lysis or promoting apoptosis and necrosis[18]. This hypothesis has been supported after identifying typical ultrastructural features and histopathological lesions of viral infection in postmortem liver biopsies of 2 cases with elevated transaminases[149]. However, the low expression level of angiotensin converting enzyme 2 (ACE2) receptors on the surface of hepatocytes, as opposed to the enriched ACE2 expression in cholangiocytes, could not support the theory of SARS-CoV-2 hepatotropism; instead, it rather implied that cholangiocytes could be targeted by SARS-CoV-2 or that alternative receptors on hepatocytes other than ACE2 could serve as the cell entry points of the virus [150]. Subsequent research revealed the presence of three SARS-CoV-2 interacting host receptors in different parts of the liver tissue, namely ACE2, transmembrane serine protease 2 and paired basic amino acid cleaving enzyme (FURIN), thus endorsing the possibility that SARS-CoV-2 may actually cause direct cytopathic injury to hepatocytes[151]. To date, the direct cytopathic viral effect has not been firmly established yet, whereas in the ICU setting this proposed mechanism does not seem to play a prominent role in the observed ALI.

It needs to be emphasized that, although there are several viruses displaying some form of hepatotropism, there may be considerable heterogeneity among them. This could be due to the fact that the immune responses mounted by the host against the virus may differ significantly depending on whether the virus is cytopathic or not, as well as on which immune evasion mechanisms are adopted by the virus, in conjunction with other factors, like impaired immunity or high viral load[92]. Furthermore, the pattern of the observed changes in transaminases during SARS-CoV-2 infection differs from the liver injury pattern of other epidemic viruses, which result in a much steeper curve of aminotransferase elevations owing to massive parenchymal necrosis[18].

Given that the mechanisms of COVID-19 induced ALI still remain largely unclear, our current understanding is limited with regard to the exact pathophysiology behind the liver injury caused by SARS-CoV-2 and how this differs or resembles the effects of other hepatotropic viruses. Even more so, data regarding the potential discrepancies in the hepatotropism of the different SARS-CoV-2 strains are lacking; hence, it would be intriguing for researchers to investigate the possible different effects that are exerted by different strains of SARS-CoV-2 on the liver and identify any associated variations in the

pathophysiology and clinical course of ALI.

LIVER HISTOPATHOLOGY IN COVID-19: IS THERE A LINK WITH PATHOPHYSIOLOGICAL MECHANISMS IN ALI?

It could be assumed that the underlying pathophysiological mechanism of ALI might be postulated based on the findings of pathological studies. However, such studies are generally inconclusive, since a firm and indisputable cause of liver injury cannot be safely deduced from histopathological reports.

As a matter of fact, histopathological findings from postmortem liver biopsy specimens have shown moderate microvesicular steatosis and mild lobular and portal activity[152]. These findings could be compatible either with direct SARS-CoV-2 infection of the liver or with DILI, but they are not conclusive in terms of supporting a definite cause of liver injury. Besides, the concomitant finding of overactivated T cells in the peripheral blood, with increased expression of proinflammatory markers and high concentration of cytotoxic granules, most likely points towards an immune-mediated injury rather than a direct cytopathic effect[152]. In another liver biopsy, the findings of mild vesicular steatosis and watery degeneration in some hepatocytes were attributed to ischemia and hypoxia, while the investigators also reported the presence of a few inflammatory cells in the hepatic sinuses, namely neutrophils, plasma cells and Kupffer cells[25].

Postmortem liver tissue biopsies from two COVID-19 cases with elevated transaminases have actually shown typical lesions of viral infection, suggestive of a direct cytopathic effect of SARS-CoV-2 on liver cells[149]. In particular, ultrastructural examination *via* transmission electron microscopy revealed that typical SARS-CoV-2 particles with corona-like spike structures were abundantly present in the cytoplasm of hepatocytes, indicating that the virus can both enter and replicate in hepatocytes. These infected hepatocytes showed cytopathic features, such as marked mitochondria swelling, endoplasmic reticulum dilation, decrease of glycogen granules and damage of the cell membrane. Histologically, plenty apoptotic hepatocytes were observed, as well as binuclear or a few multinuclear syncytial hepatocytes. Other findings included moderate microvesicular and mild macrovesicular steatosis, moderate focal lobular inflammation and mild portal inflammation with predominantly lymphocytic infiltrates[149].

Besides, in post-mortem needle core biopsies from livers, centrilobular sinusoidal dilation, mild lobular lymphocytic infiltration and patchy hepatic necrosis were mainly observed[153]. Sinusoidal dilation is a rather frequent non-specific finding observed in liver biopsies of terminally ill hospitalized patients and is often attributed to reduced venous ouflow in the hepatic veins as a result of passive liver congestion in the setting of congestive heart failure[13].

Finally, an Italian study of 48 postmortem liver biopsies reported histopathological findings suggestive of diffuse vascular alterations characterized by marked derangement of the intrahepatic vasculature and varying degrees of occlusive thrombosis[154]. Specifically, there was an increase in the number of portal vein branches, coupled with severe luminal dilation and wall fibrosis. This proliferative process was accompanied by partial or complete luminal thrombosis of portal and sinusoidal vessels, portal vein endotheliitis and portal vein wall fibrosclerosis. Steatosis was also observed in more than half of the specimens, while SARS-CoV-2 was detected in most of the samples either within blood clots or in the cytoplasm of endothelial cells[154].

PREDISPOSING FACTORS FOR ALI

COVID-19 severity has been reported to be one of the main risk factors for the development of ALI in COVID-19 patients[155]. Indeed, in a systematic review and meta-analysis it was found that increasing levels of COVID-19 severity were associated with a higher risk of developing ALI and more pronounced gastrointestinal symptoms[156]. ICU patients are more prone to developing liver impairment. This was evidenced by a meta-analysis which reported that biomarkers of liver function were significantly elevated in patients with severe and fatal forms of COVID-19 and could potentially portend a progression towards multiple organ failure, when combined with other hematologic, biochemical and immunological biomarkers[157]. Besides, higher incidence of ALI has been observed in patients with more extensive pulmonary lesions on computed tomography (CT) imaging reflected by higher CT scores, which in turn correlate with COVID-19 clinical severity[158,159].

Furthermore, pre-existing liver disease has been recognized as a predisposing factor for ALI, especially in critically ill patients with COVID-19, given the fact that patients with pre-existing liver disease, particularly those with cirrhosis, run a greater risk of hospitalization and mortality[160]. Metabolic-associated fatty liver disease has been shown to be an independent predictor for the development of mild and moderate ALI[161]. Likewise, in another patient series, metabolic-associated fatty liver disease, together with high body mass index, prevailed in the majority of patients with persistent liver injury[162]. However, it still remains controversial whether metabolic-associated fatty

liver disease is also a marker of disease severity, progression and mortality[161,162]. Shao *et al*[163] reported that the presence of fatty liver disease was associated with a higher risk of ALI in COVID-19 patients, while the presence of cirrhosis incurred an increased risk of disease progression[163]. Along the same lines, in another single-center retrospective study from Shanghai, low liver CT density, suggestive of fatty infiltration and steatosis, was shown to be a risk factor of liver injury, together with COVID-19 severity, male sex and medications, such as lopinavir/ritonavir, glucocorticoids and thymopeptides[164].

In their multicenter study involving 112 liver transplant recipients, Rabiee *et al*[37] found that ALI was associated with a higher risk for ICU admission and higher mortality in COVID-19 liver transplant recipients[37]. Interestingly, when compared to a matched control group of non-transplant patients with chronic liver disease, the incidence of ALI was higher in patients with chronic liver disease (47.5%) than in liver transplant recipients (34.6%). On multivariate analysis, younger age, metabolic syndrome, Hispanic ethnicity, administration of vasopressors and antibiotic use emerged as independent risk factors for ALI. On the other hand, non-Hispanic white liver transplant recipients had a lower risk of ALI. With regard to immunosuppressants, reducing tacrolimus or withholding mycophenolate did not increase the risk of ALI, while acute cellular rejection occurred in only one patient of the cohort. Overall, it was concluded that reduction in immunosuppression therapy was not associated with ALI or risk of mortality, thus immunosuppression can be safely modified if deemed necessary[37].

Several prediction models have been proposed in order to estimate the risk of liver injury. For instance, it has been reported that a prediction model incorporating plateletcrit, retinol-binding protein and carbon dioxide combining power could sufficiently predict the occurrence of ALI in patients with moderate COVID-19 in a timely manner[165]. Another risk scoring system was proposed by Shao *et al*[163] with the intention to predict ALI risk, which included three variables upon admission, namely ALT, CRP and lactate dehydrogenase[163]. In the same study, the authors noted that male patients were more likely to develop ALI. Interestingly, the presence of hypertension was found to convey an increased risk of ALI only for patients without any prior liver disease, but not for patients suffering from pre-existing chronic liver disease[163].

In a prospective cohort study, male sex, older age, diabetes mellitus and lymphopenia emerged as independent risk factors that could predict liver dysfunction among COVID-19 patients[166]. Other investigators concluded that male sex, along with high D-dimers and high neutrophil percentage, were the most important risk factors that could predict the development of ALI in COVID-19 patients[167]. Similarly, it was demonstrated that male sex and CPR were independently associated with liver injury [168], while another study found that male sex, high levels of serum CRP and a high neutrophil to lymphocyte ratio were potential risk factors for ALI[169]. A subsequent systematic review and meta-analysis corroborated the above findings by confirming that male sex and low lymphocyte count were associated with ALI occurrence[170].

Male sex has almost invariably been associated with the risk of liver injury and the severity of the disease[166-170]. The observed discrepancy between the genders may be related to genetic and hormonal factors, which result in lower viral load levels, milder grades of inflammation and better immune responses in women than men[171].

Finally, metabolic syndrome, which is a constellation of hypertension, hyperglycemia, dyslipidemia and obesity, has also been proposed as a predisposing factor for ALI[172]. In a recent retrospective cohort study, being overweight was a risk factor for liver injury, whereas obesity was associated with severe liver injury[173].

THERAPEUTIC STRATEGY

It should be emphasized that no specific therapy for COVID-19 induced ALI exists. Therefore, the therapeutic approach initially lies in prevention and is exclusively supportive once ALI ensues.

In the ICU setting, the main pillar of the overall ALI therapeutic strategy for critically ill COVID-19 patients consists of preventive measures. First and foremost, a detailed medical history should be obtained. This should include a comprehensive review of the medical background for concomitant liver diseases, other underlying comorbidities, use of drugs in the near past that are known to induce ALI, or exposure to alcohol, herbs and chemicals[22]. Next, high level of clinical vigilance is warranted. ICU physicians should perform meticulous clinical examination on a regular daily basis, or even at repeated intervals throughout the day if deemed necessary, in order to check for and recognize early signs and symptoms of impeding organ dysfunction. In parallel, COVID-19 patients should have regular lab tests performed to closely monitor liver enzymes and promptly identify any potential liver test abnormalities.

Among the precautionary measures to avoid ALI, prompt restoration of central hemodynamics is of paramount importance, since it will allow the hemodynamic stabilization of the patient in due time and prevent liver ischemia. In patients with hemodynamic instability, fluid resuscitation constitutes the mainstay of treatment in the attempt to restore hypovolemia. In general, a conservative over a liberal fluid strategy is advocated in critically ill COVID-19 patients with shock, provided that hypovolemia

has been addressed. Caution should be exercised so as to avoid volume overload, especially in patients with cardiac dysfunction. In order to achieve optimal loading conditions, a multimodal approach should be adopted, whereby fluid resuscitation should be guided by physical examination, follow-up of vital signs, lactate levels, dynamic parameters and point-of-care ultrasonography[120]. It has been proposed that a systematic approach incorporating focused echocardiography, lung scanning and abdominal ultrasound may facilitate the bedside evaluation of cardiac function, volume status and fluid responsiveness, while at the same time it can aid in the differential diagnosis of an undifferentiated shock, as well as in the detection of biliary or hepatic sepsis, intraabdominal fluid collection and other acute pathologies[174-176].

When hemodynamic stability is not achieved after fluid resuscitation, the administration of vasopressors is indicated. Norepinephrine should be the first-line vasopressor, while vasopressin is recommended as a second-line agent if hemodynamic status does not improve. In case of persistent hypoperfusion despite adequate fluid loading and use of vasopressors, initiation of intravenous inotropes, preferably dobutamine, is advised, particularly if there is evidence of cardiac dysfunction. Excessively high doses of vasopressors and inotropes should be avoided. Titration of vasoactive agents should generally target a mean arterial pressure of 60-65 mmHg. The ultimate goal is to achieve and maintain adequate end-organ perfusion by restoring arterial pressure and optimizing cardiac output [120].

Furthermore, increased awareness is warranted for the early identification and management of sepsis. Immediate control of the source of sepsis is crucial, along with the optimization of the patient's hemodynamic profile. Prompt and appropriate antibiotic therapy is the cornerstone in the management of patients with sepsis. In case of suspected infection, ICU physicians should start early antibiotic treatment, initially with an empiric broad spectrum antibiotic and subsequently modify antimicrobial therapy according to culture results and susceptibilities, by de-escalating or changing to a narrow spectrum antimicrobial which targets the specific pathogen[177]. The appropriate antibiotic regimen should be selected with caution, as several common antibiotic agents may carry a high risk for ALI or other adverse events. Moreover, the clinicians should be aware of drug-drug interactions and be alert for potential hepatotoxicity reactions[178]. In any case, judicious antibiotic stewardship is highly recommended.

Besides, the importance of using pharmacologic thromboprophylaxis in critically ill patients cannot be overemphasized. As a matter of fact, thromboprophylaxis is strongly recommended in the recently published surviving sepsis campaign guidelines on the management of adults with COVID-19 in the ICU[120].

Hypoxia should be consistently addressed and corrected by administering the minimum amount of supplemental oxygen that will achieve a target of peripheral oxygen saturation (SpO_2) between 92% and 96%. Regarding patients who are on invasive MV, specific attention ought to be paid to the implementation of lung-protective ventilation strategies. Low tidal volumes (4-8 mL/Kg of predicted body weight) should be applied, coupled with a target of plateau pressure < 30 cm H_2O and a driving pressure < 14 cm H_2O[120]. It should be emphasized that the volume- and pressure- limited ventilation strategy may lead to hypercapnia. Despite the fact that the strategy of permissive hypercapnia is frequently adopted in the ICU, clinicians should bear in mind that hypercapnia, combined with persistent hypoxia which is frequently observed in ARDS patients with COVID-19, may cause profound pulmonary arterial vasoconstriction, increase right ventricular afterload and jeopardize hemodynamic status with grave consequences for the liver[69,177]. It should also be noted that oftentimes ventilatory management needs to be individualized, given that different patient profiles exist with diverse lung mechanics and discrepant responses to a certain ventilatory strategy[179]. In these cases, the ventilatory approach will need to diverge from the conventional form of ventilation in ARDS. Along these lines, although a higher over a lower PEEP strategy is generally recommended in ARDS, clinicians should avoid using very high PEEP, since this could compromise right ventricular function and adversely affect liver function. Thus, close monitoring is advised so that impending cardiopulmonary deterioration is promptly recognized[179]. Furthermore, appropriate ventilator settings should be meticulously adjusted in order to minimize or prevent auto-PEEP, which can further aggravate right ventricular performance[72]. Prone positioning for 12-16 h *per* day is also recommended as a measure to improve hypoxia[120]. In cases of severe hypoxia with persistent patient-ventilator dyssynchrony or persistently elevated plateau pressures, neuromuscular blocking agents should be employed either as intermittent boluses or continuous infusions, as needed[120]. Finally, after other options have failed to enhance oxygenation, it is reasonable to apply rescue strategies. Alveolar recruitment maneuvers could be used with caution and under close monitoring for barotrauma and cardiovascular collapse, while a trial of an inhaled pulmonary vasodilator might also be attempted[120]. In cases of refractory ARDS with or without hemodynamic shock, veno-venous ECMO might be considered as a last resort to improve oxygenation and restore hemodynamic stability[120,179]. It is also considered prudent to monitor intra-abdominal pressure for potential persistent MV-related increases and address accordingly[86].

Besides, specific attention should be paid to sedation practices used in the ICU setting. If possible, administration of multiple sedative agents should be avoided in order to decrease potential risk of side-effects and drug-drug interactions. Moreover, sedatives should be titrated to the lowest effective doses and should not be administered for prolonged duration[134]. Daily sedation intervals should be applied

when appropriate[180]. Regarding ketamine, a strategy of withholding use of this agent is generally advised due to its reported adverse effects. If used, ketamine should be administered at the lowest possible doses and for brief time periods[135,136].

Nutritional status should be regularly assessed and early enteral nutrition should be initiated[181]. Parenteral nutrition should be refrained and used only in cases where enteral nutrition is not possible or contraindicated[181,182]. Caution should be exercised in order to avoid under- or over-feeding. Any nutrient deficiencies should be replaced[181]. Glycemic control should be optimized. Provided that it does not cause hypoglycemic episodes, early and intensive intravenous insulin therapy is encouraged in the ICU setting, since it reduces the risk of cholestatic liver dysfunction and the formation of biliary sludge[183,184].

In the event of ALI occurrence, every effort should be focused on restraining its progression and thus minimizing any ALI-associated consequences. Once ALI arises, it is important to seek and recognize the underlying etiology and address the responsible precipitating factor in a timely fashion. Currently, there are no specific therapies that mitigate or reverse COVID-19 induced ALI. Nevertheless, prompt diagnosis of ALI and initiation of supportive treatment is crucial. In cases of mild or moderate ALI, general supportive measures with close monitoring of liver chemistries should suffice and, in most cases, ALI will eventually subside[185].

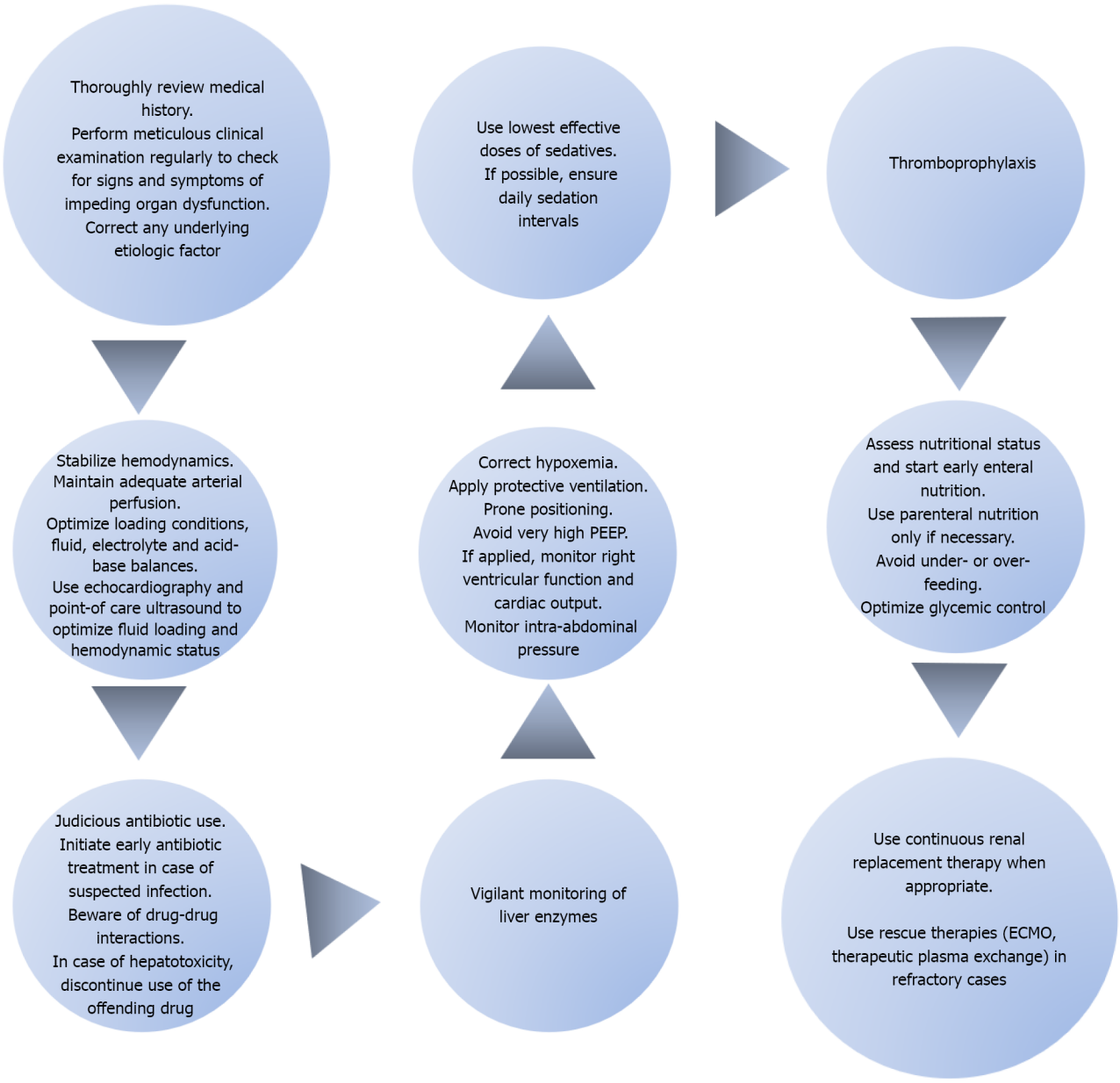
Infrequently, cases of severe ALI may occur. In such a scenario, supportive treatment should aim at preserving liver perfusion and microcirculation, by optimizing circulatory and respiratory conditions, relieving liver congestion, restoring electrolyte and acid-base balances and eliminating any underlying precipitating factor. If DILI is suspected, the administration of the causative pharmacologic agent should be discontinued[178]. Time of withdrawal of the culprit hepatotoxic factor is of major importance; hence, high level of clinical awareness is advised along with vigilant monitoring of liver enzymes[185]. With regard to acetaminophen toxicity, therapy with N-acetylcysteine should be promptly instituted by administering either an intravenous or an oral acetylcysteine regimen[186].

During the COVID-19 pandemic, the notion of continuous renal replacement therapy as a blood purification method to combat cytokine storm has been revived. In COVID-19 clinical practice, continuous renal replacement therapy can be used for septic patients with volume overload, patients with severe metabolic acidosis and those with electrolyte disturbances or progressive azotemia. Apart from its well-established indications, it has been proposed that continuous renal replacement therapy might have a potential role in critical COVID-19 cases with an excessive cytokine storm by removing cytokines and other inflammatory mediators[187,188]. Therapeutic plasma exchange has also been put forward as a promising adjunctive rescue therapy in the battle of COVID-19 critical illness, which could mitigate cytokine storm effects and thus reverse end-organ failure[189,190]. Finally, ECMO has been used as a salvage therapy for both respiratory and circulatory support[191].

Figure 2 depicts the steps of the therapeutic approach that should be consistently followed and addressed by ICU physicians both for the prevention and treatment of COVID-19 induced ALI.

CONCLUSION

Despite the conflicting estimates of its prevalence, ALI represents a common and often under-recognized complication of COVID-19 in ICU patients that deserves more clinical attention, considering the fact that it is intertwined with significant clinical ramifications and poor patient outcomes. COVID-19 induced ALI may present with varying degrees of clinical severity. Increased clinical vigilance is therefore advised, since it can easily be overlooked. In the ICU setting, several pathophysiological mechanisms may be implicated in the development of COVID-19 induced ALI. These may act either independently or more frequently synergistically. In the latter case, the effect exerted on the liver is cumulative, thus increasing the severity of ALI. Therefore, it is of utmost importance for ICU clinicians to ensure that they consistently comply with certain predefined bundles of preventive and therapeutic measures in order to alleviate the burden of COVID-19 associated ALI in ICU patients. Ongoing research on the particular scientific field will further elucidate the pathophysiology behind ALI and address unresolved issues, in the hope of mitigating the tremendous health consequences imposed by COVID-19 on ICU patients.



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Figure 2 Steps of the intensive care unit therapeutic approach in patients with coronavirus disease 2019 induced acute liver injury. The intensive care unit (ICU) therapeutic approach for coronavirus disease 2019 patients with acute liver injury (ALI) lies in preventive measures and is exclusively supportive once ALI ensues. After obtaining a detailed medical history, a thorough physical examination should be performed on a regular basis. Upon recognition of any signs of impeding liver dysfunction, the possible etiologic factor should be systematically sought and appropriately addressed. Hemodynamic stabilization of the patient is of paramount importance. The goal is to maintain adequate arterial perfusion in order to prevent liver ischemia. The use of echocardiography and point-of-care ultrasonography will help determine the hemodynamic status and optimize loading conditions. It is important to constantly maintain fluid, electrolyte and acid-base balances. Furthermore, early identification and management of sepsis cannot be overemphasized. Prompt initiation of appropriate antibiotic therapy is the mainstay of sepsis management. Antibiotics should be used judiciously with an increased level of awareness for possible drug-drug interactions. In case of hepatotoxicity, the administration of the responsible pharmacologic agent should be discontinued. Close monitoring of liver enzymes is advised at all times. Furthermore, hypoxia should be consistently addressed and corrected. In mechanically ventilated patients, a lung-protective ventilation strategy should be implemented with the use of low tidal volumes coupled with a target of plateau pressure < 30 cm H_2O. The application of very high positive end-expiratory pressure should be avoided because it could compromise right ventricular performance and precipitate passive liver congestion. Moreover, prone positioning is recommended in order to improve hypoxia. Intra-abdominal pressure should also be monitored so as to prevent any potential increases related to mechanical ventilation which could jeopardize liver function. Additionally, optimal sedation strategies should be applied with the lowest effective doses and daily sedation intervals. Thromboprophylaxis is strongly recommended as a preventive measure in all ICU patients. Besides that, nutritional status should be regularly assessed and efforts should focus on initiating early enteral feeding while avoiding parenteral nutrition. Under- or over- feeding should be avoided and glycemic control should be optimized. Finally, renal replacement therapy can be used whenever indicated. In refractory cases, rescue therapies may be employed with the use of extracorporeal membrane oxygenation and therapeutic plasma exchange in an attempt to provide circulatory and respiratory support and reverse end-organ failure. PEEP: Positive end-expiratory pressure; ECMO: Extracorporeal membrane oxygenation.

FOOTNOTES

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REVIEW

Alterations of the gut microbiota in coronavirus disease 2019 and its therapeutic potential

Hui Xiang, Qi-Ping Liu

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) poses a serious threat to global health. SARS-CoV-2 infects host cells primarily by binding to angiotensin-converting enzyme 2, which is coexpressed in alveolar type 2 cells and gut epithelial cells. It is known that COVID-19 often presents with gastrointestinal symptoms and gut dysbiosis, mainly characterized by an increase in opportunistic pathogens and a decrease in beneficial commensal bacteria. In recent years, multiple studies have comprehensively explored gut microbiota alterations in COVID-19 and highlighted the clinical correlation between dysbiosis and COVID-19. SARS-CoV-2 causes gastrointestinal infections and dysbiosis mainly through fecal-oral transmission and the circulatory and immune pathways. Studies have shown that the gut microbiota and its metabolites can regulate the immune response and modulate antiviral effects. In addition, the gut microbiota is closely related to gastrointestinal symptoms, such as diarrhea, a common gastrointestinal symptom among COVID-19. Therefore, the contribution of the gut microbiota in COVID-19 should not be overlooked. Strategies targeting the gut microbiota *via* probiotics, prebiotics and fecal microbiota transplantation should be considered to treat this patient population in the future. However, the specific alterations and mechanisms as well as the contributions of gut microbiota in COVID-19 should be urgently further explored.

Key Words: COVID-19; SARS-CoV-2; Angiotensin-converting enzyme 2; Gut microbiota; Dysbiosis; Lung

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Core Tip: Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a global health threat. SARS-CoV-2 infects host cells through binding to angiotensin-converting enzyme 2. COVID-19 patients exhibit gut dysbiosis. Here, the gut microbiota alterations in COVID-19 are summarized. The pathways and possible mechanisms of dysbiosis caused by SARS-CoV-2, as well as the impact of the gut microbiota and its metabolites on the inflammatory response and antiviral effects during the course of the disease are also described. Therefore, targeting the gut microbiota should be considered a promising strategy for COVID-19 prevention, treatment, and prognostic assessment.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a new acute infectious disease that is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to the *Coronaviridae* family[1]. SARS-CoV-2 infects host cells through the binding of the S protein to angiotensin-converting enzyme 2 (ACE2) and interacts with transmembrane serine protease 2 (TMPRSS2), which cleaves the viral S-protein, allowing efficient viral fusion[2,3]. Interestingly, ACE2 is coexpressed in alveolar type 2 cells and intestinal and colonic epithelial cells, especially in small intestinal enterocytes[4,5]. Correspondingly, in addition to infecting the respiratory system and causing respiratory symptoms (*e.g.*, fever, dry cough, dyspnea, myalgia, headache, *etc.*), SARS-CoV-2 also infects the gastrointestinal tract, where it replicates abundantly[6]. Overwhelming evidence substantiates the detection of viral RNA in fecal samples or rectal swabs from COVID-19 patients[6-8]. It is estimated that up to 48.1% of COVID-19 patients had fecal samples positive for viral RNA[7], even when the virus was not detected in respiratory and/or sputum samples[9]. Moreover, researchers have found that the gut microbiota composition of COVID-19 patients exhibits significant alterations (dysbiosis), mainly characterized by an increase in the abundance of opportunistic pathogens and a decrease in the abundance of beneficial commensal bacteria[10]. Gut dysbiosis is closely associated with gastrointestinal symptoms and disease severity[11,12]. Therefore, the crosstalk between the gut microbiota and COVID-19 is gaining attention.

The gut microbiota has become a hot research topic in recent years. The resident microbial composition of the human gut mainly includes bacteria, archaea, viruses and fungi[13]. The human gut microbiota consists of more than 10^{14} bacteria and comprises approximately 500 to 1000 species. Gut bacteria in healthy individuals are mainly *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*[13]. The complex gut microbiota communities have important genomic and enzymatic properties and perform a critical role in the immune system, which protects against pathogens and helps maintain gut microbiota homeostasis. Gut microbiota homeostasis is essential for maintaining human health. Conversely, dysbiosis can lead to metabolic disturbance, immune dysfunction and systemic inflammation and has been linked to various diseases[14].

Therefore, this review mainly summarizes the gut microbiota alterations and the possible mechanisms of dysbiosis in COVID-19. Furthermore, we highlight the theoretical basis that the gut microbiota can be considered a promising therapeutic target in COVID-19, potentially interfering with immune and inflammatory responses and antiviral effects. Finally, we also reviewed multiple interventions targeting the gut microbiota, such as prebiotics, probiotics and fecal microbiota transplantation (FMT), which could optimize COVID-19 treatment.

GUT DYSBIOSIS EXISTS IN COVID-19 AND IS ASSOCIATED WITH DISEASE SEVERITY

Gut dysbiosis in COVID-19 patients has received widespread attention in recent years (Table 1)[10,12,15-22]. The gut microbiota is significantly altered in COVID-19 patients receiving and not receiving medication compared to that in non-COVID-19 individuals[12]. Gut dysbiosis persists throughout the course of the disease and even after viral clearance[10,12]. Yeoh *et al*[12] suggested that members of the *Bacteroidetes* phylum and *Actinobacteria* were predominant in COVID-19 and non-COVID-19 individuals, respectively. In an American cohort study, *Peptoniphilus*, *Corynebacterium*, and *Campylobacter* were identified as the most enriched genera in COVID-19 patients[23]. Immunomodulatory gut bacteria, such as *Faecalibacterium prausnitzii* (*F. prausnitzii*), *Bifidobacterium adolescentis* and *Bifidobacterium longum*, were depleted in COVID-19, and their depletion was correlated with an elevation in the levels of inflammatory cytokines and markers (CXCL10, IL-10, TNF-α, CCL2, and CRP)[12]. However, Tao *et al*[16]

Table 1 Alterations of the gut microbiota composition in coronavirus disease 2019 patients

Study	Method	Increased abundance of gut microbiota	Decreased abundance of gut microbiota	Main conclusion
Gu *et al* [15], 2020	16S rrna	*Streptococcus*, *Rothia*, *Veillonella*, *Actinomyces*, *Erysipelatoclostridium*	*Ruminococcaceae family*, *Lachnospiraceae family*, *Agathobacter*, *Fusicatenibacter*, *Roseburia*	Gut microbiota has potential value as a diagnostic biomarker and therapeutic target for COVID-19
Zuo *et al* [10], 2020	Shotgun	*Clostridium hathewayi*, *Actinomyces viscosus*, *Bacteroides nordii*	*Eubacterium*, *Faecalibacterium prausnitzii*, *Roseburia*, *Lachnospiraceae*	Fecal microbiota alterations are associated with fecal virus levels and COVID-19 severity; symbionts were depletion and opportunistic pathogens were enrichment in COVID-19 patients; gut dysbiosis persists in COVID-19 patients after virus clearance
Tao *et al* [16], 2020	16S rrna	*Streptococcus*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*	*Bacteroidetes*, *Roseburia*, *Faecalibacterium*, *Coprococcus*, *Parabacteroides*	IL-18 level was higher in the fecal samples from COVID-19 patients; dysbiosis may contribute to SARS-CoV-2-induced production of inflammatory cytokines and cytokine storm in the gut
Tang *et al*[17], 2020	Q-PCR	*Enterococcus*, *Enterobacteriaceae*	*Faecalibacterium prausnitzii*, *Clostridium butyricum*, *Clostridium leptum*, *Eubacterium rectale*	Specific gut microbiota can be considered diagnostic biomarkers for COVID-19; the Ec/E ratio can be used to predict death in critically ill patients
Zuo *et al* [18], 2020	Shotgun	*Candida albicans*, *Candida auris*, *Aspergillus flavus*	-	The guts of COVID-19 patients are accompanied by massive fungal blooms
Yeoh *et al*[12], 2021	Shotgun	*Actinobacteria*, *Ruminococcus gnavus*, *Ruminococcus torques*, *Bacteroides dorei*	*Bifidobacterium adolescentis*, *Faecalibacterium prausnitzii*, *Eubacterium rectale*	Immunomodulatory gut bacteria were depleted in COVID-19 patients; gut dysbiosis persists in COVID-19 patients after virus clearance; gut microbiota composition was associated with disease severity
Wu *et al* [19], 2021	16S rrna	*Streptococcus*, *Weissella*, *Enterococcus*, *Rothia*, *Lactobacillus*, *Actinomyces*, *Granulicatella*	*Blautia*, *Coprococcus*, *Collinsella*	Personalized microbiome affects disease outcomes in COVID-19 patients; targeting the gut microbiota has potential to prevent and treat COVID-19
Zuo *et al* [20], 2021	Shotgun	*Collinsella aerofaciens*, *Collinsella tanakaei*, *Streptococcus infantis*, *Morganella morganii*	*Parabacteroides merdae*, *Bacteroides stercoris*, *Alistipes onderdonkii*, *Lachnospiraceae bacterium 1_1_57FAA*	Elimination of gut SARS-CoV-2 activity and modulation of gut microbiome composition should be considered new treatments for COVID-19; the 3′ end of SARS-CoV-2 genome was highly covered than the 5′ end
Lv *et al* [21], 2021	ITS sequencing	-	Ascomycota (*Aspergillaceae*, *Candida parapsilosis*, *Talaromyces wortmannii*); Basidiomycota (*Malassezia yamatoensis*, *Rhodotorula mucilaginosa*, *Moesziomyces aphidis*, *Trechispora sp. and Wallemia sebi*)	Total gut fungal burden was significantly elevated in patients infected with SARS- CoV-2; altered gut fungi and microbiota are closely related to patient clinical characteristics
Suskun *et al*[22], 2022	16S rrna	*Bifidobacterium adolescentis*, *Dorea formicigenerasus*, *Eubacterium dolichum*, *Eggerthella lenta*	*Faecalibacterium prausnitzii*	First evaluate the microbiota composition in multisystem inflammatory syndrome in children cases

COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IL: Interleukin.

found that COVID-19 patients had higher abundances of *Clostridium*, *Veillonella*, *Streptococcus*, *Fusobacterium*, *Lactobacillus*, *Escherichia* and *Bifidobacterium* and lower abundances of *Bacteroidetes*, *Sutterella*, *Faecalibacterium*, *Coprococcus* and *Parabacteroides* than controls. The increased richness of *Streptococcus* exacerbates the risk of opportunistic pathogenic infections[24].

Moreover, it is widely thought that gut dysbiosis is closely associated with COVID-19 severity. The basal gut microbiota of healthy individuals and its alterations during SARS-CoV-2 infection influence host susceptibility to SARS-CoV-2 and disease recovery. The abundances of *Coprobacillus*, *Clostridium ramosum*, and *Clostridium hathewayi* were significantly and positively associated with COVID-19 severity, while the abundances of *Bifidobacterium bifiduz*, *Alistipes onderdonkii* and *F. prausnitzii* were negatively associated[10,12]. However, even different members of the same phylum, such as Firmicutes, have opposing correlations in influencing disease severity and regulating ACE2 expression[10]. ACE2 presents a critical role in gut microbial ecology, gut inflammation and innate immunity[25]. Moreover, the abundances of other bacteria, including *Bacteroides dorei* (*B. dorei*), *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*), *Bacteroides ovatus* (*B. ovatus*), *Ruminococcus*, *Clostridium citroniae*, *Bifidobacterium*, and *Haemophilus parainfluenzae*, were negatively associated with viral load in fecal samples from SARS-CoV-2-infected patients[10]. Interestingly, *B. dorei*, *B. thetaiotaomicron*, and *B. ovatus* can downregulate ACE2 expression in the murine gut[10,26], suggesting that Bacteroides species may play a protective role against SARS-CoV-2 infection by interfering with ACE2 production. The abundances of *Erysipelotrichaceae bacterium*, *Prevotella copri*, and *Eubacterium dolichum* have been reported to be positively correlated with fecal viral load[10,19]. Furthermore, *Prevotella*, *Enterococcus*, *Enterobacteriaceae*, and

Campylobacter can contribute to higher infectivity and worse prognosis in COVID-19[27]. For instance, *Prevotella* is associated with enhanced T helper 17 (Th17)-mediated mucosal inflammation, stimulating the production of cytokines and subsequently promoting neutrophil recruitment and inflammation[28]. Although it has been established that an altered gut microbiota composition is prevalent in COVID-19, conflicting results have been reported due to the heterogeneity of the gut microbiota itself, the sample size, and so on. Geographic and demographic differences also appear to affect the conclusions and dysbiosis recovery after SARS-CoV-2 infection[10,29]. Therefore, targeting specific gut microbiota alterations represents a potential strategy to alleviate disease severity in COVID-19.

POTENTIAL PATHWAYS AND MECHANISMS OF SARS-COV-2-INDUCED GUT INFECTION AND DYSBIOSIS

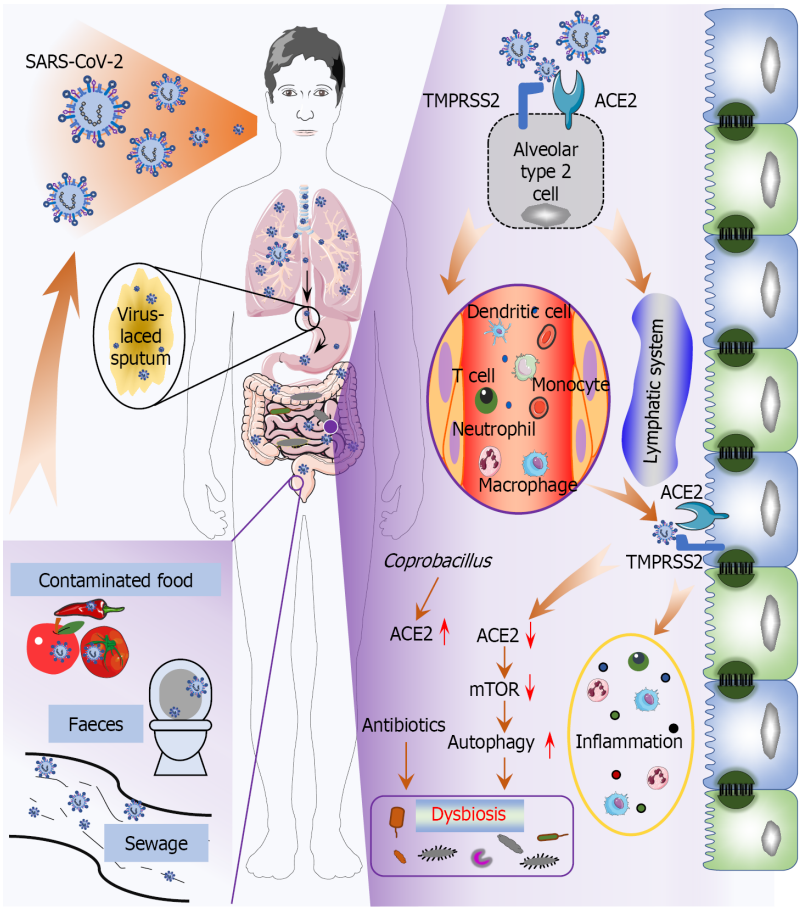
Generally, SARS-CoV-2 is principally transmitted *via* respiratory droplets and close contact, subsequently inducing diverse symptoms[30]; however, this paradigm does not explain how SARS-CoV-2 causes gut infection and dysbiosis. An *in vitro* study reported that the virus could be transmitted by the fecal-oral route through contaminated water, food, *etc*[30,31]. Since then, a new SARS-CoV-2 transmission route has been revealed (Figure 1). Notably, many viral activities are greatly diminished or even lost after passing through the gastrointestinal tract since gastric and intestinal fluids (low pH, rich in bile and digestive enzymes) can destroy the viral lipid envelope, inhibiting infectivity. For example, SARS-CoV has long been thought to be inactivated under acidic conditions (pH < 3). However, SARS-CoV-2 seems to overcome this obstacle since virus have been detected in the feces of infected individuals[30,31], and viruses isolated from feces can survive for an additional 1-2 d[32]. Therefore, SARS-CoV-2 may remain infectious in feces, particularly when patients have diarrhea[1]. Furthermore, virus-containing sputum swallowed by COVID-19 patients may be another pathway of gut infection because viscous sputum can protect the virions, preserving virus infectivity[33]. However, in the absence of evidence of fecal viral titers and viral viability in sewage and contaminated food, the capability of SARS-CoV-2 to be transmitted by the fecal-oral route requires further confirmation.

Moreover, circulatory and immune pathways are reportedly critical for SARS-CoV-2 to cause gut infection and dysbiosis (Figure 1). Studies conducted on SARS showed that coronaviruses damage lung tissue and then migrate to the systemic circulation, where they migrate to gut cells through the circulatory and lymphatic systems[34]. The virus can be found in the blood samples and gut of COVID-19 patients; in addition to those in epithelial cells, viral components are mainly present in intestinal lymphocytes and macrophages[31,35]. Therefore, it is speculated that SARS-CoV-2 may be transported from the lungs to other tissues, including the gastrointestinal tract, through transport *via* immune cells, similar to influenza virus[35,36]. Subsequently, SARS-CoV-2 invades gut epithelial cells by binding to AEC2[37], causing the release of cytokines and chemokines and triggering a gut inflammatory response characterized by neutrophil, macrophage, and T-cell infiltration, which further promotes dysbiosis[38-40]. In addition, the amino acid transport function of ACE2 is related to the microecology in the gut. B^0AT1, a molecular ACE2 chaperone, mediates the absorption of neutral amino acids in the intestinal epithelium[41]. Studies have confirmed that SARS-CoV-2-induced downregulation of B^0AT1 on gut epithelial cells contributes to gut barrier disruption and dysbiosis, promoting pathogen invasion and COVID-19 exacerbation[42-44]. de Oliveira *et al*[45] hypothesized that the internalization of SARS-CoV-2 causes ACE2 downregulation, leading to mechanistic target of rapamycin (mTOR) inhibition and intestinal autophagy activation. Interestingly, autophagy can regulate the gut microbiota, and increased autophagy has been associated with diarrhea[45,46]. There is currently no evidence, however, that the ACE2/mTOR/autophagy pathway is involved in the pathogenesis of COVID-19. Paradoxically, *Coprobacillus*, which is mostly correlated with the COVID-19 severity, has been shown to increase colonic expression of ACE2[26,47]. Therefore, the net expression of ACE2 in the gut remains largely unclear, warranting further investigation.

Misuse and overuse of antibiotics, common in initially treating COVID-19 patients[48], significantly impact the gut microbiota (Figure 1). Yeoh *et al*[12] found that COVID-19 patients treated with and without antibiotics had different gut microbiota composition. In addition, antibiotics attenuated the antiviral activity of commensal-enhanced type I interferons (IFN-I)[49]. Therefore, antibiotics are unlikely to improve patient outcomes without comorbid bacterial infections but instead exacerbate and prolong gut dysbiosis in this patient population.

IMPACT OF THE GUT MICROBIOTA AND ITS METABOLITES ON THE INFLAMMATORY RESPONSE AND ANTIVIRAL ACTIVITIES

Immune and inflammatory responses are important pathophysiological mechanisms in the pathogenesis of COVID-19. SARS-CoV-2 invades cells through ACE2 and TMPRSS2, where it replicates rapidly, producing and releasing large numbers of viruses and inducing excessive inflammatory

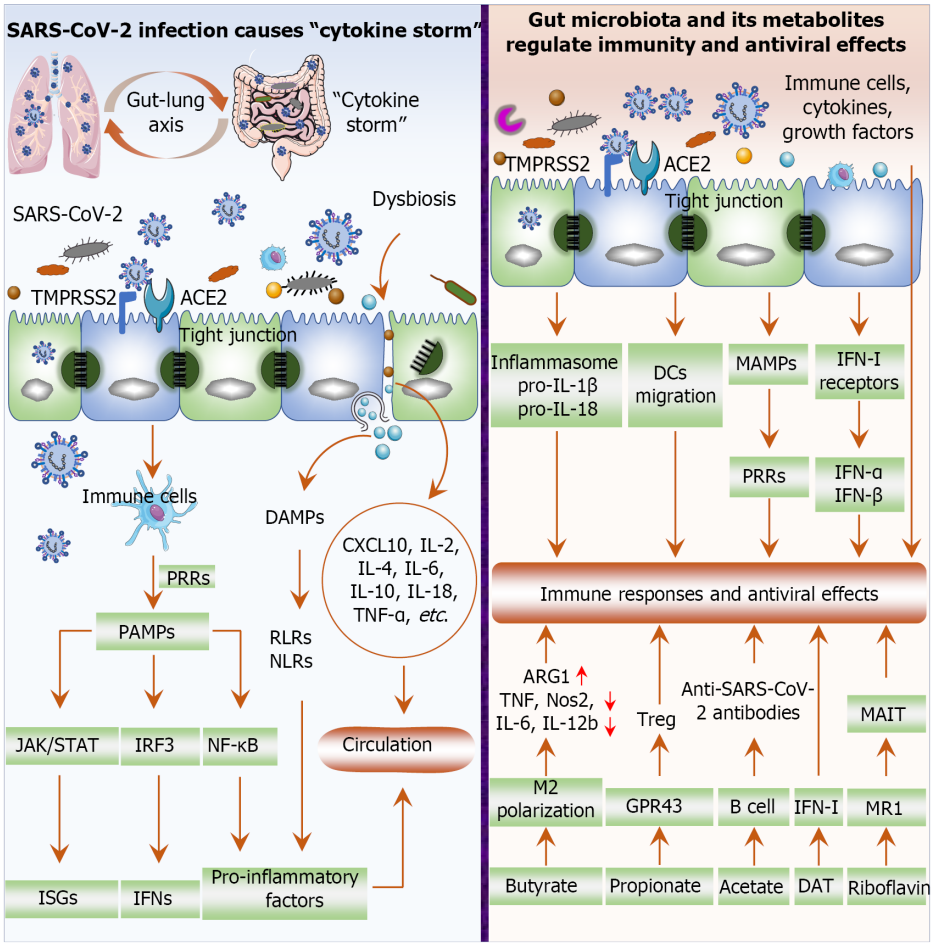


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Figure 1 Potential pathways and mechanisms of gut infection and dysbiosis induced by severe acute respiratory syndrome coronavirus 2. Fecal-oral transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in contaminated water and food, or by swallowing virus-laced sputum, results in gut infection and dysbiosis. SARS-CoV-2 infects alveolar type 2 cells and damages lung tissue before invading the gut through circulating immune cells and the lymphatic system and infiltrating gut epithelial cells *via* angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2, subsequently triggering gut inflammation and further promoting dysbiosis. Internalization of SARS-CoV-2 Leads to downregulation of ACE2, resulting in inhibition of mechanistic target of rapamycin and subsequent activation of gut autophagy, which modulates the gut microbiome. However, gut microbiota top associations with disease severity in coronavirus disease 2019 patients, such as *Coprobacillus*, have been shown to upregulate ACE2 expression in the gut. The improper use of antibiotics can also lead to dysbiosis. TMPRSS2: Transmembrane serine protease 2; ACE2: Angiotensin-converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; mTOR: Mechanistic target of rapamycin.

cytokine release "cytokine storm", such as IL-6, IL-1β, TNF-α, and IFN[41,50] (Figure 2). Excessive production of proinflammatory cytokines is pathologically associated with acute respiratory distress syndrome, extensive tissue damage, and even death. Viral replication after SARS-CoV-2 invades cells induces immune cells to recognize and bind viral pathogen-associated molecular patterns through pattern recognition receptors (PRRs), followed by the activation of the NF-κB, IRF3 and JAK/STAT signaling pathways to induce the expression of proinflammatory factors, IFNs and IFN-stimulated genes (ISGs)[41,51,52]. In addition, killing or damage of cells by SARS-CoV-2 results in the release of danger-associated molecular patterns, activating RIG-I-like receptors and NOD-like receptors and subsequently facilitating proinflammatory factor expression[41,51]. Dysbiosis after SARS-CoV-2 infection further damages the gut barrier and promotes the production of inflammatory factors such as CXCL10, IL-2, IL-4, IL-6, IL-10, IL-18, and TNF-α[16,53,54]. For example, *B. dorei* and *Akkermansia muciniphila* were positively associated with IL-1β, IL-6 and CXCL8[12]. Subsequently, opportunistic pathogens and inflammatory factors infiltrate the circulation and cause systemic inflammation and infection[55]. Therefore, SARS-CoV-2 infection promotes gut inflammation to aggravate dysbiosis, which in turn exacerbates inflammation and disease progression, forming a vicious cycle.

The gut microbiota and its metabolites influence the host immune response, inflammation and the development and regression of pulmonary infectious diseases, such as influenza A virus and *Streptococcus* pneumonia[56]. A healthy microbiome protects against respiratory viral infections[57,58]. During respiratory viral infection, the gut microbiota regulates the host immune response *via* the gut-lung axis, which refers to the crosstalk between the gut microbiota and lung. Therefore, endotoxins and microbial metabolites can affect the lung through the blood circulation, and conversely, lung inflammation will affect the gut microbiota. It has been reported that gut dysbiosis significantly increases mortality from



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Figure 2 Severe acute respiratory syndrome coronavirus 2 infection causes "cytokine storm" and the impact of gut microbiota and its metabolites on inflammatory response and antiviral effects. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) invades cells through angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) and replicates frantically, inducing innate immune cells [*e.g.*, macrophages, dendritic cells (DCs)] to recognize and bind pathogen-associated molecular patterns through pattern recognition receptors (PRRs). Subsequently, the expression of pro-inflammatory factors, interferons (IFNs) and IFN-stimulated genes is induced through the NF-κB, IRF3, and JAK/STAT signaling pathways, thereby promoting excessive inflammatory cytokine release. Killing or damaging cells by SARS-CoV-2 releases danger-associated molecular patterns that facilitate the expression of pro-inflammatory factors *via* activation of RIG-I-like receptors and NOD-like receptors. In addition, dysbiosis damages the gut barrier (tight junctions) and promotes the production of inflammatory factors such as CXCL10, IL-2, IL-4, IL-6, IL-10, IL-18, and TNF-α. Gut microbiota facilitates inflammasome activation, pro-IL-1β and pro-IL-18 expression, and DCs migration, thereby promoting protective immunity post viral infection. Microbial-associated molecular patterns are transmitted to the parenteral tissues to activate PRRs and affect innate immune responses. Gut microbiota regulates IFN-I receptors expression in respiratory epithelial cells and exerts antiviral effects through IFN-α and IFN-β. Immune cells, cytokines, and growth factors in the gut mucosa reach the respiratory tract to regulate immunity and exert antiviral effects. Butyrate promotes M2 macrophage polarization, upregulating arginase 1, and downregulating TNF, Nos2, IL-6, and IL-12b exerts anti-inflammatory activity. Propionate promotes Treg cell proliferation by activating G-protein-coupled receptor 43, thereby inhibiting autoinflammatory responses and protecting tissues from damage caused by pathological immune responses. Acetate promotes the production of SARS-CoV-2 antibodies by B cells, thereby inhibiting the development of COVID-19. The gut microbiota produces deaminotyrosine to enhance IFN-I signaling and protect the host from viral infection. Riboflavin, produced by the gut microbiome, activates mucosal-associated T cells *via* major histocompatibility complex-related protein-1. Mucosal-associated T cells participate in the immune response against SARS-CoV-2 through the gut-lung axis, thereby exerting antiviral efficacy. TMPRSS2: Transmembrane serine protease 2; ACE2: Angiotensin-converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; PRR: Pattern recognition receptors; IFN: Interferons; ISG: Interferons-stimulated gene; MAIT: Mucosal-associated T cells; MAMP: Microbial-associated molecular pattern; DAT: Deaminotyrosine; RLR: RIG-I-like receptors; NLR: NOD-like receptors; DAMP: Danger-associated molecular pattern; PAMP: Pathogen-associated molecular pattern; DC: Dendritic cell; MR1: Major histocompatibility complex-related protein-1; GPR43: G-protein-coupled receptor 43.

respiratory viral infections, which may be associated with dysregulated immune responses; increased secretion of IFN-γ, IL-6, and CCL2; and decreased Treg cell counts in the lung and gastrointestinal tract [59]. The gut microbiota facilitates inflammasome activation, pro-IL-1β and pro-IL-18 expression, and dendritic cell (DC) migration, which are critical for protective immunity post-influenza virus infection [60] (Figure 2). In addition, microbial-associated molecular patterns are transmitted to parenteral tissues to activate PRRs in immune cells and influence innate immune responses[61]. The gut microbiota reportedly regulates the expression of IFN-I receptors in respiratory epithelial cells that limit viral replication and increase resistance to viral infections through IFN-α and IFN-β[58,62]. The gut commensal microbiota promotes the IFN-I response and ISG expression by inducing IFN-β expression

mediated by TLR4-TRIF signaling through colonic lamina propria DCs, thereby enhancing antiviral capacity[62]. The gut microbiota can also affect respiratory mucosal immunity through multiple mechanisms. On the one hand, activated immune cells within the mucosa can reach and affect distant mucosal sites, highlighting the beneficial effects of the gut microbiota during respiratory viral infections [63,64]. On the other hand, the gut microbiota affects the secretion of cytokines and growth factors by the gastrointestinal mucosa, which reach the systemic circulation and act on other mucosal tissues[63, 65]. Furthermore, severe COVID-19 clinical symptoms or complications and higher mortality are more likely to arise in elderly individuals or in patients with concomitant underlying disease, such as cardiovascular disease, diabetes, and cancer[66]. Critically ill patients with COVID-19 often require invasive ventilator-assisted ventilation. However, gut microbiota intervention can reduce the demand for invasive mechanical ventilation in critically ill patients and reduce the incidence of ventilator-associated pneumonia[67], although this finding needs further validation in COVID-19 patients. Therefore, restoring gut microbiota homeostasis is essential for inhibiting the inflammatory response and enhancing antiviral effects.

Gut microbiota metabolites are signaling molecules and substrates for metabolic reactions[68]. Microbial metabolites are absorbed by the gut mucosa and participate in mucosal immune regulation, a process known as "metabolic reprogramming"[69,70]. For example, short-chain fatty acids (SCFAs, mainly including acetate, propionate, and butyrate) can reach distant organs through the bloodstream to exert immunomodulatory and immunoglobulin expression-inducing effects, as well as anti-inflammatory and antiviral effects[70,71] (Figure 2). Butyrate facilitates the polarization of M2 macrophage and exerts anti-inflammatory effects by increasing arginase 1 (ARG1) and decreasing TNF, Nos2, IL-6, and IL-12b expression[41,72]. Butyrate also inhibits histone deacetylase activity or increases the Foxp3 promoter transcription in naive T cells, thereby promoting the naive T cells differentiation into Treg cells[73,74]. Treg cells can suppress autoinflammatory responses and protect tissues from damage caused by pathological immune responses[41]. Furthermore, butyrate restored $CD8^+$ T-cell function in mice and inhibited proinflammatory cytokine production as well as eosinophilic lung infiltration[49]. Unfortunately, a reduced richness of butyrate-producing bacteria such as *F. prausnitzii* and *Clostridium* species has been found in COVID-19 patients[15,17,20,75]. Propionate promotes Treg cell proliferation by activating G-protein-coupled receptor 43 (GPR43)[76]. Moreover, microbe-produced SCFAs (acetate) enhance B-cell metabolism and gene expression, promoting the production of anti-SARS-CoV-2 antibodies and thereby inhibiting disease progression[77]. Deaminotyrosine (DAT) produced by *Clostridium orbiscindens* augments IFN-I signaling to protect the host from viral infection[57]. In addition, riboflavin is a product of gut microbiota constituents, which can activate mucosal-associated T cells (MAIT) *via* restrictive major histocompatibility complex (MHC)-related protein-1 (MR1)[78,79]. MAIT cells, as innate sensors and mediators of the antiviral response, exert antiviral response to SARS-CoV-2 through the gut-lung axis[80]. Antimicrobial peptides secreted by bacteria can promote virolysis, block cell virus fusion, and induce adaptive immune responses, and thus they have been proposed as viable alternative therapeutic treatments for infections by viruses, such as MERS-CoV[81]. Therefore, COVID-19 severity is tightly correlated with the levels of gut microbiota metabolites. However, studies on the direct action of gut microbiota metabolites in COVID-19 remain scarce.

REPROGRAMMING THE GUT MICROBIOTA AS A STRATEGY TO AMELIORATE GASTROINTESTINAL SYMPTOMS AND REDUCE DISEASE SEVERITY

The gut microbiota is closely related to various human gastrointestinal diseases and symptoms, such as gastrointestinal cancer, stomachache, diarrhea, and flatulence[82,83]. For instance, *Escherichia coli*, *Shigella*, *Salmonella*, *Campylobacter*, *Clostridium difficile*, and *Aeromonas* are the main pathogens that cause diarrhea[84,85]. Reprogramming the gut microbiota *via* probiotics, prebiotics and FMT is well documented to effectively treat gastrointestinal diseases. Moreover, probiotic/prebiotic administration has been shown to protect against viral infections in multiple studies (*e.g.*, those caused by influenza virus, rhinovirus, respiratory syncytial virus and coronaviruses). For example, Johnson *et al*[86] found that *Bacillus subtilis* peptidoglycans reduced the infectivity of coronavirus. Peptidoglycan-associated surfactin can disrupt virion integrity, such as in influenza, Ebola, Zika, and Mayaro[86]. Therefore, probiotics, prebiotics and FMT can restore ecological homeostasis by regulating the gut microbiota, representing an effective alternative approach to ameliorate or suppress COVID-19 severity.

Probiotics, such as *Lactobacillus*, yeast, *Bifidobacterium*, *Enterococcus*, and *Bacillus*, are live microorganisms that benefit the host by colonizing the human body. Probiotics maintain healthy gut homeostasis and exert antiviral effects through the gut-lung axis[87,88] and have been widely used to treat gastrointestinal diseases, including diarrhea (acute, antibiotic-associated and *C. difficile*-associated) and adult inflammatory bowel disease (IBD)[89-91]. On the one hand, probiotics can strengthen Treg cell and natural killer cell activity and suppress proinflammatory cytokines, such as TNF-a, CRP, IL-1b, IL2, IL-6, IL7, MCP1, and LDH[92,93]. *Lactobacillus* exerts antiviral activity through direct probiotic-virus interactions, production of antiviral metabolites, and stimulation of the immune system[94]. On the other hand, probiotics are beneficial for enhancing epithelial barrier function and improving gut

microbial diversity. Furthermore, probiotics combat and block harmful bacterial strains in the gut or enhance beneficial signaling pathways[95,96]. Existing evidence shows that probiotic miRNA modulation and regulation of signaling pathways such as NF-kB and STAT1 ameliorate COVID-19 complications[97,98]. Probiotics have the potential to interact with ACE2; for example, some lactobacilli release peptides with a high affinity for ACE2[93,99]. *Lactobacillus spp.* and *Bifidobacterium spp.* exhibit the strongest anti-respiratory virus activity *via* an immunomodulatory mechanism[56]. In January 2020, relevant Chinese government departments recommended the addition of probiotics in COVID-19 treatment to improve gut microbiota homeostasis and protect against subsequent bacterial infections. However, evidence detailing the efficacy of probiotics in treating COVID-19 is limited, although relevant clinical trials are being conducted. Moreover, the safety of probiotics in COVID-19 patients should be emphasized to ensure that their use does not induce new gastrointestinal symptoms or secondary infections.

Prebiotics are substrates that are selectively utilized by host microorganisms to provide health benefits[100]. Unfortunately, little information is available on using prebiotics to treat respiratory infections. In a clinical trial of prebiotics, KB109 was found to regulate gut microbiome composition and increase the production of SCFAs in the gut (NCT04414124). For instance, butyrate and propionate obtained from the fermentation of prebiotics affect the differentiation or function of T cells, macrophages, and DCs[93]. In addition, prebiotics selectively stimulate beneficial bacterial growth or enhance the activity of indigenous probiotics[93]. Accordingly, the role of prebiotics in COVID-19 patients should be further emphasized.

FMT refers to the transplantation of a functional microbiota from healthy individuals into the gastrointestinal tract of patients to treat intestinal and extraintestinal diseases[84]. An expanding body of evidence suggests that FMT can effectively treat many diseases, such as cancer, liver diseases, *C. difficile* infection, irritable bowel syndrome (IBS), and IBD[101-103]. Since COVID-19 patients exhibit a depletion of beneficial commensal bacteria, FMT represents a theoretically promising strategy to mitigate disease severity. Regrettably, convincing clinical evidence for the effect of FMT in COVID-19 is lacking, and its safety and efficacy warrant further investigation.

CONCLUSION

COVID-19 was previously identified as a respiratory infectious disease. However, accumulating clinical studies have subsequently found that a large proportion of patients have gastrointestinal symptoms, such as abdominal pain, diarrhea, vomiting, and acid reflux, as well as significant gut microbiota alterations[7,30]. The gut microbiota not only significantly affects the COVID-19 development and disease severity but also reflects the susceptibility of COVID-19 patients to long-term complications [104]. Studies have confirmed that dysbiosis increases the poor prognosis of COVID-19[104]. However, controversial results exist regarding gut microbiota alterations in COVID-19 patients. A variety of factors, such as sex, age, basic health status, medication use, genetics, ethnicity, and geographic location, can affect the composition of the gut microbiota and lead to individual differences and varying responses to SARS-CoV-2 infection. Therefore, further exploration of the specific gut microbiota alterations in COVID-19 patients and the clinical correlation between the gut microbiota and COVID-19 will be a very challenging and valuable research direction in the future.

Unfortunately, direct evidence for the contribution of the gut microbiota in COVID-19 remains lacking. For example, the exact mechanism of dysbiosis remains unclear. Moreover, the regulation of host immune and inflammatory responses and antiviral effects by the gut microbiota during SARS-CoV-2 infection relies largely on inferences or conjectures from previous studies. Given that specific drugs for COVID-19 remain enigmatic, vaccines represent the most effective prevention and control strategy; however, the continuous mutation of the virus has exacerbated this conundrum. Therefore, gut microbiota intervention through probiotics, prebiotics and FMT is undoubtedly one of the promising cosupplementation strategies for the future treatment of COVID-19, but large-scale studies are lacking and there are no corresponding treatment guidelines. The therapeutic prospects of the gut microbiota in COVID-19 are promising, but there is still a long way to go to realize its potential.

FOOTNOTES

Author contributions: Xiang H and Liu QP contributed equally to this work; Xiang H drafted the manuscript; Xiang H and Liu QP edited the manuscript, conceived and supervised the work; all the authors have read and approved the final manuscript.

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MINIREVIEWS

Microbiota in the stomach and application of probiotics to gastroduodenal diseases

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Abstract

The stomach is a hostile environment for most microbes because strong gastric acid kills indigenous microorganisms. Thus, the mass of indigenous microbes detected by traditional culturing method in a highly acidic stomach is reported to be very small. However, in a stomach with less acidity due to atrophic changes of the gastric mucosa, the number of live gastric microbiota dramatically increases and their composition changes. A probiotic is defined as a live microorganism that, when administered in adequate amounts, confers a health benefit on the host. The administration of probiotics to the stomach has thus far been considered impractical, mainly due to the strong acidity in the stomach. The identification of candidate probiotic strains with sufficient resistance to acidity and the ability to achieve close proximity to the gastric mucosa could enable the application of probiotics to the stomach. The utilization of probiotics alone for *Helicobacter pylori* (*H. pylori*) infection significantly improves gastric mucosal inflammation and decreases the density of *H. pylori* on the mucosa, although complete eradication of *H. pylori* has not yet been demonstrated. The use of probiotics in combination with antimicrobial agents significantly increases the *H. pylori* eradication rate, especially when the *H. pylori* strains are resistant to antimicrobial agents. While *H. pylori* has been considered the most important pathogenic bacterium for the development of gastric cancer, bacteria other than *H. pylori* are also suggested to be causative pathogens that promote the development of gastric cancer, even after the eradication of *H. pylori*. Increased non-*H. pylori* Gram-negative bacteria in the stomach with weak acidity accompanying atrophic gastritis may perpetuate gastric mucosal inflammation and accelerate carcinogenic progression, even after *H. pylori* eradication. Probiotics restore the acidity in this stomach environment and may therefore prevent the development of gastric cancer by termination of Gram-negative bacteria-induced inflammation. Functional dyspepsia (FD) is defined as the presence of symptoms that are thought to originate in the gastroduodenal region in the absence of any organic, systematic or metabolic diseases. Accumulating evidence has pointed out the duodenum as a target region underlying the pathophysiology of FD. A randomized placebo-controlled clinical trial using a probiotic strain (LG21) demonstrated a significant improving effect on major FD symptoms. One of the possible mechanisms of this effect is

protection of the duodenal mucosa from injurious intestinal bacteria through the resolution of small intestinal bacterial over growth.

Key Words: Stomach; Microbiota; Probiotics; *Helicobacter pylori*; Post-eradication gastric cancers; Functional dyspepsia

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Core Tip: Gastric microbiota and application of probiotics to the gastroduodenal diseases have so far been unfamiliar because the mass of live microbes is so small in the stomach with high acidity. However, in the subject whose stomach is low acidity due to atrophic gastritis or proton pump inhibitor long-use, the number of live bacteria increases so much in the stomach thus they can significantly influence the pathophysiology of gastroduodenal diseases.

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INTRODUCTION

The intestine is colonized by a complex and dynamic microbial ecosystem with a high density of bacteria whose cell number can reach as high as approximately 10^{12}/g feces. Their total number is therefore estimated to be 10-times greater than the number of eukaryotic cells in the human body, and the genes of these microbes outnumber human genes more than 100-fold[1]. As the early definition of probiotics emphasized their effects on improving the ecology of intestinal microbiota, their actions on the intestinal tract and gut mucosal immunity have received a great deal of attention.

On the other hand, the size of gastric microbiota, in which probiotic bacteria exert their beneficial effects, is very small [around 10^3 colony-forming units (CFU)/mL gastric fluid (GF)[2]] because of the strong acidity and frequent peristalsis to the intestine in the stomach. Such high acidity due to secreted gastric acid kills probiotic strains as well as gastric commensal microbes. Therefore, the application of probiotics to the stomach or proximal small intestine has historically been considered impractical.

Based on the outline of microbiota and probiotics in the stomach, this article reviews *Helicobacter pylori* (*H. pylori*) infection and functional dyspepsia (FD). The former includes the pathogenicity of *H. pylori*, the suppressive mechanism of probiotics on this bacterium and the present status of the application of probiotics in eradication therapy. In addition, this review argues a possible role of probiotics in the prevention of post-*H. pylori* eradication gastric cancer. In the description, basic and clinical data reported by the author's group are emphasized because they are indispensable for communicating the author's ideas in relation to the theme of this review article.

MICROBIOTA IN THE STOMACH

Probiotics are considered to exert beneficial effects on the host by improving the indigenous microbiota [3]. Thus, a brief description of the gastric microbiota is necessary to understand the effects of probiotics in the stomach. The stomach is a hostile environment for most microbes because strong gastric acid kills indigenous microorganisms. Therefore, when examined using traditional culturing methods, bacterial numbers in the gastric mucosa-associated or GF are reported to contain only approximately 10^3 CFU per g or mL[2]. Moreover, this method can only detect microbes that are able to grow in the media components and the atmospheric conditions of the culturing assay. Thus, in the stomach-where the acidity is high enough to kill indigenous bacteria-the investigation of gastric microbiota by traditional culturing methods makes no sense as the stomach contains few live bacteria. However, the introduction of DNA sequencing using high performance next-generation sequencers has markedly enhanced the analysis of microbiomes in the stomach as well as in the intestine. In a stomach with less acidity due to the continuous use of acid-suppressive agents [*e.g.*, proton-pump inhibitors (PPIs)] or atrophic change of the gastric mucosa, the number of bacteria in the live gastric bacterial mass dramatically increases and the composition changes, respectively[4,5]. In these low acidity settings, the gastric microbiota will exert a significant effect on the pathophysiology of upper gastrointestinal (GI) tract disorders.

The mouth is located at the entrance of the GI tract, and contains complex anatomical sites, including the teeth, gingiva and tongue. The oral microbiota flows downstream into the stomach by swallowing of saliva and mastication of foods. These are expected to exert a great influence on the microbiota in the stomach. While the gut harbors a very large and complex microbial community, it is conceivable that such intestinal microbiota can be significantly affected by the gastric microbiota through continuous inflow. This is especially true after a large increase in the bacterial mass in a stomach with low acidity. This raises the hypothesis that the GI tract has a common microbial ecosystem in which the gastric microbiota plays the role of a relay base between the oral and gut microbiotas. Therefore, in this chapter, the microbiota in the stomach is described and compared to the microbiotas of the oral cavity and gut. *H. pylori* is not an indigenous resident but a pathogen of exogenous origin. Accordingly, it is not described in the present chapter.

In 2006, Bik *et al*[6] identified 128 bacterial phenotypes based on a 16S rRNA gene analysis of gastric microbiomes with 1833 sequences obtained from 23 human gastric endoscopic biopsy specimens. A few years later, Li *et al*[7] also performed a 16S rRNA gene analysis using 1223 non-*H. pylori* sequences of 10 biopsy samples from the stomach, which were then classified into 133 phylotypes. Despite examining racially distinct populations (North America and China, respectively), both studies investigating the gastric mucosa-associated microbiomes revealed similar results. *Streptococcus* and *Prevotella* were the predominant genera, and they accounted for approximately half of the total detected species detected in their studies. In 2015, Tsuda *et al*[2] performed a meta-16S analysis of the gastric luminal microbiome with far greater sequencing depth. Their analysis was performed using GF samples obtained from Japanese subjects in a fasting state in the morning. They obtained roughly 40000 high-quality reads for the analysis from 45 GF specimens and also identified *Streptococcus* and *Prevotella* as the most prevalent genera, accounting for approximately 50% of all the species detected in the stomach (Figure 1). Moreover, in all three of these studies, *Neisseria* and *Rothia* ranked among the top 5 most prevalent genera. This similarity in the bacterial composition between the mucosa-associated[6,7] and luminal[2] samples suggested that the former bacteria moved back into the lumen, while the latter continuously colonized the mucosa. *H. pylori* is a predominant inhabitant of the mucous layer and also inhabits the gastric epithelial cells. Accordingly, it is mainly found in the mucosa-associated specimens.

Tsuda *et al*[2] also compared three different bacterial communities along the alimentary tract (oral cavity, stomach and colon) using stimulated saliva, GF, and feces specimens. There was no significant difference in the degree of species richness (α-diversity) among the three types of specimens. While the median log CFU bacterial number was only 3.4/mL (determined by culturing), the bacterial log genome copy number was as high as 7.8/mL (median) in GF samples. This large discrepancy between the CFU and genome copy numbers implied that > 99.9% of the GF bacteria were dead or viable but non-culturable. The analysis of bacterial genome copies also suggested that-if microbes were alive and metabolically active in the stomach with weak acidity-the mass size of the GF microbiota may be high enough to significantly affect the pathophysiology of the stomach and its downstream organs. A bacterial composition analysis at the genus level showed high similarity between the salivary and GF microbiota (Figure 1). Indeed, the five most prevalent genera in these microbiomes (in descending order) were as follows: *Streptococcus, Prevotella, Neisseria, Rothia* and *Veillonella,* and *Streptococcus Prevotella, Actinomyces, Neisseria* and *Rothia*. In contrast, the composition of the fecal microbiota was markedly different.

In a meta-16S analysis, which was performed to investigate the influence of gastric acidity on the microbiome composition in the stomach without atrophic change of the mucosa, PPI treatment was found to significantly increase the amount of *Streptococcus* in the mucosa-associated gastric microbiota [8]. This increase occurred independently of *H. pylori* infection. The compositions of other bacteria at the genus level showed no significant alteration. In another study, in which a bacterial DNA analysis study was conducted using GF samples, PPI-treated subjects whose GF acidity was > pH 4, showed lower α-diversity than subjects without PPI treatment[9]. No other significant changes in the GF microbiome composition were observed in GF samples with weak acidity. Furthermore, the presence of *H. pylori* was not associated with the difference in the microbiome composition. Moreover, in a study of PPI-users by Tsuda *et al*[2], no significant changes were observed in the bacterial composition (at the genus level) in the GF of PPI users, with the exception that *Streptococcus* tended to be more prevalent (Figure 1). In their study, the average pH values of GF samples obtained from the PPI-users and PPI-nonusers were 3.2 and 1.6, respectively. Of note, the average log copy number of bacterial cells (/mL) in these GF samples (measured by quantitative polymerase chain reaction) was almost the same in PPI-users (8.0) and PPI-nonusers (8.1), while the CFU number (determined by culturing) was > 1000-fold greater in PPI-users with weak acidity. This result implies that PPI-induced low acidity protected the gastric microbiota from strong acid.

Bacterial overgrowth in the stomach with weak acidity has been suggested to occur due to the restoration of active growth of relatively acid-resistant indigenous bacteria, which are kept alive (in small numbers) due to their suppressed growth in the strongly acidic stomach. However, the high similarity in the bacterial community structure between the GF and saliva, and the high similarity of their bacterial genome copy numbers, suggests that no "bacterial overgrowth" occurred. Instead, the bacteria that moved from the oral cavity to the stomach with weak acidity simply avoided being killed by gastric acid. Although PPI use was not associated with any significant alteration in the composition

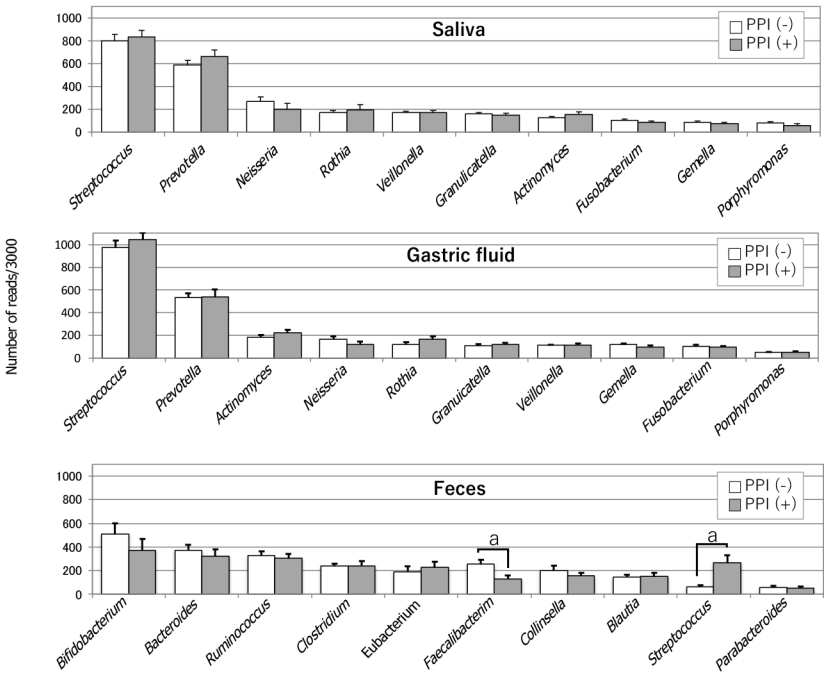


Figure 1 Comparison of the microbiota in the saliva, gastric fluid and feces, and the influence of proton-pump inhibitors. Bacterial compositions at the genus level in saliva (top), gastric fluid (median) and feces (bottom) are shown. The average of read numbers of the top 10 major genera are indicated in each group. Open and filled bars represent proton-pump inhibitor (PPI)-nonusers and PPI-users, respectively. Asterisks show a significant difference according to Student *t*-test. $^{a}P < 0.05$. PPI: Proton-pump inhibitor. Citation: Tsuda A, Suda W, Morita H, Takanashi K, Takagi A, Koga Y, Hattori M. Influence of Proton-Pump Inhibitors on the Luminal Microbiota in the Gastrointestinal Tract. *Clin Transl Gastroenterol* 2015; 6: e89. Copyright ©Wolters Kluwer Health, Inc. 2015. Published by Wolters Kluwer Health, Inc.

of the gastric microbiota, a significant decrease of *Faecalibacterium* and a significant increase of *Streptococcus* were found in the feces of PPI-users (Figure 1). A reduction in the abundance of *Faecalibacterium* was also reported in the feces of dogs treated with PPIs[10]. Whether the large increase of live bacteria induced by PPI treatment significantly influences the intestinal bacterial community structure remains to be elucidated.

PROBIOTICS FOR THE STOMACH

In 1989, Fuller defined probiotics as "a live microbial feed supplement that beneficially affects the host animals by improving its intestinal microbial balance"[3]. This early and influential definition was refined by a standard definition proposed by the Joint (Food and Agricultural Organizations of the United Nations)/World Health Organization Expert Consultation in 2001[11]: "a live microorganism that, when administered in adequate amounts, confers a health benefit on the host". The International Scientific Association for Probiotics and Prebiotics consensus statements reported in 2014 exclusively retained the body of these definitions[12].

The main beneficial effects of probiotics on the host include inhibition of potential pathogens in the GI tract, modulation of immunity, and reinforcement of the mucosal barrier. Competition with bacteria for binding sites on the mucosa by probiotic strains is the dominant mechanisms underlying the protection of the host from pathogenic bacteria in the intestine. Organic acids secreted by probiotic strains (*e.g.*, lactic acid) and short-chain fatty acids (*e.g.*, acetic acid) are considered to exert a significant bactericidal effect on the pathogens. As underlined in the definitions of probiotics, a "living" state is indispensable for probiotics to protect the hosts from pathogens, because dead probiotic strains no longer have the ability to specifically bind to the mucosa or generate organic acids.

Because the early definition of probiotics emphasized their beneficial effects on improving intestinal microbial ecology, it has been considered that probiotics should be exclusively applied to the gut. Actually, the gut is colonized with a high density of bacteria, the cell number of which can reach as high as 10^{12}/g feces[13]. In contrast, the size of bacterial mass in the stomach, in which probiotic strains could work, is small. The number of indigenous bacteria in the gastric fluid of healthy stomach-when examined by culturing methods- is at most 10^{3}/mL[2,4]. The strong acidity in the stomach due to secreted gastric acid, which causes a marked reduction in the size of such resident gastric bacteria also suppresses or terminates the beneficial effects exerted by probiotic strains soon after they move to the stomach. For this reason, the application of probiotics to the stomach or proximal small intestine has so

far been considered impractical.

The proximity of probiotic strains to the mucosa-which will be achieved by bearing affinity to the surface mucus layer, or by adhering to the epithelial cell layers-is crucial for beneficial effects to be exerted in the GI tract[14]. Those effects include competitive inhibition of the pathogenic bacteria's attachment to the mucosa, secretion of organic acids in effective concentrations, and contact-dependent immunomodulation. Peristaltic movements in the stomach are more frequent and active in comparison to the intestine; thus, it is difficult for probiotic strains to come into close proximity to the gastric mucosa. This factor also makes the use of gastric probiotics difficult.

From another point of view, the small size of resident microbiota in the stomach can easily be affected by the introduction of exogenous microorganisms. These transient bacteria (*e.g.*, ingredients of foods or accidental contaminants) exert a much greater effect on the microbiota in the upper GI tract than on the gut microbiota due to its much smaller size. The same situation is encountered with the administration of probiotics to the upper GI tract, where they are not disturbed by large amounts of robust and resilient microbiota, which are present in the gut. The application of probiotics to the upper GI tract may therefore be more advantageous in comparison to application to the gut, as long as the candidate strains are able to resist the strong acidity and achieve close proximity to the gastric mucosa.

The stomach is a harsh environment for most microorganisms because strong gastric acid kills many ingested microbes. In a fasting state in the morning, GF has a peak acidity of pH 1-2. Such strong acidity is quite reasonable because one major physiological role of gastric acid is to kill exogenous pathogenic bacteria that move to the digestive tract through the mouth. At present, the most prevalent probiotic strains belong to genera *Lactobacillus* or *Bifidobacterium*[11]. In general, *Lactobacilli* and *Bifidobacteria* show considerably high resistance to acidity. While both bacterial groups can survive acidic conditions of approximately pH 3 to 4[15], this is not as strong as the peak gastric acidity (pH 1-2). Thus, screening using candidate probiotic strains is necessary to identify a suitable probiotic strain for the stomach that can tolerate approximately pH 2.

Genus *Lactobacillus* is one of the predominant resident bacterial groups found in the stomach (when examined by culturing methods)[16]. Accordingly, *Lactobacillus* strains might be the most appropriate for use as probiotics in the stomach. Furthermore, in adult mice with a specific pathogen-free (SPF) environment, no *H. pylori* infection was found in the stomach after oral inoculation of the bacteria[16]. In contrast, mice bred in a germ-free environment were easily infected by oral inoculation of *H. pylori*. In this animal study, *H. pylori* infection was prevented in SPF mice with a large number of indigenous lactobacilli in the stomach (> 10^8 CFU/g tissue). A representative probiotic strain that can be used for the stomach is *Lactobacillus gasseri* OLL2716 (LG21). This was selected out of approximately 2000 strains of lactobacilli[17]. The criteria for selection were both resistance to acidity and the ability to bind to the gastric mucosa. In the stationary growth phase, LG21 can survive in culture broth at pH 2.5, which is similar to the acidity of GF. LG21 has several defense mechanisms that enable it to withstand acid stress, including the up-regulation of the cation ATP-binding cassette transporter genes and the downregulation of the genes associated with transcription and protein synthesis[18]. The acid stress response is generally indispensable for lactobacilli, because they always secrete large amounts of organic acids to the external environment when they grow with metabolic activity. Without this defense mechanism, the acidic milieu induces the arrest of the growth of lactobacilli and it may even cause their death.

In a handful of trials, endoscopy directly demonstrated mucosal colonization by probiotics. Using biopsy samples obtained by upper GI endoscopy, LG21 strains administered through a yogurt drink were shown to be able to enter the mucous layer of the human stomach[19]. The laser-assisted microdissection and non-contact pressure catapulting method enabled this fine topical analysis.

H. PYLORI INFECTION

H. pylori and its pathogenicity

H. pylori is a gram-negative and microaerophilic bacterium that can move in the mucus layer on the surface epithelial cells of the stomach using several of flagella that are located at one end (Figure 2). As much as half of the people in the world are infected with *H. pylori*[20]. *H. pylori* infection causes inflammation of the gastric mucosa and then leads to a gradual loss of hydrochloric acid-secreting parietal cells of the stomach. This ultimately results in a condition known as atrophic gastritis. Atrophic gastritis is a chronic inflammatory and low gastric acidity state that has a high risk of progressing to gastric cancer[21].

H. pylori can tightly bind to epithelial cells by multiple bacterial-surface components. The best-characterized adhesin, BabA, is a 78 kD outer-membrane protein that binds to the fucosylated Lewis B blood group antigen on the host cell[22]. Firm contact between *H. pylori* and the host cell through adhesion is considered a prerequisite for the *H. pylori* to transport effecter molecules (*e.g.*, CagA) into the host cell using the *cag* PAI-encoded type IV secretion system[23]. This event is regarded as the pathway leading to the generation of proinflammatory cytokines (*e.g.*, IL-8 and IL-1β) by host cells. Thus, the adhesion of *H. pylori* to epithelial cells is a critical event in the development of an inflammatory response and the establishment of infection in the stomach.



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Figure 2 Observation of *Helicobacter pylori* by scanning electron microscopy. The bar at the bottom shows 1 μm.

H. pylori eradication

Mechanism of the suppressive effect of probiotics on *H. pylori*: The major mechanisms of probiotics against *H. pylori* infection are thought to be competition with *H. pylori* for binding sites on gastric epithelial cells, reinforcement of the mucosal barrier, and secretion of bactericidal organic acids (*e.g.*, lactic acid). These are the principle anti-bacterial effects exerted by probiotics. As for the mechanism of competitive binding, *L. reuteri* is reported to inhibit the attachment of *H. pylori* on the epithelial cell surface by competitive binding to asialo-GM1 and surface receptors[24]. Moreover, other probiotic species (*e.g.*, *L. acidophilus*, *L. johnsonii* and *L. salivarius*) were reported to prevent *H. pylori* colonization through specific adhesion molecules[25,26]. Specific binding of *H. pylori* to the host cell then induces the production of IL-8 through the type IV secretion system. Tamura *et al*[27] also demonstrated competition for binding sites between *H. pylori* and a probiotic strain LG21 using a coculture system with MKN45 cells (a human gastric epithelial cell line) and *H. pylori*. Large amounts of IL-8 were produced in the gastric epithelial cells cocultured with *H. pylori* (10^6 CFU) alone. When 10^6 CFU of non-treated live LG21 (equivalent to the number of *H. pylori*) was added to the coculture system, the amount of IL-8 secreted into the culture supernatant significantly decreased. However, UV- or heat-treated LG21 could not exert any suppressive effect on *H. pylori*-induced IL-8 production, even at 10^8 CFU (100 times the amount of non-treated LG21). An adherence assay in their study supported that LG21 competitively inhibited the binding of *H. pylori* to MKN45 cells, which suppressed the production of IL-8. Moreover, they demonstrated that the suppressive effect of LG21 also worked in the human stomach. The measurement of the IL-8 Level in gastric biopsy specimens from *H. pylori*-infected subjects also revealed that the oral intake of probiotic LG21 significantly suppressed the generation of IL-8 in the gastric mucosa[27].

Application of probiotics in the eradication therapy: The clinical application of probiotics in the treatment of *H. pylori* infection has been performed in many countries for more than 20 years. Now the utilization of probiotics alone for *H. pylori* infection has almost been settled. Both early and recent reviews[28,29] concluded that probiotics significantly improved gastric mucosal inflammation, and decreased the density of *H. pylori* on the mucosa. However, to our knowledge, the complete eradication of *H. pylori* colonizing the stomach by probiotic treatment alone has not been demonstrated. One representative trial of probiotics alone for the treatment of *H. pylori* infection was reported by Sakamoto *et al*[17] in 2001. In their study, 31 *H. pylori*-infected subjects (mean age 50 years) ingested yogurt containing 10^9 CFU of LG21 or placebo yogurt without LG21 every day for 8 wk. The results of ^{13}C-urea breath tests (UBT) and assays of serum pepsinogens I and II (PGI/II) showed a significant clinical improvement after LG21 yogurt treatment. The ^{13}C-UBT result and the PGI/II ratio are known to indirectly represent *H. pylori* density and the degree of mucosal inflammation in the stomach, respectively[30,31]. A bacterial examination of gastric mucosal biopsy specimens (by culturing) revealed 2-100-fold decreases in the number of *H. pylori*. However, there were no subjects in whom *H. pylori* was completely eliminated. Pantoflickova *et al*[32] reported the effects of the administration of *L. johnsonii* La1 (LC-1) to 50 *H. pylori*-positive healthy volunteers in a randomized controlled, double-blind study. The subjects received 125 g of fermented milk containing 10^6-10^7 CFU/g of LC-1 or placebo milk without LC-1 every day for 16 wk. The severity/activity of antral gastritis (assessed histologically) and the *H. pylori* density (assesses by a ^{13}C-UBT) showed significant improvement. The histological examination of the mucous mucosa also revealed a significant increase in the mucous thickness in the LC-1-treated group. This suggested that the stabilization of the mucosal barrier by probiotics also enhanced the suppression of *H. pylori*.

Recently, the *H. pylori* eradication rate in patients treated using anti-microbial agents is decreasing. This is mainly due to antimicrobial resistance. In the early 1990s, the standard triple therapy achieved an eradication rate of > 90%. In contrast in the past decade, the effectiveness of this regimen often falls to < 70%[33,34]. According to an ITT analysis by Deguchi *et al*[35] in 2012, the successful eradication rate

using the same regimen was just 69.3%. In those subjects, the rate of infection with clarithromycin-resistant strains of *H. pylori* was as high as 27.1%. This increase in resistance to antimicrobials like clarithromycin is thought to have reduced the eradication rate. Actually, the clarithromycin resistance rates of *H. pylori* isolated from children in North America and Europe were reported to be 10.6%-25% and 1.7%-23.4% respectively[36,37]. These studies also reported the increasing prevalence of *H. pylori* isolates that are resistant to metronidazole, which is frequently used in the first-line regimen.

The use of probiotics in combination with antimicrobial agents significantly increased the eradication rate, especially for bacteria with antimicrobial resistance. Both the suppressive effect on *H. pylori* by probiotics and the compliance-promoting effect of ameliorating the side effects of antimicrobials are thought to significantly increase the eradication rate. Actually, Deguchi *et al*[35] reported that a group treated with one week-triple therapy supplemented with LG21 yogurt and a group with triple therapy alone showed cure rates of 82.6% and 69.3%, respectively. The difference in the intention-to-treat analysis was statistically significant ($P = 0.018$). In their study, 112 g of yogurt containing 10^9 CFU of LG21 was given twice daily for 4 wk (3 wk of pretreatment and 1 wk during eradication therapy). According to a recent meta-analysis of 40 eligible studies with 8924 patients[29], the use of probiotics before and throughout the eradication treatment was associated with a superior eradication effect. Patients who received supplementary probiotics showed a higher eradication rate [relative risk (RR) 1.14, 95%CI: 1.10-1.18, $P < 0.001$] and lower incidence of total side effects (RR 0.47, 95%CI: 0.39-0.57, $P < 0.001$) in comparison to the control group without probiotics. In a sub-analysis, *Lactobacillus* was the best choice among the probiotic strains, and probiotics combined with bismuth quadruple regimen was suggested to be the best combination.

Possible role of probiotics in preventing post-eradication gastric cancer: The lifetime risk of gastric cancers in *H. pylori*-infected individuals is estimated to be 3%-5%[38]. In *H. pylori*-infected patients, colonization with *H. pylori* on the gastric mucosa is known to gradually decrease overtime and often becomes undetectable in patients who develop gastric cancer[39]. Furthermore, during long-term follow-up (up to approximately 20 years) of patients who had been cured of *H. pylori* infection at the start of observation, 0.35% of subjects developed gastric cancer per year. That is, 7% is estimated to have developed gastric cancer at 20 years[40]. These findings strongly suggest that there are some causative factors other than *H. pylori* can also promote the development of gastric cancer even after *H. pylori* eradication. However, *H. pylori* is currently considered the most important pathogen for the development of gastric cancer.

According to the Correa pathway[41], chronic *H. pylori* infection progresses over the decades through the following stages: chronic gastritis, atrophy, intestinal metaplasia, and cancer. Gastric adenocarcinomas are classified as both well-differentiated (intestinal-type) and undifferentiated (diffuse-type) ones[42]. The development of gastric atrophy is recognized as a critical step to the development of intestinal-type gastric cancer in the Correa pathway. Mucosal atrophy is usually accompanied by inflammation, and is thus recognized as atrophic gastritis. Accordingly, atrophic gastritis appears to be the strongest risk factor for gastric cancer[21]. The histological characteristics of the gastric mucosa (*e.g.*, inflammation, atrophy, and intestinal metaplasia) were analyzed to identify risk factors for gastric cancer after *H. pylori* eradication[43]. The mucosal inflammation score of the group who developed gastric cancer after successful *H. pylori* eradication ($n = 61$) was significantly higher than the group without cancer after eradication ($n = 122$). The RR and 95%CI were 5.92 and 2.11-16.6, respectively ($P < 0.01$). Neither atrophy nor intestinal metaplasia itself was a direct risk factor for post-eradication cancer.

The gastric corpus and antrum predominantly contain acid-secreting parietal cells and gastrin-secreting G cells, respectively. Thus, the mucosal atrophy in the corpus caused by *H. pylori* infection rapidly leads to a reduction in the gastric acid production. In contrast, the production of gastrin (an acid secretion stimulating hormone) remains relatively unchanged. Of note, patients who develop *H. pylori*-associated duodenal ulcers seem to be somewhat protected from the occurrence of gastric cancers[44]. The predominant mechanism of this protection in patients with duodenal ulcer from the cancers appears to be a higher basal level of gastric acid secretion. On the contrary, it was reported that the long-term suppression of gastric acid secretion by proton pump inhibitors (PPIs) was associated with a significantly increased risk of gastric cancer in *H. pylori*-infected subjects[45]. During approximately 8 years of follow-up, Cheung *et al*[46] evaluated the gastric cancer risk in patients treated with PPI using a Cox proportional hazards model. The study population consisted of approximately 63000 subjects, who had received clarithromycin-based triple therapy for *H. pylori* eradication. The use of PPIs was associated with an increased gastric cancer risk (HR 2.44, 95%CI: 1.42-4.20). This result demonstrated that the long-term use of PPIs was still associated with an increased risk of gastric cancer, even after *H. pylori* eradication. Accordingly, the stomach with low acidity accompanied by gastritis with atrophy and/or intestinal metaplasia must be considered a high-risk environment that predisposes the gastric mucosa to the development of gastric cancer.

Gastric acid reduction invariably results in a marked increase in the number of non-*H. pylori* bacteria. Due to the low acidity, these bacteria are still viable and show metabolic activity in the stomach. It therefore appears likely that such an enlarged bacterial mass causes the development of gastric cancers even after *H. pylori* eradication. Recent studies on the characteristics of the gastric microbial changes associated with gastric carcinogenesis revealed a reduction of species richness, the enrichment of

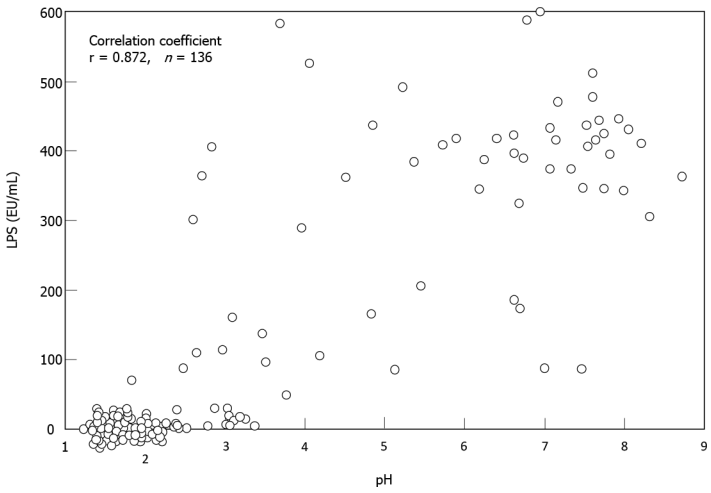
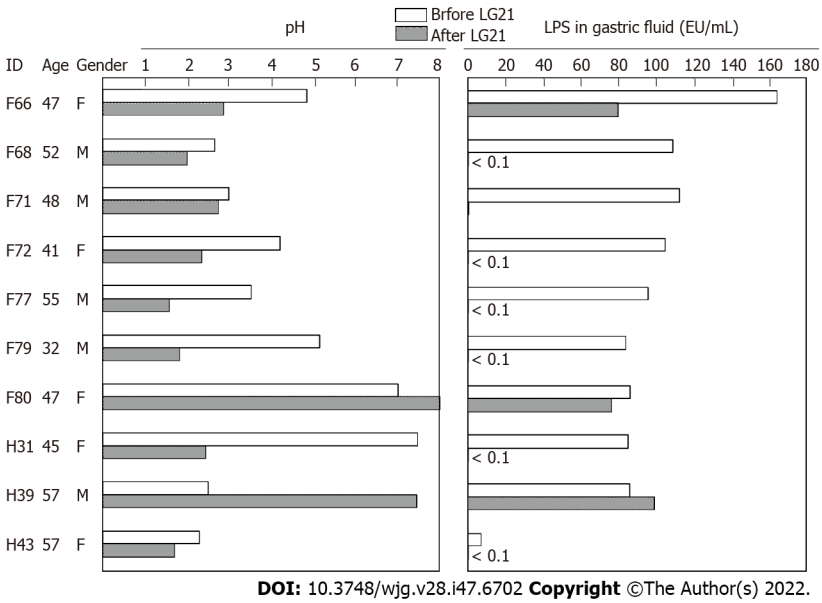


Figure 3 Correlation between pH and lipopolysaccharide activity in gastric fluid. The pH values and lipopolysaccharide activity of gastric fluid samples from 136 subjects were examined using a recombinant factor C assay kit. The correlation coefficients of the both parameters by Spearman test (*r*) is shown on the upper left. LPS: Lipopolysaccharide. Citation: Sano M, Uchida T, Igarashi M, Matsuoka T, Kimura M, Koike J, Fujisawa M, Mizukami H, Monma M, Teramura E, Yoshihara S, Sato H, Morimachi M, Ito A, Ueda T, Shiraishi K, Matsushima M, Suzuki T, Koga Y. Increase in the Lipopolysaccharide Activity and Accumulation of Gram-Negative Bacteria in the Stomach With Low Acidity. *Clin Transl Gastroenterol* 2020; 11: e00190. Copyright ©Wolters Kluwer Health, Inc. 2020. Published by Wolters Kluwer Health, Inc.

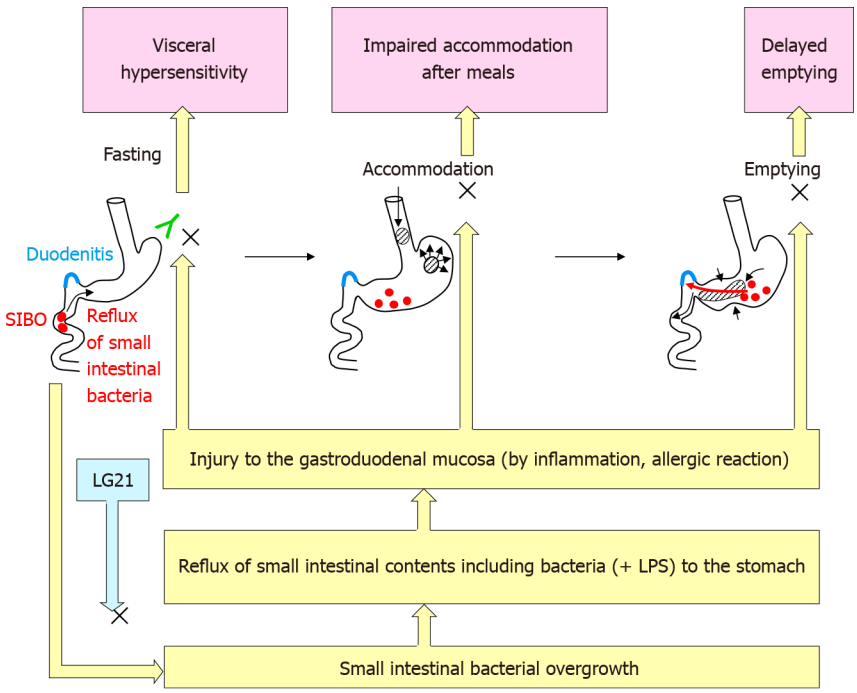


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Figure 4 Effect of LG21 administration on the pH and lipopolysaccharide activity in the gastric fluid. Ten subjects who had gastric fluid (GF) with low acidity and high lipopolysaccharide (LPS) activity consumed yogurt containing 10^9 CFU of LG21 every day for 3 mo. The pH value and LPS activity in the GF were measured before and after LG21 treatment. LPS: Lipopolysaccharide.

intestinal bacteria or an increase of bacterial species of oral cavity origin[47,48]. It seems unlikely that the deoxidization of dietary nitrates to nitrite by such dysbiotic bacteria could rapidly convert dietary amines into carcinogenic N-nitro compounds, because this conversion requires a sufficient amount of acid (which is not present in the stomach with mucosal atrophy)[49].

Sung *et al*[5] analyzed gastric microbes associated with gastric mucosal inflammation-which is considered to be the strongest risk factor for post-eradication gastric cancer-at one year after *H. pylori* eradication. They identified several of bacterial groups that were significantly associated with persistent inflammation. These bacteria included the genera *Acientobacter, Ralstonia, Actinobacillus* and *Erwinia,* which are all Gram-negative bacteria. Miyata *et al*[50] isolated several types of Gram-negative bacteria from the *H. pylori*-infected gastric mucosa, including *Fusobacterium, Haemophilus, Neisseria* and *Veillonella* species. Coculture of a gastric epithelial cell line with the lipopolysaccharide (LPS) specimens extracted from these bacterial groups stimulated a significant amount of IL-8 production. Sano *et al*[51] found high LPS activity in gastric fluid samples with weak acidity (pH > 4), whereas there was little or no activity in those with strong acidity (pH < 2). Spearman's test demonstrated a close correlation between



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Figure 5 Pathophysiology of functional dyspepsia and possible mechanisms of the effects of LG21 treatment. SIBO: Small intestinal bacterial overgrowth; LPS: Lipopolysaccharide.

pH and LPS activity in their 136 samples (r = 0.872) (Figure 3). These findings suggested that LPS from such non-*H. pylori* Gram-negative bacteria may perpetuate gastric inflammation and accelerate neoplastic progression in the hypochlorhydric stomach after *H. pylori* eradication.

To examine a possible preventive effect of probiotics on post-eradication gastric cancer, we administered a probiotic LG21 strain to subjects with successful eradication who still suffered from atrophic gastritis. In a fasting state in the morning, the pH value and LPS activity of their gastric fluids' samples were > 3.0 and > 10 EU/mL, respectively (Figure 4). Then, they received 10^9 CFU of LG21 in yogurt every day for 3 mo. In 8 of 10 subjects, the pH value considerably decreased after LG21 treatment. Lactic acid secreted by the probiotic LG21 strain is thought to restore acidity in the stomach with low acidity. Interestingly, the LPS activity of these subjects, in whom the gastric acidity partially recovered, almost disappeared or markedly decreased. The possible termination of LPS-induced inflammation by LG21 suggests a possible role of probiotics in preventing the development of gastric cancer after *H. pylori* eradication[52].

FD

FD is defined as the presence of symptoms that are thought to originate in the gastroduodenal region, in the absence of any organic, systemic or metabolic disease that is likely explain the symptoms. Because of the high prevalence and recurrent nature of symptoms, FD is a clinical problem of considerable magnitude for healthcare. According to the Rome IV criteria[53], there are two subtypes of FD: Postprandial distress syndrome (PDS) with postprandial fullness or early satiation, and epigastric pain syndrome (EPS) with epigastric pain or epigastric burning. The symptoms must be severe enough to affect daily activities, and must be present for > 3 mo with the onset of symptoms at least 6 mo before the diagnosis. While the exact pathophysiology of FD remains to be clarified, gastric motility disturbance (*e.g.*, impaired gastric accommodation and delayed gastric emptying) and visceral hypersensitivity have been suggested as critical underlying mechanisms (Figure 5)[54]. Recently, accumulating evidence supports that the duodenum is a target region underlying the pathophysiology of FD[55]. Impaired mucosal integrity and low-grade inflammation in the duodenum are thought to be associated with altered neuronal signaling and mucosal immune activation in this region. This eventually result in the uncontrolled motile and sensory mechanisms in FD. In addition, gastric acid, bile, food and microbiota are considered to induce and/or aggravate such underlying disorders in FD.

There is evidence to suggest that dysbiosis of intestinal microbiota is involved in the pathogenesis of irritable bowel syndrome (IBS) [a functional gastrointestinal disorder (FGID) originating in the intestine] [56]. However, the role of the gastroduodenal microbiota in the pathophysiology of FD (an FGID originating from the stomach and possibly the proximal small intestine) remains to be clarified. *H. pylori*

infection had been considered to be involved in the pathogenesis of FD-like symptoms that are often observed in these subjects. While FD-like symptoms in some *H. pylori*-infected patients are alleviated by antimicrobial eradication therapy, the improvement of the symptoms might not be mediated by the elimination of *H. pylori* but by the effect of antimicrobials on non-*H. pylori* bacteria in the stomach and proximal small intestine[57]. Indeed, Miwa *et al*[58] demonstrated that the curative treatment of *H. pylori* infection in eradication therapy was not significantly accompanied by the improvement of symptoms in a double-blind placebo-controlled clinical test. The involvement of an *H. pylori*-independent mechanism in the pathogenesis of FD is also suggested by a clinical study of probiotics. When the effect of an LG21 strain on FD-like symptoms was examined in *H. pylori*-infected patients, the severity of PDS after LG21 treatment was significantly lower than that was before treatment, while laboratory tests indicating the number and activity of *H. pylori* colonizing the stomach showed no significant difference between before and after the treatment[59]. These results suggested that bacteria other than *H. pylori,* which are resident in the GI tract, play an important role in the pathophysiology of FD. Tan *et al*[60] reported that the oral administration of the antimicrobial refaximin to patients with FD induced adequate relief of PDS. This implied the involvement of dysbiotic microbiota in the pathogenesis of FD. Nakae *et al*[61] compared the structure of the microbiota in GF between 44 FD patients and 44 healthy control subjects. A PERMANOVA test showed that the overall bacterial community structures of the two groups were significantly different (P = 0.001). In the bacterial composition analysis using those samples[62], the accumulation of bacteria that usually colonize the intestine, such as *Bacteroides*, *Bifidobacterium* and *Escherichia,* was often found in the GF of FD patients. As bile acids are also detected in these GF samples, the reflux of small intestinal contents, including bile and proximal small intestinal bacteria, to the stomach was suggested to induce such changes in the bacterial composition. Small intestinal bacterial overgrowth (SIBO) is broadly defined as an increase in the number of bacteria in the proximal small intestine together with various types of GI symptoms[63]. More than 60% of Japanese patients with FD have been reported to have overlapping IBS, in which SIBO is considered a critical etiological factor[64]. Thus, FD patients, whose GF contained large numbers of intestinal-type bacteria, might also suffer from SIBO. It is likely that the duodenal mucosa is injured by bile and/or the bacteria-especially Gram-negative bacteria such as *Escherichia*-in the content that is refluxed from the small intestine. Such mucosal damage would cause both deranged duodenal mucosal integrity and low-grade inflammation in the mucosa (Figure 5). Their studies also showed that 12-wk treatment of FD patients with an LG21 strain was effective for significantly improving symptoms, and shifted the composition of the GF microbiota to that observed in healthy subjects, whose GF microbiota no longer included any intestinal-type bacteria[61,62]. Therefore, the disappearance of such dysbiotic intestinal-type bacteria may be attributable to the resolution of SIBO after LG21 treatment.

Interestingly, there was also a significant inverse correlation between the differential abundance of *Prevotella* and the improvement of PDS symptoms in the FD patients treated with LG21[61]. That is; a greater increase in the relative abundance of *Prevotella* in GF after treatment was associated with a higher degree of symptom improvement. A significantly higher abundance of *Prevotella* on the duodenal mucosa was also observed in healthy control subjects in comparison to patients with FD[65]. Given that the genus *Prevotella* is sensitive to bile[66], the increase in the abundance after the treatment in FD patients may reflect a lower frequency of bile reflux, while lower abundance in FD patients may reflect a higher frequency of bile reflux.

To evaluate the clinical efficacy of treatment with a probiotic LG21 strain in FD patients, a randomized placebo-controlled clinical trial was performed using 116 individuals without *H. pylori* infection[67]. Participants were assigned to ingest yogurt containing 10^9 CFU of LG21 (LG21 group) or LG21-free yogurt (placebo group) every day. According to a questionnaire on the severity of FD symptoms, a trend toward a positive overall ameliorative effect on FD symptoms was observed in the LG21 treatment group (P = 0.07). Moreover, after treatment, the elimination rate for 4 major FD symptoms (postprandial fullness, early satiety, epigastric pain and epigastric burning) was 17.3% in the placebo group and 35.3% in the LG21 group, respectively (P = 0.048).

Although the beneficial effect of LG21 on FD was demonstrated by the clinical trial, the underlying mechanism remains to be clarified. One possible mechanism is protection of the duodenal mucosa from injurious intestinal bacteria and bile by through the resolution of SIBO and/or frequent duodeno-gastric reflux, as mentioned above (Figure 5). Considering that acid-suppressive therapy is so effective and has thus been recommended as the first-line treatment for FD[68], another mechanism underlying the effects of LG21 treatment may be a reduction of gastric acid production. Nakae *et al*[61], reported that the mean pH values (IQR) of GF of FD patients (n = 44) before and after the treatment were 1.58 (1.43-1.85) and 1.84 (1.56-3.81), respectively. Although this difference was not so great, it was statistically significant (P = 0.012). Given that hypersensitivity of the gastroduodenal mucosa is a critical pathophysiology underlying FD, it is reasonable that even a small reduction of gastric acidity by LG21 treatment can attenuate the gastric sensory and motor disturbances, which would then lead to an improvement of PDS and EPS symptoms. Similarly, in addition to PPI treatment, H-2 receptor antagonist (H2RA) treatment is effective for FD, although the efficiency of acid suppressive by H2RA is considerably lower in comparison to PPIs[55]. The moderate decrease in the secretion of gastric acid observed with LG21 treatment may be attributable to a reduction in the expression of gastrin (an acid secretion stimulating hormone) as the oral administration of LG21 has been reported to reduce the gastrin exression in the

murine system[69]. However, LG21 treatment did not reduce the serum gastrin concentration at all in long-term PPI users, who showed very high gastrin levels (> 200 pg/mL)[70]. This means that the suppressive effect of LG21 is no longer exerted in subjects with high gastrin levels such as PPI users and possibly patients with corpus-dominant atrophic gastritis, in whom the serum gastrin concentration is abnormally high due to the secondary response to very low intragastric acidity. Emerging data increasingly point toward the role of gastroduodenal microbiota in the pathophysiology of FD. Accordingly, the application of probiotics in the treatment of this regions is expected to be successful.

CONCLUSION

Probiotics for the stomach have been demonstrated to suppress *H. pylori* in the stomach, and thus improve eradication rate in patients who receive antimicrobial treatment. If probiotics strains are sufficiently resistant to the gastric acidity and able to achieve close proximity to the gastric mucosa, they are also expected to prevent the development of gastric cancer, even after *H. pylori* eradication, through the correction of the dysbiotic gastric microbiota. If a deranged gastric bacterial population is involved in the pathophysiology of functional dyspepsia, the use of such probiotics may be useful for the treatment of this functional gastroduodenal disorder.

FOOTNOTES

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MINIREVIEWS

Liver injury in COVID-19: A minireview

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Abstract

Coronavirus disease 2019 (COVID-19), caused by infection with the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has escalated into a global tragedy afflicting human health, life, and social governance. Through the increasing depth of research and a better understanding of this disease, it has been ascertained that, in addition to the lungs, SARS-CoV-2 can also induce injuries to other organs including the liver. Liver injury is a common clinical manifestation of COVID-19, particularly in severe cases, and is often associated with a poorer prognosis and higher severity of COVID-19. This review focuses on the general existing information on liver injury caused by COVID-19, including risk factors and subpopulations of liver injury in COVID-19, the association between pre-existing liver diseases and the severity of COVID-19, and the potential mechanisms by which SARS-CoV-2 affects the liver. This review may provide some useful information for the development of therapeutic and preventive strategies for COVID-19-associated liver injury.

Key Words: Liver; SARS-CoV-2; Angiotensin-converting enzyme 2; Transmembrane serine protease 2; Chronic liver disease

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Core Tip: The global pandemic of coronavirus disease 2019 (COVID-19) has imposed a great threat to human health and become a medical and social challenge. Although severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) mainly affects the respiratory tract, it also frequently damages the liver especially in severe and critical cases. Direct hepatotoxicity of SARS-CoV-2, or indirect hepatic injury caused by immune overactivation and systemic inflammation, drug-induced injury, ischemia/reperfusion and hypoxia/reoxygenation injuries, and worsening of preexisting liver diseases, are potential contributing factors to liver damage in COVID-19.

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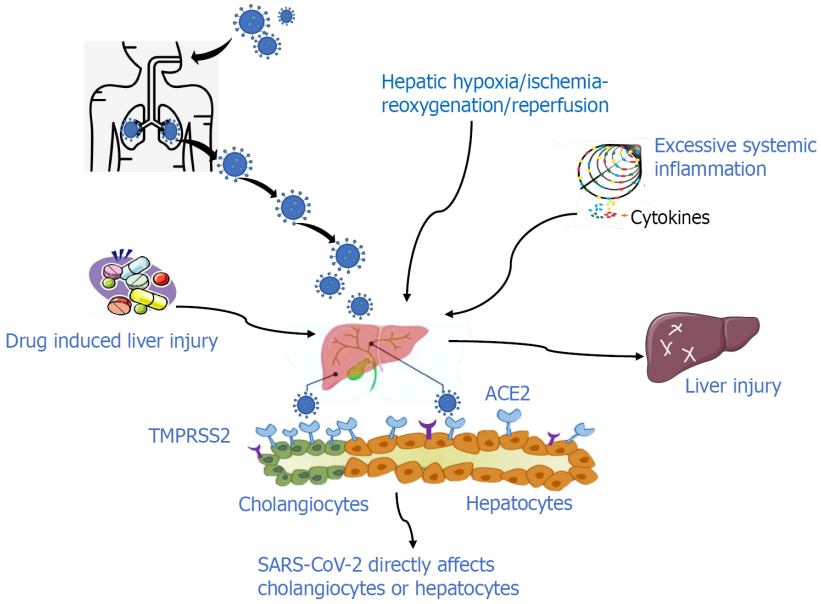
INTRODUCTION

In December 2019, an outbreak of the novel coronavirus disease 2019 (COVID-19) was reported in Wuhan, Hubei Province, China, induced by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). COVID-19 has evolved into a global health challenge[1], posing an enormous threat to human health and economic development. Severe COVID-19 patients may present symptoms of acute respiratory distress syndrome (ARDS), requiring admission to the intensive care unit (ICU) and oxygen ventilation therapy. The shortest time from admission to ARDS is approximately 2 d. At this stage, COVID-19 mortality is extremely high[2]. Global SARS-CoV-2 infection is now widespread, with 601189435 confirmed COVID-19 cases, including 6475346 deaths reported until September 3, 2022 by the WHO[3]. Most COVID-19 patients exhibit mild symptoms (fever, cough, shortness of breath, fatigue, vomiting, diarrhea, anosmia, and headache), whereas critical cases may develop into severe illness and even death due to severe lung injury and respiratory failure, liver injury, cardiac injury, septic shock, and even multi-organ failure[4].

Although COVID-19 mainly affects the respiratory tract, researchers have focused on the impacts of SARS-CoV-2 on other organs[5]. Liver injury is common in COVID-19 and is associated with poor prognoses. Liver test abnormalities, including higher levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), are frequently observed in severe and critical COVID-19 cases compared to mild and moderate cases[6]. The most common manifestation of liver injury in COVID-19 is acute hepatitis with elevated AST, ALT, and total bilirubin levels[7]. Moderate microvascular steatosis and slight lobular activity are commonly observed in the liver biopsies[8]. Possible mechanisms include direct SARS-CoV-2 infection in hepatocytes or bile duct epithelial cells, excessive inflammation, drug-induced liver injury (DILI), ischemia/reperfusion syndrome, and liver injury associated with preexisting liver disease. Angiotensin-converting enzyme 2 (ACE2) is known to be the most important receptor for SARS-CoV-2 in contact with the host cell membrane, while transmembrane serine protease 2 (TMPRSS2) also plays an essential role in mediating viral entry. TMPRSS2 can prime the spike protein (S protein), and has been considered as a target for designing TMPRSS2 inhibitors to block virus entry as a new therapeutic approach[9]. When cells are infected by SARS-CoV-2, the S protein of the viral particles must be cleaved by TMPRSS2 and then binds to ACE2, so that viral particles can fuse with the plasma membrane and enter host cells[10]. Lower ACE2 expression has been detected in hepatocytes, while biliary epithelial cells express abundant ACE2 and thus can serve as a binding site for SARS-CoV-2. It has also been shown that most viruses that infect the respiratory tract can damage hepatocytes by affecting the $CD8^+$ mediated immune response[11]. Common risk factors, such as older age, male sex, and a range of potential comorbidities, including hypertension, obesity, diabetes, and underlying liver diseases, can also lead to varying degrees of liver damage, critical illness, and even death. However, the exact connection between comorbidities and liver injury caused by SARS-CoV-2 remains unclear. This review summarizes the pathophysiology, possible mechanisms, clinical manifestations, risk factors, and special populations of liver injury in COVID-19 patients.

PATHOPHYSIOLOGY OF LIVER INJURY IN COVID-19

The mechanism of liver injury in COVID-19 remains largely unknown. SARS-CoV-2 may induce direct hepatotoxicity after entering into the liver *via* bile duct cells which express high levels of the ACE2 receptor. SARS-CoV-2 may also indirectly injure the liver *via* immune overactivation, systemic inflammation, drug toxicity, and hepatic hypoxia/reoxygenation or ischemia/reperfusion due to respiratory failure and endothelial damage (Figure 1). The liver is the main organ for detoxification and metabolism, and liver injury can reflect the severity and clinical course of COVID-19. Therefore, it is



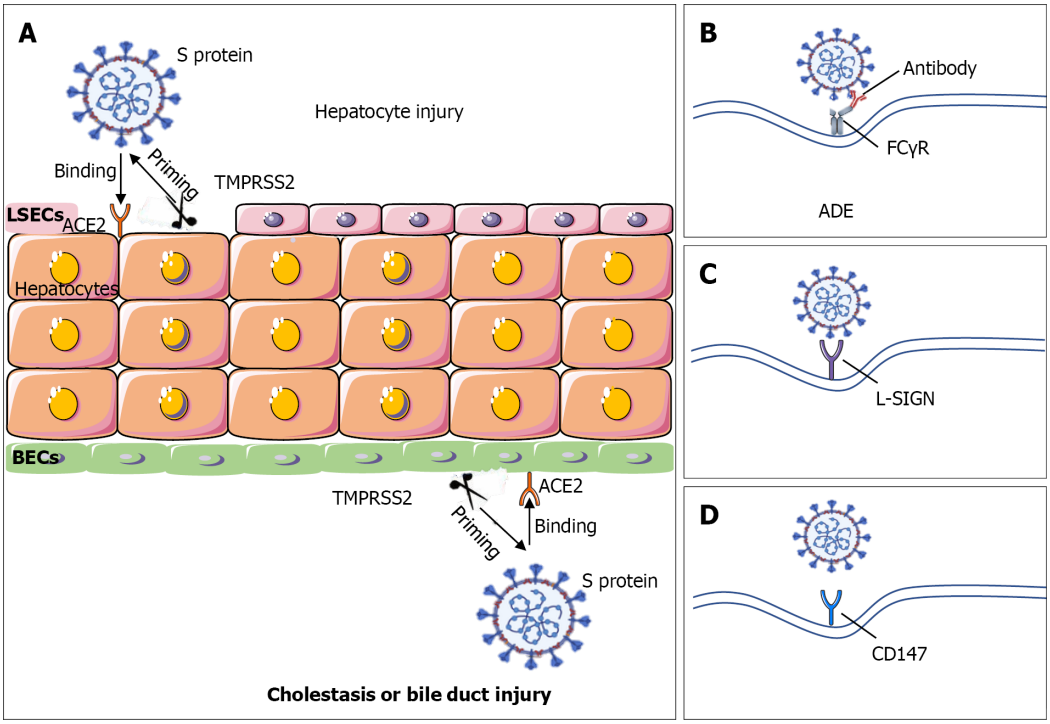
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Figure 1 Mechanisms of liver injury caused by SARS-CoV-2. The mechanisms include direct hepatoxicity (severe acute respiratory syndrome coronavirus 2 affects cholangiocytes or hepatocytes) or indirect hepatic injury (drug-induced liver injury, excessive systemic inflammation and cytokine storm, and deterioration of pre-existing liver disease). SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane serine protease 2.

crucial to understand the mechanisms underlying liver injury in COVID-19 to develop effective treatments.

Direct effect of SARS-CoV-2 on the liver

Previous research has established that the ACE2 receptor can specifically bind to the S protein, which has a receptor binding domain (RBD) to mediate SARS-CoV-2 entry into host cells. Although the binding pattern of the SARS-CoV-2 RBD-ACE2 complex is greatly analogous to that of the SARS-CoV RBD-ACE2 complex, ACE2 has a higher affinity for the RBD of SARS-CoV-2[12]. This may be one possible reason why the SARS-CoV-2 is more dangerous than the other emerging SARS-CoVs. However, the level of ACE2 in the liver tissue is much lower than that in the bile duct based on previous data analysis, which suggests that SARS-CoV-2 may bind to ACE2-positive bile duct cells, but not hepatocytes[13]. Another possibility is that ACE2 can sense viral entry and upregulate its expression in hepatocytes[14]. Current evidence indicates that bile duct cells are actively involved in immune defense, inflammatory response, and liver regeneration, which may be a possible explanation for virus-induced liver injury once these cells are damaged. Cholangiocytes can co-express ACE2 and TMPRSS2, and are susceptible to viral infections[13]. Elevated levels of gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) are caused by bile duct cell injury[15]. A previous study reported the histological, ultrastructural, and immunohistochemical staining of liver biopsies performed on two patients who died of COVID-19. The researchers discovered a mass of viral particles of SARS-CoV-2 in the hepatocyte cytoplasm, and most of the viral particles had an intact coronoid envelope, indicating that SARS-CoV-2 can not only enter, but also replicate in the hepatocytes[14]. Another report also presented detailed liver histological results from two patients with acute COVID-19, and found extensive mitosis especially in the cholangiocytes, in addition to mixed inflammatory infiltrates in the portal region, endodermatitis, and severe bile duct injury[16]. Another study of three patient cohorts provided evidence of SARS-CoV-2 liver tropism. In autopsy reports from the third cohort of patients, viral RNA was detected in 69% of autopsy liver specimens and SARS-CoV-2 carrying infectiousness was detected in post-mortem liver tissues[17]. Regardless of whether SARS-CoV-2 directly affects cholangiocytes or hepatocytes, all the above studies support that the liver injury caused by SARS-CoV-2 is a direct cytopathic injury[16] (Figure 2A). Furthermore, obvious mitochondrial swelling, endoplasmic reticulum dilatation, and impaired cell membranes were also observed in two COVID-19 cases, suggesting cytopathic damage. However, pathological changes including moderate microvascular steatosis and slight lobular and portal inflammatory infiltration are non-specific in viral infection and can be caused by DILI or chronic liver disease (CLD) such as non-alcoholic fatty liver disease (NAFLD) [18]. In addition, no viral inclusion bodies were observed in the liver tissue of COVID-19 patients. In summary, it is still unclear whether SARS-CoV-2 directly causes cytopathic changes in liver cells.



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Figure 2 Possible pathways of SARS-CoV-2 entering into the liver. A: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects host cells through angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) receptors by its S protein. Direct SARS-CoV-2 infection targeted to hepatocytes or billiary epithelial cells results in hepatocyte injury or bile duct injury; B and C: CD147 (B) and L-SIGN (C) may be alternative receptors for SARS-CoV-2 entry into the liver; D: Antibody dependent enhancement is a pathway which can enhance interaction of virus-based antibody and the CR and/or FC receptor complements further making virus easily entry and infection. ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane serine protease 2; LSECs: Liver sinusoidal endothelial cells; BECs: Biliary epithelial cells.

Systemic inflammatory response and cytokine storm in COVID-19

Severe COVID-19 is characterized by a systemic inflammatory response that may cause a cytokine storm leading to multi-organ failure. Current evidence has shown that serum inflammatory cytokine levels are positively correlated with the indicators of liver dysfunction in COVID-19 patients[19]. This suggests that systemic inflammatory response and cytokine storm are also involved in liver injury, with a possible underlying mechanism between them. A previous study has shown that the activation and dysregulation of $CD8^+$ T cells in severe patients may be an important factor for the pathogenesis of SARS-CoV-2 infection, as $CD8^+$ T cells in critically ill patients express high levels of cytotoxic molecules [20]. A cohort study of 133 COVID-19 patients with liver damage reported high levels of inflammatory cytokines [TNF-α, interleukin (IL)-2, IL-6, and IL-10] and low levels of T lymphocyte subsets ($CD3^+$, $CD4^+$, and $CD8^+$ T cells). IL-2, IL-6, IL-10, $CD4^+$, and $CD8^+$ T cells can be regarded as possible independent predictors of hepatic injury in COVID-19 patients[21]. This is consistent with an alternative study, which also revealed that the elevated levels of IL-2, IL-6, and IL-10 in the serum of COVID-19 patients were associated with the progression of severe disease[22]. Notably, IL-6 is particularly important for liver injury, because IL-6-mediated procoagulant endotheliopathy with increased hepatic von Willebrand factor (vWF) expression and platelet accumulation are linked to liver injury and liver inflammation (elevated ALT and neutrophil infiltration). In addition, IL-6 plays a potential role in hepatic endothelial dysfunction and inflammation because its level is correlated with vWF level[23]. IL-6 is associated with elevated liver enzymes, but the relationship is not necessarily causal, as IL-6 can be used to accurately detect inflammatory responses to liver injury[24]. IL-6 is activated primarily by the pathway of JAK (Janus kinase)/STAT (signal transducer and activator of transcription). Baricitinib, an inhibitor of the JAK/STAT pathway, can improve the clinical outcomes of COVID-19[25]. JAK inhibition can affect the viral entry and inflammation of COVID-19[26]. Previous studies have established an association between increased levels of endotoxin, ILs, and TNF-α in COVID-19 patients with liver function damage compared with those with normal liver function[27]. COVID-19 patients with elevated ALT levels also have increased IL-6, ferritin, lactate dehydrogenase (LDH), and C-reactive protein (CRP) [14]. Furthermore, significantly increased levels of IL-2, IL-7, IL-10, granulocyte colony-stimulating factor (G-CSF), and IP-10 (interferon-inducible protein-10) have been observed in severe patients compared with mild or moderate patients[22]. In conclusion, patients with severe COVID-19 may exhibit intense inflammation and cytokine storm syndrome leading to liver injury.

DILI in COVID-19

An additional opinion is that liver injury in COVID-19 patients is related to hepatotoxicity. DILI cannot be overlooked, as it may contribute to abnormal liver function, such as elevated ALT and ALP levels, and subsequently affect drug metabolism and excretion[28]. In addition, histopathologic findings from liver biopsies of COVID-19 patients, such as microvascular steatosis and liver inflammation, may also be associated with DILI[18]. Recent evidence suggests that DILI may occur secondarily to the drugs commonly used for COVID-19 treatment, such as paracetamol, antiviral therapies, low molecular weight heparin, anti-IL-6 receptor agents, and antibiotic treatments[29]. This is consistent with another study, in which corticosteroids and immune modulators were also mentioned[18]. Notably, NAFLD patients are more likely to develop DILI, because NAFLD can increase the sensitivity of the liver to hepatotoxicants, such as acetaminophen[30]. Antibiotics and nonsteroidal anti-inflammatory drugs are considered one of the most common causes of DILI[31]. One previous study showed that, when receiving glucocorticoid therapy, more patients had a liver injury (58.1%) than those without (39%)[32], and, tocilizumab (TCZ)-induced liver injury was reported in one COVID-19 patient. TCZ, also known as an IL-6 inhibitor, is recommended for the treatment of COVID-19 due to its vital role in inducing cytokines, and increased IL-6 can predict the fatal outcome of COVID-19. In this case, when TCZ was administrated, serum aminotransferase levels increased by nearly 40-fold on the first day, and after 10 d of DILI formation, aminotransferase levels returned to normal. Therefore, it is no doubt that TCZ has positive effects on other clinical and laboratory parameters of cytokine storm, such as CRP, IL-6, fibrinogen, and D-dimer, resulting in transaminase levels close to the normal range[33]. In brief, it is necessary to emphasize the importance of drug-related liver damage in patients with COVID-19, especially in those with underlying liver diseases.

Hypoxia/reoxygenation and ischemia/reperfusion-induced liver damage

COVID-19 is primarily characterized by respiratory failure, thus, severe cases of hypoxic hepatitis are commonly seen, and 10% of the cases suffer from a hypoxic liver injury in the ICU[34]. Hypoxic hepatitis, also known as ischemic hepatitis or shock liver[35], is accompanied by a rapid elevation of aminotransferases in cases of respiratory failure, shock, or heart failure. One possible reason is that the complex vascularization of the liver makes it susceptible to changes in circulation, resulting in decreased liver perfusion[31]. It has been shown that SARS-CoV-2 also causes liver damage by producing diffuse endodermatitis. Viral inclusion structures can also be observed in endothelial cells. Liver ischemia/reperfusion, including ischemia-induced cell injury and reperfusion-induced inflammatory responses resulting from the activation of neutrophils, Kupffer cells, and platelets, can induce the generation of reactive oxygen species (ROS) and calcium overload. Endothelial cells are involved in liver ischemia/reperfusion damage, which can promote oxidative stress through ROS and derivatives of nitric oxide[18]. ROS and lipid peroxidation products can mediate the production of redox-sensitive transcription factors, which in turn induce the release of abundant pro-inflammatory factors, resulting in hepatic injury[31]. Furthermore, hepatic sinusoidal endothelial cell damage has been reported to further aggravate hepatic ischemia and hypoxia by disturbing microcirculation[36]. In addition, a high level of positive end-expiratory pressure may be a possible contributor to liver injury in COVID-19 patients, because it can increase right atrial pressure and obstruct venous return, leading to hepatic stasis[37]. These findings indicate that hepatic hypoxia/reoxygenation and ischemia/reperfusion may be potential etiologies of COVID-19-related liver injury.

The ACE2-independent pathway of liver injury

In addition to receptor-mediated viral entry, antibody dependent enhancement (ADE) may also partially associated with hepatic injury in COVID-19 patients. ADE is a pathway that can enhance the interaction of virus-based antibodies, and the CR and/or FC receptor complements allow the virus to easily come into contact with macrophages, granulocytes, and monocytes. This results in the virus multiplying and increasing production, causing the infection to worsen. It has been identified that ADE can be activated by SARS-CoV antibodies, thus, SARS-CoV can trigger ADE activity in immune cells, even though the immune cells do not express ACE receptors (Figure 2B). However, there is a lack of discussion concerning the ADE pathway of liver injury, thus the ACE2-independent mechanisms of liver damage in COVID-19 remain unclear and warrant further study[27].

There is alternative hypothesis that liver cells express two receptors, CD147 and L-SIGN, which have an affinity for the S protein of SARS-CoV-2, and thus may mediate the infection of liver cells by SARS-CoV-2. The presence of CD147 on the surface of host cells may be a new way for SARS-CoV-2 invasion [38]. It has been experimentally verified that CD147 directly interacts with SARS-CoV-2 and that blocking CD147 can prevent SARS-CoV-2 replication, whereas massive expression of CD147 can promote SARS-CoV-2 replication[39]. These findings suggest that CD147 mediates the entry of SARS-CoV-2 into liver cells. However, this requires further exploration (Figure 2C). Additionally, L-SIGN serves as a liver-specific membrane receptor associated with viral capture. Autopsy studies have shown that L-SIGN receptors were present in SARS-CoV-2-infected sinusoidal cells[40] (Figure 2D). Therefore, L-SIGN may provide an alternative way for SARS-CoV-2 to invade the liver tissue.

Clinical manifestation of liver injury in COVID-19

Liver injury is common in severe COVID-19 cases, as shown by significant circulatory elevations in hepatocyte enzymes including ALT and AST, and a slight increase in cholangiocyte-related enzymes such as GGT and ALP. Mao *et al*[41] analyzed 35 studies involving 6686 COVID-19 patients, among which one in five patients developed liver function abnormalities, especially in severe cases. Therefore, paying close attention to liver function in COVID-19 patients before and during admission is necessary to control the severity of COVID-19. The main findings of the liver biopsy were moderate microvascular steatosis and mild lobular and portal activity. Other specific clinical manifestations of liver injury warrant further investigation.

Abnormal liver function tests

Liver test abnormalities are frequent in COVID-19 patients and often progress to severe illness. Liver function tests include hepatocyte injury markers ALT and AST, cholestasis or bile duct injury markers GGT and ALP, and measures of synthetic capacity [prothrombin time (PT) and albumin (ALB)]. One comprehensive study has described the results of liver tests from 417 patients with COVID-19 and found elevated levels of ALT and GGT (24%), AST and total bilirubin (TBIL, 12% and 15%), and no increase in ALP[42]. Such elevated GGT and normal ALP cannot be attributed to bile duct type but are possibly drug-induced liver injuries. GGT elevation is also considered to be a marker of substitution of excessive oxidative stress and increased inflammation due to acute inflammatory stress. However, by analyzing a small subset of COVID-19 patients with possible persistent abnormalities over 60 d, one study found that the predominant abnormality was a cholestatic pattern, as indicated by elevation of the ALP/GGT ratio[43]. Although GGT is not a specific marker of liver injury, liver damage may result from direct bile duct cell injury and cholestasis induced by SARS-CoV-2[43]. Many studies in China revealed that COVID-19 patients who used lopinavir/ritonavir during hospitalization had liver injuries that influenced liver tests[44]. A cohort study including 1040 COVID-19 patients (mean age 38 years old, 54% men) revealed that drugs used for treatment, such as lopinavir-ritonavir, interferon beta, and/or corticosteroids, are correlated with an elevation in the ALT/AST ratio, which acts as an independent contributor to worse clinical outcomes[45]. Using a random effect method and an inverse variance approach, a meta-analysis was conducted using 128 studies, identifying that the most common abnormalities were hypoalbuminemia [61.27% (48.24-72.87)], increased levels of GGT [27.94% (18.22-40.27)], ALT [23.28% (19.92-27.01)] and AST [23.41% (18.84-28.70)][46]. Notably, hypoalbuminemia is an independent risk predictor of liver dysfunction and mortality upon admission[47]. One previous study analyzed 2623 confirmed COVID-19 cases at different risk levels, including non-critically ill, critically ill, and deceased groups[48]. This study revealed that hypoalbuminemia may be due to hepatotoxicity of the cytokine storm. In addition, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels were significantly lower in the critically ill and dead groups than in the non-critically ill group. Furthermore, patients with abnormal liver function had higher inflammatory indices, such as a high level of CRP which is associated with disease severity. Patients with an increased level of ALP have significantly elevated CRP levels, and CRP levels are correlated with ALT levels[49]. Notably, D-dimer, LDH, creatine kinase, and troponin levels are also higher in patients with abnormal liver function tests than in those with normal liver function[50]. A retrospective study analyzed 216 patients diagnosed with COVID-19 and concluded that elevated LDH is an independent contributing factor to ICU admission and may require mechanical ventilation[51]. However, the increase in serum LDH does not specifically reflect cellular damage in the liver, because LDH is present in many organs.

In summary, abnormal liver function tests are frequent in COVID-19 patients, which are secondary to other injuries, mostly ischemia or drug-induced liver damage. Patients with abnormal liver tests have extended hospital stay and are at higher risk of developing severe disease; however, this does not directly leading to death[52].

Hepatic histopathological findings in COVID-19

Hepatic histopathological findings in patients who died from COVID-19 revealed moderate microvascular steatosis in addition to mild lobular and portal activity. This is consistent with other studies suggesting that the changes are non-specific, and liver injury may be due to SARS-Cov-2 or drugs, but not caused by cholestasis[8,53,54]. Some studies have found sinusoidal thrombosis, which is less common and is mostly observed in severe COVID-19, because COVID-19 is a prothrombotic disease [55,56]. A case study of the liver autopsy of an elderly patient with COVID-19 found no obvious inflammation in the portal area, but observed a few hepatocytes with slight vesicular steatosis and watery degeneration, possibly associated with ischemia and hypoxia[42]. Inflammatory cells, including neutrophils, plasma cells, and Kupffer cells, were observed in the hepatic sinuses at a higher magnification. In addition, numerous scattered or apoptotic hepatocytes characterized by nuclear condensation or formation of apoptotic bodies were also observed in liver biopsy tissues, but no viral inclusions were observed[14], similar to the results of Wang *et al*[32]. Based on these studies, direct effect of SARS-CoV-2 on hepatocytes seems less convincing. Notably, a report on the histopathological findings of 40 patients who died from COVID-19 revealed that mild lobular necroinflammation was present in 20 (50%) cases, with few infiltrations of lymphocytes and histiocytes[55]. Hepatocyte

degeneration with focal necrosis and low infiltrations of leukocytes in the lobular and portal areas have also been observed in an alternative liver biopsy study of deceased COVID-19 patients[57]. Liver biopsies from three patients and necropsies from three others revealed striking iron accumulation in hepatocytes, and an abundance of ferritin particles in damaged mitochondria[58], suggesting that hepatic iron overload could have a potential relationship with liver injury. Post-mortem wedge liver tissues from 48 deceased COVID-19 patients suggest that different degrees of liver injuries are present, including liver blood involvement and acute (thrombosis and lumen expansion) and chronic (fibrous thickening of vessel wall and portal vein fibrosis) liver injuries[59]. In conclusion, the most common seen liver histopathological findings were hepatic steatosis, portal inflammation, and varying degrees of liver injury. However, related investigations into the mechanisms and etiologies of hepatic histopathological changes are still lacking.

Other special clinical manifestations

In addition to the elevated liver enzymes and histopathologic changes described above, other specific manifestations of liver damage in COVID-19 patients have also been reported, including a darkened face and pigmentation. Pigmentation may be a result of abnormal liver metabolism *via* the following mechanisms. First, liver dysfunction can increase estrogen levels, thereby damaging the inhibitory effect of thiamine on tyrosinase, so that more tyrosine is transformed into melanin. Second, abnormal liver function may result in hypofunction of adrenocortical hormones, and melanin secretion is increased due to the elevation of the melanocyte-stimulating hormone. In addition, more iron may enter into the blood vessels due to liver damage, increasing the ion level in the blood circulation to the facial skin and resulting in a darkened face[27].

COVID-19-INDUCED LIVER INJURY IN SPECIAL POPULATIONS

Risk factors of severe COVID-19 and liver injury

A growing body of studies suggests that male sex, older age, and potential comorbidities, such as hypertension, obesity, diabetes, cardiovascular diseases, and respiratory diseases, and particularly obesity and type 2 diabetes, are more likely to be associated with critical COVID-19 after infection by SARS-CoV-2[60]. One study involving 174 consecutive COVID-19 patients shows that 24 of them had no other comorbidities but diabetes. By comparing the diabetic group with the non-diabetic group, it was found that COVID-19 patients with diabetes more easily experienced an overactivated inflammatory response and a hypercoagulable state, resulting in a worse COVID-19. The mechanism could be that COVID-19 patients with diabetes are easy to develop inflammatory storm that rapidly worsens COVID-19 due to high blood levels of inflammatory factors, such as IL-6, CRP, serum ferritin, coagulation index, and D-dimer[61]. These proinflammatory cytokines were strongly correlated with liver injury, so it is worth paying attention to whether patients with a history of diabetes are more susceptible to liver injury. Age is by far the most associated risk factor of COVID-19, with the risk increasing gradually after 65 years[62]. This may be due to T cell and B cell functions which show age-dependent defects, as well as inadequate control of viral replication due to overproduction of type 2 cytokines and prolonged inflammatory responses[63]. Compared to infected adults, infected children usually present with mild clinical symptoms, and are mostly asymptomatic[64]. This may be because of their immature immune systems. Past reports have confirmed that children with abnormal liver function tests such as increased ALT usually have a preexisting condition such as an immunocompromised state (including malignancy) or CLD[65]. Patients with poor underlying liver conditions, such as cirrhosis, hyperplasia, non-alcoholic steatohepatitis, and mild steatosis, could have high expression of ACE2 than those without liver diseases[66]. Preexisting liver disease and age ≥ 60 years are risk factors for progressing to severe COVID-19[67]. Besides, male sex is linked to the severity of COVID-19 and has been identified as an important etiology of liver injury, possibly due to the lack of the protective effect of estrogen on the liver [68], or the enhancement of male innate immunity and the activation of T cells[62].

Notably, patients with obesity represent a dysregulated hepatic innate immunity, adaptive immune response, and pro-inflammatory state, thus aggravating the cytokine storm caused by SARS-CoV-2 infection, resulting in worse prognoses of COVID-19[60]. To evaluate the relationship between obesity and COVID-19, a meta-analysis was conducted using 50 studies in total with a total of 18260378 eligible subjects. It was found that obesity increased the chance of contracting SARS-CoV-2 and developing critical COVID-19. In addition, patients with a body mass index (BMI) ≥ 30 were 1.39 times more susceptible to SARS-CoV-2 infection compared with those with normal body weight, and the hospitalization rate of COVID-19 became higher with increasing BMI[69]. However, fatty liver is not a predisposing factor for hepatic injury after SARS-CoV-2 infection compared with the susceptibility rate of the general population[70].

In addition to the above risk factors, chronic heart disease, excessive inflammatory response, extended prothrombin time (PT), elevated liver enzymes, and high levels of bilirubin may also be related to severe COVID-19 and mortality[71]. In conclusion, the sex of the patient (males), older age, and comorbidities were independent risk factors for the rapid aggravation of COVID-19. Patients older

than 60 years with a history of diabetes have an increased risk of mortality[72]. However, the exact association between liver injury and risk factors remains unclear.

Preexisting liver diseases and severity of COVID-19

CLD is deemed to be a major burden of disease and a threat to human worldwide. CLD mainly includes cirrhosis, alcoholic liver disease, non-alcoholic fatty liver disease, and chronic hepatitis B, which affects nearly 300 million people in China. Because of this high burden, it is necessary to carefully assess the relationship between underlying liver disease and liver injury in patients with COVID-19[73]. However, the extent to which CLD affects the liver function and the disease ending in COVID-19 is controversial. On the one hand, patients with CLD are thought to be more susceptible to viral infection and developing liver injury due to an altered immune status. On the other hand, previous studies reveal that CLD is rarely related to the progression of liver injury or critical/fatal outcomes of COVID-19 (Table 1)[74]. Therefore, the connection between the underlying liver disease and COVID-19 requires further investigation.

Cirrhosis and COVID-19

Cirrhotic patients have complex immune disorders and are more susceptible to infection, and the leading causes of mortality are not only respiratory complications but also deterioration of liver function, leading to end-stage liver disease. Patients with cirrhosis are not only at risk of related immune dysfunction, but also are more likely to have comorbidities that predispose them to severe COVID-19, such as diabetes, chronic kidney disease, and heart disease[75]. However, there is a debate about the risk of cirrhosis on the clinical outcomes of COVID-19. A multicenter retrospective study revealed that cirrhotic patients infected with COVID-19 have a high 30-d mortality rate[76]. In this study, elevated transaminases may have had an adverse influence on the process of cirrhosis, and ALT levels in cirrhotic patients were affected by SARS-CoV-2. Additionally, nearly half of the patients who previously had normal transaminases developed to an acute liver injury. Indeed, patients with impaired liver function have a higher 30-d mortality rate and more studies suggested that cirrhotic patients are more susceptible to infection resulting from immune dysfunction. SARS-CoV-2 damages the lymphocytes, especially T cells, resulting in impaired immune function and increased sensitivity to the viruses. Moreover, an elevated neutrophil-to-lymphocyte ratio (NLR) increases the risk of bacterial infection, which is related to stress and sepsis, particularly in decompensated liver cirrhosis leading to a worse outcome[67]. In a study of 386 patients with cirrhosis, 32% had a growing risk of mortality after SARS-CoV-2 infection. Patients with CLD without cirrhosis just have a similar risk of mortality as patients without liver disease[77]. In addition, cirrhotic patients with SARS-CoV-2 infection appear to have a high risk of developing acute hepatic decompensation (46%), as well as a 2-fold increased rate of mortality. These studies suggest that cirrhosis is closely linked to the disease progression from COVID-19.

Contrary opinions argue that CLD and cirrhosis have little correlation with the critical and mortality rates of COVID-19. One study found that there was no increase in liver damage after SARS-CoV-2 infection in CLD patients[18]. However, the results may be relatively low persuasive, because only three patients with cirrhosis were studied.

Hepatitis B and COVID-19

Recent evidence suggests that liver injury in patients co-infected with SARS-CoV-2 and hepatitis B virus (HBV) leads to increased disease severity and worse clinical outcomes[78]. In addition, liver test abnormalities often appear in patients co-infected with SARS-CoV-2 and HBV, with elevated levels of ALT, AST, ALP, and TBIL.

In addition, the largest multicenter retrospective study revealed several novel risk factors such as high levels of LDH, D-dimer, and reduced ALB or ALB/globulin (GLO), which increase the risk of COVID-19 severity and mortality in patients with chronic hepatitis B (CHB)[79]. An alternative study showed similar results: High levels of LDH, D-dimer, and IL-6 were more likely to worsen liver function in COVID-19 patients following HBV coinfection. Two weeks after the onset of symptoms, serum LDH levels were increased only in patients with COVID-19 and HBV coinfection compared with patients without HBV coinfection[80]. The conditions of four chronic HBV-infected patients were further aggravated after SARS-CoV-2 coinfection, as presented by progressive jaundice elevation, coagulation disorders, and ascites, and developed acute-on-chronic liver failure[78]. These findings suggest that SARS-CoV-2 and HBV coinfection is related to disease severity and poor clinical presentation, and affects liver function tests and that HBV inactive carriers have more severe liver damage. This may be due to the altered immune status caused by SARS-CoV-2 coinfection. Other possible reasons include inactive HBV carriers, who are more prone to HBV reactivation and liver injury with ALT flares caused by SARS-CoV-2 coinfection, and these patients have increased sensitivity to hepatotoxic antiviral drugs used for COVID-19 treatment[80].

There are opposing opinions that chronic HBV coinfection is not a predictive factor for the disease severity or poor prognosis of COVID-19. A study using a prognostic model with a nomogram indicated that HBV infection was not linked to the mortality risk of COVID-19[81]. Several studies have supported

this hypothesis. A cohort study of 326 confirmed COVID-19 patients shows that 20 (6.1%) had HBV coinfection, and the outcomes of the 20 patients (including rates of severe/critically ill, mortality, and discharged and hospital stays) showed no difference from those of patients without coinfection, and HBV coinfection did not increase the degree of liver injury[82]. This is consistent with the results of a previous review indicating that the degree of hepatic damage in patients with HBV and SARS-CoV-2 coinfection is not significantly different from that in patients with SARS-CoV-2 infection alone[83]. Therefore, whether or not HBV infection affects the clinical manifestations and outcomes of COVID-19, and how, requires further study.

Metabolic dysfunction-associated fatty liver disease and COVID-19

Metabolic dysfunction-associated fatty liver disease (MAFLD), previously defined as NAFLD, has been reported to be associated with increased ICU admissions and the need for mechanical ventilation after infection with SARS-CoV-2. However, it was not a predictive factor for death in COVID-19[18]. MAFLD is the most frequently occurring chronic disease in the world, with a common incidence of 30%, and is regarded as a liver performance of the metabolic syndrome. There is a distinct lack of research regarding the history of liver disease in COVID-19 patients and the connection between MAFLD and COVID-19[84]. A meta-analysis of COVID-19 comorbidities showed upregulation of ACE2 receptor expression in NAFLD[85]. A cohort study of 202 consecutive COVID-19 patients showed that patients with NAFLD had prolonged virus-shedding time and increased risk of disease severity compared to non-NAFLD patients[86]. In addition, a previous study has shown that younger patients (age < 60 years) with MAFLD have increased disease severity, possibly due to the higher incidence of cytokine storm caused by MAFLD in younger patients[87]. This is consistent with an alternative multicenter study that compared the risks of developing severe COVID-19 between younger and older patients with MAFLD. Results showed that 55.9% of the younger and 24% of the elderly developed severe COVID-19 (P = 0.01)[88], suggesting that younger MAFLD patients have an higher risk of developing severe COVID-19, while MAFLD in older patients appears to have little association with disease severity.

The mechanism underlying age-related disease severity in COVID-19 remains unclear. Possible explanations include: (1) Younger MAFLD patients are more likely to exhibit liver and systemic immune responses, and thereby a cytokine storm after SARS-CoV-2 infection; (2) older MAFLD patients usually have a higher burden of comorbidities which may harm COVID-19 and mask the link between MAFLD and COVID-19 severity[88]; (3) cytokine storm has been shown to increase in obese patients, especially in those with preexisting MAFLD[89]; (4) BMI is much higher in patients with NAFLD than in those without[90]; (5) MAFLD is also linked to abnormal levels of aminotransferases and GGT[89]; and (6) the connection between MAFLD and the severity of COVID-19 may be related to the release of pro-inflammatory mediators, such as, tumor necrosis factor-alpha and IL-6[91]. Notably, one study reported that the prognosis of MAFLD is closely related to the degree of fibrosis, and thus could affect the outcomes of COVID-19[63]. When MAFLD is accompanied by severe advanced liver fibrosis, it may exacerbate the cytokine storm caused by the virus, thus the release of proinflammatory cytokines leads to more severe COVID-19. In conclusion, unhealthy lifestyles contribute to the high morbidity of MAFLD and high risk of severe COVID-19.

Alcoholic liver disease and COVID-19

A previous study revealed an unprecedented increase in both ALD and liver transplant rates during the spread of COVID-19[92]. Therefore, more research focus should be placed on ALD, in relation to the COVID-19 pandemic. It was reported by the Centers for Disease Control and Prevention in the US that the mortality due to ALD has increased rapidly during the COVID-19 pandemic. One possible reason for this is the high consumption of alcohol and the increase in alcohol-related comorbidities before and during the COVID-19 pandemic. Other possible reasons include increased obesity/metabolic syndrome and NAFLD, which also contribute to the risk of ALD development. In addition, a study found that females and young adults showed the highest relative increases in ALD-related mortality, while older adults over the age of 85 years presented a smaller increase in overall female mortality and even a decrease in male mortality[93]. A comprehensive study of 978 CLD patients with SARS-CoV-2 infection from 21 institutions in the United States revealed a novel association between ALD and poor survival or COVID-19 related mortality. It has been confirmed that patients with preexisting ALD may be more susceptible to contracting COVID-19 due to immune system dysregulation. ALD is associated with a sterile inflammatory state caused by an injury-related molecular pattern, resulting in the spread of pro-inflammatory cytokines throughout the body. In addition, SARS-CoV-2-induced cytokine storm may exacerbate inflammation in patients with ALD, leading to worse clinical outcomes[94]. In a report on the trends in alcohol consumption and liver disease before and after COVID-19, more than a third of patients with CLD and 50% with ALD consumed alcohol daily, suggesting that high alcohol consumption may indicate a relationship with poor prognosis of COVID-19. There are many possible reasons why alcohol abuse leads to poor outcomes. Alcohol abuse and related liver disease can disrupt the innate and acquired immune systems due to impaired immune cell function and survival, which participate in defending against viral infections. Another reason may be that patients with chronic alcohol consumption are more likely to develop ARDS, thus leading to a worse outcome[95].

Liver transplant and COVID-19

Liver transplant recipients are more likely to develop severe COVID-19, due to chronic immunosuppression and related comorbidities, which may lead to mortality[96]. A systematic search of 15 studies consisting of 223 Liver transplant patients, among which 77.7% required hospitalization, found that 36% experienced more severe disease, with a higher mortality rate observed in the cohort compared with approximately 1%-4% in the general population. In addition, liver transplant recipients often present with fever and dyspnea which are similar to common COVID-19 manifestations; however, liver transplant recipients are more likely to have concurrent diarrhea symptoms. Furthermore, older liver transplant recipients (aged > 60 years) with diabetes are at an increased risk of mortality[72]. Another study compared 151 Liver transplant patients (68% male, median age of 60 years) with 627 non-transplanted patients (52% male, median age of 73 years), showing that groups of liver transplant patients tended to have ICU admission (28% *vs* 8%) and needed treatment with invasive ventilation (20% *vs* 5%), while mortality was lower in the liver transplant cohort than in the non-transplant cohort (19% *vs* 27%). Several statistical analyses have shown age-related mortality among liver transplant recipients[18]. Similarly, the first nationwide study compared the morbidity and outcomes of 111 Liver transplant patients infected with SARS-CoV-2 with those of the general population, matched for age and sex[97]. Liver transplant recipients have a doubled risk of developing COVID-19, but have a lower standardized mortality rate compared to the general population. They conclude that chronic immunosuppression and increased comorbidity make the liver transplant recipients more likely to suffer from the infection of the virus and develop to COVID-19, whereas chronic immunosuppression can resist the most severe forms of COVID-19 and play a protective role against the virus, resulting in reduced mortality rates.

DISCUSSION

COVID-19 caused by SARS-CoV-2 infection is a worldwide epidemic, which has had catastrophic impacts on healthcare systems and social management. Although SARS-CoV-2 mainly affects the respiratory tract, current studies have also focused on multi-organ injury particularly liver injury, with liver test abnormalities identified as an important extrapulmonary manifestation related to SARS-CoV-2 infection[98]. As liver dysfunction is associated with disease severity and poor clinical presentation, understanding the pathogenesis of liver injury and its associated risk factors is extremely important for the prognosis and treatment of the disease. This review briefly summarizes the onset, mechanism, risk factors, susceptible groups, and correlation between liver injury and the clinical outcomes of COVID-19.

According to previous studies, hypoalbuminemia and elevated AST and ALT levels are frequently observed in severe COVID-19 cases, indicating abnormal liver function. These elevations are usually accompanied by elevated LDH. GGT levels were also generally elevated, but ALP elevation was not evident in most studies. Although it is debated whether the pattern of liver injury is hepatocellular or cholestatic, the vast majority of studies support the hepatocellular injury. However, because of the high expression of ACE2 receptors in cholangiocytes, the hypothesis that liver injury results from the direct cytopathic effect of SARS-CoV-2 on hepatocytes seems unconvincing. Liver injuries likely reflect the severity of the disease. Liver histopathological studies predominantly showed moderate microvascular steatosis and slight lobular activity and inflammatory infiltration of the portal area in the biopsy and autopsy of COVID-19 cases. However, the exact mechanisms of liver injury in COVID-19 are still unclear, perhaps more cases are due to indirect liver injury.

Predisposing risk factors for liver damage in COVID-19 include male sex, older age, other comorbidities, and underlying CLDs. Men are more likely to develop liver injury than women because of the stronger innate immune system in men and estrogen protection in women. Patients aged > 65 years appear to exhibit a progressive increase in the risk of developing severe disease. Obesity is also a contributing factor to severe COVID-19, because dysregulated hepatic innate immunity, adaptive immune response, and pro-inflammatory state are usually present in fatty cases, which may aggravate the cytokine storms. Cytokine storms can be enhanced in patients with preexisting MAFLD. The contributions of other preexisting CLDs (including cirrhosis, ALD and chronic hepatitis B) to the progression of disease severity and liver injury in COVID-19 remain controversial. Most studies have emphasized that CLDs are more susceptible to severe COVID-19 due to immune disorders, and CLDs are often associated with abnormal liver function tests. However, CLD is not a predictor of mortality. Liver transplant recipients have many potential risk factors for developing worse outcomes in COVID-19, but it appears that chronic immunosuppression can protect COVID-19 patients from developing severe disease and thus reduce the mortality. Older liver transplant recipients (aged > 60 years) with diabetes are at increased risk of mortality. The exact link between CLD and COVID-19 is unclear, and whether CLD aggravates liver dysfunction and worsens the clinical outcomes of COVID-19 remains ambiguous.

CONCLUSION

Liver injury is frequent in patients with COVID-19 and abnormal liver function tests are more often observed in severe cases. Risk factors and populations for liver dysfunction in COVID-19 patients include male sex, older age, underlying comorbidities (particularly diabetes, hypertension, and obesity) and preexisting liver diseases (CLD and liver transplant). The mechanisms underlying liver injury in COVID-19 patients remain unclear, which are more likely due to the direct effect of the virus on hepatocytes or the biliary epithelium, and the indirect effects of excessive inflammation, drugs, and ischemia/hypoxia syndromes. Collectively, liver injury in COVID-19 patients is associated with increased disease severity and requires additional attention and effective treatments. In addition, targeted therapy is needed for patients with preexisting liver diseases, especially older patients with comorbidities. Further research is needed to determine whether the underlying disease coexists with COVID-19 to cause severe diseases that causes liver damage, or whether the underlying conditions directly contribute to liver damage. Attention should also be paid to DILI during the treatment of COVID-19, especially the conventional and new drugs used for specific groups. The information discussed in this review may aid in establishing recommendations and guidance for the treatment of liver injury in COVID-19 to reduce liver burden.

Table 1 Abnormal liver function with existing liver disease and effects of COVID-19

Ref.	No. of patients included	No. of patients with preexisting liver disease	Liver function markers	Impact on disease
Cirrhosis				
Iavarone *et al* [76], 2020	399	50	Bilirubin and ALT significantly increased, while albumin significantly decreased	Elevated transaminases may have an adverse impact on the process of cirrhosis and the 30-d mortality rate was higher in those patients who had impaired liver function
Marjot *et al* [77], 2021	745	386	-	SARS-CoV-2 infection in patients with cirrhosis appears to be associated with high rates of acute hepatic decompensation (46%) and patients would have a 2-fold increased rate of mortality
Hepatitis B				
Zou *et al*[78], 2021	105	105	Elevated levels of ALT (22, 20.95%), AST (29, 27.62%), total bilirubin (7, 6.67%), GST (7, 6.67%), and ALP (1, 0.95%)	Liver injury in patients with SARS-CoV-2 and chronic HBV co-infection was associated with disease severity
Chen *et al* [82], 2020	376	20	No significant increase	Coinfection with SARS-CoV-2 and HBV slightly affected liver function and had no effect on COVID-19 outcomes
MAFLD				
Zhou *et al* [88], 2020	327	93	-	Younger COVID-19 patients (aged < 60 years) with MAFLD have a more than 2-fold higher prevalence of severe COVID-19 while MAFLD in older patients appears to have no relation to the severity of the disease
Tripon *et al* [89], 2022	719	445	-	SARS-CoV-2-induced cytokine storm can be enhanced in patients with a preexisting liver disease like NAFLD
ALD				
Kim *et al*[94], 2021	367	94	-	Patients with ALD were at higher risk of contracting COVID-19 due to their immune system dysregulation and SARS-CoV-2-induced cytokine storm may exacerbate inflammation in ALD patients
Liver transplant				
Fraser *et al* [72], 2020	223	223	-	In liver transplant recipients with COVID-19, 77.7% required hospitalization, 36% experienced more severe disease, and the mortality rate observed in the cohort was 19.3%
Colmenero *et al*[97], 2021	111	111	-	In liver transplant patients, chronic immune-suppression increases the risk of developing COVID-19 but it could reduce disease severity and the mortality

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GST: Gamma-glutamyl transpeptidase; MAFLD: Metabolic dysfunction-associated fatty liver disease; ALD: Alcoholic liver disease.

FOOTNOTES

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MINIREVIEWS

Obstructive and secretory complications of diverting ileostomy

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Abstract

This review aimed to highlight the etiology, diagnosis, treatment, and prevention of obstructive and secretory complications associated with diverting ileostomy (DI). Obstructive complications at the stoma site are termed stoma outlet obstruction (SOO) or stoma-related obstruction (SRO). The incidence of SOO/SRO is 5.4%-27.3%, and the risk factors are multifactorial; however, the configuration of the stoma limb and the thickness of the rectus abdominis muscle (RAM) may be of particular concern. Trans-stomal tube decompression is initially attempted with a success rate of 33%-86%. A thick RAM may carry the risk of recurrence. Surgical refinement, including a wider incision of the anterior sheath and adequate stoma limb length, avoids tension and immobility and may decrease SOO/SRO. Secretory complications of DI are termed high output stoma (HOS). Persistent HOS lead to water and sodium depletion, and secondary hyperaldosteronism, resulting in electrolyte imbalances, such as hypomagnesemia. The incidence of HOS is 14%-24%, with an output of 1000-2000 mL/d lasting up to three days. Treatment of HOS is commenced after excluding postoperative complications or enteritis and includes fluid intake restriction, antimotility and antisecretory drug therapies, and magnesium supplementation. Intensive monitoring and surveillance programs have been successful in decreasing readmissions for dehydration.

Key Words: Small bowel obstruction; Stoma outlet obstruction; Stoma-related obstruction; High output stoma; High output syndrome; Dehydration

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Core Tip: This review highlights the etiology, diagnosis, treatment, and prevention of obstructive and secretory complications associated with diverting ileostomy (DI). Obstructive complications at the stoma site (stoma outlet obstruction/stoma-related obstruction, SOO/SRO) affect 5.4%-27.3% of patients with DI. Trans-stomal tube decompression is effective in most cases. Surgical refinement is important for reducing SOO/SRO. Secretory complications (high output stoma, HOS) lead to water and sodium depletion and secondary hyperaldosteronism with electrolyte imbalances. The incidence of HOS is 14%-24%, with an output of 1000-2000 mL/d. HOS treatment includes fluid intake restriction, antimotility and antisecretory drug therapies, and magnesium supplementation. Intensive monitoring and surveillance programs may decrease the readmission rates for dehydration.

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INTRODUCTION

Diverting ileostomy (DI) is often performed in patients undergoing low anterior resection for rectal cancer or restorative proctocolectomy with ileal-pouch anal anastomosis (IPAA) for ulcerative colitis. The purpose of DI creation is to protect the anastomosis from leakage and mitigate the severity of symptoms relative to anastomotic complications. However, the efficacy of DI is solely limited to a significant decrease in anastomotic leakage, and no other advantages may be found in the short term[1,2]. Moreover, stoma formation significantly increases the risk of small-bowel obstruction, postoperative ileus, dehydration, and electrolyte imbalance[2-4]. Some of these complications are specific to DI and can be classified into "obstructive" and "secretory" complications. The risk factors for developing these complications have been widely discussed, but the fundamental mechanisms and management of these complications are not fully understood. This review aimed to highlight the etiology, diagnosis, treatment, and prevention of obstructive and secretory complications associated with DI.

METHODOLOGY

An electronic English literature search was performed on PubMed/MEDLINE database from the inception to September 15, 2022. The search items included "diverting ileostomy" or "covering ileostomy", "small bowel obstruction", "stoma outlet obstruction" (SOO) or "outlet obstruction", "stoma-related obstruction" (SRO), "high output stoma" (HOS) or "high output syndrome", and "dehydration". Inclusion criteria for the article type were systematic review and meta-analyses, randomized controlled studies, retrospective observational studies, and narrative reviews for nursing aspects. Case reports were not included.

OBSTRUCTIVE COMPLICATIONS

Etiology

Small bowel obstruction (SBO) is one of the most common complications associated with colorectal surgery. The increased incidence of SBO when DI is created at the time of the initial surgery is of particular concern[4-8]. Since DI is brought up extracorporeally through the abdominal wall by splitting the rectus abdominis muscle (RAM), scar formation or tissue inflammation at the anterior rectus sheath (where the incision is made) may lead to stenosis of the stoma opening (outlet). Furthermore, the stoma outlet is physiologically vulnerable to the risk of obstruction, and its underlying mechanisms can be explained by the following speculations. First, the stoma is edematous, and the bowel lumen tends to be narrower in the early postoperative period. Second, the intraluminal pressure of the small bowel is lower than that of the colon, suggesting relative stenosis at the RAM level[9]. Third, if a high volume of bowel content flows into the lumen, the stoma outlet may have a change in caliber, leading to relative narrowing[10].

Obstructive complications at the stoma site have been termed SOO and SRO in the literature. Okita *et al*[11] proposed a definition of SBO at the stoma site that has the following criteria: (1) Radiographically confirmed dilatation of the oral stoma limb; (2) Increase in stomal output and relief of symptoms after trans-stomal tube decompression; and (3) Exclusion of SBO other than that at the stoma site. Some of the subsequent reports have followed these criteria for diagnosis[10,12-14], while others have used more

simplified criteria, such as SBO at the stoma site with radiographic confirmation excluding paralytic ileus[15-17].

Risk factors

SOO/SRO occurs in 5.4%-27.3% of patients with DI[10-18]. Risk factors are classified into the following categories: patient characteristics[10,11,13,16,19] (young age, low body mass index, thick subcutaneous fat, thick RAM, and long distance between the superior mesenteric artery root and the bottom of the external anal sphincter), disease[15] (ulcerative colitis), surgical factors[12,14,20-22] (laparoscopic surgery, rotation of stoma limb, IPAA, two-stage surgery for ulcerative colitis, and short distance from the anastomosis to the stoma site), and stoma functions[10,17] (high output from stoma). The risk factors are summarized in Table 1.

Young age (less than 16 years old) and low body mass index (less than 21 kg/m^2) in patients with ulcerative colitis were reported as risk factors by Okita *et al*[11]. The mechanisms and interpretation of the results were not clearly shown, although they assumed that the small abdominal cavity or small amount of mesenteric fat in these individuals allowed volvulus or kinking. A contradictory result was reported by Tamura *et al*[16], who found that increased subcutaneous fat [vertical distance of 20 mm or more at the stoma site marking on computed tomography (CT)] in obese patients was a significant predictor of SOO/SRO. However, the different pathologies in the study population (inflammatory disease *vs* colorectal cancer) make this comparison difficult. The authors assumed that the tension and twisting in the loop stoma might have been caused by the minimum size of stoma apertures at the skin and tight with a narrow subcutaneous cavity in obese patients, and thus surgeons should be aware of it. Mori *et al*[19] reported that SOO/SRO was more common in patients with a long distance between the superior mesenteric artery root and the bottom of the external anal sphincter (height-adjusted, 191 mm/m on CT), suggesting that the mesentery may be under tension. The authors encouraged surgeons to reduce tension in the mesentery using all applicable surgical techniques. Thick RAM at the stoma passage (vertical length of 10 mm or more on CT) has been shown to increase the risk of SOO/SRO[10,13], and it is speculated that the intraluminal pressure of the stoma may be overwhelmed by the increased pressure of the thick RAM[10]. The authors assumed that surgeons need to create a wider split of the RAM for these patients.

As a disease-related factor, ulcerative colitis has been reported as an independent risk factor for SOO/SRO, but the specific reason remains unclear[15]. It is speculated that the risk may not be due to the disease itself; rather, it refers to the surgical procedure of total proctocolectomy with IPAA with DI (also known as the first of the two-stage surgery)[12,20]. If the length of the diverted bowel (between the stoma and pouch) is too short, strong tension in the mesentery may occur, and it is difficult to revert if bowel twisting or mesenteric torsion occurs. If the length is too long, bowel twisting, kinking, or angulation may occur; however, a spontaneous resolution is expected in such cases[12,18]. Several studies have shown an increased risk of SOO/SRO in laparoscopic surgery[18,22]. Laparoscopic surgery essentially reduces adhesion formation and may induce torsion of the mesentery or bowel as a result of increased bowel mobility. Regarding rotation, the risk of SOO/SRO remains controversial. Forced stoma rotation by 180-degree with IPAA significantly increases the incidence of SBO; bowel kinking at or below the fascia is presumably responsible for the obstruction[21]. In contrast, recent reports have shown that stoma rotation did not increase the incidence of SOO/SRO[11,12,15,18,20]. The reason for the SOO/SRO associated with high output from the stoma has already been described in the *Etiology* section.

Diagnosis

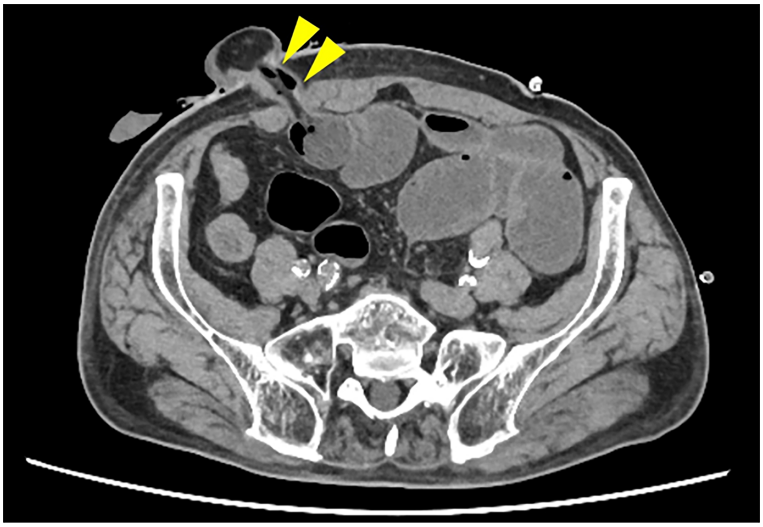
To examine whether the outlet is stenotic or obstructive, clinicians can simply insert their fingers into the stoma[23]. An abdominal CT scan or contrast enema study through the stoma may be useful for precise diagnosis, as they can demonstrate the location responsible for SOO/SRO and exclude other sites of SBO (Figures 1 and 2). Other pathologies causing similar symptoms, such as paralytic ileus, enteritis, volvulus, internal hernia, and parastomal hernia, should also be excluded.

Treatment

In cases of SBO, fasting, bowel rest, and intravenous fluid administration are fundamental recommendations. When nausea, vomiting, or abdominal bloating/distention is present, a nasogastric tube may be placed. Although uncommon in Western societies, long nasointestinal tubes may be indicated to achieve quicker and more effective intraluminal decompression[11,24,25]. Trans-stomal tube decompression is attempted as an initial treatment for SOO/SRO. The success rate of local management using trans-stomal tubes ranges from 33%-86%[11,12,15,17]. Redo treatment may be indicated in cases of recurrent obstructions. It has been shown that thick RAM carries the risk of recurrent obstruction[17]. Importantly, adverse events related to trans-stomal tube decompression have also been reported. Bowel injury occurred in 3.7% of the intubated patients requiring emergency surgery. Stoma closure is the ultimate solution for SOO/SRO in patients for whom non-surgical, conservative management, or repeated tube decompressions were not successful.

Table 1 Risk factors of obstructive complication of diverting ileostomy

Risk factors
Patient characteristics
Young age (less than 16 yr old)[11]
Low body mass index (less than 21 kg/m^2)[11]
Thick subcutaneous fat at the stoma marking site (20 mm or more) [16]
Thick rectus abdominis muscle at the stoma passage (10 mm or more)[10,13]
Long distance between the superior mesenteric artery root and the bottom of the external anal sphincter (height-adjusted, 191 mm/m)[19]
Disease
Ulcerative colitis (compared with colorectal cancer)[15]
Surgical factors
Laparoscopic surgery (compared with open surgery)[18,22]
Rotation of stoma limb (180-degree rotation, the oral limb situated on the caudal side)[21]
Ileal-pouch anal anastomosis (compared with low anterior resection or intersphincteric resection)[12]
Two-stage surgery for ulcerative colitis[20]
Short distance from the ileal pouch to the stoma site (< 30 cm)[12,18]
Stoma functions
High output from stoma (2000 mL or more per day)[10,17]

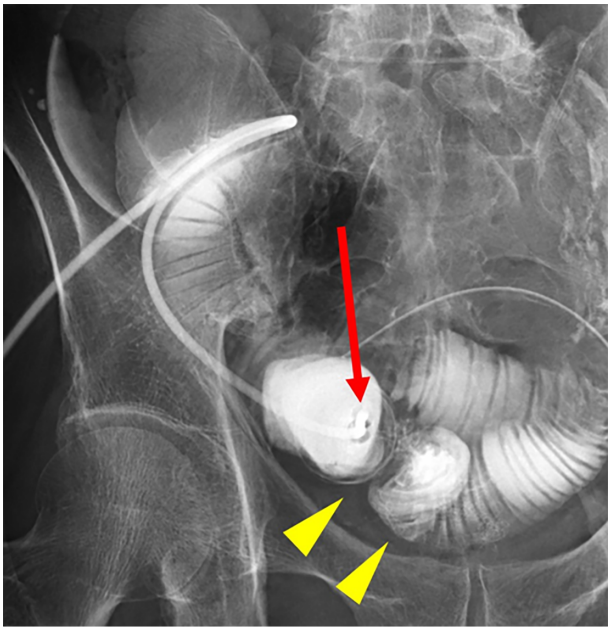


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Figure 1 Computed tomography shows stenosis at the ileostomy site (arrowheads).

Prevention

Some of the risk factors for SOO/SRO, such as patient, disease, anatomical, and stoma function, may not be preventable, and surgical refinement must be fully considered. When creating DI, the anterior sheath is adequately incised, and the RAM is split and dilated to accommodate the stoma limbs. In the case of thick RAM, creating the tunnel by the conventional "two finger breadths rule" may not be sufficient; thus, wider dissection is necessary[13]. Moreover, care must be taken in laparoscopic surgery because the stoma is constructed under the effect of pneumoperitoneum with muscle relaxation[14]. In the case of IPAA, the distance between the ileal pouch and anastomosis must be longer than 30 cm to mitigate the risk of tension in the mesentery[12,18]. Rotation of the stoma limb is carefully performed if necessary. Although they did not advocate stoma rotation, Takehara *et al*[14] proposed that the oral stoma limb should be placed on the cranial side to avoid gravitational compression by the anal stoma limb. Some authors have recommended intra-abdominal suture fixation between the stoma limb and abdominal wall[26,27]; however, it might also carry the risk of immobility, tension, and fecal impaction



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Figure 2 Contrast enema study shows a decompression tube inserted at the stenotic stomal opening (arrow) and dilated small bowel proximal to the stoma site (arrowheads).

in the stoma.

SECRETORY COMPLICATION (HIGH OUTPUT)

Etiology

In healthy adults, approximately 1500 mL of intestinal fluid enters the colon from the ileum[28]. Theoretically, the same amount of fluid is drained from the newly established ileostomy; however, the normal output of ileostomy has been proposed as 600-1200 mL per day and may decrease over time[29-31]. This reduction in the quantity is called "ileostomy adaptation," which suggests a compensatory increase in anti-diuretic hormones such as renin and aldosterone[28]. The consistency of the ileostomy output is generally watery when created and thickens in the next 2-3 mo[29]. Stool consistency, amount, and ileostomy output may be altered by the patient's body weight, disease for the index surgery, liquid and food intake, and volume of gastrointestinal secretions[29,32].

Secretory complications of DI occur when the output exceeds the aforementioned normal limit. It is commonly termed "HOS" in the literature. This term may be considered similar to "dehydration'. Dehydration has been shown to be a cause of readmission in 9.3%-43% of the patients after ileostomy creation without preventive protocols[33-35]. The most recent meta-analysis showed that the pooled incidence of readmission due to dehydration was 6% regardless of HOS prevention and was accompanied by increased medical costs[36]. Moreover, HOS results in electrolyte imbalance and acute kidney injury in the early postoperative period and may lead to malnutrition and chronic renal impairment in the long term[30,37]. The prevalence of *Clostridium difficile* infection in the development of postoperative diarrhea/HOS was investigated, but it was found that the infection rate was low (1.6%) and that the patient outcomes were not affected by the infection[38]. Contradictory results have also been reported[39].

The ileostomy output includes large amounts of sodium (85-180 mmol/L per day)[40]. Persistent HOS can lead to sodium depletion, dehydration, and secondary hyperaldosteronism, which may cause sequential hypokalemia and hypomagnesemia, which are the main features of electrolyte imbalances. As the ileum and colon are the main sites of magnesium absorption, ileostomy patients may be susceptible to hypomagnesemia. Other reasons for hypomagnesemia include free fatty acid malabsorption and the use of proton pump inhibitors[31]. Baker *et al*[29] reported that nearly half of the patients with HOS exhibit hypomagnesemia.

Definitions

The incidence of HOS varies from 14% to 24%[29,41-43] with various definitions. The majority of previous reports have defined using specific values of output volume with a timeframe, such as 2000 mL per day[38,44,45], 1500 mL per day for two days[41], 1000 mL per day for three days[42,46], and

2000 mL per day for three days[29,43]. Some authors have used a combination of output volume with laboratory findings of renal impairment and/or physical signs of dehydration[36,47].

HOS can occur in the early or late postoperative periods[29]. In a study, early HOS occurs within three weeks after stoma creation and resolves spontaneously in 49% of patients with HOS, while 7% receive persistent treatment. Late HOS occurs more than three weeks after stoma creation and resolves spontaneously in 15% of patients with HOS, while 47% receive persistent treatment. In that study, the diagnosis of cancer, followed by perforation and short bowel (less than 200 cm), correlated with the incidence of early HOS, whereas inflammatory bowel and obstruction were deemed to have an impact on late HOS[29]. Therefore, secretory complications of DI are an ongoing problem after stoma creation, and patients are always at risk, even after hospital discharge. This issue has become more relevant in the era of enhanced recovery after surgery.

Causes and risk factors

The causes of HOS are multifactorial, including factors associated with patients[42,48], disease[29,42,43], anatomy[29], surgical procedure[29,42,43,48], medication[29,33], nutrition[29], enteritis/metabolism[29,39] and those related to postoperative complications[29,44,49]. Older age was identified as an independent risk factor of HOS in two studies: however, the cut-off values were not shown because the comparisons were made without dichotomization in their studies[42,48]. The reasons were not specified in either of the studies. Assaf *et al*[48] also showed that higher American Society of Anesthesiologists-physical status and elevated creatine levels were independently associated with HOS. Again, the cut-off values were not shown for the same reason as the older age. The authors assumed that impaired kidney functions resulted in less adaptation to fluids and electrolyte loss in these groups of patients.

Takeda *et al*[43] found that diabetes was one of the risk factors for HOS, with the following explanations. Diabetes may cause autonomic nervous system impairment that decreases the motor function of the bowel, followed by abnormal proliferation of intestinal bacteria and an augmented intestinal pressure with increased gas proliferation. Inflammatory bowel diseases, particularly Crohn's disease, have been shown to be significantly associated with HOS[29,42,43]. Crohn's disease presents an impaired intestinal permeability/barrier increased with altered gut microbiota and inflammatory tissue damage[42]. These factors can lead to HOS. Shortening of the small bowel with a more proximal ileostomy can also lead to HOS, and this may explain the reason why some surgical procedures (right-side colectomy, separate ileostomy, small bowel resection, and IPAA) were independent risk factors of HOS. Takeda *et al*[43] proposed another reason for HOS in patients who underwent IPAA: Inhibition of lipids absorption leads to hydroxylation or desaturation of unabsorbed long-chain acids, which triggers intestinal fluid and electrolyte secretion. Open surgery was significantly associated with HOS compared to laparoscopic surgery[42,48]. The possible explanation is that open surgery itself may not be the direct cause but patients undergoing more complex surgery with open laparotomy[48]. A short bowel (less than 200 cm) frequently causes HOS[29], but this may not be applicable in DI formation, where a stoma is created at the distal ileum.

Perioperative medication is an important underlying pathology for the development of HOS. The use of diuretics may easily result in dehydration because the body fluid balance is vulnerable in patients with ileostomy, even without any medications[33]. Administration of prokinetic drugs (*e.g.*, metoclopramide) induces HOS; therefore, care must be taken when the patients suffer from nausea/vomiting with gastric stasis[31]. Sudden withdrawal of opiates and steroids induces reactive intestinal secretion[29-31]. Diarrhea with dehydration is commonly seen in patients who receive chemotherapy consisting of cytotoxic agents.

Regarding nutritional factors, intake of hypotonic fluids such as water, tea, coffee, fruit juice, and alcohol precipitates dehydration, and thus it is generally restricted or avoided[29-31]. Enteric infections, including *Clostridium difficile* or *Salmonella*, typically present with acute and severe diarrhea[29,50]. Chronic diarrhea may be caused by bacterial overgrowth from diverticula or blind loop fermentation[29,50].

Postoperative complications may induce secondary HOS, including prolonged ileus[44] (presented with nausea, vomiting, intolerance to oral feeding, abdominal distension, or failure to pass flatus or bowel movements within postoperative 7 d), SBO[29], SOO/SRO[49], or intra-abdominal sepsis/deep surgical site infection[29,49]. These postoperative complications must be excluded clinically and radiologically when suspicious of HOS. The details of the causes and risk factors of HOS are summarized in Table 2.

Diagnosis

The key clinical symptoms of HOS are due to the loss of water and sodium and include thirst, cramps, muscle weakness, and faintness[31,50]. Patients may also present with loss of appetite, rapid weight loss, fall in postural blood pressure, or a decrease in urinary output[31]. The stomal output volume is measured every 8 h to facilitate early recognition of HOS. The stoma color and stool consistency should be inspected. Because stenosis or obstruction at the stoma outlet is one of the causes of HOS, a digital examination of the stoma is suitable for diagnosis. Laboratory findings may include elevated serum urea/creatinine ratio, hyponatremia, hypokalemia, and hypomagnesemia. A decrease in urinary sodium (less than 10 mmol/L) reflects sodium depletion more accurately than serum sodium level[31,

Table 2 Causes and risk factors of high output stoma

Causes and risk factors of high output stoma
Patient
[1]Older age[42,48]
[1]Higher ASA-PS[48]
[1]Elevated baseline creatine[48]
Disease
[1]Diabetes[43]
Inflammatory bowel disease (*i.e.*, ulcerative disease, Crohn's disease)[29,42,43]
Anatomy
Short bowel (less than 200 cm)[29]
Surgical procedure
Open surgery (*vs* laparoscopic surgery)[42,48]
Total proctocolectomy (with ileal-pouch anal anastomosis)[42]
Right-side colectomy[42]
Separate ileostomy[42]
Small bowel resection[29,42,43]
Medication
Preoperative use of diuretics[31,33]
Prokinetic drugs (*i.e.*, metoclopramide)[29,31]
Sudden withdrawal of corticosteroids or opiates[29,31]
Postoperative adjuvant chemotherapy[29]
Nutrition
Hypotonic liquids (low sodium): water, tea, coffee, fruit juice, alcohol[29,30,31]
Enteritis/metabolism
Clostridium difficile infection[29,50]
Salmonella infection[29,50]
Bacterial overgrowth from diverticula or blind loop fermentation[29,50]
Relative to postoperative complications
Postoperative ileus (symptoms of nausea, vomiting, intolerance to oral feeding, abdominal distension, or failure to pass flatus or bowel movements within postoperative 7 d)[44]
Intra-abdominal sepsis (pelvic sepsis, organ/space infection)[29,49]
Small bowel obstruction[29]
Stoma outlet obstruction[49]

[1]No specific values or definitions were shown in the referenced articles. ASA-PS: American Society of Anesthesiologists-physical status.

50]. Long-lasting HOS may decrease the absorption of vitamin B12 and folic acid and increase the incidence of renal calculi and gallstones[40].

Treatment

Exclusion of possible causes: As shown in Table 2, HOS occurs secondary to the underlying intra-abdominal complications. CT is useful for identifying these factors, and treatment of the identified cause must be prioritized. The administration or cessation of certain drugs is responsible for HOS and is corrected accordingly after the diagnosis. Enteritis induced by *Clostridium difficile* or *Salmonella* is excluded by taking stool cultures from the output.

Restriction of fluid intake: Since patients with dehydration complain of thirst in the early phase of HOS, increasing fluid intake may be inappropriately advised to relieve symptoms[30]. Consumption of excessive hypotonic fluids (less than 90 mmol/L of sodium) leads to worsening symptoms with sodium depletion caused by a net efflux from the plasma into the bowel lumen[29,50]. Hypertonic fluids containing glucose may also lead to increased stomal output[50]. Previous reports have suggested that hyper- and hypotonic fluid intake must be restricted to 0.5-1.0 mL per day[29,31,50]. Oral intake may not be possible when presenting with nausea/vomiting, and intravenous fluid rehydration containing 100-150 mmol/L of sodium (*e.g.*, normal saline) is necessary to avoid acute kidney injury[31]. If oral intake is possible, it is recommended to sip 1 L or more of a glucose-saline solution containing at least 90 mmol/L sodium in a small quantity at intervals[31,50].

Drug therapies: Antimotility drugs include loperamide and codeine phosphate. Both drugs act against intestinal motility, and it has been shown that the ileotomy output reduces by 20%-30%[31]. Loperamide is preferred over codeine phosphate because it is not addictive. Loperamide can be prescribed at 4 mg/d to 16 mg/d, while codeine phosphate can be prescribed at 15 mg/d to 60 mg/d, and the effect becomes greater if both are taken together[31,41,50].

Antisecretory drugs include H_2 antagonists and proton pump inhibitors that reduce gastric acid secretion. The somatostatin analog, octreotide, reduces gastric and pancreaticobiliary secretions and delays gastric emptying and small-bowel transit. Antisecretory drugs may be used when the stomal output exceeds 2 L/d, but octreotide is not preferred because the proton pump is as effective as octreotide in reducing the stomal output[29,31,41].

Hypomagnesemia in patients with HOS is caused by the chelation of magnesium with unabsorbed fatty acids and increased magnesium secretion due to secondary hyperaldosteronism[50] and reduced absorption of fatty acids. Magnesium is preferably supplemented orally; however, it can be slowly administered intravenously to avoid a flushing sensation.

Stoma closure: HOS may persist in 5.0%-15% of the affected patients until the stoma is reversed[29,35]. Stoma closure is planned for patients who do not respond to non-surgical conservative treatment. In a study, patients with readmission due to dehydration underwent stoma closure earlier than those without readmission[33]. Ihnát *et al*[5] reported that 3.8% of patients with DI underwent acute surgery for HOS.

Prevention

Intensive monitoring and surveillance programs have been proposed recently. Most protocols consist of preoperative patient education, in-hospital monitoring and intervention protocols, post-discharge hospital visits, and surveillance using telephone or telemedicine platforms[51-54]. For example, Shaffer *et al*[54] proposed a pilot program for outpatient follow-ups using regular visits and telephone interviews. The triggers of intervention included tachycardia with > 100 beats/min, ileostomy output > 1200 mL/d, major changes in weight, fever, nausea, poor oral intake, dry mouth, and low urinary output. The response by the team included an assessment of the basic metabolic profile, intravenous hydration at home, and phone calls to the doctor's office. This program successfully reduced the incidence of readmission from 21% to 8.7% and the cost of readmission by more than 80%.

These intense programs have been mostly successful in decreasing readmissions for dehydration (15%-21% before intervention *vs* 5.0%-8.8% after intervention)[52-54]; however, a conflicting result has also been reported, where the program failed to decrease readmissions for dehydration (8.2% *vs* 5.9%) despite obtaining better phone follow-up and an increase in outpatient intravenous fluid in the intervention group[51].

LIMITATIONS

There are several limitations in this review. First, this is a narrative review without a systematic approach where a literature search was performed according to the authors' experiences using their preferred keywords of choice. Furthermore, inclusion for the selection was purely based on the authors' judgment considering their relevance to the topics. Second, the selected articles were mostly non-randomized, retrospective studies resulting in selection bias. Since the causes and risk factors of obstructive and secretory stoma complications are often multifactorial, it may be difficult to design prospective trials to investigate the efficacy of specific surgical or medical interventions. Instead, recent prospective studies have shown that postoperative monitoring and surveillance programs may facilitate early recognition and decrease the adverse events relative to the complications[51-54]. Third, the conclusions of the selected articles were based on their population-based analyses. The research findings may not be instantly applicable or comparable to the current practice of readers due to the differences in the definitions (diagnostic criteria), patient characteristics, disease, surgical settings, social background, and healthcare systems. The research findings must be carefully interpreted, considering these variables in the communities of interest.

CONCLUSION

Obstructive and secretory complications of DI occur both in the early and late postoperative periods and require intensive monitoring and intervention. Non-surgical conservative treatment is mostly effective; however, stoma closure may be considered in recurrent or refractory cases. To facilitate the diagnosis and treatment for patient safety, seamless communication and close collaboration in a multidisciplinary team are necessary.

Interestingly, obstructive and secretory complications may also occur simultaneously. However, the causative factors of these complex pathologies are various and inconsistent: the potential reason is that most evidence has been obtained by retrospective, observational studies with a small sample size. Further prospective randomized trials are needed to assess the efficacy of interventions or protocols to improve the outcomes of obstructive and secretory complications of ileostomy.

FOOTNOTES

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Role of the combination of biologics and/or small molecules in the treatment of patients with inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is a group of chronic diseases that includes ulcerative colitis, Crohn's disease, and indeterminate colitis. Patients with IBD require prolonged treatment and high utilization of healthcare resources for proper management. The treatment of patients with IBD is focused on achieving therapeutic goals including clinical, biochemical, and endoscopic variables that result in improvement of the quality of life and prevention of disability. Advanced IBD treatment includes tumor necrosis factor inhibitors, integrin antagonist, antagonist of the p40 subunit of interleukin 12/23, and small molecule drugs. However, despite the multiple treatments available, about 40% of patients are refractory to therapy and present with persistent symptoms that have a great impact on their quality of life, with hospitalization and surgery being necessary in many cases. Dual therapy, a strategy sometimes applicable to refractory IBD patients, includes the combination of two biologics or a biologic in combination with a small molecule drug. There are two distinct scenarios in IBD patients in which this approach can be used: (1) Refractory active luminal disease without extraintestinal manifestations; and (2) patients with IBD in remission, but with active extraintestinal manifestations or immune-mediated inflammatory diseases. This review provides a summary of the results (clinical response and remission) of different combinations of advanced drugs in patients with IBD, both in adults and in the pediatric population. In addition, the safety profile of different combinations of dual therapy is analyzed. The use of newer combinations, including recently approved treatments, the application of new biomarkers and artificial intelligence, and clinical trials to establish effectiveness during long-term follow-up, are needed to establish new strategies for the use of advanced treatments in patients with refractory IBD.

Key Words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Dual-therapy biologic therapy; Small molecule drugs; Clinical remission

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Core Tip: Patients with inflammatory bowel disease (IBD) require prolonged treatment and high utilization of healthcare resources. About 40% of patients are refractory to different treatments with an increase need for hospitalization and surgery. Dual therapy, a strategy applicable to refractory IBD patients, includes the combination of two biologics or a biologic in combination with a small molecule drug. There are two distinct scenarios in IBD therapy in which this approach can be used: (1) Refractory active luminal disease without extraintestinal manifestations; and (2) patients with IBD in remission, but with active extraintestinal manifestations or immune-mediated inflammatory diseases.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic diseases that includes ulcerative colitis (UC), Crohn's disease (CD), and indeterminate colitis. Patients with IBD require prolonged treatment and high utilization of healthcare resources for its proper management[1]. Medical treatment includes the use of so-called conventional drugs (mesalazine, immunosuppressants such as azathioprine or methotrexate and corticosteroids) and biologics [anti-tumor necrosis factor (anti-TNF), anti-integrins, and anti-interleukins (IL)], with small molecules (Janus kinase inhibitors and sphingosine 1-phosphate receptor modulators) having recently been added to the possible advanced treatments[2-5].

After the onset of therapy, the treatment of patients with IBD is focused on achieving therapeutic goals, which include improvement or normalization of clinical, biochemical, endoscopic variables, and also the quality of life and disability[6]. Despite the multiple treatments available, about 40% of patients are refractory to several treatments with different mechanisms of action, and these patients present with persistent symptoms that often have a great impact on their quality of life, due to the need for hospitalization and the requirement of surgery, which has to be carried out several times in some cases[7].

Extraintestinal manifestations (EIM) are present in about one-third of patients after diagnosis[8]. These mainly involve osteoarticular and dermatological manifestations. Some EIM are independent of IBD activity and require independent therapeutic management. In addition, some patients have multiple comorbidities throughout the course of the disease associated with prolonged corticosteroid treatment (diabetes, osteoporosis, adrenal insufficiency, and others), which is frequently used in these patients with a suboptimal response to advanced treatments[9].

Different studies have described a therapeutic window of opportunity, which implies the early use of advanced treatment in patients with IBD, especially in patients with early CD (< 2 years)[10]. These interventions are associated with a decrease in the progression of intestinal damage and complications such as stenosis and fistulas, and consequently reducing the need for hospitalization and surgery[10]. Finally, patients with long-standing IBD with persistent inflammatory activity represent a group at higher risk for the development of colorectal cancer, which develops by a different sequence to that of non-IBD colorectal cancer[11]. It has also been described that a better control of inflammatory activity may have an impact the development of this complication during long-term evolution[12,13].

DEFINITION AND INDICATIONS OF DUAL THERAPY

The development of new molecules and the implementation of new strategies are necessary to achieve better control of IBD activity in patients who are refractory to currently available treatments[14]. However, there are multiple pathways of inflammatory activity activated in patients with IBD, and for this reason, treatment with monotherapies may not be sufficient for the management of all patients[15]. Related to this, there are many scenarios in medicine in which dual therapy is used in both the induction and maintenance of treatment. This strategy involves the combination of two or more treatments with the aim of achieving optimal control of pathologies with different therapeutic targets. Indeed, this modality has seen great development in oncological or hematological treatments[14]. Similarly, in patients with rheumatologic pathologies, this approach is used in some patient subgroups [16]. This approach is also applicable to patients with refractory IBD to advanced treatments (dual therapy) by using two biologics simultaneously or a biologic in combination with a small molecule[16,17]. In patients with IBD, there are two distinct scenarios in which it can be used: (1) Patients with

refractory IBD without EIM; and (2) patients with IBD in remission, but with active EIM or immune-mediated inflammatory diseases (IMID)[18].

EVIDENCE RELATED TO DUAL THERAPY

The first clinical trial that assessed a combination of biologics was developed in 2007[19,20]. Later, in 2010, the SONIC trial demonstrated that the association of infliximab and azathioprine is more effective compared with either infliximab or azathioprine monotherapy in CD patients, since which time multiple publications have described the results of different combinations of advanced drugs in patients with UC and CD, both in adults and in the pediatric population[21-35] (Figure 1). These combinations have varied according to the availability and practical experience of the drugs that were approved after the anti-TNFs. Table 1 shows the data from publications related to drug combination in patients with IBD. A major limitation of the dual therapy data is that they are mostly retrospective[19]. For this reason, the definitions of response evaluation (clinical, endoscopic and biochemical) are abbreviated and with the exception of few series are only described for short periods[33]. In addition, the definition of complications and the requirement for hospitalization and surgery can be subject to biases related to the follow-up time and the clinical condition prior to the start of the combined treatment[16]. Also, the differential evaluation of this strategy in patients with UC *vs* CD is not reported in many publications, which makes assessment difficult in some cases. Finally, some series include data on patients who received more than one combination, and it is possible that the effectiveness and adverse events could be different depending on the sequencing order of these combinations.

Effectiveness

The partial or complete response in patients with indication for dual therapy for refractory IBD has been evaluated using different meta-analyses[16,19,36]. In these studies, the patients included were mainly those with CD (70%), and in the great majority, the indication for dual therapy was for refractory endoluminal activity[16]. Overall, the observed clinical response varied between 60% and 84% in most of the publications[16,19]. However, clinical remission, which is a difficult clinical situation to achieve considering that these are multi-refractory patients, ranged between 47% and 80% of the patients who received combined therapy[16,18]. The therapeutic response of the different combinations has not been reported to reveal significant variation with respect to the main indication (refractory luminal activity *vs* active EIM or IMID)[16]. Persistence in the treatment of dual therapy varies according to the follow-up period. It has been published that globally 45% of patients may discontinue the dual scheme during its evolution, with loss of response being the main cause (64%) and intolerance together with adverse effects representing a smaller percentage (12%)[33]. It is noteworthy that in a recent study, 21% of patients were able to discontinue one of the drugs in the combination without impacting the subsequent evolution[33]. It is important to mention that many series have included a recycling strategy. This involves the use in the combination of a drug, which the patient did not respond to[14]. Several publications have mentioned such a situation, and have observed that the response in these patients was similar to that observed in those who had not been previously exposed to that drug[18]. This strategy requires further evolution, especially in areas with limited resources for access to new advanced treatments.

Safety

The combination of two biologics or a biologic plus a small molecule has been associated with a higher rate of complications in other indications[17,18]. This has been observed in studies of patients with rheumatologic diseases who received combination therapy[14]. However, in these series, a significant percentage of patients received different treatments with medications that present a higher rate of adverse events, such as the use of rituximab, abatacept, and tocilizumab[18]. On the other hand, in patients with IBD, most of the proposed combinations include drugs with a high relevant safety profile such as vedolizumab or ustekinumab, which are used in both the pediatric and adult populations[25,29]. In a recent meta-analysis, the presence of adverse events varied from 6%-24% according to the combinations[16]. However, the presence of severe adverse events with indication for hospitalization or surgery was only present in 0%-12% of patients[16]. Within these severe adverse events, 75% were due to both intestinal and soft tissue infections[16]. In a recently published European series, a higher number of infections requiring hospitalization was observed in patients who received anti-TNF, corticosteroids, and immunomodulators, and who had a concomitant diagnosis of IMID/EIM (most frequently ankylosing spondylitis)[33]. Nevertheless, in this series, these complications developed only in patients with CD. Importantly, no case of reactivation of herpes zoster has been reported in any publication. Although one case of herpetic meningoencephalitis was diagnosed in a 43-year-old patient with CD who had received a combination including certolizumab, vedolizumab, and methotrexate, this was resolved after treatment[33]. Finally, one incident case of benign skin neoplasia (clear cell acanthoma) and one case of recurrence of basal cell skin cancer were reported[33,35]. No other cancers or treatment-related deaths have been reported.

Table 1 Publications including 2 or more adult or pediatric inflammatory bowel disease patients with use of combination therapy

Ref.	Study type (type of patients)	Disease (number of patients)	Combinations	Efficacy	Adverse events
Sands *et al* [20], 2007	Randomized controlled trial (adults)	CD (52)	NAT + IFX	Remission 37%	Headache, CD exacerbation, nausea, nasopharyngitis
Buer *et al* [22], 2018	Prospective cohort (adults)	CD (4), UC (6)	9 IFX + VDZ, 1 ADA + UST	Remission 100 %	3 upper airway infections
Mao *et al* [23], 2018	Case series (adults)	CD (4)	1 TNFi + UST/VDZ, 1 VDZ + UST, 2 VDZ + GOL	Remission 3/4	1 hand, foot and mouth disease, 1 influenza, 1 *Clostridiodes difficile*
Kwapisz *et al*[24], 2020	Retrospective cohort (adults)	CD (14), UC (1)	8 VDZ + TNFi, 5 VDZ + UST, 2 UST + TNFi	Improvement 11/15	*Salmonella, Clostridiodes difficile*, 4 infections, arthralgia
Olbjørn *et al* [25], 2020	Retrospective cohort (pediatrics)	CD (9), UC (4)	8 IFX + VDZ, 5 IFX + UST	Remission 9/13	Elevated transaminases, eczema, skin infection
Fumery *et al* [26], 2020	Case series (adults)	CD (5), UC (2)	5 TNFi + UST, 2 TNF + VDZ	Remission 6/7	No
Glassner *et al*[27], 2020	Retrospective cohort (adults)	CD (30), UC (18), IBD-U (1)	7 VDZ + TNFi, 25 VDZ + UST, 9 TOF + TNFi, 8 TOF + VDZ, 3 TOF + UST	Remission 50%	3 bacterial enteric infections (*E. coli*), 3 *Clostridiodes difficile*, 1 peristomal cellulitis, 2 abdominal wall abscesses
Privitera *et al*[28], 2020	Retrospective cohort (adults)	CD (11), UC (5)	3 VDZ + UST, 9 VDZ + TNFi/other, 3 VDZ + UST	Clinical response 43%	1 cutaneous reaction, 1 drug-induced liver injury, 1 perianal abscess
Yang *et al* [29], 2020	Retrospective cohort (adults)	CD (22)	8 VDZ + UST, 13 VDZ + TNFi, 3 UST + TNFi	Remission 41%	1 drug induced lupus, 1 pneumonia, 1 *Clostridiodes difficile*, 1 acinetobacter bacteremia
Alayo *et al* [30], 2021	Retrospective cohort (adults)	CD (10), UC (25)	24 VDZ + TOF, 5 TOF + UST	Remission 70% at 26 wk	1 *Clostridiodes difficile*, 1 candida esophagitis, 1 abnormal lipid profile
Dolinger *et al*[31], 2021	Retrospective cohort (pediatrics)	CD (7), UC (8), IBD-U (1)	9 VDZ + TOF, 4 VDZ + UST, 3 UST + TOF	Remission 12/16	1 septic arthritis, 1 deep vein thrombosis
Llano *et al* [32], 2021	Retrospective cohort (adults)	CD (3), UC (10), IBD-U (1)	3 UST + VDZ, 2 VDZ + TNFi, 9 VDZ + TOF	Clinical or biochemical remission 50%	2 *Clostridiodes difficile*, 2 pneumonia, 3 abnormal lipid profile
Goessens *et al*[33], 2021	Retrospective cohort (adults)	CD (58), UC (40)	41 VDZ + TNFi, 21 VDZ + UST, 11 UST + TNFi, 1 TOF + TNFi, 13 TOF + VDZ, 17 other	Clinical response 44%	10 serious or opportunistic infections
Howard *et al* [34], 2022	Case series (pediatrics)	CD (3)	3 VDZ + UST	Clinical remission 100%	Not reported
Lee *et al*[35], 2022	Retrospective cohort (adults)	CD (19)	7 TOF + VDZ, 11 TOF + UST, 1 TOF + TNFi	Remission 60%	1 basal cell carcinoma

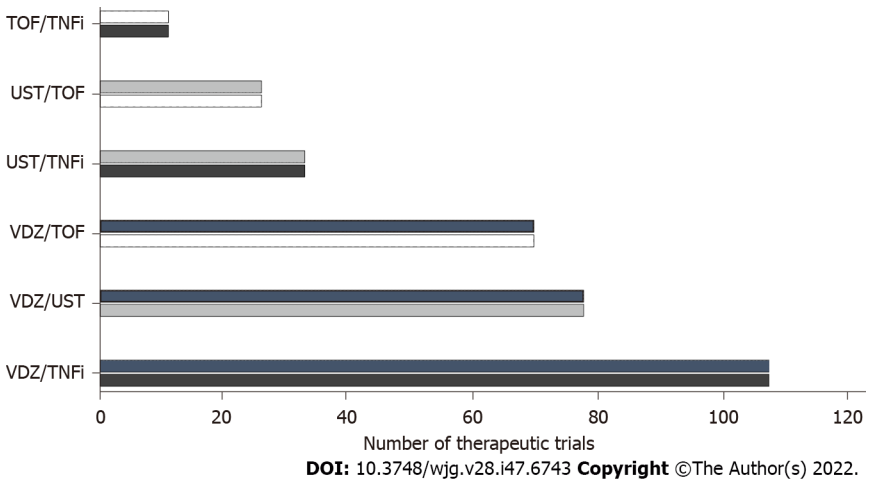
ADA: Adalimumab; CD: Crohn's disease; GOL: Golimumab; IBD-U: Inflammatory bowel disease-unclassified; IFX: Infliximab; NAT: Natalizumab; TNFi: Tumor necrosis factor inhibitor; TOF: Tofacitinib; UC: Ulcerative colitis; UST: Ustekinumab; VDZ: Vedolizumab.

Data in pediatric population

Different case series in pediatric patients have reported results with various combinations in both CD and UC[37]. In one study, 75% of patients with luminal activity achieved a clinical remission free of corticosteroids at 6 mo, with the median time to achieve this goal being 88 d[31]. Interestingly, another potential indication that has been described in pediatric patients is the use of dual therapy (vedolizumab and tofacitinib) in patients with acute severe UC[31]. Nevertheless, more data are needed to explore this indication in an urgent and severe situation in patients with IBD. Different adverse events have been described in pediatric patients, but in general there are less frequent than in adult patients[25]. In a series of 16 pediatric patients, 1 (6%) patient presented septic arthritis and subsequent deep vein thrombosis[31].

NEW HORIZONS

It is necessary to establish new strategies for the use of advanced treatments in patients with refractory IBD, which must take into account health costs in order to be sustainable[14]. The sequencing of



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Figure 1 Number of therapeutic trials described in studies including 2 or more inflammatory bowel disease patients. TNFi: Tumor necrosis factor inhibitor; TOF: Tofacitinib; UST: Ustekinumab; VDZ: Vedolizumab.

biologics or small molecules in patients in remission is a strategy that probably results in a better cost balance. Related to this, some series described patients who achieved remission with two biologics, with the subsequent suspension of one of these (usually anti-TNF) not leading to the presence of disease reactivation during follow-up[22]. In addition, other studies have shown that patients in remission on infliximab were able to maintain their clinical status after initiation of vedolizumab and discontinuation of anti-TNF[38]. The implementation of these strategies requires further research, and in particular, clinical trials are needed to establish their effectiveness during long-term follow-up.

The use of artificial intelligence and the implementation of new biomarkers in the future will possibly be able to differentiate the patients who will benefit from certain combination schemes. Artificial intelligence may also enable remote monitoring to provide new data as well as algorithms to ensure better decision making in refractory patients[39]. In addition, biomarkers might improve patient stratification. Recent data have shown that HLA-DQA1*05 is non-uniformly distributed in patients with or without anti-TNF failure[40]. Likewise, IL-23 receptor expansion is a mechanism of anti-TNF resistance and is reflected as a secondary loss of response[41]. According to this, the use of ustekinumab may allow to regained response in patients with prior anti-TNF.

It is possible that in the near future new combinations with different effectiveness and safety profile will be described, with the use of ozanimod, upadacitinib, risankizumab, guselkumab, and mirikizumab, among others, expanding the current options[18]. In this regard, it is important to note that future clinical trials will be developed to compare current therapy with the combination of two biologic treatments (golimumab and guselkumab) or the combination of two biologics (vedolizumab and adalimumab) and an immunomodulator (methotrexate)[42-44]. Moreover, the design of the new pivotal studies has been modified. Recently, a phase 2 study in patients with CD compared different doses of guselkumab with placebo but also included a ustekinumab arm as this provides better comparative information[45].

Another point to consider is that some good results have been reported after the change of formulation (from intravenous to subcutaneous) of the same drug such is the case of as infliximab or vedolizumab[46,47]. This could be important in future combinations, since it would facilitate logistics and reduce associated costs. In addition to the combination of biological drugs or small molecules, the future role of other approaches should be determined, such as the use of probiotics and gut flora regulators as well as the role of microbiota transplantation[48,49].

Finally, the development of more real-life evidence will be of great importance. Currently most of the data comes from Europe and North America[16]. In this sense, it would be very useful to develop international registries involving several countries currently experiencing a clear increase in the incidence of IBD, such as Latin America and Asia, and which have greater difficulty in accessing advanced treatments[50-52]. In this regard, the costs associated with dual therapy are the main limitation to access, which restrict the provision of a personalized treatment in patients with indication for this strategy[53]. Moreover, it is of great relevance to inform the health insurance of these patients about the objectives and advantages of the dual therapy strategy to obtain the appropriate approval in a timely manner for the indication.

CONCLUSION

The combination of biologics and/or small molecules is a strategy applicable to refractory IBD patients in two distinct scenarios: (1) Refractory active luminal disease without extraintestinal manifestations; and (2) patients with IBD in remission, but with active extraintestinal manifestations or immune-mediated inflammatory diseases. The observed clinical response using this strategy varied between 60% and 84% in most of the publications, and severe adverse events were observed in a few patients. However, most of the data on dual therapy are retrospective and with short-term follow-up. New clinical trials are needed to establish dual therapy effectiveness and safety during long-term follow-up. Finally, it is expected that new combinations using new drugs with different efficacy and safety profiles will be described in the coming years, expanding the current options.

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ORIGINAL ARTICLE

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Interleukin-34 deficiency aggravates development of colitis and colitis-associated cancer in mice

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Abstract

BACKGROUND

Although expression of interleukin (IL)-34 is upregulated in active ulcerative colitis (UC), the molecular function and underlying mechanism are largely unclear.

AIM

To investigate the function of IL-34 in acute colitis, in a wound healing model and

in colitis-associated cancer in IL-34-deficient mice.

METHODS

Colitis was induced by administration of dextran sodium sulfate (DSS), and carcinogenesis was induced by azoxymethane (AOM). Whether the impact of IL-34 on colitis was dependent on macrophages was validated by depletion of macrophages in a murine model. The association between IL-34 expression and epithelial proliferation was studied in patients with active UC.

RESULTS

IL-34 deficiency aggravated murine colitis in acute colitis and in wound healing phase. The effect of IL-34 on experimental colitis was not dependent on macrophage differentiation and polarization. IL-34-deficient mice developed more tumors than wild-type mice following administration of AOM and DSS. No significant difference was shown in degree of cellular differentiation in tumors between wild-type and IL-34-deficient mice. IL-34 was dramatically increased in the active UC patients as previously reported. More importantly, expression of IL-34 was positively correlated with epithelial cell proliferation in patients with UC.

CONCLUSION

IL-34 deficiency exacerbates colonic inflammation and accelerates colitis-associated carcinogenesis in mice. It might be served as a potential therapeutic target in UC.

Key Words: Interleukin-34; Ulcerative colitis; Mucosal healing; Colitis-associated cancer; Macrophage; Murine model

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Core Tip: This study highlights the role of interleukin (IL)-34 in acute experimental colitis and wound healing phase in mice. We found that IL-34 did not drive inflammatory response and tissue destruction in physiological conditions, but protects the host from inflammatory injury and reduces the risk of colitis-associated cancer. IL-34 might serve as a potential therapeutic target for inducing mucosal healing in treatment of ulcerative colitis (UC) and reducing colitis-associated cancer in UC.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic progressive recurrent intestinal inflammatory disorder characterized by bloody diarrhea and abdominal pain[1]. UC is an important promoter of colorectal cancer[2]. The exact pathogenesis of UC remains unclear. It is currently assumed that it involves immunological derangement of the gut microbiota in genetically predisposed individuals after exposure to environmental factors[1]. Cytokines exert multiple effects and participate in the pathogenesis of colitis[3]. Identification of distinctive cytokines involved in immunopathogenesis of UC has become a hot spot for the development of biological therapies[4].

Colony-stimulating factor-1 receptor (CSF-1R) signaling regulates intestinal and colon development, gut homeostasis and inflammatory reaction[5]. Interleukin (IL)-34 was discovered in 2008 as a specific ligand for CSF-1R independent of CSF-1[6,7]. Human IL-34 is a homodimeric glycoprotein that consists of 242 amino acids produced by various cell types including epithelial, endothelial and immune cells, and fibroblasts[8]. Secreted IL-34 binds to the extracellular domains of CSF-1R and protein-tyrosine phosphatase, the other receptor for IL-34, which is regulated by syndecan-1, which results in autophosphorylation of its intracellular tyrosine residues and activates several signaling pathways controlling cell biological function[8,9]. IL-34 functions as a pivotal regulator in cell differentiation, proliferation and survival in the mononuclear phagocyte system[10]. Additionally, IL-34 mediates the crosstalk between the innate and adaptive immune systems during inflammation[8,11].

Under normal circumstances, IL-34 is primarily expressed in a tissue-specific manner in keratinocytes and neurons[8]. In pathological status, the expression pattern is changed. Increased IL-34 expression positively associates with disease progression, severity and chronicity in autoimmune diseases such as

rheumatoid arthritis and Sjogren's syndrome[12]. In contrast, IL-34 has demonstrated beneficial effects in other diseases. IL-34 has been identified as a new mediator to induce transplant tolerance by targeting suppressive T regulatory cells[13]. Therefore, the multiple function of IL-34 in different diseases is complex, disputable, and context-dependent[8].

The involvement of IL-34 in inflammatory bowel disease (IBD) has also drawn attention recently. Higher expression of IL-34 in human ileum compared with colon has been revealed[14]. In human and experimental colitis, the mRNA and protein level of IL-34 is markedly increased in inflamed mucosa compared to that in the matched normal mucosa and in healthy controls[14]. Recently, it has been proposed that IL-34 plays a prominent role in intestinal fibrogenesis *via* a p38 mitogen-activated-protein-kinase-dependent mechanism in Crohn's disease[15]. *In vitro,* tumor necrosis factor (TNF)-α significantly upregulates expression of IL-34 in lamina propria mononuclear cells (LPMCs) *via* NF-kB signaling. Intriguingly, LPMCs treated with IL-34 increase expression of proinflammatory cytokines such as IL-6, TNF-α and chemokine CC ligand 20[14]. Accordingly, IL-34 neutralization decreases synthesis of TNF-α and IL-6 in IBD mucosal explants[14]. Therefore, it has been noted that IL-34 sustains gut inflammation *via* regulating positive feedback of proinflammatory cytokine production *in vitro*[8, 14]. On the contrary, IL-34 has previously demonstrated immunosuppressive characteristics that contribute to inflammation improvement by inducing differentiation of monocytes into M2 phenotype [16]. M2 macrophages mediate immunotolerance and promote wound healing in experimental colitis *in vivo*[17,18]. IL-34 is upregulated in colon cancer and sustains the protumorigenic signals by inducing proliferation of cancer cells *via* extracellular signal-regulated kinase (ERK) 1/2 or cancer-associated fibroblasts[19,20]. Whether IL-34 is "friend or foe" in the pathogenesis of UC remains to be explored. In this study, we investigated the potential role of IL-34 in experimental colitis, colitis-associated carcinogenesis and UC.

MATERIALS AND METHODS

Animal experiment

IL-34-deficient mice were generated in C57BL/6J mice by using CRISPR/Cas9 technology (Beijing Cas Gene Biotech, Beijing, China). Gene targeting technology was applied to delete exons 3-5 in the *IL-34* gene and led to frameshift mutation of *IL-34* gene. IL-34-deficient and C57BL/6J wild-type mice were bred and maintained in a specific-pathogen-free animal facility in the Laboratory Animal Center of Nantong University. All animal experiments were approved by the Institutional Ethics Committee of Nantong University (Date: 21/12/2015, Number: S20151221-908).

Acute colitis was induced by oral administration of 3% (M/V) dextran sulfate sodium (DSS, MW: 36 000-50 000; MP Biologicals, LLC, California, United States) in drinking water for 7 d. The mice were killed to obtain colon tissues at the indicated time or until day 16 to record the mortality. The murine wound healing model was established by oral administration of 3% DSS for 5 d and then switched to normal water for the following 5 d. The mice were killed on day 8 or 10. Colitis-associated cancer was induced by administration of the carcinogen azoxymethane (AOM; Sigma, Darmstadt, Germany) and repeated administration of DSS. The mice were given a single injection of AOM (10 mg/kg). Seven days later, mice were administrated 1.25% DSS (w/v) in drinking water for seven consecutive days, and fresh drinking water for 14 d. Seven days of DSS and 14 d of fresh water was repeated four times as a cycle [21]. The mice were killed, and the incidence rate of tumors was analyzed. Macrophage depletion from murine colons *in vivo* was performed as described previously[22]. In brief, 200 μL clodronate liposomes (Liposoma Research, Amsterdam, Netherlands) were intraperitoneally injected into mice 2 d prior to onset of experimental colitis and every 2 d during the process.

The percentage of body weight change of each mouse was recorded throughout the duration of DSS administration. Fresh feces from mice were collected for occult blood tests using a fecal occult blood kit (Nanjing Jiancheng Bioengineering, Jiangsu, China). The clinical score index of the murine model consisted of stool consistency and fecal occult blood, as previously described[23].

Human tissue

Human tissue specimens were obtained from 40 adult patients with active UC and 20 healthy controls during colonoscopy in Nantong Third People's Hospital Affiliated to Nantong University and Affiliated Hospital of Nantong University between January 2014 and August 2015. Histopathology was evaluated by an experienced pathologist. This study was approved by the Ethics Committee of Nantong Third People's Hospital Affiliated to Nantong University and Affiliated Hospital of Nantong University, and all people signed an informed consent form. All patients met the diagnostic criteria that were in line with the Consensus on Diagnosis and Management of Inflammatory Bowel Disease (2018, Beijing, China). The patients did not receive any therapy before colonoscopy. More details on the patients' characteristics can be found in Supplementary Table 1.

Histopathology

The entire colon was harvested for measuring the length. The colon was washed in phosphate-buffer saline and fixed in 10% formaldehyde solution for 24 h. Hematoxylin-eosin (H&E) staining was performed on the tissue sections. The inflammatory score was as follows: Presence of occasional inflammatory cells in the lamina propria was scored as 0; increased numbers of inflammatory cells in the lamina propria was scored as 1; confluence of inflammatory cells extending into the submucosa was scored as 2; and transmural extension of the infiltrate was scored as 3. For tissue damage score, no mucosal damage was scored as 0; lymphoepithelial lesions were scored as 1; surface mucosal erosion or focal ulceration was scored as 2; and extensive mucosal damage and extension into deeper structures of the bowel wall was scored as 3. The combined histological score was calculated by inflammatory and tissue damage score, and ranged from 0 (no changes) to 6 (extensive infiltration and tissue damage).

Immunohistochemistry

The colonic tissues were sectioned into 4-μm slices following fixation with 10% formaldehyde and embedded in paraffin. Immunohistochemical staining was conducted as in our previous study[24]. Tissue slices were incubated with 3% H_2O_2 for 15 min after deparaffinization. Antigen was retrieved in citrate buffer (pH 6.0; Maxim Biotechnologies, Fuzhou, China) in a microwave for 10 min. Nonimmune 10% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) was used to block nonspecific reactions. The sections were incubated with primary antibodies: rabbit anti human/mouse IL34, 1:100 (Abcam, Cambridge, MA, United States); rabbit anti human/mouse Ki-67 antibody, 1:200 (Abcam); rabbit anti mouse CSF1-R 1:1000 (Abcam); and rabbit anti-mouse CD68, 1:100 (Boster Biological Technology, Wuhan, China) overnight at 4 °C. On the second day, the sections were incubated with horseradish-peroxidase-conjugated secondary antibody (Shanghai Long Island Biotechnology, Shanghai, China) for 30 min at room temperature. 3, 3′-diaminobenzidine (Maxim Biotechnologies) was applied for 30 s. Hematoxylin was applied for contrast staining. Proliferation index of Ki-67 was defined as the percentage of Ki-67-positive cells in crypts (CK4) within the random visual scope. Positive index of CSF1-R was defined as the percentage of CSF1-R-positive cells in colonic mucosa within the random visual scope. All the tissue sections were analyzed by optical microscopy (IX73; Olympus, Tokyo, Japan) by experienced pathologists.

TUNEL assay

Colonic tissue sections were prepared as described above. Apoptosis was analyzed by fluorescence microscopy according to standard procedure using *in situ* cell death detection (Roche, Basel, Switzerland). Five randomly optical fields were chosen for further analysis.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from the indicated murine colonic tissues by Trizol Reagent (TaKaRa Bio, Dalian, China). RNA samples were reverse-transcribed into cDNA with a PrimeScript™RT Master Mix kit (TaKaRa Bio). cDNA samples were detected using a SYBR®Premix Ex Taq™II kit (TaKaRa Bio) on CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, United States). PCR was performed at 95 °C for 30 s, and the samples were subjected to 40 cycles of amplification at 95 °C for 5 s and 60 °C for 30 s. Expression of target genes was normalized to β-actin. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All the primers are listed in Supplementary Table 2.

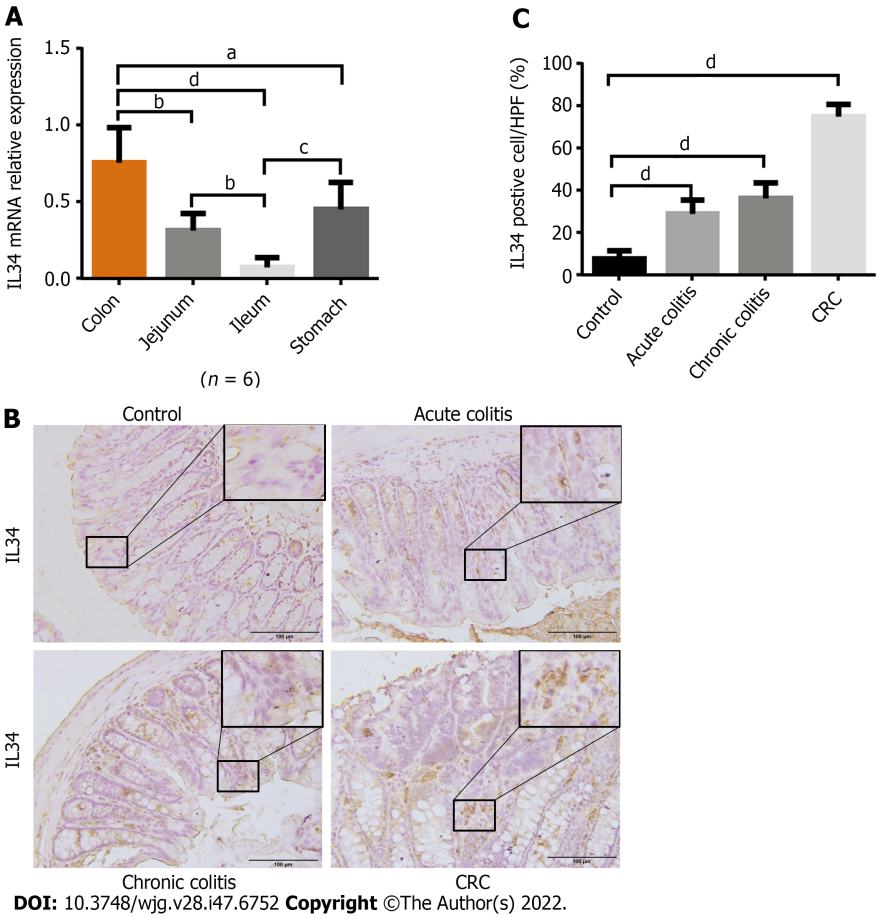
Statistical analysis

Data are presented as mean ± SD. The difference between more than two groups was analyzed using one-way analysis of variance and comparison of two groups was carried out using a *t*-test. Differences in survival between two groups were analyzed by Kaplan-Meier test. All statistical analyses were performed using GraphPad Prism 6.0 (San Diego, CA, United States). $P < 0.05$ was considered statistically significantly.

RESULTS

IL-34 is elevated in colitis and colitis-related cancer

We analyzed IL-34 mRNA expression in gastrointestinal mucosal epithelium in wild-type C57BL/6J mice. Expression of IL-34 was highest in the colon compared with other parts of the digestive tract (Figure 1A). We investigated expression of IL-34 in DSS-induced colitis and AOM-DSS-induced colitis-associated cancer in wild-type mice. Immunohistochemical staining of murine colon tissue revealed that expression of IL-34 was elevated in both acute and chronic colitis, with the highest expression in colitis-associated cancer (Figure 1B and C).

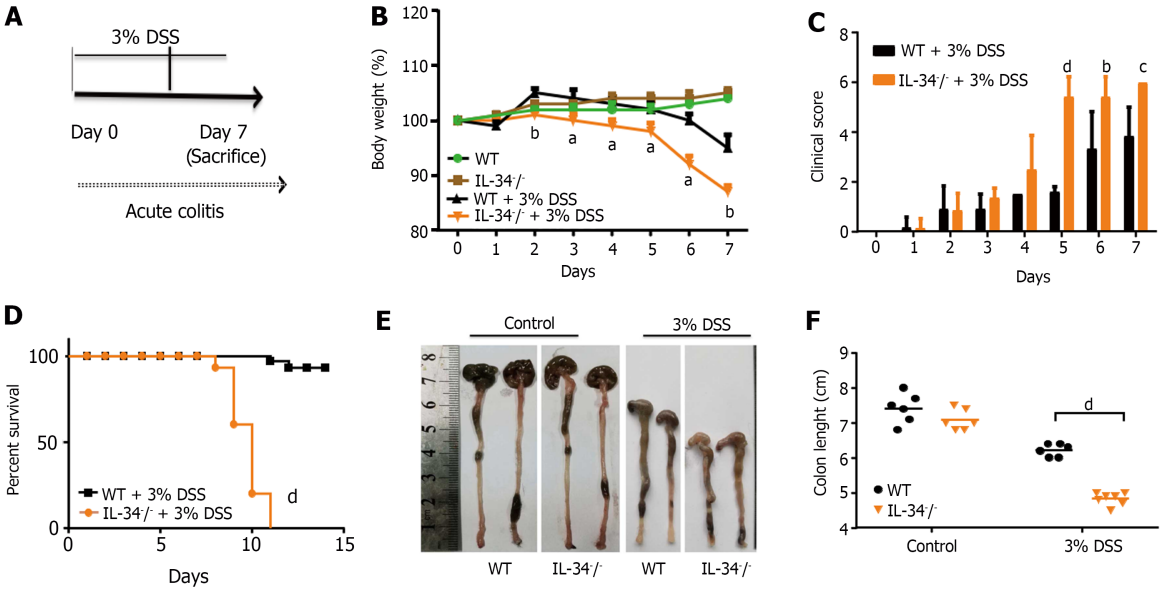


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Figure 1 Interleukin-34 is elevated in colitis and colitis-related cancers. A: Interleukin-34 (IL-34) relative mRNA expression in stomach, jejunum, ileum and colon in wild-type mice (C57BL/6J) (n = 6); B and C: The dynamic of IL-34 expression in dextran sodium sulfate (DSS)-induced colitis and azoxymethane-DSS-induced colorectal cancer in wild-type mice (n = 6 per group); representative IL-34 immunohistochemical staining for healthy control, acute colitis, chronic colitis and colorectal cancer mice (B); the percentage of IL-34-positive cells per high-power field were quantified in colonic tissue of four groups of mice (n = 6 per group) (C). AOM: Azoxymethane; CRC: Colorectal cancer; DSS: Dextran sodium sulfate; IL-34: Interleukin-34. Scale bars = 100 μm. Data depict the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$, $^{d}P < 0.001$.

IL-34 deficiency increases susceptibility to acute DSS-induced colitis

To determine the role of IL-34 in murine acute experimental colitis, IL-34$^{-/-}$ and wild-type mice were fed with 3% DSS in drinking water for 7 d and then killed (Figure 2A). DSS-fed IL-34$^{-/-}$ mice showed significantly greater body weight loss compared to DSS-fed wild-type mice. The average weight loss in DSS-fed IL-34$^{-/-}$ mice was approximately double than that in the wild-type mice (Figure 2B). IL-34$^{-/-}$ mice displayed a significantly higher clinical score compared to wild-type mice (Figure 2C). Strikingly, the mortality of IL-34$^{-/-}$ mice was 100% (10/10), whereas only 20% (2/10) of the wild-type mice died during the experiment, as illustrated by Kaplan-Meier curves (Figure 2D, $P < 0.001$). Following DSS administration, IL-34$^{-/-}$ mice showed remarkably shorter colon compared to wild-type mice (4.83 cm ± 0.13 cm *vs* 6.27 cm ± 0.14 cm, $P < 0.001$), suggesting more severe colitis in IL-34$^{-/-}$ mice (Figure 2E and F). DSS-fed IL-34$^{-/-}$ mice exhibited more severe inflammation with ulceration and necrotic lesions compared to wild-type mice, while there was no difference in the wild-type or IL-34$^{-/-}$ mice fed with normal water. More inflammatory cells infiltrated the lamina propria and submucosa in DSS-fed IL-34$^{-/-}$ mice. More importantly, the normal tissue architecture damage was more severe in DSS-fed IL-34$^{-/-}$ mice compared with wild-type mice (Figure 3A). Semiquantitative score of histopathology confirmed more severe colitis in DSS-fed IL-34$^{-/-}$ mice compared to DSS-fed wild-type mice (9.17 ± 0.31 *vs* 5.60 ± 1.10, $P < 0.001$) (Figure 3B). The expression of CD68, which serves as a characteristic marker for macrophages, was indistinguishable between IL-34$^{-/-}$ and wild-type mice untreated with DSS (Figure 3C and D). CD68 expression was significantly increased in DSS-fed IL-34$^{-/-}$ mice compared to DSS-fed wild-type controls (Figure 3C and D), which indicated worsening colitis. IL-1β, IL-23, and macrophage colony-stimulating factor levels were significantly upregulated in DSS-treated IL-34$^{-/-}$ mice compared to wild-type mice treated with DSS (Figure 3E).



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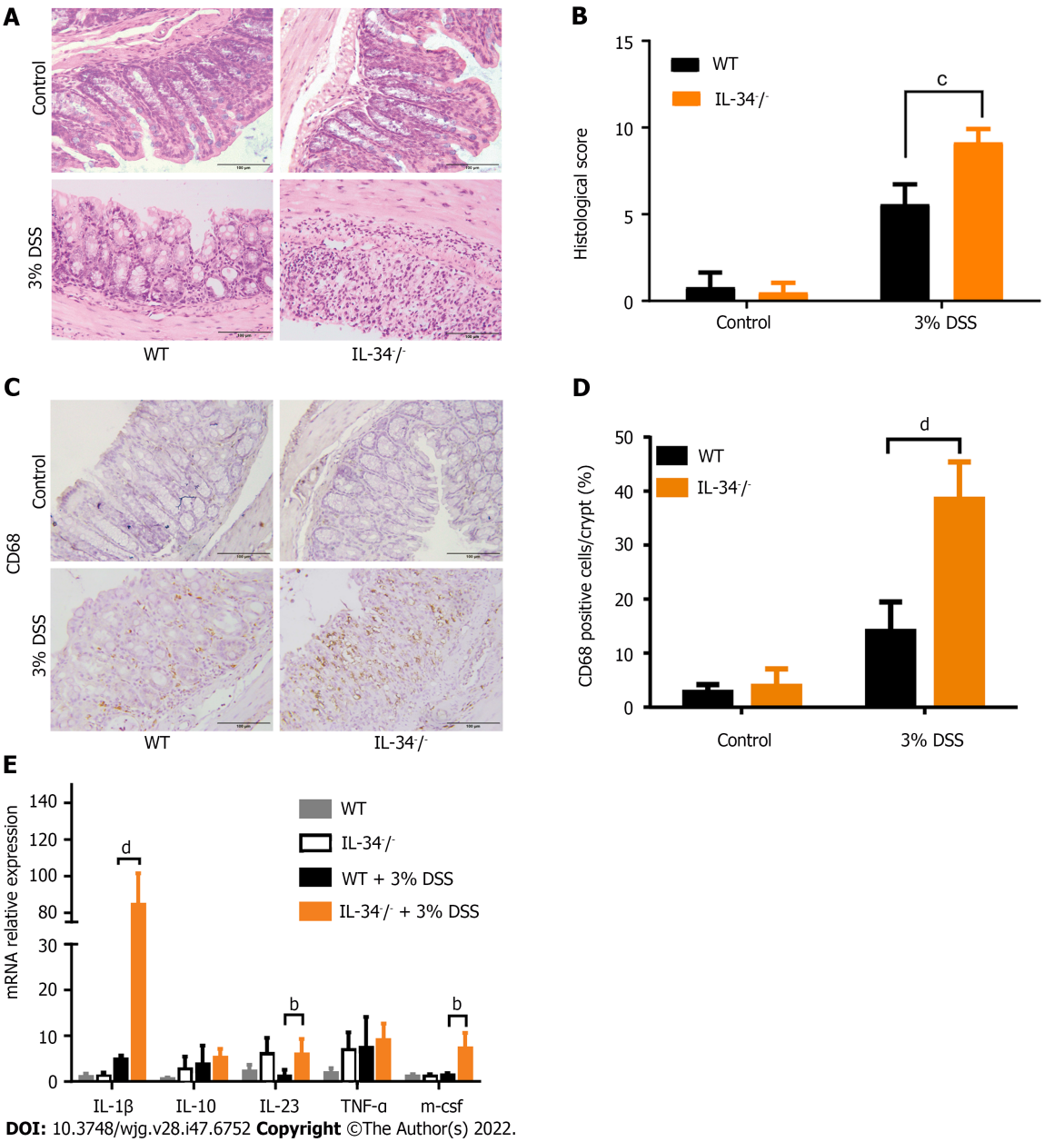
Figure 2 Interleukin-34 deficiency aggravates acute colitis induced by dextran sodium sulfate. A: Schematic of the design of acute dextran sodium sulfate (DSS)-induced colitis model. IL-34$^{-/-}$ and WT mice were fed with a 3% DSS solution in drinking water for 7 d and then sacrificed (n = 6 or 7 per group); B: Body weight was represented as a percentage of starting weight in IL-34$^{-/-}$ and WT mice after administration of 3% DSS (n = 6 or 7 per group); C: The clinical score of IL-34$^{-/-}$ and WT mice treated with 3% DSS (n = 6 or 7 per group). Data depict the mean ± SD; D: Kaplan-Meier survival curves were plotted in IL-34$^{-/-}$ and WT mice administrated with 3% DSS for 15 d (n = 10 per group); E and F: Colon length was measured in IL-34$^{-/-}$ and WT mice fed with 3% DSS for 7 d (n = 6 or 7 per group). DSS: Dextran sodium sulfate; IL-34: Interleukin-34; WT: Wild-type. Data depict the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$, $^{d}P < 0.001$.

IL-34 deficiency inhibits proliferation and promotes apoptosis of colonic epithelium in DSS-induced colitis

Prior to DSS administration, the levels of colonic epithelium proliferation and apoptosis were both comparable between wild-type and IL-34$^{-/-}$ mice (Figure 4A-D). A marked reduction in colonic epithelial cells stained positive for Ki-67 was detected in DSS-fed IL-34$^{-/-}$ mice compared to DSS-fed wild-type mice (Figure 4A and B). A marked increased in colonic epithelial cell apoptosis was noted in DSS-fed IL-34$^{-/-}$ mice compared to the DSS-fed wild-type mice (Figure 4C and D). We detected expression of three receptors of IL-34 in mouse colonic tissue by real-time quantitative PCR. In DSS-fed IL-34$^{-/-}$ mice, we only observed an increase in the expression of CSF1-R, but the other two receptors of IL34, ptprz1, and syndecan-1, did not change significantly (Figure 4E). By immunohistochemical staining of the mouse colonic tissues, we found that CSF1-R was expressed in epithelial and mesenchymal cells. Compared to the control group, the mice in the DSS-fed group showed an increasing positive index of CSF1-R. Expression of CSF1-R did not show a significant difference between IL-34$^{-/-}$ and wild-type mice untreated with DSS ($P > 0.05$, Figure 4F and G). DSS-fed IL-34$^{-/-}$ mice exhibited a higher index of CSF1-R positivity compared to DSS-fed wild-type mice ($P < 0.01$, Figure 4F and G). CSF1-R acts as a receptor for IL-34 and was compensatorily increased when colitis developed and IL-34 was absent. The results were consistent with the results of quantitative real-time PCR of CSF1R.

Protective effect of IL-34 against acute DSS-induced colitis is not dependent on macrophages

Macrophages were increased in the IL-34$^{-/-}$ mice treated with 3% DSS compared with the wild-type mice. We investigated whether the protective effect of IL-34 in colitis was dependent on macrophages. It is known that IL-34 plays a role in macrophage polarization. We studied the potential role of IL-34 in macrophage polarization by examining the selected markers for M1 and M2 macrophages. Most importantly, there was no difference in the ratio of colonic M2/M1 macrophage markers between DSS-treated wild-type and IL-34$^{-/-}$ mice (Figure 5A). It was speculated that the effect of IL-34 on experimental colitis was not attributed to macrophage polarization. In order to confirm that the protective effect of IL-34 in colitis was not dependent on macrophages, we depleted macrophages by intraperitoneal injection of clodronate liposomes (Clo-lips) in IL-34$^{-/-}$ and wild-type mice (Figure 5B). The macrophage cell marker F4/80 was significantly reduced in colonic mucosa of mice treated with Clo-lips, which confirmed that the liposomes effectively depleted macrophages in the colon (Figure 5C). Despite macrophage depletion, DSS-fed IL-34$^{-/-}$ mice still showed significantly higher clinical score and shorter colon length compared with DSS-fed wild-type mice (Figure 5D-F). Similarly, the histopathological manifestation showed that colitis severity in DSS-fed IL-34$^{-/-}$ mice was more pronounced compared to that in DSS-fed wild-type mice (Figure 5G and H). The inflammatory cytokines remained significantly higher in colonic mucosa of DSS-fed IL-34$^{-/-}$ mice compared with DSS-fed wild-type mice (Figure 5I).

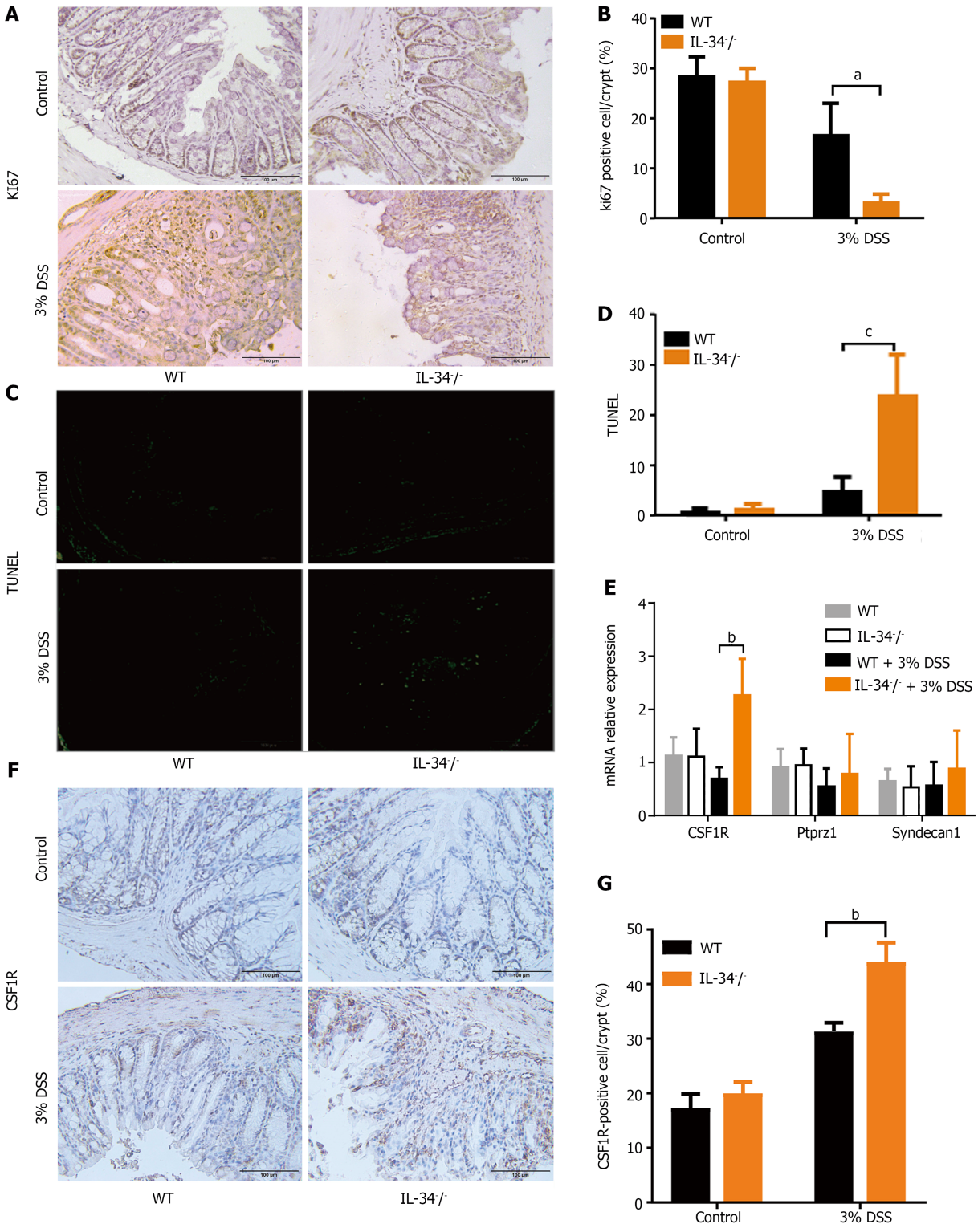


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Figure 3 Interleukin-34 deficiency aggravates acute colitis and increase proinflammatory cytokines. A: Representative microscopic pictures of hematoxylin and eosin-stained colon sections of Interleukin-34 (IL-34)$^{-/-}$ and WT mice fed with 3% DSS for 7 d (n = 6 or 7 per group); B: Histological score for IL-34$^{-/-}$ and WT mice fed with 3% dextran sodium sulfate (DSS); C: Representative photomicrographs of macrophage staining in colon sections of IL-34$^{-/-}$ and WT mice treated with DSS; D: Statistical analysis of CD68-positive cells in IL-34$^{-/-}$ and WT mice treated with DSS; E: mRNA expression of proinflammatory cytokines including IL-1β, IL-10, IL-23, TNF-α, and M-CSF in IL-34$^{-/-}$ and WT mice fed with 3% DSS. DSS: Dextran sodium sulfate; IL: Interleukin; M-CSF: Macrophage colony-stimulating factor; TNF-α: Tumor necrosis factor-α; WT: Wild-type. Scale bars = 100 μm. Data depict the mean ± SD. $^{b}P < 0.01$, $^{c}P < 0.005$, $^{d}P < 0.001$.

IL-34 deficiency delays mucosal healing in a murine model induced by DSS

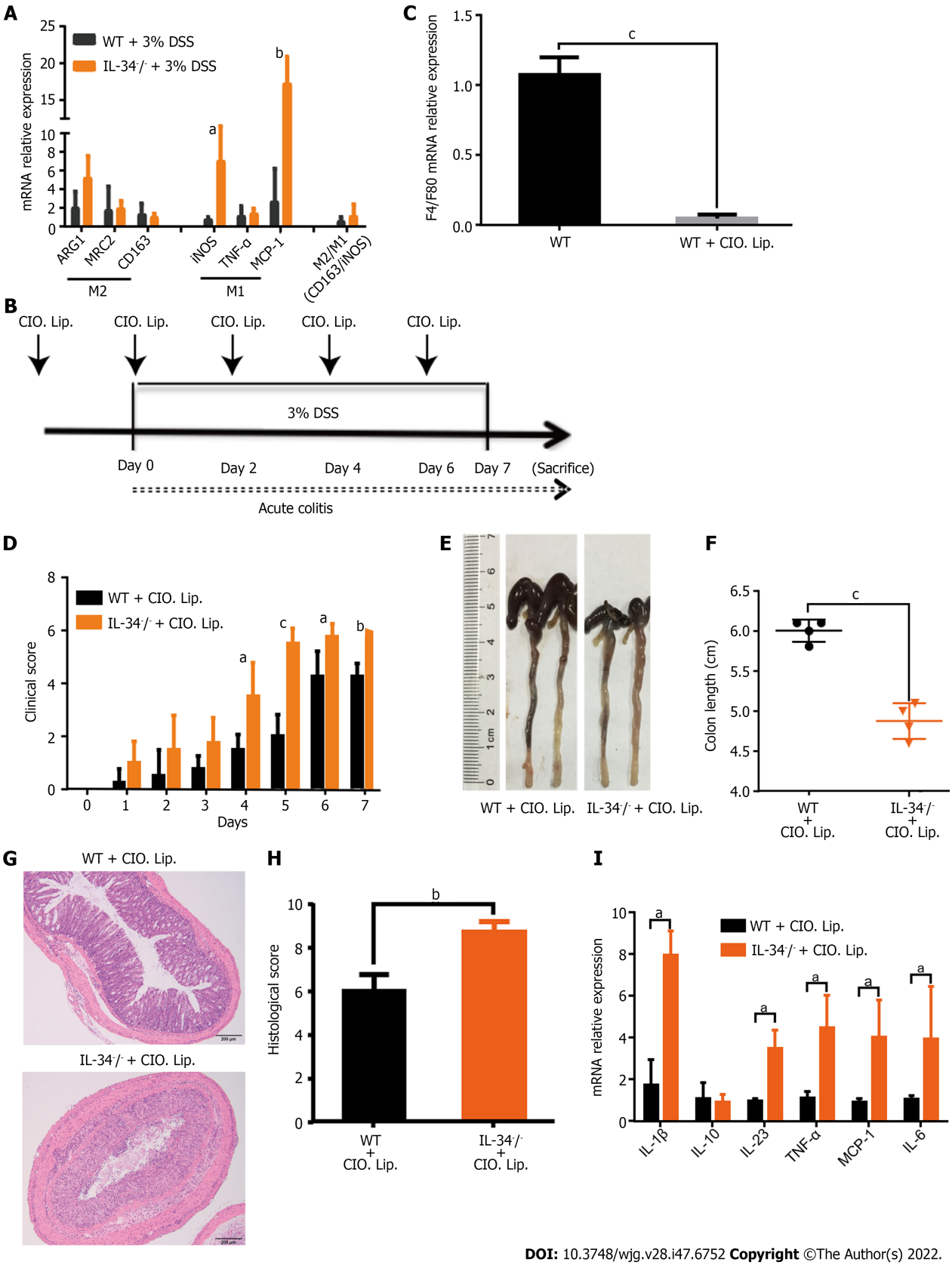
IL-34$^{-/-}$ and wild-type mice were fed with 3% DSS for 5 d and then switched to normal water for the following 5 d to establish a mucosal healing model. The mice in both groups were killed on day 8 or 10 (Figure 6A). The body weight loss was significantly greater in IL-34$^{-/-}$ mice compared to the wild-type control mice. The body weight increased quickly on day 8 in wild-type mice, while it did not recover until day 10 in IL-34$^{-/-}$ mice (Figure 6B). The clinical score was higher in IL-34$^{-/-}$ mice compared to wild-type mice from day 5. The clinical score decreased rapidly from day 7 in wild-type mice, while it was falling slowly from day 8 in IL-34$^{-/-}$ mice (Figure 6C). IL-34$^{-/-}$ mice showed remarkably shorter colon length compared to wild-type controls on day 8 (5.25 cm ± 0.32 cm *vs* 6.30 cm ± 0.25 cm, $P < 0.01$) and day 10 (5.03 cm ± 0.49 cm *vs* 7.30 cm ± 0.47 cm, $P < 0.005$) (Figure 6D and E). Histopathological analysis showed that IL-34$^{-/-}$ mice exhibited more severe inflammation and tissue damage compared to wild-type mice on days 8 and 10, respectively (Figure 6F). Semiquantitative scoring of histopathology showed that colitis severity in IL-34$^{-/-}$ mice was significantly higher than that in wild-type mice on day 8 (8.13 ± 0.66 *vs* 3.63 ± 0.58, $P < 0.01$) and day 10 (8.45 ± 061 *vs* 2.60 ± 0.58, $P < 0.01$), respectively (Figure 6G). The



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Figure 4 Interleukin-34 deficiency inhibits proliferation and promotes apoptosis of colonic epithelium in dextran sodium sulfate-induced colitis. A: Representative photomicrographs of Ki-67 immunohistochemical staining for colon sections of IL-34$^{-/-}$ and WT mice treated with dextran sodium sulfate (DSS); B: Percentage of proliferation marker Ki-67 per crypt; C and D: Representative microscopic pictures of TUNEL staining (C) and quantification of TUNEL-positive cells per field (D) in IL-34$^{-/-}$ and WT mice treated with DSS as before; E: mRNA expression of three receptors for IL-34 (CSF1R, ptprz1 and syndecan-1) was detected in IL-34$^{-/-}$ and WT mice treated with DSS (n = 6 or 7 per group); F and G: Representative photomicrographs of CSF1R immunohistochemical staining (F) and percentage of CSF1R-positive cells (G) in colon sections of IL-34$^{-/-}$ and WT mice treated with 3% DSS. CSF1R: Colony-stimulating factor-1 receptor; DSS: Dextran sodium sulfate; IL-34: Interleukin-34; WT: Wild-type. Scale bars = 100 μm. Data depict mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$.

number of colonic epithelial cells positive for Ki-67 was markedly decreased in IL-34$^{-/-}$ mice compared to wild-type mice on days 8 and 10 (Figure 6H and I).



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Figure 5 Protective effect of interleukin-34 against acute dextran-sodium-sulfate-induced colitis is not dependent on macrophages. A: mRNA expression levels of M1 macrophage markers (iNOS, TNF-α and MCP-1) and M2 macrophage markers [ARG1, MRC2 (CD163)] in the colonic mucosa of IL-34$^{-/-}$ and WT mice treated with 3% DSS. M2/M1 ratio (CD163/iNOS) was calculated; B: Experimental design. To deplete macrophages, IL-34$^{-/-}$ and WT mice were treated with Clo-lips 2 d prior to 3% dextran sodium sulfate (DSS) administration and continuing once every 2 d until death (n = 4 per group); C: mRNA expression of macrophage cell marker (F4/80) was detected in colonic mucosa of WT mice treated with Clo-lips (n = 4 per group); D: Clinical score of IL-34$^{-/-}$ and WT mice treated with 3% DSS and Clo-lips (n = 4 per group); E and F: Colon length of IL-34$^{-/-}$ and WT mice treated with 3% DSS and Clo-lips (n = 4 per group); G and H: Representative microscopic pictures (G) and histological scores (H) of IL-34$^{-/-}$ and WT mice fed with 3% DSS and Clo-lips (n = 4 per group); I: mRNA expression of

inflammatory cytokines was detected in IL-34$^{-/-}$ and WT mice treated with 3% DSS and Clo-lips (n = 4 per group). ARG1: Arginase; Clo-lip: Clodronate liposome; CSF1R: Colony-stimulating factor-1 receptor; DSS: Dextran sodium sulfate; IL-34: Interleukin-34; iNOS: Inducible nitric oxide synthase; MCP-1: Monocyte chemoattractant protein-1; MRC2: Mannose receptor C type 2; TNF-α: Tumor necrosis factor-α; WT: Wild-type. Scale bars = 200 μm. Data depict mean ± SD. ^{a}P < 0.05, ^{b}P < 0.01, ^{c}P < 0.005.

IL-34 deficiency promotes AOM/DSS-induced colitis-associated tumorigenesis

To investigate the role of IL-34 in colitis-associated tumorigenesis, wild-type and IL-34$^{-/-}$ mice were treated with AOM and DSS (Figure 7A). No wild-type mice died, whereas a mortality rate of 30% was noted in IL-34$^{-/-}$ mice (Figure 7B). More importantly, a marked difference in tumor number was observed between the two groups. IL-34$^{-/-}$ mice developed a greater number of colon tumors than wild-type mice (Figure 7C and D). Representative H&E staining of colitis-associated cancer in wild-type and IL-34$^{-/-}$ mice is shown in Figure 7E.

IL-34 expression was elevated in inflamed mucosa and was associated with colonic epithelium proliferation in UC patients

We investigated the IL-34 expression pattern and its relationship with colonic epithelium proliferation in active UC patients. There was no age or sex difference between the groups (Supplementary Table 1). Consistent with previous studies, we also observed elevated expression of IL-34 in diseased mucosa of UC patients compared with the normal controls as detected by immunohistochemistry. Importantly, IL-34 was suspected to be predominantly expressed in the colonic stromal tissue (Figure 8A and B). Significantly enhanced colonic expression of proliferation index Ki-67 was detected in UC patients with higher IL-34 expression in colonic mucosa (Figure 8C). There was a positive correlation between IL-34 and Ki-67 expression in UC-inflamed mucosa with a correlation coefficient of 0.60 (P < 0.0001) (Figure 8D). No correlation was found between IL-34-positive and TUNEL-positive cells (data not shown).

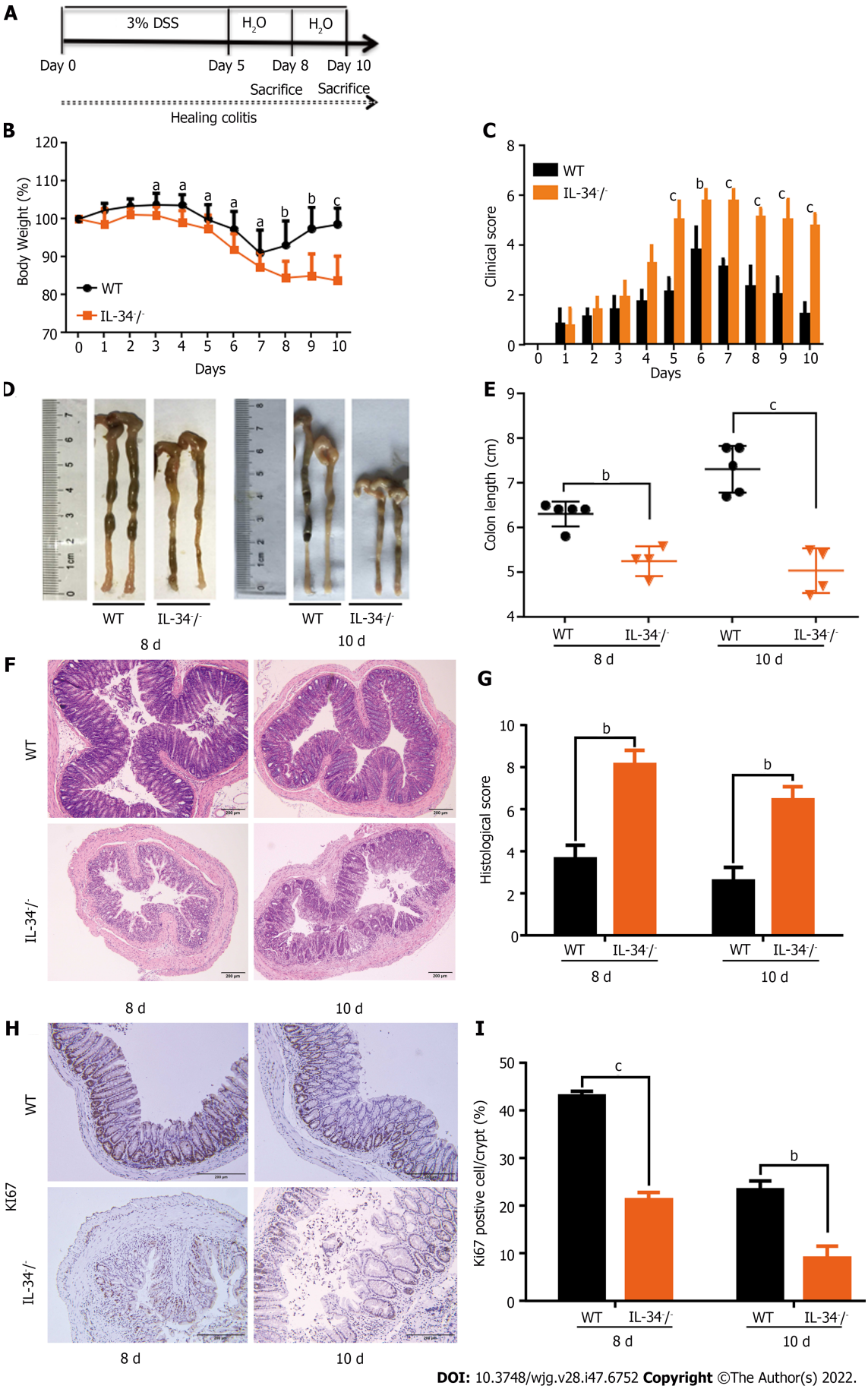
DISCUSSION

Mucosal healing has become the goal of therapy because of its association with better prognosis and improved quality of life[25]. However, the underlying molecular mechanism is unclear. Here, we revealed that IL-34 deficiency strongly increased susceptibility to acute DSS-induced colitis and intestinal wounding. Additionally, IL-34 deficiency enhanced AOM/DSS-induced colitis-associated tumorigenesis. Different from the previous reports, our findings suggest the protective role of IL-34 in UC- and colitis-associated cancer and may help to establish a new potential approach for management of UC.

The IL-34 knockout mice used in our study facilitated us to illustrate the role of IL-34 in pathogenesis of UC. Prior to DSS administration, no difference in colon morphological and histological manifestations was observed between IL-34-deficient and wild-type control mice. The results suggest that IL-34 is dispensable for colon homoeostasis under steady state.

In past studies, overexpression of IL-34 stimulated by inflammatory cytokines augmented the production of proinflammatory cytokines *in vitro*, probably raising IL-34 as a pathogenic contributor to sustained colon inflammation[14]. However, the increased expression of IL-34 might be a consequence of inflammation as a protective mechanism rather than a disease cause[16]. Indeed, IL-34 transgenic mice do not display aggravating inflammatory responses in the colon[26]. The precise mechanistic role of IL-34 during colon inflammation has not been clarified yet. Until now, there has been a lack of research on IL-34 involved in UC *in vivo*. A recent study has reported that IL-34 is critical for the suppressive function of CD4^{+} T regulatory cells, and its deficiency leads to increased susceptibility to autoimmunity[27]. We demonstrated for the first time that IL-34 deficiency exacerbates DSS-induced colitis during acute and delayed mucosal healing process and is associated with high colitis-related mortality. We assumed that significantly increased expression of IL-34 in mucosa from patients with active UC might act as an urgent requirement for alleviating inflammation and promoting mucosal healing. Our findings support the protective characteristic of IL-34 during DSS-induced colitis and early healing stage.

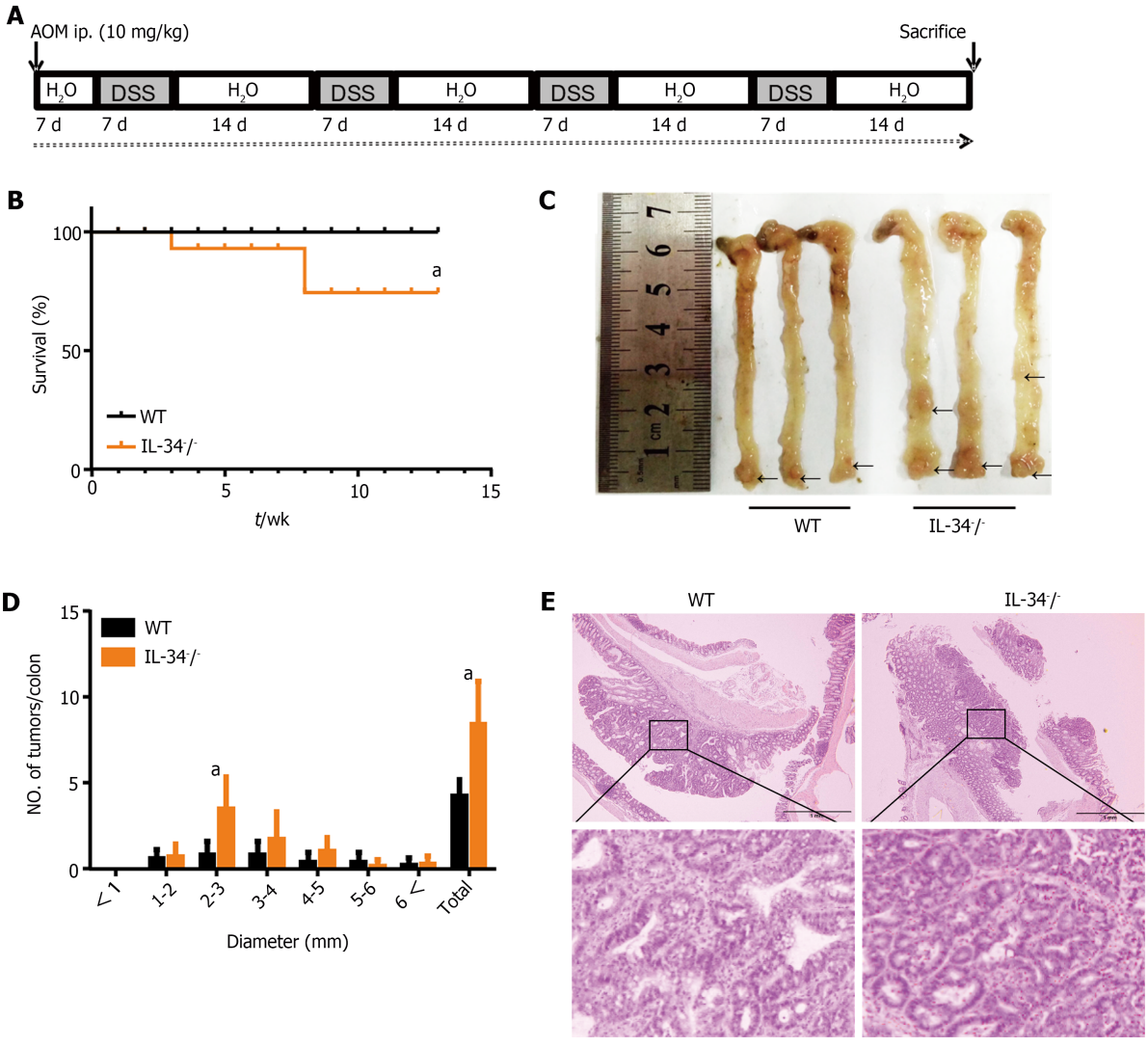
Macrophages are critical to mucosal homeostasis in orchestrating innate and adaptive immunity[28]. Depending on the local microenvironment, macrophages differentiated from the peripheral blood monocytes develop towards M1-like or M2-like phenotype macrophages[29]. M1 macrophages secreting proinflammatory cytokines such as TNF-α or IL-12 are considered to amplify the inflammatory response, whereas M2 macrophages producing anti-inflammatory mediators such transforming growth factor-β or IL-10 suppress the inflammatory response[29]. IL-34 has been identified to trigger CSF-1R signaling and induce macrophage differentiation to M2 phenotype *via* activation of the ERK1/2/AKT/AMPK signaling pathway[8], which contributes to mucosal homeostasis maintenance and



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Figure 6 Interleukin-34 deficiency delays the mucosal healing in murine model induced by dextran sodium sulfate. A: Establishing healing

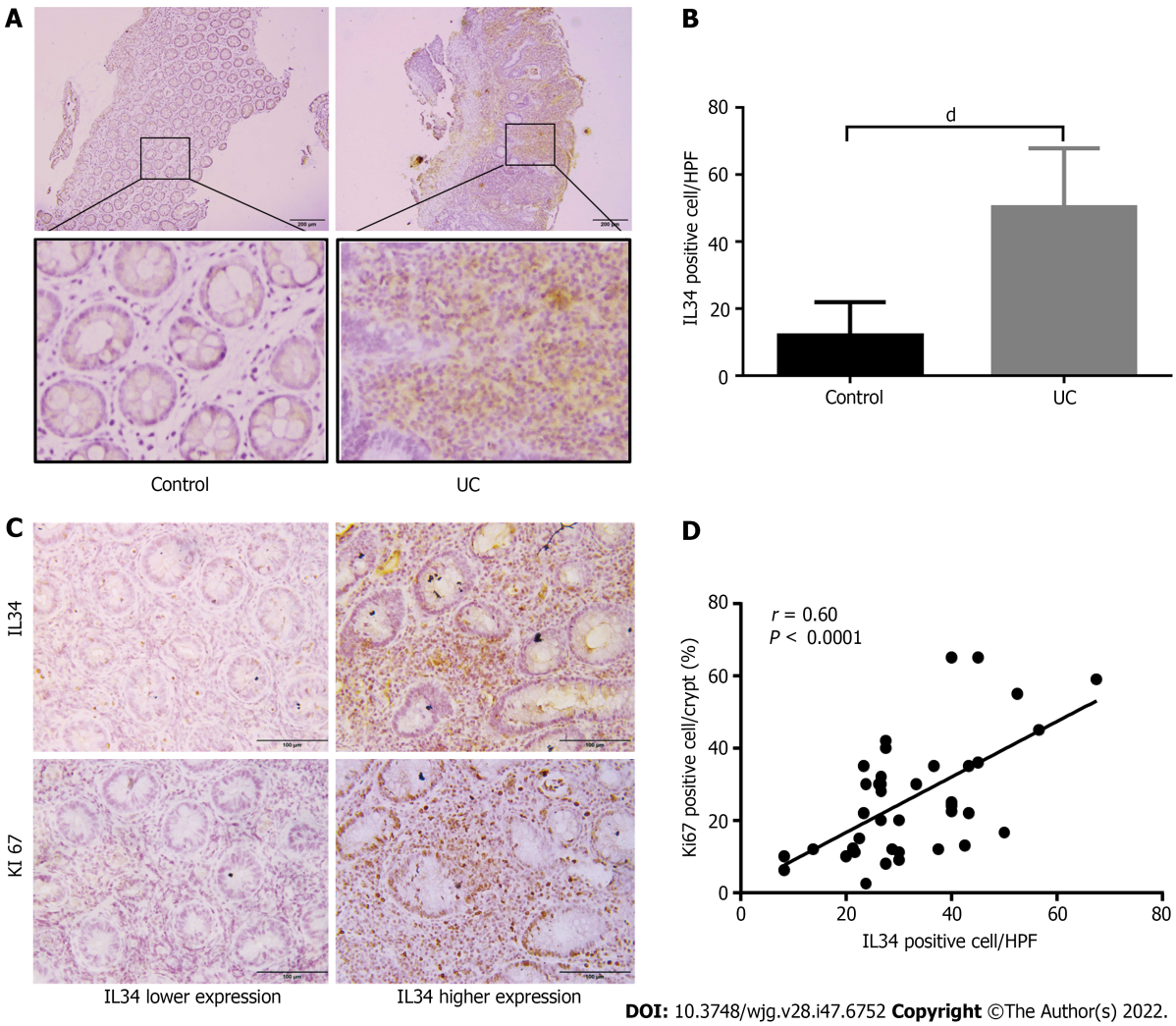
model chart. The Interleukin-34 (IL-34)$^{-/-}$ and WT mice were fed with 3% dextran sodium sulfate (DSS) for 5 d and switched to normal drinking water for the following 5 d. The mice in both groups were killed on day 8 or 10 (n = 4 or 5 per group); B and C: Body weight (B) and clinical score (C) were determined daily in IL-34$^{-/-}$ and WT mice until day 10 (n = 4 or 5 per group); D and E: The colon length was measured in IL-34$^{-/-}$ and WT mice on day 8 and 10, respectively (n = 4 or 5 per group); F and G: Representative microscopic pictures (F) and semiquantitative histological scores (G) of IL-34$^{-/-}$ and WT mice on day 8 and 10, respectively (n = 4 or 5 per group); H and I: Representative photomicrographs of Ki-67 immunohistochemical staining (H) and percentage of Ki-67-positive per crypt (I) in IL-34$^{-/-}$ and WT mice on day 8 and 10, respectively. DSS: Dextran sodium sulfate; IL-34: Interleukin-34; WT: Wild type. Scale bars = 200 μm. Data depict the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$.



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Figure 7 Interleukin-34 deficiency promotes azoxymethane /dextran-sodium-sulfate-induced colitis-associated tumorigenesis. A: Experimental design was shown as diagram. WT and Interleukin-34 (IL-34)$^{-/-}$ mice were given a single injection of azoxymethane (AOM, 10 mg/kg). Seven days later, mice were treated with 1.25% dextran sodium sulfate (DSS) (w/v) in drinking water for seven consecutive days, and given fresh drinking water for 14 d. Seven days of DSS and 14 d of fresh water as a cycle, repeated four times; B: Kaplan-Meier survival curves were plotted in IL-34$^{-/-}$ and WT mice treated with AOM/DSS (n = 10 per group); C and D: Representative photo of colon (C) and tumor number in colon (D) in IL-34$^{-/-}$ and WT mice treated with AOM/DSS (n = 10 per group); E: Representative hematoxylin and eosin-stained colon sections in IL-34$^{-/-}$ and WT mice treated with AOM/DSS (n = 10 per group). AOM: Azoxymethane; DSS: Dextran sodium sulfate; WT: Wild type. Bars: 1 mm (top) and 100 μm (bottom). Data depict mean ± SD. $^{a}P < 0.05$.

tissue remodeling[17]. Regulation of M1 to M2 phenotype switch has been confirmed to promote the proliferative phase of wound healing and ameliorates DSS-induced colitis, indicating a possible role of IL-34 in alleviation of colonic inflammation[17,18]. In our study, IL-34-deficient mice treated with DSS presented with worse colitis with significant accumulation of macrophages compared to DSS-treated wild-type control mice. It is speculated that downregulation of the M1 to M2 macrophage phenotypic switch results in more severe colitis. However, M2/M1 macrophage ratio was not significantly decreased in DSS-treated IL-34-deficient mice. We subsequently depleted the macrophages in the murine colitis model by administration of Clo-lips, which is a well-established method for macrophage



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Figure 8 Interleukin-34 expression was elevated in inflamed mucosa and associated with colonic epithelium proliferation in active ulcerative colitis. A: Representative microscopic pictures of interleukin-34 (IL-34) immunohistochemical staining in colonic biopsies of healthy controls or individuals with active ulcerative colitis (UC); B: Percentage of IL-34-positive cells per high-power field were quantified in colonic biopsies of healthy controls (n = 20) and active UC patients (n = 40); C: Representative photomicrographs of immunostaining of Ki67 in colonic biopsies of active UC patients in IL-34 Lower expression and higher expression groups; D: Correlation analysis of colonic IL-34 expression and Ki 67-positive cells in active UC patients. IL-34: Interleukin-34; UC: Ulcerative colitis. Scale bar indicates 100 μm or 200 μm. $^{d}P < 0.001$.

depletion[30], showing that IL-34 deficiency aggravated murine colitis whether or not macrophages were depleted. Our results suggested that the protective effect of IL-34 on experimental colitis was not primarily dependent on macrophage polarization.

Mucosal healing largely relies on controlled colonic epithelial proliferation and apoptosis, which are manipulated by multiple growth factors, gut peptides, cytokines, as well as signaling pathways[25]. Given different DSS-induced injury levels in normal and IL-34-deficient colonic mucosa, we should be cautious to draw the conclusion that IL-34 deficiency inhibits the mucosal healing process *in vivo*. Based upon immunohistochemical staining, IL-34 was predominantly localized in the colon stromal tissue in our study. The specific cellular source of IL-34 needs to be further explored. In the acute colitis model, epithelial cell proliferation was inhibited but epithelial cell apoptosis was induced in IL-34$^{-/-}$ mice. CSF-1R was compensatorily increased in the IL-34$^{-/-}$ mice treated with DSS. Furthermore, it has been proved that IL-34 expression is positively correlated with epithelial proliferation in mucosal biopsies from UC patients. No correlation was shown between IL-34 expression level and apoptosis in UC patients. It is speculated that IL-34 might be produced from colon stromal tissue and promote colonic epithelial cell growth and wound closure by binding to CSF-1R in inflammatory conditions. Further studies are warranted to investigate the potential function of IL-34 in colonic epithelial cells.

Emerging data have demonstrated the multidimensional role of IL-34 in tumor progression and metastasis. The function of IL-34 in cancer may vary among different types of tumors[31]. A recent study on the relationship between IL-34 and gastric cancer proposed that the reduction of IL-34 in gastric cancer was inversely related to the degree of tumor differentiation and was closely related to the poor survival rate of patients[32]. However, the relationship of IL-34 to colorectal cancer remains contro-

versial. It has been recently reported that IL-34 stimulates colorectal adenocarcinoma cell proliferation *via* an ERK1/2-dependent pathway. The connection between chronic colon inflammation and colitis-associated cancer has been documented in UC patients. Repeated mucosal damage and repair in the inflamed microenvironment can result in uncontrolled epithelial cell proliferation and induce cancer events[33]. In a cohort study, Wang *et al*[34] showed that low expression of IL-34 is associated with poor survival in colorectal cancer. Accumulating evidence suggests that chronic inflammation enhances the development of colitis-associated cancer through multiple mechanisms, including oxidative stress, DNA damage, abnormal immune response, and gut microbiome dysbiosis[35]. In our study, tumor burden was markedly enhanced in IL-34$^{-/-}$ mice treated with AOM/DSS compared to wild-type control mice. We assumed that IL-34 deficiency worsened the colonic inflammation response and tissue damage, and thus sustained inflammatory injury increased the risk of colitis-associated cancer.

CONCLUSION

In summary, our work has highlighted the role of IL-34 during acute experimental colitis and wound healing. We found that IL-34 does not drive the inflammatory response and tissue destruction under physiological conditions, but protects the host from inflammatory injury and reduces the risk of colitis-associated cancer. IL-34 might serve as a potential therapeutic target for inducing mucosal healing in treatment for UC and reducing colitis-associated cancer in UC.

ARTICLE HIGHLIGHTS

Research background

The exact pathogenesis of ulcerative colitis (UC) remains unclear. Identification of distinctive cytokines involved in immunopathogenesis of UC has become a hot spot for the development of biological therapies. The expression of interleukin (IL)-34 is upregulated in active UC but the molecular function and underlying mechanism are largely unknown.

Research motivation

Whether IL-34 is a "friend or foe" in the pathogenesis of UC remains be explored. In this study, we investigated the potential role of IL-34 in experimental colitis, colitis-associated carcinogenesis and UC.

Research objectives

To investigate the function of IL-34 in acute colitis, in a wound healing model and in colitis-associated cancer, and the IL-34 expression pattern and its relationship with colonic epithelium proliferation in active UC patients.

Research methods

We conducted a controlled study using IL-34 knockout mice and wild-type mice (C57BL/6J). Colitis was induced by administration of dextran sodium sulfate, and carcinogenesis was induced by azoxymethane. Whether the impact of IL-34 on colitis was dependent on macrophages was validated by depletion of macrophages in a murine model. The association between IL-34 expression and epithelial cell proliferation was determined in patients with active UC.

Research results

IL-34 deficiency *in vivo* exacerbated colitis in mice during acute and wound healing phases and increased tumor susceptibility in the mouse colon. The effect was independent of macrophage differentiation and polarization. IL-34 was markedly increased in patients with active UC and the expression was positively correlated with epithelial cell proliferation in UC.

Research conclusions

IL-34 deficiency exacerbates colonic inflammation and accelerates colitis-associated carcinogenesis in mice.

Research perspectives

IL-34 might serve as a potential therapeutic target for inducing mucosal healing in treatment of UC and reducing colitis-associated cancer in UC.

FOOTNOTES

Author contributions: Liu ZX performed the main part of the study; Chen WJ and Wang Y performed the main part of the study and wrote original draft; Liu YC, Cheng TC, Luo LL, Chen L, and Ju LL provided technical support and analyzed the data; Chen BQ, Liu Y, Li M, and Feng N contributed to part of the experiments; Shao JG designed the study and guided the manuscript writing; Bian ZL Conceived and designed the study; Liu ZX, Chen WJ, and Wang Y contributed equally to this work; all authors have read and approve the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Dickkopf-related protein 1/cytoskeleton-associated protein 4 signaling activation by *Helicobacter pylori*-induced activator protein-1 promotes gastric tumorigenesis *via* the PI3K/AKT/mTOR pathway

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Abstract

BACKGROUND

Gastric cancer (GC) is a common malignant tumor with high incidence and mortality rates globally, especially in East Asian countries. *Helicobacter pylori* (*H. pylori*) infection is a significant and independent risk factor for GC. However, its underlying mechanism of action is not fully understood. Dickkopf-related protein (DKK) 1 is a Wnt signaling antagonist, and cytoskeleton-associated protein (CKAP) 4 is a newly identified DKK1 receptor. Recent studies found that the binding of DKK1 to CAKP4 mediated the procancer signaling of DKK1 independent of Wnt signaling. We hypothesize that *H. pylori*-induced activation of DKK1/CKAP4 signaling contributes to the initiation and progression of GC.

AIM

To investigate the interaction of *H. pylori* infection, DKK1 and CAKP4 in GC, as well as the underlying molecular mechanisms.

METHODS

RNA sequencing was used to identify differentially expressed genes (DEGs) between *H. pylori*-infected and uninfected primary GC cells. Gain- and loss-of-

function experiments were performed to verify the *H. pylori*-induced upregulation of activator protein-1 (AP-1) in GC cells. A dual-luciferase reporter assay and co-immunoprecipitation were used to determine the binding of AP-1 to the DKK1 promoter and DKK1 to CKAP4. Western blotting and immunohistochemistry detected the expression of DKK1, CKAP4, and phosphatidylinositol 3-kinase (PI3K) pathway-related proteins in GC cells and tissues. Functional experiments and tumorigenicity in nude mice detected malignant behavior of GC cells *in vitro* and *in vivo*.

RESULTS

We identified 32 DEGs between primary GC cells with and without *H. pylori* infection, including JUN, fos-like antigen-1 (FOSL1), and DKK1, and confirmed that the three proteins and CKAP4 were highly expressed in *H. pylori*-infected GC cells, *H. pylori*-infected gerbil gastric tissues, and human GC tissues. JUN and FOSL1 form AP-1 to transcriptionally activate DKK1 expression by binding to the DKK1 promoter. Activated DKK1 bound to CKAP4, but not the most common Wnt coreceptor low-density lipoprotein receptor-related protein 5/6, to promote GC cell growth, colony formation, migration, invasion, and xenograft tumor growth in nude mice. All these effects were driven by activation of the PI3K/AKT/mammalian target of rapamycin (mTOR) pathway. Targeting the PI3K signaling pathway by LY294002 inhibited DKK1-mediated CKAP4/PI3K signaling activity and the malignant behavior of GC cells.

CONCLUSION

H. pylori induces JUN and FOSL1 expression to form AP-1, which transcriptionally activates DKK1. Binding of DKK1 to KAKP4 contributes to gastric tumorigenesis *via* the PI3K/AKT/mTOR pathway.

Key Words: Gastric cancer; *Helicobacter pylori*; Dickkopf-related protein 1; Cytoskeleton-associated protein 4; Phosphatidylinositol 3-kinase pathway

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Core Tip: *Helicobacter pylori* (*H. pylori*) infection is the most significant risk factor for gastric cancer (GC). More than half of the global population has *H. pylori* infection, and 1%-3% of the infected individuals develop GC, but the mechanism behind this link remains unclear. Here, we identified 32 highly expressed genes in *H. pylori*-infected GC cells and demonstrated that *H. pylori*-induced high expression of JUN and fos-like antigen-1 formed activator protein-1 to transcriptionally activate dickkopf-related protein (DKK) 1, which by binding to cytoskeleton-associated protein 4 (CKAP4) receptor activated the PI3K/AKT/mammalian target of rapamycin pathway and, consequently, gastric tumorigenesis. Targeting the DKK1/CKAP4 interaction may be a novel strategy to treat GC.

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INTRODUCTION

Gastric cancer (GC) is the fifth most prevalent tumor and fourth leading cause of tumor-related mortality worldwide[1]. Although the incidence and mortality rates of this malignancy have steadily decreased over the past several decades, GC remains a significant health problem in developing nations [2]. Most patients with GC present at an advanced stage when diagnosed, and metastasis and recurrence in individuals are commonly noted[3]. *Helicobacter pylori* (*H. pylori*) are Gram-negative and microaerophilic pathogenic bacteria. *H. pylori* infection is the most significant risk factor for GC. The International Agency for Research on Cancer designated it a Group I carcinogen for GC in 1994[4]. More than half of the global population has *H. pylori* infection, and 1%-3% of infected individuals develop GC [5]. Globally, an estimated 89.4% and 20% of new noncardia and cardia GC cases were attributable to *H. pylori* infection in 2018[6]. However, this figure was 78.5% for noncardia GC and 62.1% for cardia GC in China[7]. Although many studies have investigated the link between *H. pylori* infection and GC[8], the

molecular mechanisms are not fully understood.

By RNA sequencing, we discovered that JUN (also known as c-JUN), fos-like antigen-1 (FOSL1, a FOS family member encoding FRA-1), and Dickkopf-related protein (DKK) 1 were elevated in primary GC cells infected with *H. pylori*. JUN and FOSL1 are the transcription factor activator protein (AP)-1 complex components and form the JUN::JUN homodimer and the JUN::FOSL1 heterodimer to regulate target gene transcription *via* binding to the promoters and enhancers of target genes[9]. *In vitro* and *in vivo* studies showed that activator protein-1 (AP-1) controlled tumor growth, progression and drug resistance[10,11]. DKK1 is a potent antagonist that suppresses oncogenic Wnt/β-catenin signaling and tumors by binding to the Wnt coreceptor: low-density lipoprotein receptor-related protein (LRP) 5/6[12,13]. However, recent studies demonstrated that DKK1 behaved like an oncogene in a variety of cancers [14,15]. Several studies consistently indicated that DKK1 levels were elevated in GC patient cancer tissues and serum, and higher DKK1 levels were significantly associated with worse outcomes[16-18]. However, little is known about the transcriptional regulation of DKK1, its relationship with *H. pylori*, and the mechanism of DKK1 promotion of gastric carcinogenesis.

Cytoskeleton-associated protein (CKAP) 4 is a newly identified DKK1 receptor[19]. The binding of DKK1 to CKAP4 recruits phosphatidylinositol 3-kinase (PI3K) *via* association between the proline-rich region of CKAP4 and the Src homology 3 domain of PI3K, which leads to the activation of serine/threonine-protein kinase AKT[20]. Although there is no evidence that the DKK1/CKAP4 axis exists in GC, the PI3K pathway involved in the malignant transformation of cells is activated in most GC patients[21]. AKT and p-AKT were overexpressed in > 74% of GC patients[22], and phosphorylated mammalian target of rapamycin (p-mTOR) expression was found in 60% of gastric adenocarcinoma specimens[23]. Therefore, we postulate that DKK1 contributes to GC through the PI3K pathway but not the Wnt pathway.

Here, we revealed that *H. pylori* infection induced high expression of JUN and FOSL1, which formed AP-1 to activate DKK1 transcriptionally. DKK1 binding to CKAP4, but not the most common Wnt coreceptor LRP5/6, promoted GC growth and invasion by triggering the PI3K/AKT/mTOR pathway. The results provide novel insight into the molecular mechanism underlying *H. pylori*-induced gastric carcinogenesis.

MATERIALS AND METHODS

H. pylori strains and cell lines

H. pylori GZ7 is a typical East Asian strain (*cagA*$^+$) that was isolated from the gastric mucosa of a GC patient by our group[24]. *H. pylori* 26695 (ATCC 700392) is a typical western strain (*cagA*$^+$) that was purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States). Two strains were cultured on Columbia agar plates supplemented with *H. pylori* selective supplement (Oxoid, United Kingdom) and 10% sheep blood at 37 °C in a microaerobic environment (5% O_2, 10% CO_2, 85% N_2).

The human GC cell lines AGS, NCI-N87 and SNU-16 cells were purchased from ATCC, and SGC-7901 and BGC823 cells were obtained from the tissue bank in Shanghai, China. Primary GC cells were separated and identified in our laboratory[25]. All cell lines were grown in 5% CO2 at 37 °C in RPMI-1640 with 10% FCS (Gibco, United States).

Human GC tissues

From June to December 2021, 12 pairs of GC and paracancer normal tissues were collected during surgery at the Affiliated Hospital of Guizhou Medical University in China. Tissues were immediately formalin-fixed and paraffin-embedded for pathological evaluation and immunohistochemistry. All subjects provided their written informed consent. The study was performed following the Declaration of Helsinki. The Guizhou Medical University Ethics Committee approved the study protocol, No. 2017(43).

Mongolian gerbil stomach tissues

In our earlier work, Mongolian gerbils were intragastrically infected with *H. pylori* NCTC 11637 (ATCC 43504, *cagA*$^+$) for 2 years. At 3-24 mo after infection, gerbils developed erosion, atrophy, intestinal metaplasia, and well-differentiated GC in the gastric mucosa[26]. The stomach tissues of gerbils (n = 3 at each timepoint) were removed after decapitation and immediately fixed and embedded. The embedded tissues were sliced into sections (5 μm thick) and stained for immunohistochemistry.

RNA-sequencing

Three primary GC cell lines (5 × 10^7) were cultured with 50 multiplicity of infection (MOI) *H. pylori* GZ7 for 6 h before being harvested. TRIzol (Invitrogen, Carlsbad, CA, United States) was used to extract overall RNA, which was then purified by DNase I treatment. After being enriched by Oligo (dT) beads, eukaryotic mRNA was interrupted into short fragments and synthesized into cDNA. Using the Illumina

TruSeq library construction kit, purified cDNA was amplified by PCR to construct the RNA-sequencing (RNA-seq) library. After the library was tested for quality using the Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System, the library was sequenced on the Illumina HiSeqX10 platform by Beijing Genomics Institute (China). Sequence reads were mapped to the reference human genome (hg19), and the gene and transcript expression levels were calculated. Differentially expressed genes (DEGs) with a fold-change > 2 and false discovery rate (FDR) < 0.05 were identified between *H. pylori*-infected and uninfected cells. The DEGs shared by three datasets were selected for further bioinformatics analysis. The R heatmap package (version 0.7.7) was used to generate a heatmap. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and protein-protein interaction (PPI) analyses were performed using the Gene Ontology Consortium (http://geneontology.org/), KOBAS (http://kobas.cbi.pku.edu.cn/), and the STRING tool (https://string-db.org/), respectively. KEGG pathways and PPI networks were visualized using Cytoscape software (version 3.7.2). Gene set enrichment analysis (GSEA) was carried out using GSEA software (version 4.1.0). A significant gene set was defined as having an FDR < 0.25 and $P < 0.01$.

Dual-luciferase reporter assay

The expression vectors of JUN and FOSL1 were purchased from Hanheng Biotech (Shanghai, China). The human DKK1 promoter region from -1100 to + 1 bp was cloned into the luciferase reporter vector pGL4.29 (Promega, Charbonnières-les-Bains, France), which was cotransfected with pcDNA3.1-JUN and/or pcDNA3.1-FOSL1 into AGS cells for 48 h using Lipofectamine 2000 (Invitrogen). The activities of firefly luciferase and Renilla luciferase were determined using the dual luciferase reporter system (Promega). The ratio of firefly luciferase activities to Renilla luciferase activities (Fluc/Rluc) was used to describe the reporter activities.

Western blotting

Proteins (25 g) were resolved *via* SDS-PAGE and transferred by electroblotting onto the PVDF membrane (Millipore, Billerica, MA, United States). After blocking with 5% nonfat milk, primary antibodies were used to probe the membrane overnight at 4 °C. The membrane was incubated with horseradish-peroxidase-conjugated secondary antibodies. The bands were visualized using electro-chemiluminescence. Details of the antibodies are provided in Supplementary Table 1.

RT-qPCR

RNA isolation was performed by TRIzol reagent. cDNA was synthesized through the process of reverse transcription (RT) using a standard protocol according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using fluorogenic SYBR Green (BioRad, Hercules, CA, United States). The $2^{-\Delta\Delta CT}$ method was used for relative mRNA quantitation of target genes. GAPDH gene served as the loading control. RT-qPCR primers are listed in Supplemental Table 2.

Immunohistochemistry and immunofluorescence

Gerbil stomachs, nude mouse xenografts, and human GC tissues were cut into 5-μm thick sections. Immunohistochemistry was used to detect expression of JUN, DKK1, CKAP4 and Ki67 in these tissues, and immunofluorescence imaging was used to assess β-catenin expression and nuclear translocation in DKK1-silenced AGS cells, as described previously[25]. The quantification of immunohistochemical staining was performed by Image J software. Immunohistochemistry score was determined by multiplying the intensity score and the percentage score[27]. Details of the antibodies are provided in Supplementary Table 1.

Cell growth curve

Cell viability was determined using Cell Counting Kit-8 kit (CCK-8, Dojindo, Japan) in accordance with the kit instructions. Cells (1000 cells per well) were placed in a 96-well plate in sextuplicate per condition and cultured for 1-6 d. CCK-8 solution (10 μL) was added to each well at the corresponding time points, and the cells were cultured for an additional 2 h. CCK-8 was used to detect cell viability.

Colony formation assay

Cells (500 cells per well) were placed in a 6-well plate in triplicate per condition and cultured for 2 wk. The growth medium was changed once every 4 d. Two weeks later, 0.1% crystal violet (Solarbio, China) was used to stain the colonies after being fixed with 4% paraformaldehyde. Colony count was performed using a microscope.

Transwell assay

Transwell migration and invasion assays were performed using a 24-well Transwell insert (pore size of 8 μm) with and without Matrigel (Biosciences, San Jose, CA, United States). A total of 1×10^4 cells in medium with 1% FBS was added to the upper chamber of the Transwell, and 800 μL of medium with 10% FBS was added in lower chamber. At 24 h and 72 h after culture, 4% paraformaldehyde was used to fix the inserts for 30 min at room temperature before being stained in a 0.1% crystal violet solution and

imaged. Cells that migrated or invaded to the lower chamber were counted using a microscope.

Cell cycle and apoptosis

Cell cycle and apoptosis analyses were performed using flow cytometry, as previously described[25]. Cell cycle distribution and apoptosis were analyzed using FloJo software.

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed using an Absin Co-IP kit (Shanghai, China). BGC823 cells (3×10^6) with or without *H. pylori* infection were sonicated three times for 20 s each in ice-cold lysis buffer before being centrifuged at 14000 × g for 10 min at 4 °C. The supernatant was then collected as whole-cell lysates. Cell lysates were incubated overnight at 4 °C with a CKAP4 antibody (2 μg) and control IgG (1 μg). Protein A- and protein G-Sepharose (10 μL) (Sigma-Aldrich, St. Louis, MO, United States) were added to the cell lysates and left on for 8 h at 4 °C. After 1 min of centrifugation at 12000 × g, the beads were collected and washed three times with wash buffer. The beads were boiled in 2 × Laemmeli sample buffer for 5 min and centrifugated for 1 min at 14000 × g and 4 °C. SDS-PAGE was used to resolve proteins in the supernatants (15 μL) after being collected. Western blotting was used to detect DKK1 by an anti-DKK1 antibody (1:400).

Tumorigenicity in nude mice

The ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines (https://www.nc3rs.org.uk/arrive-guidelines) were followed for all animal experiments to minimize pain and discomfort for the animals. Guizhou Medical University Animal Care Welfare Committee approved the animal study (No. 1702155). Ten BALB/c nude mice (male, age 3-4 wk) were provided by Chongqing Tengxin Biotechnology (Chongqing, China). Mice were accustomed to the laboratory environment that was a 12-h light/12-h dark cycle at 23 °C with 50% humidity and were given water and food *ad libitum*. After 2 wk, BGC823 cells (2×10^6) stably overexpressing DKK1 and negative control cells were transplanted subcutaneously into the flanks of the mice (5 mice per group), and the tumor size was measured every 3 d. After 16 d, all mice were killed by intravenous injection of barbiturate overdose (150 mg/kg), and the tumors were removed. Tumor weights and volumes were determined using the formula (length × width^2)/2.

Lentivirus infection

The lentiviruses containing shJUN and shDKK1, overexpression lentiviruses for JUN and DKK1, and control lentiviruses were obtained from Shanghai Jikai Gene Co. Ltd. (China). These lentiviruses were used to infect AGS, SGC-7901, NCT-N87, SNU-16 and BGC823 cells (2×10^5) in a 12-well plate at MOI 10-30 for 48 h. Polybrene (8 μg/mL; Sigma) was added to the 12-well plate to increase the efficiency of lentivirus infection. After 24 h, the medium containing lentiviruses and polybrene was changed with fresh medium and cultured for a further 48 h. After that, the stable cell lines were selected for 2 wk using 2 μg/mL puromycin, in which the medium containing puromycin was changed every 2-3 d. The cells were maintained in 1 μg/mL puromycin. Western blotting and RT-qPCR were used to ensure that target genes were stably knocked down or overexpressed.

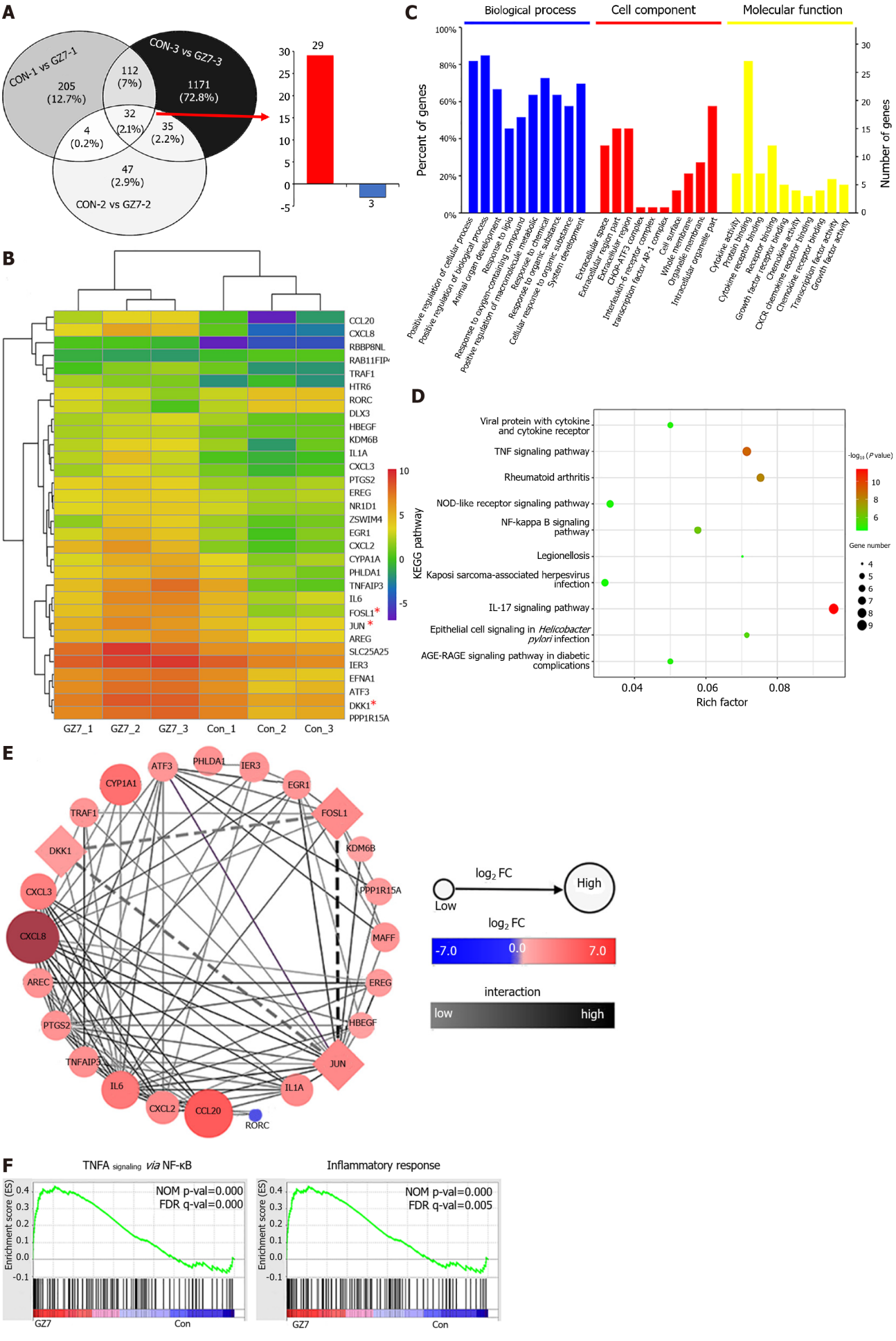
Statistical analysis

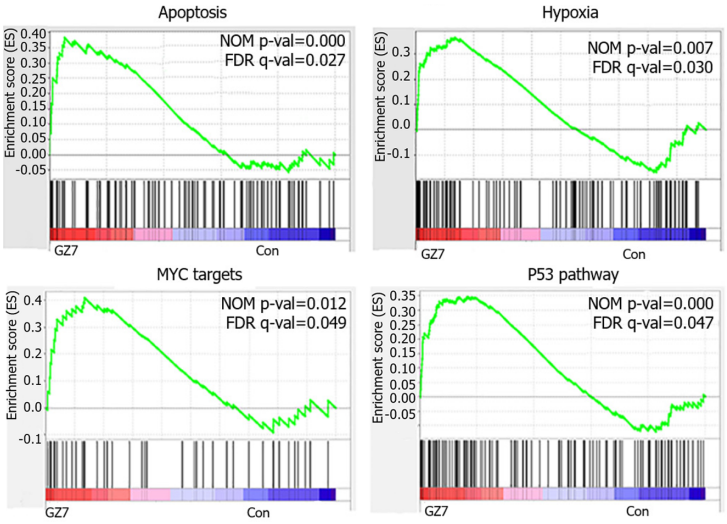
SPSS 22.0 (IBM, Armonk, NY, United States) was used for all statistical analyses. ImageJ software (National Institutes of Health, Bethesda, MD, United States) was used for the quantification of images from immunohistochemistry. GraphPad Prism 8.0 (La Jolla, CA, United States) was used to create statistical figures. All presented images were representative of three or more individual experiments. One-way or two-way analysis of variance was performed for comparisons between multiple groups. All results were expressed as mean ± SD. $P < 0.05$ indicated statistically significant differences.

RESULTS

Analysis of DEGs between primary GC cells with and without H. pylori infection

RNA-seq analysis identified 32 DEGs, including 29 upregulated and three downregulated genes that were shared by three pairs of primary GC cells infected with or without *H. pylori* (Figure 1A and B). These DEGs were mostly enriched in the regulation of biological processes, cytokine activity, protein binding, and tumor necrosis factor (TNF), nuclear factor (NF)-κB, and interleukin (IL)-17 pathways according to GO and KEGG pathway enrichment analysis (Figure 1C and D). The PPI network identified the hub genes *JUN*, *CXCL8*, *CCL20*, and *FOSL1* and their interactions, and JUN and FOLS1 were directly connected to DKK1 (Figure 1E). Based on the GSEA of DEGs, TNFA signaling *via* NF-κB, the inflammatory response, apoptosis, hypoxia, MYC targets, and the P53 pathway were enriched in *H. pylori*-infected GC cells (Figure 1F). These molecular events are closely associated with GC development and progression. JUN, FOLS1, and DKK1 were selected for further investigation.


A
CON-1 vs GZ7-1
CON-3 vs GZ7-3
CON-2 vs GZ7-2
205 (12.7%)
112 (7%)
1171 (72.8%)
32 (2.1%)
4 (0.2%)
35 (2.2%)
47 (2.9%)
29
3
B
CCL20
CXCL8
RBBP8NL
RAB11FIP4
TRAF1
HTR6
RORC
DLX3
HBEGF
KDM6B
IL1A
CXCL3
PTGS2
EREG
NR1D1
ZSWIM4
EGR1
CXCL2
CYPA1A
PHLDA1
TNFAIP3
IL6
FOSL1*
JUN*
AREG
SLC25A25
IER3
EFNA1
ATF3
DKK1*
PPP1R15A
GZ7_1
GZ7_2
GZ7_3
Con_1
Con_2
Con_3
C
Biological process
Cell component
Molecular function
Percent of genes
Number of genes
Positive regulation of cellular process
Positive regulation of biological process
Animal organ development
Response to lipid
Response to oxygen-containing compound
Positive regulation of macromolecule metabolic
Response to chemical
Response to organic substance
Cellular response to organic substance
System development
Extracellular space
Extracellular region part
Extracellular region
CHOP-ATF3 complex
Interleukin-6 receptor complex
transcription factor AP-1 complex
Cell surface
Whole membrane
Organelle membrane
Intracellular organelle part
Cytokine activity
Protein binding
Cytokine receptor binding
Receptor binding
Growth factor receptor binding
Chemokine activity
CXCR chemokine receptor binding
Chemokine receptor binding
Transcription factor activity
Growth factor activity
D
KEGG pathway
Viral protein with cytokine and cytokine receptor
TNF signaling pathway
Rheumatoid arthritis
NOD-like receptor signaling pathway
NF-kappa B signaling pathway
Legionellosis
Kaposi sarcoma-associated herpesvirus infection
IL-17 signaling pathway
Epithelial cell signaling in Helicobacter pylori infection
AGE-RAGE signaling pathway in diabetic complications
Rich factor
-log10 (P value)
Gene number
E
ATF3
PHLDA1
IER3
CYP1A1
EGR1
TRAF1
FOSL1
DKK1
KDM6B
CXCL3
PPP1R15A
CXCL8
MAFF
AREC
EREG
PTGS2
HBEGF
TNFAIP3
JUN
IL6
IL1A
CXCL2
CCL20
RORC
log2 FC
Low
High
-7.0
0.0
7.0
interaction
low
high
F
TNFA signaling via NF-κB
NOM p-val=0.000
FDR q-val=0.000
Inflammatory response
NOM p-val=0.000
FDR q-val=0.005
Enrichment score (ES)
GZ7
Con



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Figure 1 Comparison of differentially expressed genes between primary gastric cancer cells with and without *Helicobacter pylori* infection. A: Venn diagram of differentially expressed genes (>DEGs); B: DEGs heatmap analysis by R heatmap package. Asterisks indicate the genes investigated in this study; C: Gene Ontology analysis of the DEGs; D: Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the DEGs; E: Protein-protein interaction network of the DEGs; F: Gene set enrichment analysis (GSEA) enrichment analyses of the DEGs with GSEA version 4.1.0. Con: Control; GZ7: *Helicobacter pylori* GZ7. TNF: Tumor necrosis factor; FOSL1: Fos-like antigen-1; DKK1: Dickkopf-related protein 1.

H. pylori infection induces JUN and FOSL1 upregulation to activate DKK1 expression via binding to the DKK1 promoter

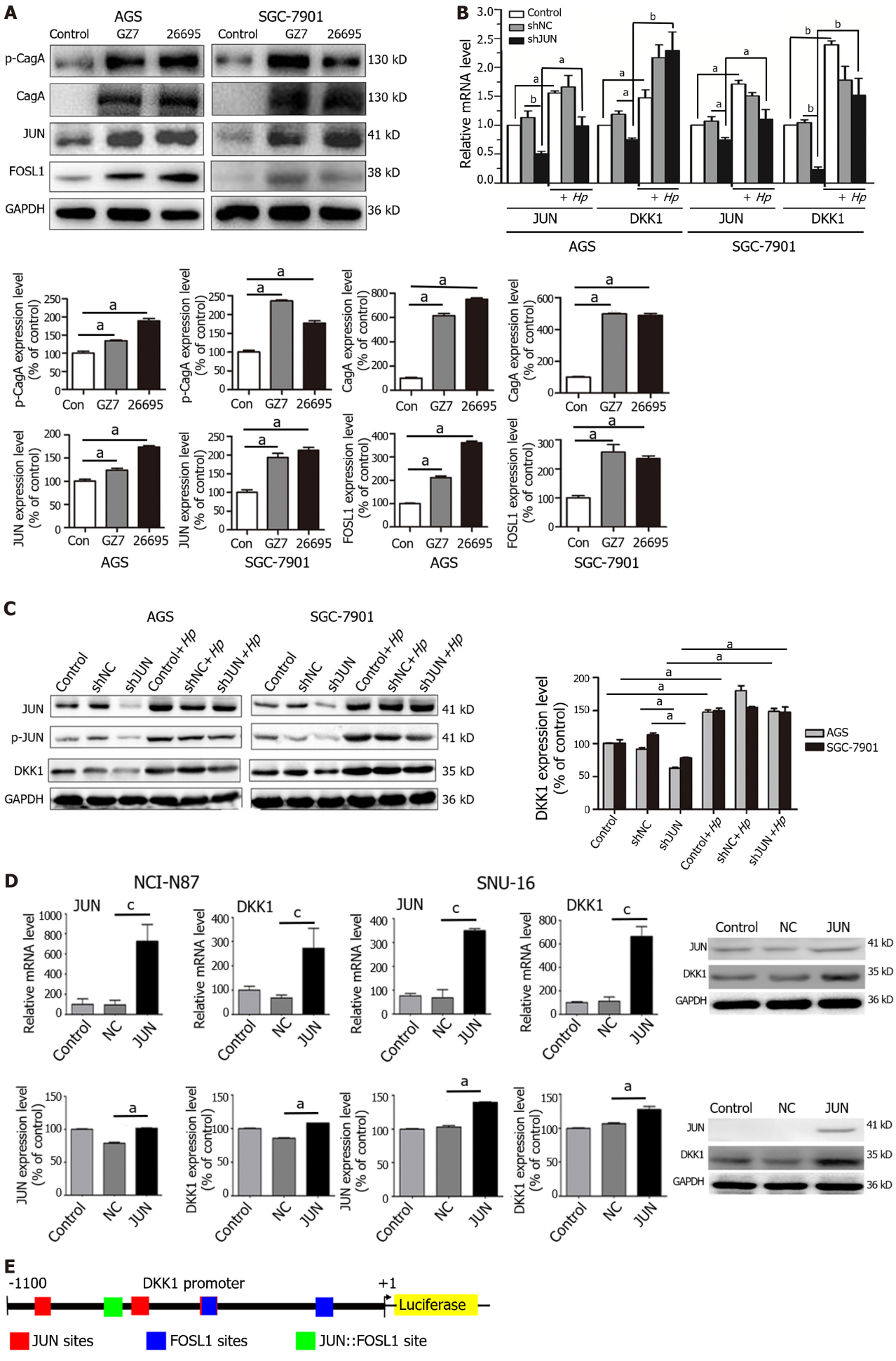
To confirm the results of RNA-seq, AGS and SGC-7901 cells were infected with *H. pylori* GZ7 and 26695. We discovered that JUN and FOSL1 proteins were highly expressed in *H. pylori*-infected cells compared to the control cells, which supported the RNA-seq resultss (Figure 2A). The *H. pylori* virulence factor CagA and its phosphorylation levels were dramatically elevated in *H. pylori*-infected cells (Figure 2A).

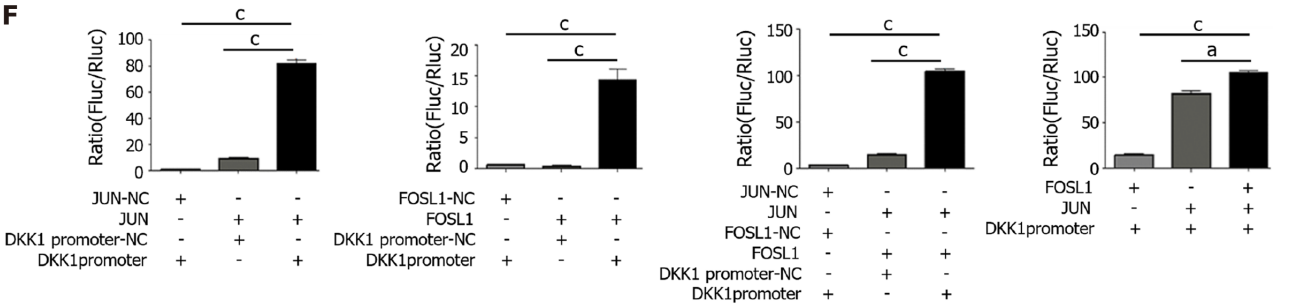
We determined JUN expression in eight GC cell lines. JUN was highly expressed in AGS and SGC-7901 cells but weakly expressed in NCI-N87 and SNU-16 cells (Supplementary Figure 1). JUN was knocked down using short hairpin RNA (shRNA) in AGS and SGC-7901 cells and overexpressed using lentivirus infection in NCI-N87 and SNU-16 cells. RT-qPCR and western blotting revealed a substantial decrease in DKK1 expression after JUN knockdown. When the JUN-knockdown cells were infected with *H. pylori*, the mRNA and protein levels of DKK1 recovered and JUN expression and phosphorylation increased (Figure 2B and C). In contrast, the mRNA and protein level of DKK1 was significantly increased in SNU-16 and NCI-N87 cells after JUN overexpression (Figure 2D). These findings revealed that *H. pylori*-induced JUN enhanced DKK1 expression.

JUN and FOSL1 comprise AP-1 *via* their basic leucine zipper domain[28]. AP-1-binding sites include the 5′-TGA(C/G) TCA-3′ (TRE motif), 5′-TCACGTCA-3′ (CRE motif), and their single-base variants[29]. To confirm that AP-1 transcriptionally regulated DKK1 expression, we predicted AP-1-binding sites in the promoter region from -1100 to + 1 bp upstream of the DKK1 transcription site, which is highly conserved in mammalian genomic DNA[30]. Three JUN sites, two FOSL1 sites, and a JUN::FOSL site were observed in the DKK1 promoter (Figure 2E). The DKK1 promoter region from -1100 to + 1 bp was inserted into a luciferase reporter plasmid, which was cotransfected into AGS cells with JUN and/or FOSL1 vectors. The dual-luciferase reporter assay indicated that transfection of AGS cells with JUN and FOSL1 markedly increased the luciferase activity, and the effect of the JUN vector was stronger. JUN and FOSL1 cotransfection exhibited the highest luciferase activity in AGS cells (Figure 2F). This result confirmed that AP-1, including the JUN::JUN and JUN::FOSL1 complexes, bound to the DKK1 promoter to initiate DKK1 transcription.

High JUN and DKK1 expression in gerbil gastric tissues infected with H. pylori and human GC tissues

We successfully established GC models in Mongolian gerbils *via H. pylori* infection in a previous study, in which erosion, atrophy, intestinal metaplasia, and well-differentiated GC were progressively detected in the stomach mucosa[26]. Positive JUN and DKK1 staining increased progressively in gerbil gastric epithelium with *H. pylori* infection from 3 mo to 24 mo in the present study (Figure 3A), which suggested that the expression of JUN and DKK1 was linked to the pathological development of GC in gerbils. Human GC genomic data were extracted from the TCGA-Stomach Adenocarcinoma database (TCGA-STAD), which included 375 cancer and 391 normal tissues. The expression of DKK1 mRNA was


A
AGS
SGC-7901
Control
GZ7
26695
p-CagA
CagA
JUN
FOSL1
GAPDH
130 kD
130 kD
41 kD
38 kD
36 kD
B
Control
shNC
shJUN
Relative mRNA level
+ Hp
JUN
DKK1
AGS
SGC-7901
p-CagA expression level (% of control)
CagA expression level (% of control)
JUN expression level (% of control)
FOSL1 expression level (% of control)
Con
C
Control
shNC
shJUN
Control+Hp
shNC+Hp
shJUN+Hp
p-JUN
DKK1
41 kD
35 kD
DKK1 expression level (% of control)
D
NCI-N87
SNU-16
Relative mRNA level
NC
E
-1100
DKK1 promoter
+1
Luciferase
JUN sites
FOSL1 sites
JUN::FOSL1 site



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Figure 2 *Helicobacter pylori* infection increases JUN and fos-like antigen-1 expression to activate the dickkopf-related protein 1 promoter. A: Western blotting for CagA, p-CagA, JUN, and fos-like antigen-1 (FOSL1) in AGS and SGC-7901 cells infected with *Helicobacter pylori* (*H. pylori*) for 6 h. $^{a}P < 0.05$; B: RT-qPCR for JUN and dickkopf-related protein 1 (DKK1) in JUN knockdown and/or *H. pylori*-infected AGS and SGC-7901 cells. $^{a}P < 0.05$; $^{b}P < 0.01$; C: Western blotting for JUN, p-JUN, and DKK1 in JUN knockdown and/or *H. pylori*-infected AGS and SGC-7901 cells. $^{a}P < 0.05$; D: RT-qPCR (top) and Western blotting (bottom) for JUN and DKK1 in NCI-N87 and SNU-16 cells with JUN overexpression. $^{a}P < 0.05$; $^{c}P < 0.001$; E: Prediction of AP-1 binding sites in the DKK1 promoter (from -1100 to + 1 bps) by JASPAR; F: JUN and/or FOSL1 promotes DKK1 promoter activity in AGS cells by dual-luciferase reporter assay. The ratio of firefly to Renilla luciferase (Fluc/Rluc) is used to display the results. $^{a}P < 0.01$; $^{c}P < 0.001$; Con: Control; *Hp*: *H. pylori*; GZ7: *H. pylori* GZ7; 26695: *H. pylori* 26695. FOSL1: Fos-like antigen-1; DKK1: Dickkopf-related protein 1; *H. pylori*: *Helicobacter pylori*.

significantly higher in GC tissues than normal tissues, and GC patients with a higher DKK1 expression had a shorter overall and disease-free survival (Figure 3B). Human GC tissues showed stronger positive staining for JUN and DKK1 than pericancer tissues (Figure 3C). However, no distinction was observed in the levels of JUN mRNA between GC and normal tissues in the TCGA-STAD database (Supplementary Figure 2).

Knockdown or overexpression of DKK1 suppresses or promotes malignant behavior of GC cells

To investigate the function of DKK1 in GC, we first established DKK1-knockdown AGS cells using two independent shRNAs (Supplementary Figure 3A) and found that cell proliferation and colony formation were significantly inhibited in AGS cells (Figure 4A and B). DKK1 knockdown also suppressed AGS cell migration and invasion (Figure 4C). Flow cytometry showed that the knockdown of DKK1 greatly increased apoptosis in AGS cells, especially early apoptosis (Figure 4D).

We generated stable DKK1-overexpressing AGS and BGC823 cells (Supplementary Figure 3B). In contrast to DKK1 knockdown, DKK1 overexpression promoted AGS and BGC823 cell growth, colony formation, migration, and invasion (Figure 4E and F). We subcutaneously implanted the DKK1-overexpressing BGC823 cells into nude mice and found that DKK1 overexpression in cancer cells increased the growth of xenograft tumors in mice. The tumor weight and volume in DKK1-overexpressing nude mice were significantly greater than normal control mice (Figure 4G). The mice were killed, and the subcutaneous tumors were removed. Immunohistochemical staining confirmed that DKK1 and Ki67 (cell proliferation marker) were expressed at higher levels in DKK1-overexpressing than control tumors (Figure 4H). These findings verified the tumor-promoting effects of DKK1 in GC.

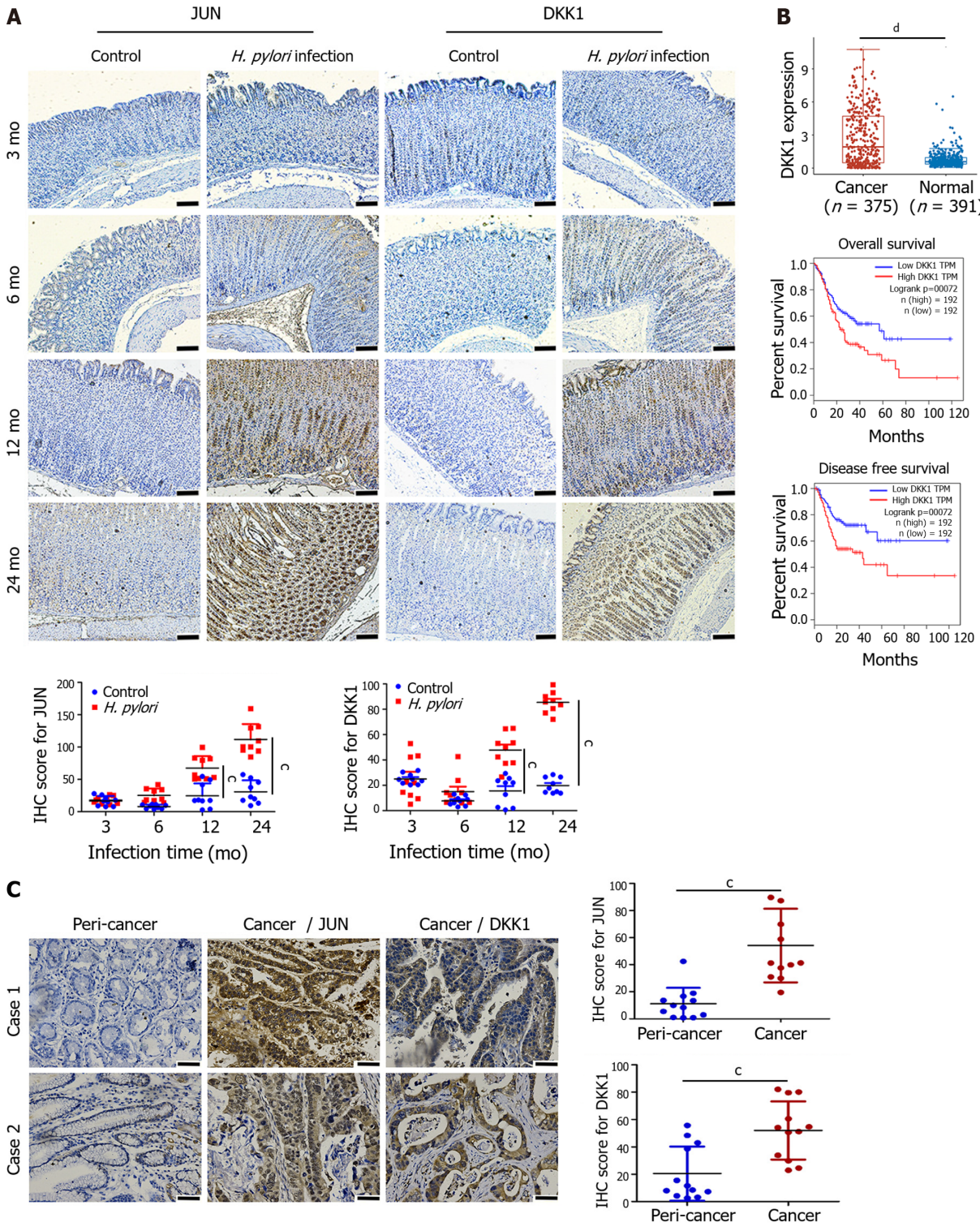
Binding of DKK1 to CKAP4, but not LRP5/6, in GC tissues and cells

DKK1 suppresses the Wnt/β-catenin pathway by interacting with Wnt coreceptors LRP5/6. Therefore, we detected β-catenin expression and nuclear translocation in DKK1-knockdown AGS cells and discovered that the levels of β-catenin mRNA and protein were reduced by DKK1 knockdown without significant change in β-catenin nuclear translocation, which was even lower in shDKK1-2# cells (Figure 5A-C). In contrast, DKK1 overexpression enhanced β-catenin mRNA levels in AGS and BGC823 cells (Figure 5D). The expression of β-catenin in GC tissues was greater than in normal tissues, but there is no correlation between β-catenin expression and GC patients' survival in the TCGA-STAD dataset (Figure 5E). The findings suggest that the effects of DKK1 are independent of Wnt/β-catenin signaling in GC.

CKAP4 was recently discovered as a DKK1 receptor. We found that CKAP4 mRNA and protein expression was increased in 12 clinical GC samples, especially in the cell surface membrane (red arrow), and 375 GC specimens from the TCGA-STAD database (Figure 5F-H). Infection with *H. pylori* also upregulated CKAP4 expression in GC cells (Figure 5I). Higher CKAP4 expression was linked to lower overall survival in GC patients (Figure 5F). The binding of endogenous DKK1 to CKAP4 was detected in *H. pylori*-infected and uninfected BGC823 cells (Figure 5J).

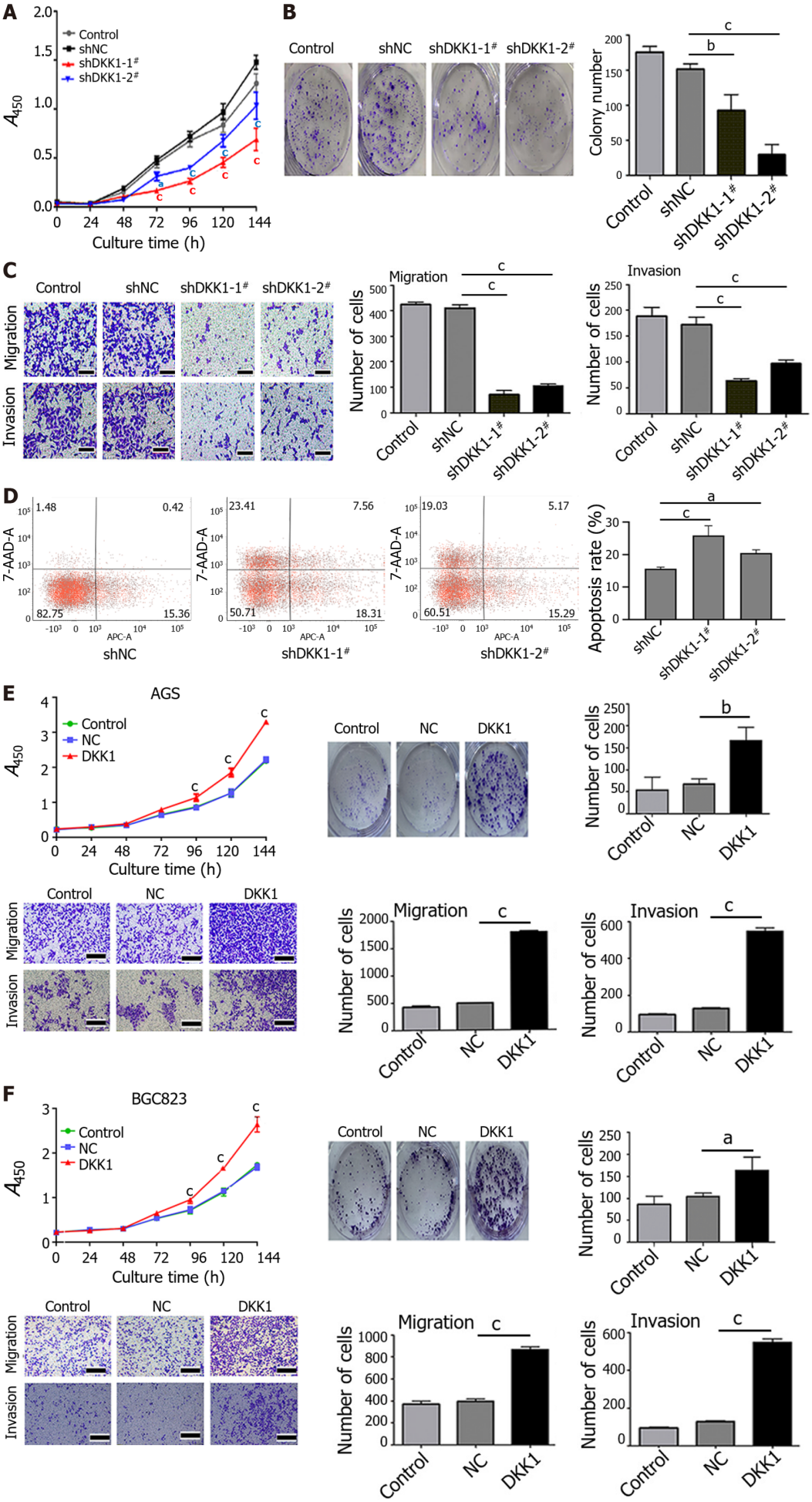
DKK1 promotes the malignant phenotype of GC cells via the PI3K/AKT/mTOR pathway

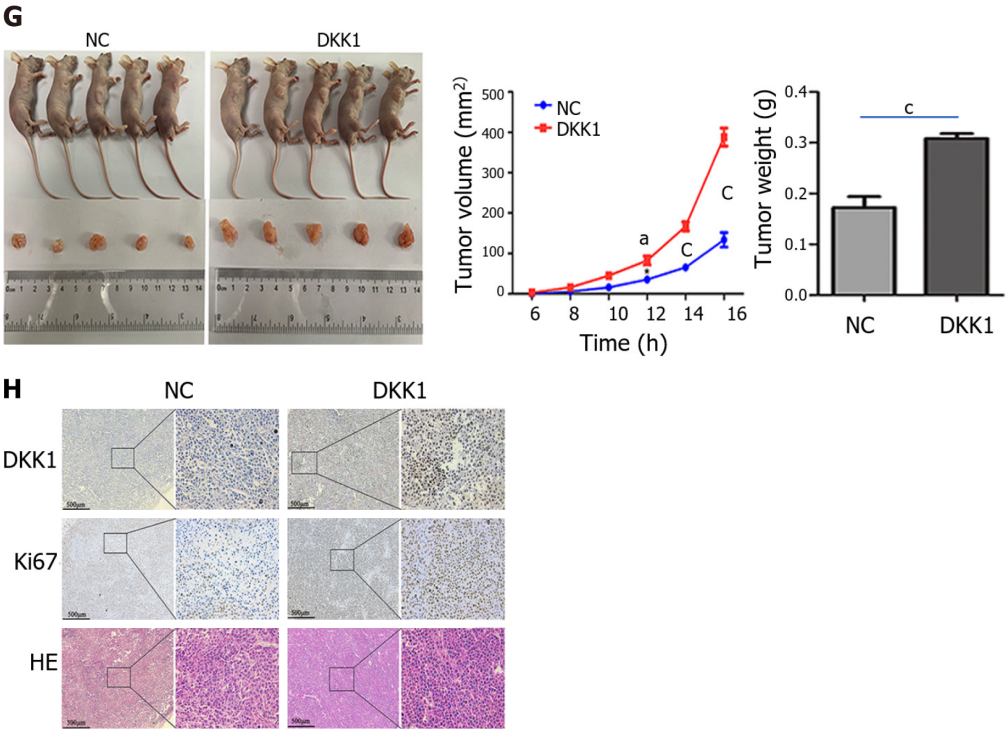
After confirming the DKK1/CKAP4 axis in GC cells, we evaluated whether DKK1 knockdown and overexpression affected the PI3K/AKT/mTOR pathway. DKK1 knockdown markedly inhibited the



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Figure 3 Immunohistochemistry for JUN and dickkopf-related protein 1 in gerbil stomach infected with *Helicobacter pylori* and gastric cancer tissues. A: Immunohistochemical analysis of JUN and dickkopf-related protein 1 (DKK1) proteins in *Helicobacter pylori* (*H. pylori*)-infected gerbil stomach tissues at 3 mo, 6 mo, 12 mo, and 24 mo post-infection (n = 3). Dot diagrams show the quantification of JUN and DKK1 staining in immunohistochemical samples. Nine sections were chosen randomly from three different samples and used to determine mean ± SD of the immunohistochemistry (IHC) score, which was calculated as described in the methods. Scale bar = 100 μm. $^{c}P < 0.001$; B: DKK1 expression and survival analysis in gastric cancer patients from the TCGA-STAD dataset. $^{d}P < 0.0001$; C: Representative images of immunohistochemical staining of JUN and DKK1 proteins in human gastric cancer specimens. Dot diagrams show the quantitation of JUN and DKK1 staining. Scale bar = 50 μm. Data from 12 clinical samples of gastric cancer patients are expressed as mean ± SD. $^{c}P < 0.001$. DKK1: Dickkopf-related protein 1; *H. pylori*: *Helicobacter pylori*.


A
Control
shNC
shDKK1-1#
shDKK1-2#
A450
Culture time (h)
B
Control
shNC
shDKK1-1#
shDKK1-2#
Colony number
C
Migration
Invasion
Number of cells
D
7-AAD-A
APC-A
1.48
0.42
82.75
15.36
23.41
7.56
50.71
18.31
19.03
5.17
60.51
15.29
Apoptosis rate (%)
E
AGS
NC
DKK1
F
BGC823



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Figure 4 The effects of dickkopf-related protein 1 knockdown and overexpression on gastric cancer cell growth, migration, invasion, apoptosis, and xenograft growth in nude mice. A: Growth curve of dickkopf-related protein 1 (DKK1) knockdown AGS cells using CCK8 assay. $^{a}P < 0.05$; $^{c}P < 0.001$; B: Colony formation assay of AGS cells with DKK1 knockdown. $^{b}P < 0.01$; $^{c}P < 0.001$; C: Migration and invasion assay of DKK1 knockdown AGS cells using Transwell system. Bar graphs show the number of migrated or invaded cells (right). $^{c}P < 0.001$. Scale bar = 200 μm; D: Flow cytometry analysis for apoptosis in DKK1 knockdown AGS cells. Bar graphs show the percentage of apoptotic cells (right). $^{a}P < 0.05$; $^{c}P < 0.001$; E and F: CCK8, colony formation, and Transwell assays were used to measure the growth curve, colony formation, migration, and invasion of AGS (E) and BGC823 (F) cells with DKK1 overexpression. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; Scale bar = 200 μm; G: DKK1 overexpression promotes xenograft tumor growth in nude mice. Line dot and bar graph show tumor volume and weight, respectively (n = 5 mice per group). $^{a}P < 0.05$; $^{c}P < 0.001$; H: Hematoxylin eosin staining of xenograft tumors and immunohistochemical analysis of DKK1 and Ki67 in xenograft tumors from nude mice. The boxed regions are magnified to the right. Scale bar = 500 μm. DKK1: Dickkopf-related protein 1; HE: Hematoxylin eosin.

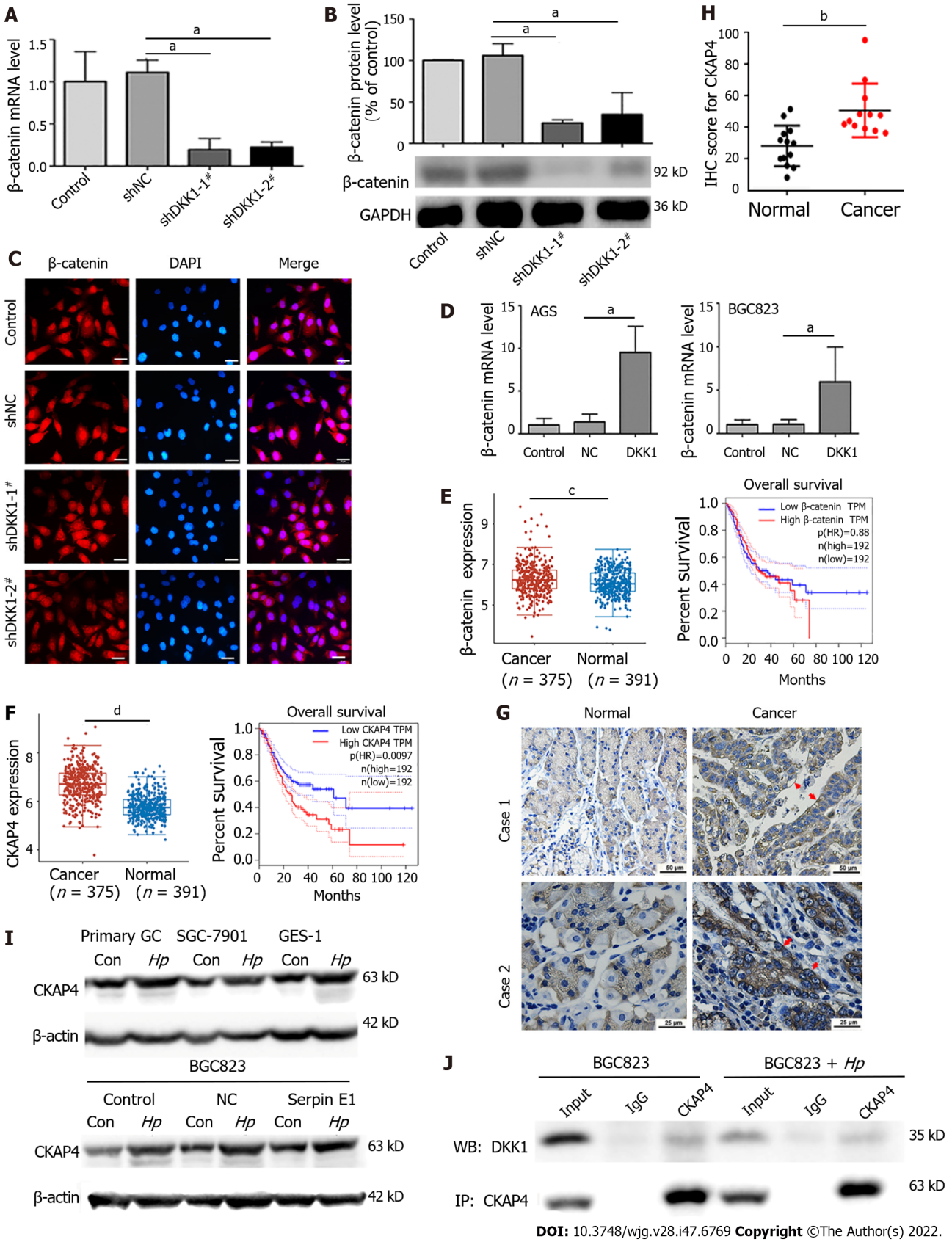
expression of p-PI3K, PI3K, p-AKT, AKT, p-mTOR, and mTOR, and p-PI3K, p-AKT, and p-mTOR were more inhibited. *H. pylori* infection restored the expression of DKK1 and increased PI3K/AKT/mTOR signaling in DKK1 knockdown cells (Figure 6A). Conversely, DKK1 overexpression increased PI3K, AKT and mTOR phosphorylation, and a specific inhibitor of PI3K, LY294002, inhibited the DKK1-induced increase in the three phosphorylated proteins in AGS and BGC823 cells (Figure 6B). These findings suggest that *H. pylori*-upregulated DKK1 activated the PI3K/AKT/mTOR pathway.

Next, we examined the effects of LY294002 in DKK1-overexpressing AGS and BGC823 cells and found that blockade of PI3K/AKT/mTOR signaling with LY294002 significantly decreased cell proliferation, colony formation, migration and invasion (Figure 6C and D), which further confirmed our hypothesis that DKK1 promoted the malignant phenotypes of GC cells *via* activation of the PI3K/AKT/mTOR pathway.

DISCUSSION

Our current study revealed that *H. pylori* infection upregulated the expression of JUN and FOSL1, which formed AP-1 to activate DKK1 transcription. Gain- and loss-of-function studies showed that DKK1 had important tumor-promoting functions in *H. pylori*-related GC *via* activation of the CKAP4/PI3K/AKT/mTOR pathway.

The gene expression profiles obtained by independent research groups using RNA-seq were inconsistent due to the use of diverse cell lines, GC tissues, *H. pylori* strains, and whole-genome expression arrays[31-33]. The upregulated expression of JUN and FOLS1 was noted in *H. pylori* NCTC11639-infected AGS cells, but not experimentally verified[34]. DKK1 was also downregulated in *H. pylori* 26695-infected AGS cells, but this observation was not tested experimentally[35]. Using RNA-seq, we identified 32 DEGs that were shared by all three models of primary GC cells infected with *H. pylori* GZ7. Of the 32 DEGs, JUN, FOSL1 and DKK1 were highly expressed in *H. pylori*-infected cells, which was confirmed in GC tissues and *H. pylori*-infected gerbil stomach tissues. The enrichment



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Figure 5 Dickkopf-related protein 1 promotes β-catenin and cytoskeleton-associated protein 4 expression and the binding of dickkopf-related protein 1 to cytoskeleton-associated protein 4 in gastric cancer tissues and cells but does not affect the nuclear translocation of β-catenin. A-C: Dickkopf-related protein 1 (DKK1) knockdown decreases β-catenin expression without changing β-catenin nuclear translocation in AGS cells, as determined using RT-qPCR (A), Western blotting (B), and immunofluorescence (C). Scale bar = 25 μm; $^{a}P < 0.05$; D: RT-qPCR for β-catenin in AGS and BGC823 cells with DKK1 overexpression. $^{a}P < 0.05$; E: β-catenin expression and its association with 10-year overall survival in gastric cancer (GC) samples of the TCGA-STAD database. $^{c}P < 0.001$; F: Cytoskeleton-associated protein 4 (CKAP4) expression and its positive association with 10-year overall survival in GC samples of the

TCGA-STAD database. [d]*P* < 0.0001; G: Immunohistochemical staining of CKAP4 in clinical GC tissues and matched normal tissues. The red arrow indicates CKAP4 expression in the cell membrane; H: CKAP4 staining quantification in G. Data from 12 independent samples are expressed as means ± SD. [b]*P* < 0.01; I: Western blotting for CKAP4 in GC cells with or without *Helicobacter pylori* (*H. pylori*) infection for 72 h; J: Co-IP of DKK1 and CKAP4 in BGC823 cells with and without *H. pylori* infection for 72 h. Con: Control; *Hp*: *H. pylori*. DKK1: Dickkopf-related protein 1; *H. pylori*: *Helicobacter pylori*; CKAP4: Cytoskeleton-associated protein 4; GC: Gastric cancer.

analysis of KEGG pathways suggested that DEGs were primarily involved in inflammatory signalings such as TNF, NF-κB and IL-17. GESA also revealed that *H. pylori* infection activates the TNFA pathway, the P53 pathway, apoptosis, hypoxia, and the inflammatory response.

DKK1 was recently found to be epigenetically downregulated by promoter hypermethylation, which resulted in the nuclear translocation of β-catenin and activation of the Wnt/β-catenin pathway in gastric intestinal metaplasia, high-grade adenoma, and adenocarcinoma[36,37]. Conversely, Kikuchi *et al*[38] observed that DKK1 promoted the proliferation, invasion and metastasis of cancer cells independent of Wnt signaling. Other studies also suggested that DKK1 was often overexpressed in diverse tumor tissues, including GC, and DKK1 overexpression strongly correlated with poor cancer patient survival [39,40]. However, the mechanism underlying this association was not clear. Our study explained, for the first time, why DKK1 was upregulated in GC. *H. pylori* infection upregulates JUN and FOSL1 expression to form AP-1. AP-1 binds to the TRE and CRE motifs in the DKK1 promoter region to transcriptionally activate DKK1 expression in GC. This finding is contradictory to two earlier studies[36,37]. We speculate that decreased expression of DKK1 *via* promotor methylation, as reported by Lu *et al*[36], is more prevalent in gastric precancerous lesions and early-stage adenocarcinoma, and increased expression of DKK1 *via* promotor binding of AP-1, as observed in our study, is more likely to occur in *H. pylori*-infected and advanced GC. Two observations provide support for our assertion. First, we discovered that high expression of JUN and DKK1 was mostly observed in the stomach tissues of gerbils at least 12 mo after *H. pylori* infection. Second, DKK1 is frequently overexpressed in GC patients with lymph node invasion and distant metastasis[41].

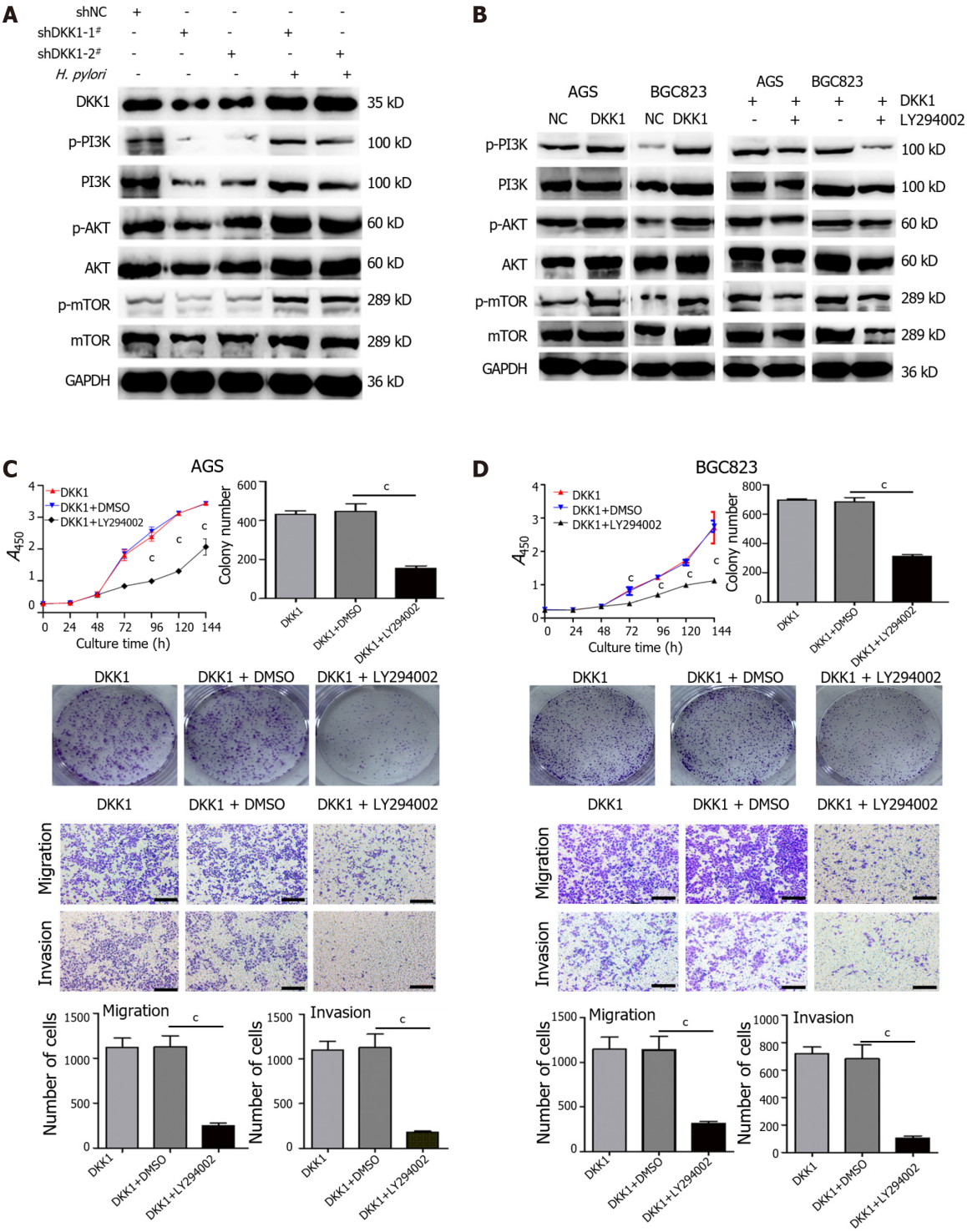
We demonstrated that the cancer-promoting function of DKK1 in GC was independent of Wnt/β-catenin signaling by observing that β-catenin expression and nuclear translocation were inhibited in DKK1-knockdown cells. CKAP4 was initially identified as an endoplasmic reticulum protein that was trafficked to the cell surface membrane after palmitoylation[42]. The combination of CKAP4 and DKK1 on the cell surface triggers pro-oncogenic or tumor-suppressive DKK1 signaling in different tumors[43, 44]. Although CKAP4 expression is high in many tumors[45,46], there is no information on CKAP4 expression in GC. We discovered that *H. pylori* infection simultaneously increased the expression of DKK1 and CKAP4 in GC cells, particularly CKAP4 expression on the cell membrane. High levels of DKK1 and CKAP4 expression were also consistently observed in GC tissues from the TCGA-STAD database and clinical GC specimens and were linked to a poor prognosis for GC patients. Notably, the interaction between DKK1 and CKAP4 was detected in GC cells. These results show that the DKK1/CKAP4 axis is present in *H. pylori*-infected GC cells and GC tissues.

There are two tandem cysteine-rich domains in the DKK1 promoter region, CRD1 and CRD2. DKK1 CRD1 domain binding to LPR6 inhibits Wnt signaling, and DKK1 CRD2 domain binding to CKAP4 activates PI3K/AKT signaling[38]. CKAP4 overexpression on the surface of cancer cells likely prevents DKK1 binding to LRP6, resulting in subsequent suppression of Wnt/β-catenin signaling[47]. This presumption is partially supported by our experimental results that *H. pylori*-induced DKK1 activated the CKAP4/PI3K/AKT/mTOR pathway rather than inhibiting LRP6/Wnt/β-catenin signaling in GC cells. Infection with *H. pylori* reversed the PI3K/AKT/mTOR signaling inhibition caused by DKK1 knockdown. PI3K inhibition with LY294002 also suppressed the DKK1-mediated activation of the PI3K pathway and the malignant phenotype of GC cells. Recent evidence has shown that the PI3K/AKT/mTOR and Wnt/β-catenin signalings are commonly activated in GC[48,49]. However, the reason for DKK1 selective activation of the CKAP4/PI3K/AKT/mTOR pathway in *H. pylori*-related GC is not known and requires further study.

There were several limitations to this study. First, the number of clinical samples was small. Therefore, further study with larger sample sizes is required to determine the expression of the DKK1/CAKP4 axis in GC tissues and its association with *H. pylori* infection in cancer tissues. Second, only two strains of *H. pylori* were used in this study: An East Asian strain (*H. pylori* GZ7) and a western strain (*H. pylori* 26695). However, *H. pylori* exhibit intrastrain and interstrain heterogeneity. More *H. pylori* strains will be required to verify our findings.

CONCLUSION

H. pylori infection upregulated JUN and FOSL1 expression, which formed AP-1 to promote DKK1 transcription that resulted in gastric tumorigenesis *via* activation of the CKAP4/PI3K/AKT/mTOR pathway. DKK1/CKAP4 interaction could become an attractive target for *H. pylori*-related GC therapy.



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Figure 6 Dickkopf-related protein 1 promotes the growth, migration, and invasion of AGS and BGC823 cells by activating the phosphatidylinositol 3-kinase/AKT/ mammalian target of rapamycin pathway. A and B: Western blotting for phosphatidylinositol 3-kinase (PI3K), p-PI3K, AKT, p-AKT, mammalian target of rapamycin (mTOR), and p-mTOR in dickkopf-related protein 1 (DKK1) knockdown and/or *Helicobacter pylori*-infected AGS cells (A) and DKK1 overexpression and/or LY294002 (50 μmol/L)-treated AGS and BGC823 cells (B); C and D: LY294002 treatment (50 μmol/L) decreases cell proliferation, colony formation, migration, and invasion in DKK1 overexpression AGS (C) and BGC823 (D) cells. Bar graphs show the quantitation of colony numbers or migrated and invaded cell numbers. Scale bar = 200 μm. $^{c}P < 0.001$. DKK1: Dickkopf-related protein 1; *H. pylori*: *Helicobacter pylori*; PI3K: Phosphatidylinositol 3-kinase; mTOR: Mammalian target of rapamycin.

The identification of small compounds and drugs targeting the DKK1/CKAP4 axis will be a crucial aspect of future studies. We will also investigate this possibility further.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is one of the most common malignant tumors with a high morbidity and mortality rate globally, especially in East Asian countries. *Helicobacter pylori* (*H. pylori*) infection is the most significant risk factor for GC. Studying their interaction can reveal the potential pathogenesis and therapeutic targets of GC.

Research motivation

Although substantial efforts have been done to link *H. pylori* infection and GC over the past decades, the molecular mechanisms of *H. pylori*-induced GC are not fully understood, which results in reduced treatment benefits.

Research objectives

The present study aimed to study the interaction of *H. pylori* infection, dickkopf-related protein (DKK) 1, and cytoskeleton-associated protein (CAKP) 4 in GC and the underlying molecular mechanisms.

Research methods

RNA sequencing identified differentially expressed genes (DEGs) between *H. pylori*-infected and uninfected primary GC cells. Dual-luciferase reporter assay and co-immunoprecipitation determined the interaction of activator protein (AP)-1, DKK1 and CKAP4. Western blotting and immunohistochemistry detected the expression of DKK1, CKAP4 and phosphatidylinositol 3-kinase (PI3K) pathway-related proteins in GC cells and tissues. Functional experiments and tumorigenicity in nude mice detected the malignant behavior of GC cells *in vitro* and *in vivo*.

Research results

H. pylori infection upregulated JUN, FOSL1, DKK1 and CKAP4 expression in GC cells, *H. pylori*-infected gerbil gastric tissues, and human GC samples. JUN and FOSL1 formed activator protein-1 (AP-1) to transcriptionally activate DKK1. DKK1 bound to CKAP4, but not Wnt coreceptor, to promote GC cell growth, migration, invasion, and xenograft tumor growth in nude mice *via* activating the PI-3K/AKT/mammalian target of rapamycin (mTOR) pathway. Targeting PI3K inhibited DKK1-mediated CKAP4/PI3K signaling activity and the malignant behavior of GC cells.

Research conclusions

H. pylori-induced AP-1 promotes the binding of DKK1 to CAKP4, which contributes to gastric tumorigenesis *via* the PI3K/AKT/mTOR pathway.

Research perspectives

The findings suggest that the DKK1/CKAP4 interaction may be a therapeutic target for *H. pylori*-induced GC. The identification of small compounds and drugs targeting the DKK1/CKAP4 axis will be a crucial aspect of future studies. We will also investigate this possibility further.

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FOOTNOTES

Author contributions: Luo M and Chen YJ contributed equally to this work; Zhou JJ and Xie Y designed the research study; Luo M, Chen YJ, Zhao Y, and Long NY performed the research; Wang QR, Xiang YN, and Yang WX collected samples and analysis data; Zhou JJ and Zhao Y wrote the manuscript; all authors have read and approve the final manuscript.

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Conflict-of-interest statement: All authors declare no competing interests for this article.

Data sharing statement: All the data presented in the study are included in the article/supplementary materials. The datasets analyzed in the study can be found online, and the names of the datasets can be found in the article. Other data are available from the corresponding author on reasonable request.

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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LETTER TO THE EDITOR

The potential role of the three-dimensional-bioprinting model in screening and developing drugs

Chao-Lin Deng, Bin Wu

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Abstract

Recently, we have read with great interest the original article used different spatial configuration models of colorectal cancer (CRC) for validating the anti-tumor efficacy with Diiminoquinone. We feel obliged to provide new insight into the drug screening models by integrating and analyzing the original method and result. These comments may provide comprehensive insights into three-dimensional drug screening models and the difference between pathologic subtypes in CRC.

Key Words: Colorectal cancer; three-dimensional-bioprinting; Mucinous adenocarcinoma; Drug screening models

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Core Tip: Chemotherapy is the main treatment option for inoperable colorectal cancer (CRC). We recently read an article about the anti-cancer effects of Diiminoquinone. We feel obliged to express our opinion on this article on drug screening models and the difference between pathologic subtypes in CRC and hope it could deepen understanding for the reader.

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TO THE EDITOR

We have read with great interest the article by Monzer *et al*[1]. The authors present a novel drug, Diiminoquinone (DIQ), with inhibitory effects for colorectal cancer (CRC) in different spatial configuration models. In vivo tests, Similar results have been obtained for drug effectiveness. In conclusion, the authors showed that DIQ may through suppresses Wnt/-catenin, AKT, and ERK pathways to the tumor and thereby inhibits tumor progression with significant potential to be translated into clinical practice.

The highlight of this study is that the authors used multiple three-dimensional (3D) models to verify the effectiveness of DIQ. The two-dimensional (2D) monolayer model has long been used *in vitro* cancer research for novel drug development and screening. However, 2D cancer cell models dramatically differ from cancer in vivo. Without spatial configurations, oncometabolite around the tumor microenvironment (TME)[2,3], and intercellular signaling between the cancer cell and other cells, the result from the 2D module may be unable to draw correct conclusions, and this causes further challenges for clinic translation. In this research, sphere formation assays with tumor cell lines and derived organoids were established and used to prove the safety and efficacy of DIQ and to reflect more accurately drug sensitivity measurements result.

We found some details through in-depth analysis and hope to express some relevant views. 3D culture models should ideally recapitulate the native TME. Despite sphere formation as a classic approach for 3D models, the limitation of this method is the lack of intercellular communication in multiple cell types. However, 3D-bioprinting provides several critical advantages over sphere formation assay in drug development or screening, such as using bio-ink to simulate the cytoskeleton or partial tumor tissue with multi-cell to a highly complex hierarchical 3D structure. These configurational were able to enhance intercellular communication and signaling factors transportation and provide a more accurate result for novel drug development[4,5]. Although the authors used organoid cultures to verify the drug's effectiveness at a later stage, the success rate of organ-like laboratory cultures is too low, which significantly limits the possibility of large-scale experimental validation. If 3D bioprinting is used, the required tissue size and culture conditions are lower than those of organoid cultures, which seems to provide more experimental samples for drug validation and enhance the data grade of this drug for clinical validation. Various 3D-bioprinting models were established, which aimed at disease modeling, novel drug development, and biological function evaluation[3,6]. Therefore, based on the current research data, tumor modeling using 3D bioprinting technology after primary cell cultures seems to be more beneficial for chemotherapy drug sensitivity screening.

Another interesting finding was that the DIQ showed chemotherapy effectivity in mucinous adenocarcinoma (MC), a unique pathological subtype of CRC[7]. In a previous study, the chemosensitivity of MC was poor either irinotecan- or oxaliplatin-based therapeutic strategies than in non-mucinous tumors[8]. One MC patient tissue was successfully grown as an organoid model in the paper, which does not seem to provide sufficient evidence for the effectiveness of DIQ for colorectal MC. Nevertheless, the authors' experimental results provide a possible research direction for chemotherapy targeting pathological subtypes.

This original article uses multiple models of CRC to demonstrate DQI as a potential novel drug for chemotherapy. However, further research is needed to support the safety and efficacy of clinical translation.

FOOTNOTES

Author contributions: Wu B designed and revised the manuscript; Deng CL wrote the manuscript.

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REVIEW

COVID-19 vaccination and liver disease

Sotaro Ozaka, Takashi Kobayashi, Kazuhiro Mizukami, Kazunari Murakami

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Abstract

Various vaccines against severe acute respiratory syndrome coronavirus 2 have been developed in response to the coronavirus disease 2019 (COVID-19) global pandemic, several of which are highly effective in preventing COVID-19 in the general population. Patients with chronic liver diseases (CLDs), particularly those with liver cirrhosis, are considered to be at a high risk for severe COVID-19 and death. Given the increased rates of disease severity and mortality in patients with liver disease, there is an urgent need to understand the efficacy of vaccination in this population. However, the data regarding efficacy and safety of COVID-19 vaccination in patients with CLDs is limited. Indeed, several organ-specific or systemic immune-mediated side effects following COVID-19 vaccination, including liver injury similar to autoimmune hepatitis, have been recently reported. Although the number of cases of vaccine-related liver injury is increasing, its frequency, clinical course, and mechanism remain unclear. Here, we review the current findings on COVID-19 vaccination and liver disease, focusing on: (1) The impact of COVID-19 in patients with CLD; (2) The efficacy, safety, and risk-benefit profiles of COVID-19 vaccines in patients with CLD; and (3) Liver injury following COVID-19 vaccination.

Key Words: COVID-19 vaccine; Liver disease; Side effect; Liver injury; Immune-related hepatitis; Autoimmune hepatitis

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Core Tip: Patients with chronic liver disease (CLD), including cirrhosis, are a high-risk group for severe coronavirus disease 2019 (COVID-19). Presently, the results of several clinical trials for measuring the efficacy and safety of the available COVID-19 vaccines in patients with CLD have been reported. Given the increased rates of severity and mortality of COVID-19 in patients with CLD, the importance of aggressive vaccination in the effective management of severe acute respiratory syndrome coronavirus 2 infection should be emphasized. Although liver injury following COVID-19 vaccination has also been reported, it is infrequent and is not a factor in vaccine hesitancy.

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INTRODUCTION

The December 2019 outbreak of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) rapidly spread worldwide and became a global health threat[1,2]. COVID-19 is a respiratory disease caused by SARS-CoV-2, which can damage not only the lungs, but also other organs, including the cardiovascular system, liver, and gastrointestinal tract[3-5]. Vaccines are the most effective prophylaxis against COVID-19, and several vaccines against SARS-CoV-2 have been developed in response to the COVID-19 pandemic, among which vaccines produced mainly by Pfizer-BioNTech, Moderna, and Oxford-AstraZeneca are now widely used[6]. These vaccines are of great importance in controlling severe COVID-19 not only in healthy individuals, but also in high-risk populations, including those with chronic diseases. Chronic liver disease (CLD) is characterized by the gradual destruction of liver tissue over time, and includes liver diseases that are caused by chronic inflammation [chronic viral hepatitis, non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, and autoimmune hepatitis (AIH)] with or without cirrhosis and hepatocellular carcinoma (HCC). Patients with CLD, particularly those with cirrhosis, and liver transplantation (LT) recipients infected with SARS-CoV-2 have been reported to have a higher risk of adverse outcomes than the general population. For example, among 2780 individuals with COVID-19 in the United States, comparison between 250 individuals with liver diseases *vs* the rest of the cases indicated a significantly higher mortality rate in those with liver disease [hazard ratio (HR), 2.8; 95% confidence interval (CI): 1.9-4.0] and a higher mortality risk in those with cirrhosis (HR: 3.0; 95%CI: 1.5-6.0)[7]. In a recent study, the mortality rate of cirrhotic patients with COVID-19 was significantly higher than that of non-cirrhotic patients (HR: 2.38; 95%CI: 2.18-2.59), and was also increased in cirrhotic patients with underlying CLD (HR: 3.31; 95%CI: 2.91-3.77)[8]. Therefore, patients with CLD are considered to be at an increased risk of SARS-CoV-2 infection and worse outcomes. Given the increased rates of severity and mortality in patients with liver disease, there is an urgent need to understand the efficacy and safety of vaccination, as well as the importance of aggressive vaccination in this population. However, since most of the phase 2/3 trials of COVID-19 vaccines mainly recruited healthy individuals, data regarding the efficacy and safety in patients with liver diseases is limited. Presently, the results of several clinical trials for measuring the efficacy and safety of the available COVID-19 vaccines in patients with CLD have been reported.

The safety profiles of each vaccine have also been extensively studied. Common side effects of COVID-19 vaccines include injection site pain, transient fever, headache and fatigue[9,10]. Recently, however, several organ specific or systemic immune-mediated side effects following COVID-19 vaccination have also been reported[11]. These side effects include immune-mediated liver injury resembling AIH.

In this review, we summarized the current knowledge, focusing on the impact of COVID-19 in patients with liver disease, as well as the efficacy and safety of COVID-19 vaccination in these patients. In addition, we analyzed case reports of acute liver injury following COVID-19 vaccination.

OVERVIEW OF COVID-19 VACCINES

Vaccines against SARS-CoV-2 can be categorized based on the platform they are developed on into mRNA, viral vector, inactivated virus, attenuated virus, protein subunit, and recombinant DNA vaccines. The major COVID-19 vaccines currently used worldwide include the vaccines produced by Pfizer-BioNTech, Moderna, and Oxford-AstraZeneca[6]. These, plus the CoronaVac vaccine (Sinovac Biotech) and Janssen-Ad26.COV2.S vaccine (Johnson & Johnson), are the five vaccines registered in the WHO Emergency Use Listing of Qualified Vaccines (Table 1)[12]. Various COVID-19 vaccines have been

Table 1 Summary of coronavirus disease 2019 vaccines

Corporation	Vaccine	Mechanism	Vaccination Group: Number of cases/number of vaccinations (%)	Invaccination Group: Number of cases/number of vaccinations (%)	Efficacy % (95%CI)
Pfizer-BioNTech [18]	BNT162b2	mRNA	8/21720 (0.037)	162/21728 (0.746)	95.0 (90.3-97.6)
Moderna[20]	mRNA-1273	mRNA	11/15210 (0.072)	185/15210 (1.216)	94.1 (89.3-96.8)
Oxford-AstraZeneca[25]	ChAdOx1 nCoV-19 (AZD122)	Vector	27/4440 (0.608)	71/4455 (1.594)	62.1 (41.0-75.7)
Johnson & Johnson[26]	Ad26.COV2.S	Vector	433/19113 (2.265)	883/18924 (4.666)	52.9 (47.1-58.1)
Sinovac Life Science[29]	Corona Vac	Inacctivated	9/6559 (0.137)	32/3470 (0.922)	83.5 (65.4–92.1)

proven to be highly effective and have good safety profiles in healthy populations. The efficacy of vaccines is evaluated based on their: (1) Immunogenicity; (2) Efficacy rate in clinical trials; and (3) Real-world efficacy rate.

mRNA vaccines

The BNT162b2 mRNA (Pfizer-BioNTech) and mRNA-1273 mRNA (Moderna) vaccines are based on mRNA encoding SARS-CoV-2 spike proteins[13]. The injected mRNA is internalized into local host cells and translated, resulting in the production of antigen proteins and antigen-specific immune responses [14,15]. mRNA based vaccines have been shown to be safe and well tolerated in clinical trials. The BNT162b2 mRNA vaccine was administered to healthy adults (18-55 and 65-85 years old) at doses of 10 μg, 30 μg, or 100 μg in a phase 1/2 trial and showed immunogenicity, tolerability, and safety profiles consistent with these doses[16,17]. A phase 3 study of 43548 individuals also showed an efficacy rate of 95% [95%CI: 90.3-97.6; 8 cases of COVID-19 (0.04%) of 21720 in the BNT162b2 group *vs* 162 cases of COVID-19 (0.75%) of 21,728 in the placebo group][18], and a 6-mo follow-up of 44616 adults aged 16 years and older and 2264 participants aged 12 to 15 years showed an efficacy rate of 91.3% (95%CI: 89.0-93.2)[19]. The mRNA-1273 vaccine also showed an efficacy of 94.1% [95%CI: 89.3-96.8; 11 cases of COVID-19 (0.07%) of 15210 in the mRNA-1273 group *vs* 185 cases of COVID-19 (1.22%) of 15210 in the placebo group] in a phase 3 study of 30420 healthy individuals aged 18 or above[20]. It should be noted, however, that the efficacy against the recent epidemic Omicron strain is reduced for both these mRNA vaccines. Neutralizing antibody titers 3 wk after two doses of the BNT162b2 vaccine were significantly lower for the Omicron strain compared with the Wuhan strain[21], and even with the mRNA-1273 vaccine, neutralizing antibody titers against the Omicron strain after two doses of the vaccine were 1/41 to 1/84 of those against the European strain[22]. On the other hand, it was also shown that the neutralizing antibody titer against the Omicron strain increased significantly after the third dose of both vaccines and was almost equivalent to that after two doses[21,22], and the booster dose of mRNA vaccines was also effective against the Omicron strain, with a good efficacy rate of 60 to 70%[23].

Viral vector vaccines

The ChAdOx1 nCoV-19 vaccine (AZD1222), which was developed by Oxford-AstraZeneca, is a viral vector vaccine. Immunity to the spike protein is induced by administration of a chimpanzee adenovirus vector containing the SARS-CoV-2 spike protein gene[24]. A clinical trial of ChAdOx1 nCoV-19 reported an efficacy of 62.1% [95%CI: 41.0-75.7; 27 cases of COVID-19 (0.6%) of 4440 in the ChAdOx1 nCoV-19 group *vs* 71 cases of COVID-19 (1.6%) of 4455 in the control group][25]. The efficacy of Ad26.COV2.S, a single-dose viral vector vaccine produced by Johnson & Johnson, has also been reported. In a phase 3 study, vaccine efficacy after 28 d was 52.9% [95%CI: 47.1-58.1; 433 cases of COVID-19 of 19113 (2.3%) in the Ad26.COV2.S group *vs* 883 cases of COVID-19 of 18924 (4.7%) in the placebo group][26,27].

Inactivated vaccines

Sinovac-CoronaVac is an inactivated vaccine developed by Sinovac Life Sciences and is being used in several countries. Its efficacy and safety were demonstrated in a phase 1/2 study by Zhang *et al*[28] and the phase 3 study of Tanriover *et al*[29]. In a population aged 18-59 years, CoronaVac was shown to be highly effective in preventing symptomatic COVID-19 (83.5% *vs* placebo) and COVID-19-related hospitalizations (100%) at least 14 d after the second dose[29].

IMPORTANCE OF COVID-19 VACCINATION IN PATIENTS WITH LIVER DISEASES

During the COVID-19 pandemic, many cases have been accumulated and the clinical course of COVID-19 in patients with CLD has been characterized. Patients with CLD, including those with cirrhosis or HCC, and LT recipients, are a high-risk group for severe COVID-19[30]. In a large cohort study using electronic health record data from more than 17 million patients in the United Kingdom, which included more than 0.1 million patients with CLD, CLD was a risk factor for death from COVID-19 (HR: 2.39; 95%CI: 2.06-2.77)[31]. A particularly high mortality rate due to COVID-19 has been reported in patients with cirrhosis[7,8,32], and recent prospective data from a multicenter study reported a high mortality rate of 32% among 729 patients with CLD from 29 countries. In particular, patients with decompensated cirrhosis were found to be at a higher risk of hospitalization, mechanical ventilation and death (overall mortality: Child-Pugh-A: 19%, Child-Pugh-B: 32%, Child-Pugh-C: 51%, non-cirrhosis: 8%)[33]. Similarly, in a North American multicenter cohort study, compensated cirrhosis had no effect on mortality in COVID-19, while mortality was increased in patients with decompensated cirrhosis[34]. Furthermore, cirrhotic patients with COVID-19 had a significantly higher mortality rate than patients with COVID-19 alone (30% *vs* 13%, $P = 0.03$) in a multicenter cohort study in the United States and Canada[35]. Patients with CLD, particularly those with cirrhosis, have multiple mechanisms of immune dysfunction that can lead to increased susceptibility to SARS-CoV-2 infection and an abnormal inflammatory response during infection. Thus, it has become clear that liver cirrhosis patients are at an increased risk of adverse COVID-19 outcomes, including death, as has been established by large observational cohorts and population-level data and international registry findings.

In addition to cirrhosis, NAFLD, alcoholic liver injury, and HCC are known to be factors affecting COVID-19 severity. Several observational cohort studies revealed a significant increase in the risk of severe COVID-19 in patients with NAFLD. In a Chinese study analyzing 202 COVID-19 cases, NAFLD complications were significantly more frequent in 39 patients whose disease progressed after hospitalization than in non-progressors (87.2% *vs* 25.8%), and the rate of severe disease was significantly higher in patients with NAFLD than in those without NAFLD (44.7% *vs* 6.6%)[36]. A meta-analysis by Pan *et al*[37] showed that NAFLD was associated with more severe COVID-19 [odds ratio (OR): 2.93; 95%CI: 1.87-4.60][37]. Two other meta-analyses have also been reported, both of which showed that NAFLD is a severity factor for COVID-19[38,39]. Patients with NAFLD have decreased hepatic innate immunity with skewed M1/M2 macrophage polarization, as well as increased levels of inflammatory mediators and cytokines. This underlying inflammatory status associated with NAFLD might lead to further exacerbation of the SARS-CoV-2 infection and can lead to a cytokine storm, which greatly increases the mortality rate[37]. Furthermore, it should be noted that diabetes mellitus, obesity, and cardiovascular diseases are also frequently present in the background of NAFLD, and these metabolic disorders might also be factors related to the increased mortality of COVID-19. Regarding alcoholic liver disease, Marjot *et al*[33] reported that it is an independent risk factor for COVID-19 related death (OR: 1.79; 95%CI: 1.03-3.13)[33], and Kim *et al*[34] also reported a 2.4-fold increase in COVID-19 mortality (HR: 2.42; 95%CI: 1.29-4.55) in these patients[34]. Their report also showed that HCC is a risk factor for death from COVID-19 (HR: 3.96; 95%CI: 1.74-8.98)[34]. Since HCC often occurs secondary to cirrhosis, the greater severity of COVID-19 in this patient group is thought to be a result of reduced immunity. Since LT recipients are required to use immunosuppressive agents for a long period of time, they are also considered to be a high-risk group for severe COVID-19. However, many reports from actual cohort studies concluded that LT is not an independent risk factor for COVID-19 related death[40-42]. Webb *et al*[43] compared mortality in 151 COVID-19 patients who underwent LT with 627 healthy subjects without LT, and reported no difference in overall mortality between the two groups (absolute risk difference 1.4%; 95%CI: 7.7-10.4)[43]. On the other hand, their study also reported that gastrointestinal symptoms, such as diarrhea, but not respiratory symptoms, were significantly increased in post-LT patients affected by COVID-19 (30% *vs* 12%, $P < 0.0001$)[43]. In addition, a report from the European Liver Transplant Association of 103 LT recipients affected by COVID-19 showed a significantly higher mortality rate in recipients older than 60 years[44].

Thus, since patients with CLD, especially those with cirrhosis, are a high-risk group for severe COVID-19, aggressive vaccination of this patient group is most important for the effective management of SARS-CoV-2 infection.

EFFICACY AND SAFETY OF COVID-19 VACCINES IN PATIENTS WITH LIVER DISEASES

Given the increased severity and mortality rates of COVID-19 in patients with liver disease, we need to understand the efficacy and safety of vaccination in this population. However, data regarding the efficacy and safety of COVID-19 vaccination in patients with CLD is limited. Previous studies on vaccine efficacy and safety in patients with liver disease that have been reported to date are shown in Table 2[45-65]. It should be noted that most of the reports in Table 2 do not take into account the influence of the omicron variant, which is a limitation of using previous data in the current clinical environment of omicron variant predominance.

Table 2 Literature review of the efficacy and safety of coronavirus disease 2019 vaccines in patients with liver disease

Ref.	Design	Vaccine	Country/region	Number of participants	Value	Major findings (efficacy)	Major findings (safety)
Cirrhosis							
John *et al*[45], 2021	Multicentre retrospective cohort study	BNT162b2 and mRNA-1273	United States	Cirrhosis group (n = 20037); Control (n = 20037)	Efficacy	64.8% decrease in the development of COVID-19 infection after the first dose and a 78.6% decrease after the second dose	NA
Thuluvath *et al*[46], 2021	Prospective cohort study	BNT162b2, mRNA-1273, and AZD1222	United States	LT (n = 62); Cirrhosis (n = 79); CLD (n = 92)	Immunogenicity	Antibody was detectable in 82.2% of LT recipients, 96.2% of cirrhosis and 95.7% of CLD without cirrhosis. 61.3% of LT recipients and 24% CLD with/without cirrhosis had poor antibody responses	No patient had any serious AEs
Ruether *et al*[47], 2022	Prospective cohort study	BNT162b2, mRNA-1273, and AZD1222	Germany	LT (n = 138); Cirrhosis (n = 48); Control (n = 52)	Immunogenicity	Immunological response rates were 36.6%, 65.4%, and 100% in LT, cirrhosis, and controls, respectively	NA
Willuweit *et al*[48], 2022	Prospective cohort study	BNT162b2	Germany	Cirrhosis (n = 166); Control (n = 79)	Immunogenicity	Antibody was detectable in 96% of cirrhosis and 99% of controls. The median SARS-CoV-2 IgG titer was significantly lower in cirrhosis compared to the controls (939 *vs* 1905 BAU/mL, P = 0.0001)	NA
Wang *et al*[49], 2022	Multicentre retrospective cohort study	Inactivated vaccine	China	Compensated-cirrhosis (n = 388); Decompensated cirrhosis (n = 165)	Immunogenicity	Antibodies were detectable in 71.6% and 66.1% in compensated-cirrhosis and decompensated-cirrhosis	The vaccines were well tolerated, most AEs were mild and transient
Ai *et al*[50], 2022	Multicentre prospective cohort study	Inactivated vaccine	China	CLD (n = 284); Compensated cirrhosis (n = 123); Decompensated cirrhosis (n = 30)	Immunogenicity	Antibody detection rates were 76.8% in noncirrhotic CLD group, 78.9% in compensated cirrhotic group, 76.7% in decompensated cirrhotic group, and 90.3% in controls (P = 0.008 *vs* CLD)	There was no significant difference in AE among subgroups
Liver transplant recipients							
Rabinowich *et al*[51], 2021	Multicentre retrospective cohort study	BNT162b2 mRNA vaccine	Israel	LT patients (n = 80); Control (n = 25)	Immunogenicity	Immunogenicity among LT recipients was significantly lower [47.5% (LT) *vs* 100% (control), P < 0.001]	No patient had any serious AEs
Herrera *et al*[52], 2021	Multicentre prospective cohort study	mRNA-1273	Spain	LT recipients (n = 58)	Immunogenicity	93% of patients developed immunologic responses to mRNA-1273 vaccine	No serious AEs were reported in LT recipients
Strauss *et al*[53], 2021	Multicentre retrospective cohort study	BNT162b2 and mRNA-1273	United States	LT recipients (n = 161)	Immunogenicity	Antibody was detectable in 34% (95%CI: 27%-42%) of participants after first dose, and in 81% (95%CI: 74%-87%) after second dose	NA
Nazaruk *et al*[54], 2021	Retrospective cohort study	BNT162b2 mRNA vaccine	Poland	LT recipients (n = 65)	Immunogenicity	Antibody detection rate was 88.9% in LT recipients after the second dose	NA
Timmermann *et al*[55], 2021	Retrospective cohort study	mRNA vaccines	Germany	LT recipients (n = 118)	Immunogenicity	The seroconversion rate was 78.0% in LT recipients. MMF for immunosuppression was risk factors for seroneg-ativity	NA
D'Offizi *et al*[56], 2022	Retrospective cohort study	BNT162b2 and mRNA-	Italy	LT patients (n = 61); Control (n =	Immunogenicity	Immunological response rates 2 wk after 2nd dose	NA

		1273		51)		were 47.5% (LT) and 100% (control) ($P < 0.001$)	
John *et al*[57], 2022	Multicentre retrospective cohort study	BNT162b2 and mRNA-1273	United States	LT patients (n = 1133); Control (n = 791)	Efficacy	Vaccination with 2 doses of an mRNA vaccine was associated with a 64% decrease in COVID-19 infection and 87% decrease in COVID-19–related death in LT recipients	NA
Davidov *et al* [58], 2022	Retrospective cohort study	BNT162b2 mRNA vaccine	Israel	LT patients (n = 76); Control (n = 174)	Immunogenicity	Immunological response rates 2 wk after 2nd dose were 72.0% (LT) and 94.2% (control) ($P < 0.001$)	AEs were reported by 51% LT recipients. No serious events were reported
Sakai *et al* [59], 2022	Retrospective cohort study	BNT162b2	Japan	LT patients (n = 56); Control (n = 42)	Immunogenicity	LT recipients showed a lower seroconversion rate (44/56; 78.6%) than healthy controls (41/42; 97.6%)	NA
Calleri *et al* [60], 2022	Retrospective cohort study	BNT162b2 and mRNA-1273	Italy	Pre-LT patients (n = 89)	Immunogenicity	In the 89 pre-LT patients, seroconversion rate was 94.4% (23 d after vaccination), and 92.0% (68 d after vaccination)	No serious AEs were reported in participants
Viral hepatitis and NAFLD							
Xiang *et al* [61], 2021	Retrospective cohort study	Inactivated vaccine	China	CHB patients (n = 149)	Immunogenicity	The seroconversion rate was 87.2% in CHB	No serious AEs were reported in participants
He *et al*[62], 2022	Cross-sectional observational study	Inactivated vaccine	China	CHB patients (n = 362); Control (n = 87)	Immunogenicity	The seroconversion rates of SARS-CoV-2 antibodies were similar between CHB patients and healthy controls	The incidence was similar between CHB patients and controls. No serious AE
Wang *et al* [63], 2021	Multicentre retrospective cohort study	Inactivated vaccine	China	NAFLD patients (n = 381)	Immunogenicity	The inactivated COVID-19 vaccine was good immunogenicity (95.5%) in patients with NAFLD	AEs within 7 d and within 28 d totaled 95 (24.9%) and 112 (29.4%), respectively. No serious AEs were recorded
Autoimmune liver disease							
Duengelhoef *et al*[64], 2022	Prospective cohort study	BNT162b2, mRNA-1273, and AZD1222	Germany	AIH (n = 103); PSC (n = 64); PBC (n = 61); Control (n = 95)	Immunogenicity	Seroconversion was measurable in 97% of AIH and 99% of PBC/PSC patients, respectively. In 14% of AIH patients antibody levels were lower compared to PBC/PSC or controls	NA
Schneider *et al* [65], 2022	Prospective cohort study	BNT162b2 mRNA vaccine	Austria	AIH (n = 12); Control (n = 24)	Immunogenicity	Patients of AIH and healty controls acquired sufficient antibodies after third vaccination	NA

AE: Adverse event; AIH: Autoimmune hepatitis; CHB: Chronic hepatitis B; CLD: Chronic liver disease; LT: Liver transplant; MMF: Mycophenolate mofetil; NA: Not available; NAFLD: Non-alcoholic fatty liver disease; PBC: Primary biliary cholangitis; PSC: Primary sclerosing cholangitis; CI: Confidence interval.

Response to vaccination in liver cirrhosis patients

Given the higher COVID-19 related mortality in individuals with decompensated cirrhosis, it is important to prioritize vaccination in this group. Patients with cirrhosis have previously shown hyporesponsiveness to hepatitis B virus and pneumococcal vaccines[66,67], and were also considered to be less responsive to COVID-19 vaccines[68]. Indeed, cirrhosis has been reported to be a high-risk factor (3.0-fold) for COVID-19 related deaths after COVID-19 mRNA vaccination in a large United Kingdom cohort study. A defect in acquired immunity in patients with cirrhosis probably predicts a low response to vaccination in this patient population[69]. However, a recent study in the United States found that 75%

of CLD patients without cirrhosis and 77% of those with cirrhosis had adequate antibody responses to COVID-19 vaccination[46]. In that study, antibody responses to the BNT162b2 mRNA vaccine and mRNA-1273 mRNA vaccine were favorable (64.4% and 76.4%, respectively), whereas those to the Ad26.COV2.S vaccine (Johnson & Johnson) were low (15.8%). In a study analyzing the immune response of 110 patients with cirrhosis after two doses of the BNT162b2 mRNA vaccine, while the antibody initial acquisition rate was 96%, which was not significantly different from that in the control group (99%) (P = 0.04), the antibody titer showed a rapid and significant reduction in patients with cirrhosis[48]. This suggests that although the initial results after vaccination with the COVID-19 BNT162b2 vaccine are favorable in patients with cirrhosis, it should be noted that the antibody response deteriorates rapidly over time, and, hence, the timing of the booster shot needs to be addressed in the near future.

The immunogenicity of COVID-19 vaccines in patients with cirrhosis appears to be inferior to that in healthy individuals, although real-world cohort studies showed that BNT162b2 mRNA and mRNA-1273 vaccines reduce the development of COVID-19 infection by 64.8% after the first dose and 78.6% after the second dose[45]. Furthermore, a recent report on BNT162b2 mRNA, mRNA-1273, and Ad26.COV2.S vaccines has shown significantly reduced COVID-19-related mortality in vaccinated patients with cirrhosis (HR: 0.21; 95%CI: 0.11-0.42)[70]. In the report on the Sinovac-CoronaVac vaccine, the immunogenicity in patients with CLD was lower (77.3%) compared to that in healthy controls (90.3%), although the presence or absence of cirrhosis in CLD patients did not affect the antibody retention rate [non-cirrhotic CLD (76.8%), compensated cirrhosis (78.9%), and decompensated cirrhosis (76.7%)]. The safety in each group was as good as in healthy controls in that report [healthy controls (16.0%), non-cirrhotic CLD (15.5%), compensated cirrhosis (16.3%), and decompensated cirrhosis (20.0%)][50]. In a study examining the efficacy of the Ad26.COV2.S vaccine, the vaccine efficacy in patients with cirrhosis was 64% (OR: 0.36; 95%CI: 0.20-0.62, P = 0.005), which was non-inferior to the results of a phase 3 trial[71].

Regarding safety, Cao *et al*[72] reported that of 85 patients with decompensated cirrhosis who received at least one dose of a SARS-CoV-2 vaccine, only one patient (1.2%) had an adverse reaction requiring hospitalization[72]. Bakasis *et al*[73] also reported that there was no significant difference in the safety of mRNA vaccines given to 87 patients with liver diseases including cirrhosis, and 40 controls [73]. The limitations of these studies[50,71-73], however, include a relatively small sample size of participants who received the vaccines and a short follow-up period. Research with larger sample sizes is, thus, required in the future.

Hence, although each vaccine is slightly inferior in immunogenicity in patients with cirrhosis compared with healthy individuals, they can be safely used with adequate efficacy in patients with cirrhosis. However, there is insufficient data to allow recommendation of one vaccine over another.

LT

COVID-19 outcomes in LT recipients are not necessarily worse than those in the general population, although they have a higher rate of admission to the intensive care unit. Thus, COVID-19 vaccination should be prioritized for these patients, since the benefits far outweigh the potential risks. Vaccination for LT recipients is an interesting area of research, with a series of reports on its efficacy and safety.

Several reports of low humoral and cellular responses after COVID-19 vaccination in LT recipients suggest that vaccine-induced immunity in this patient subgroup is lower than in the general population. As shown in Table 2, LT recipients exhibit significantly attenuated humoral and cellular immunity 2 wk after mRNA vaccination compared to healthy individuals[56,58], lower seroconversion rates[59] and significantly lower immunogenicity[51]. Indeed, combination immunosuppressive therapy, including mofetil mycophenolate, is reportedly a predictor of reduced responsiveness to vaccination[55,58,59]. The results of such vaccine responses in LT recipients were similar to the results of other studies in kidney transplant recipients[74], lung transplant recipients[75], and allogeneic hematopoietic stem-cell transplant recipients[76]. On the other hand, a recent multicenter cohort study showed that mRNA vaccines reduce SARS-CoV-2 infection rate, occurrence of symptomatic COVID-19, and mortality in LT recipients as well as in patients with cirrhosis[70]. While the immunogenicity of mRNA vaccines in LT recipients appears to be attenuated compared with that in healthy individuals, the disease status of COVID-19 is greatly attenuated in vaccinated LT recipients compared to unvaccinated recipients. Additionally, the value and safety of routine immunization in liver transplant recipients has been well established, and current guidelines recommend pre-transplant vaccination whenever possible[77]. However, since LT recipients also show a rapid decrease in antibody titers after mRNA vaccination[59], and because booster doses are reportedly extremely safe in post-organ transplant patients [78], it is recommended that all LT recipients receive a third or fourth booster dose if they have low or insufficient antibody titers[47].

HCC

HCC patients are considered a high risk group for severe COVID-19[34]. Nevertheless, there are no cohort studies investigating the immunogenicity and safety of COVID-19 vaccines in patients with HCC. According to the American Association for the Study of Liver Diseases, patients with HCC receiving locoregional or systemic therapy should also be considered for vaccination without

interruption of treatment[79]. Most patients treated for solid tumors, including HCC, show an adequate humoral response against SARS-CoV-2 after two vaccine doses. However, vaccination during chemotherapy tends to be associated with lower antibody levels, resulting in a suboptimal response in a small percentage of patients[80,81]. Furthermore, the concentration of neutralizing antibodies decreases over time, further reducing immunity[82]. Based on these reports, many countries are prioritizing a third vaccine dose in patients with solid tumors.

Viral hepatitis and NAFLD

With regard to viral hepatitis and NAFLD, several cohort studies evaluating the efficacy of inactivated vaccines have been reported from China. Xiang *et al*[61] analyzed the seroprevalence of anti-spike protein antibodies and neutralizing antibodies in chronic hepatitis B (CHB) patients who received two doses of inactivated vaccines, and reported high seropositivity rates of 87.2% and 74.5%, respectively [61]. This report showed that nucleotide analog therapy has no effect on vaccine-induced immune responses, suggesting that vaccination should be performed even during treatment of CHB. He *et al*[62] also reported that seroconversion rates of SARS-CoV-2 antibodies after 1, 2, and 3 mo in patients with CHB who received an inactivated vaccine were comparable to those in healthy controls[62]. No serious adverse reactions were reported in either study.Next, in a multicenter study of the safety and immunogenicity of inactivated vaccines in 381 individuals with NAFLD, the incidence of adverse reactions within 7 d and 28 d after vaccination was 24.9% and 29.4%, respectively. Neutralizing antibodies were detectable in 364 (95.5%) patients, and titers of neutralizing antibodies were shown to persist for a long time[63]. Given that NAFLD is a risk factor for severe COVID-19[37-39], active vaccination of this patient population would be ideal.

AIH

There have been two reports on the immunogenicity of COVID-19 vaccines in patients with AIH. Duengelhoef *et al*[64] reported that 91 of 94 (97%) AIH patients who received the second dose of a vaccine achieved seroconversion, although AIH patients showed impaired spike-specific T-cell responses and lower antibody levels at 7 mo compared with healthy individuals or patients with primary biliary cholangitis (PBC)/primary sclerosing cholangitis (PSC) (641 *vs* 1020 *vs* 1200 BAU/mL, respectively). These results were not related to the use of immunosuppressive medications, suggesting that the underlying immune abnormality of AIH might be involved in this diminished response[64]. In addition, a study that followed the antibody responses after mRNA vaccination in patients with AIH and healthy controls showed that anti-SARS-CoV-2 antibodies were sufficiently produced after the second and third vaccinations in both groups[65]. Therefore, early booster doses of vaccines should be considered in patients with AIH. Although none of the patients in these reports had serious adverse reactions, it is noteworthy that several case reports have been published reporting an increased risk of developing AIH-like syndromes after SARS-CoV-2 vaccination.

LIVER INJURY AFTER COVID-19 VACCINATION

Adverse effects of COVID-19 vaccines

Vaccination in patients with liver diseases is generally considered safe and effective and should be strongly recommended. The common side effects of COVID-19 vaccination include injection site pain, fever, headaches, and fatigue. All these side effects are mild and typically resolve 1-3 d after vaccination. However, recent worldwide dissemination of COVID-19 vaccines and post-marketing surveillance have led to an increasing number of reports of several organ-specific immune-related diseases, including myocarditis, immune thrombotic thrombocytopenia, Guillain-Barré syndrome, and pancreatitis, among others[11,83-85]. It is speculated that an abnormal immune response following vaccination is involved in the development of such diseases. Acute liver injury was not previously reported in clinical trials on COVID-19 vaccination because the sample size was insufficient to detect rare adverse events after vaccination. Recently, a large-scale population-based study on acute liver injury occurring after COVID-19 vaccination was reported from Hong Kong. In that study, among 2343288 COVID-19 vaccine recipients, acute liver injury within 56 d after the first and second vaccine dose occurred in 307 and 521 (335 and 334 *per* 100000 person-years) individuals vaccinated with BNT162b2, and 304 and 474 (358 and 403 *per* 100000 person-years) of those who received CoronaVac. The incidence of acute liver injury within 56 d of SARS-CoV-2 infection, on the other hand, was 32997 cases *per* 100000 person-years, indicating that the incidence of acute liver injury after COVID-19 vaccination was much lower than that after SARS-CoV-2 infection. It was also concluded that compared to the non-exposure period, no increased risk of acute liver injury was observed in the 56-d risk period following the first (IRR: 0.800; 95%CI: 0.680-0.942) and second (IRR: 0.944; 95%CI: 0.816-1.091) BNT162b2 dose, and the first (IRR: 0.689; 95%CI: 0.588-0.807) and second (IRR: 0.905, 95%CI: 0.781-1.048) CoronaVac dose. Thus, COVID-19 vaccines do not seem to increase the risk of acute liver injury[86]. However, since there have been case reports of acute liver injury requiring hospitalization after COVID-19 vaccination, this is an adverse effect that should not be overlooked. The current review aimed to increase awareness of this rare

adverse effect to promote its early recognition.Review of case reports on liver injury after COVID-19 vaccinationSince the summer of 2021, several case reports of liver injury after COVID-19 vaccination have been reported. The clinical and histological findings of most patients resembled AIH and the reported cases responded well to corticosteroid therapy. Previous cases of AIH-like acute liver injury after COVID-19 vaccinations are shown in Table 3[87-109]. Twenty-three reports (28 cases) of acute liver injury secondary to COVID-19 vaccines have been published in the PubMed database as of July 2022 (Table 3). The median age at the time of diagnosis was 61 (range 27-80) years, with a predominance of women (79%, females: 22, males: 6). Eight patients (29%) had no underlying disease, whereas nine patients (32%) had been diagnosed with other immune disorders (Hashimoto's disease: 5, PSC: 2, PBC: 1, sarcoidosis: 1). One patient was three months postpartum, and another patient was taking hormonal therapy due to menstrual irregularities. Liver injury occurred after vaccination following the BNT162b2 vaccine in 11 (39%) of the cases, mRNA-1273 vaccine in nine (32%), ChAdOx1 nCoV-19 vaccine in seven (25%), and CoronaVac vaccine in one case (4%). Seventeen patients (61%) developed liver injury after the first dose, 10 patients (36%) after the second dose, and two patients (7%) after the booster shot. The median time from vaccination to the onset of acute liver injury was 11 (range 3-35) d. The most common symptom was jaundice, while other symptoms, such as fatigue and anorexia, were also frequently observed. The most common pattern of liver injury was hepatocellular injury, with transaminase levels exceeding 1000 U/L in many cases. The mean alanine aminotransferase level was 848.2 (± 465.0) U/L, mean aspartate aminotransferase level was 1031.5 (± 578.3) U/L, and mean total bilirubin level was 9.08 (± 5.76) mg/dL. Serum immunoglobulin G (IgG) levels were measured in 25 patients and were elevated in 22 patients (88%). Anti-nuclear antibodies (ANA) were positive in 22 (82%) of 27 patients, while anti-smooth muscle antibodies and anti-mitochondrial antibodies (AMA) were elevated in some cases. Liver biopsy was performed in all cases. According to the simplified international diagnostic criteria published by the international AIH-group, seven cases were "typical" of AIH and 20 cases were "compatible with" AIH. Only one patient had poor findings of typical AIH and was diagnosed with drug-induced liver injury. Steroids as first-line treatment were used in 26 of the patients (93%), of whom 10 received prednisone, 14 received prednisolone, one received budesonide, and one was given methyl-prednisolone intravenously. Azathioprine was concomitantly used in five patients. The overall outcomes with corticosteroid therapy were favorable, and improvement was seen in 27 patients (96%). Only one male patient reported from India progressed to liver failure and died despite five cycles of plasma exchange[99].

All of the patients developed acute liver injury soon after COVID-19 vaccination, with findings consistent with AIH on liver biopsy, and responded well to corticosteroids. These case reports strongly suggest that the association between vaccination and the onset of AIH-like liver injury might be more than coincidental. However, it is difficult to establish a causal relationship between vaccination and liver injury with certainty. Indeed, post-pregnancy status, use of statins, and concomitant history of autoimmune diseases included in the reported cases are likely major confounding factors. It should be noted that almost all the reported cases lacked pre-vaccination laboratory data, and hence, the presence of pre-existing hepatitis cannot be ruled out. Indeed, Cao *et al*[110] reported that a patient with vaccine-induced AIH had advanced fibrosis on liver biopsy, suggesting the presence of CLD prior to vaccination [110].

Furthermore, a large international case series that provided evidence for the hepatotoxic potential of COVID-19 vaccines has recently been reported[111]. In that study, data from 18 countries on 87 patients who developed liver injury after COVID-19 vaccination were retrospectively collected. The median age at diagnosis was 48 (range 18-79) years and 63% were female. The median time from vaccination to the onset of liver injury was 15 (range 3-65) d. Liver injury occurred after vaccination with BNT162b2 in 59% of the cases, mRNA-1273 in 18% and ChAdOx1 nCoV-19 in 23%. When elevated IgG and autoantibody positivity were used to define immune-mediated hepatitis, 57% of the patients had immune-mediated hepatitis. Corticosteroids were mainly used in cases of severe liver injury and immune-mediated hepatitis (53%). In this study, there was one case of liver failure requiring LT, while the remaining cases had a good prognosis. There were no differences in the severity of liver injury, the rate of immune-mediated hepatitis, or the rate of steroid usage depending on the type of vaccine. Responses to treatment and outcomes were favorable in all groups. These results were generally consistent with the characteristics of the previous case reports shown in Table 3.

There is also a concern that hepatitis might be exacerbated by vaccination of patients originally diagnosed with AIH. Shroff *et al*[112] reported that six of 16 patients who developed vaccine-induced liver injury previously had AIH. They concluded that hepatotoxicity could be induced after vaccination by autoimmune induction[112]. However, the number of patients described in previous reports[87-109] (Table 3) is extremely small compared with the overall vaccinated population worldwide. The current review, thus, aimed to increase awareness about this rare adverse effect in order to promote its early recognition.

Mechanism of liver injury after COVID-19 vaccination

The mechanisms underlying the development of liver injury following COVID-19 vaccination remain unclear. As shown in Table 3, liver injury following COVID-19 vaccination is clinically and pathologically similar to AIH, suggesting that immune abnormalities associated with vaccination contribute to

Table 3 Reported cases of liver injury following coronavirus disease 2019 vaccination

Ref.	Age/sex	Past history	Vaccine	Onset	AST/ALT (U/L)	Total bilirubin (mg/ dL)	IgG	Antibody	Biopsy	Diagnose	Treatment	Outcome
Bril *et al*[87]	35/F	Third month postpartum	BNT162b2	7 d after the1st dose	754/2001	4.8	Normal	ANA: 1:1280	Interface hepatitis, rosette formation, eosinophil infiltration	Typical for AIH	Prednisone (20 mg/d)	Improved
Lodato *et al*[88]	43/F	Dyslipidemia	BNT162b2	15 d after the 1st dose	52/51	17.54	Normal	ANA: negative	Moderate portal inflammatory infiltrate with interface hepatitis, biliary injury	Compatible with AIH	Methyl-prednisolone (1 mg/kg/d)	Improved
Vuille-Lessard *et al*[89]	76/F	Hashimoto's disease, urothelial carcinoma	mRNA-1273	3 d after the 1st dose	811/579	3.8	Increased	ANA: 1:1280, AMA: 1:1280	Interface hepatitis, plasma cells infiltration, pseudorosettes	Compatible with AIH	Prednisolone (40 mg/d) + azathioprine	Improved
Londoño *et al*[90]	41/F	Premature ovarian failure	mRNA-1273	7 d after the 2nd dose	993/1312	2.3	Increased	ANA: 1:80	Interface hepatitis with a lymphoplasmacytic infiltrate	Typical for AIH	Prednisone (1 mg/kg)	Improved
Rocco *et al*[91]	80/F	Hashimoto's disease	BNT162b2	7 d after the 3rd dose	1401/1186	10.5	Increased	ANA: 1:160	Interface hepatitis with a lymphoplasmacytic infiltrate	Typical for AIH	Prednisone	Improved
McShane *et al*[92]	71/F	Osteoarthritis	mRNA-1273	4 d after the 1st dose	-/1067	15.7	Increased	ASMA: 1:2560	Interface hepatitis, eosinophil infiltration	Compatible with AIH	Prednisolone (40 mg/d)	Improved
Clayton-Chubb *et al*[93]	36/M	Hypertension	AZD1222	26 d after the 1st dose	633/1774	9.9	Normal	ANA: 1:160	Interface hepatitis	Compatible with AIH	Prednisolone (60 mg/d)	Improved
Tan *et al*[94]	56/F	None	mRNA-1273	35 d after the 1st dose	1124/1701	5.9	Increased	ANA: positive, ASMA: Positive	Interface hepatitis, rosette formation, eosinophil infiltration	Compatible with AIH	Budesonide	Improved
Ghielmetti *et al*[95]	63/M	Type 2 diabetes, ischemic heart disease	mRNA-1273	7 d after the 1st dose	1127/1038	11.9	Increased	ANA: 1:640	Inflammatory portal infiltrate with interface hepatitis	Typical for AIH	Prednisone (40 mg/d)	Improved
Zhou *et al*[96]	36/F	Ulcerative colitis, primary sclerosing cholangitis	mRNA-1273	11 d after the 1st dose	581/588	1.4	Increased	ANA: 1:2560	Interface hepatitis with a lymphoplasmacytic infiltrate, rosette, eosinophil	Compatible with AIH	Prednisone (50 mg/d)	Improved
Garrido *et al*[97]	65/F	JAK2 V617F-positive polycythemia	mRNA-1273	14 d after the 1st dose	1056/1092	1.1	Increased	ANA: 1:100	Interface hepatitis	Compatible with AIH	Prednisolone (60 mg/d)	Improved
Goulas *et al*[98]	52/F	None	mRNA-1273	14 d after the 1st dose	350/936	9.06	Increased	ANA: 1:320, ASMA: positive	Portal, periportal inflammation, rosette formation	Typical for AIH	Prednisolone (50 mg/d) + azathioprine	Improved
Rela *et al*[99]	38/F	Hypothyroidism	AZD1222	20 d after the 1st dose	1101/1025	14.9	Increased	ANA: positive	Multiacnar hepatic necrosis and periportal neocholangiolar proliferation	Compatible with AIH	Prednisolone (30 mg/d)	Improved
Rela *et al*[99]	62/M	None	AZD1222	13 d after the 2nd dose	1361/1094	19.2	NA	ANA: negative	Portal neocholangiolar proliferation and mild to moderate	Compatible with AIH	Prednisolone (30 mg/d) + plasma	Death

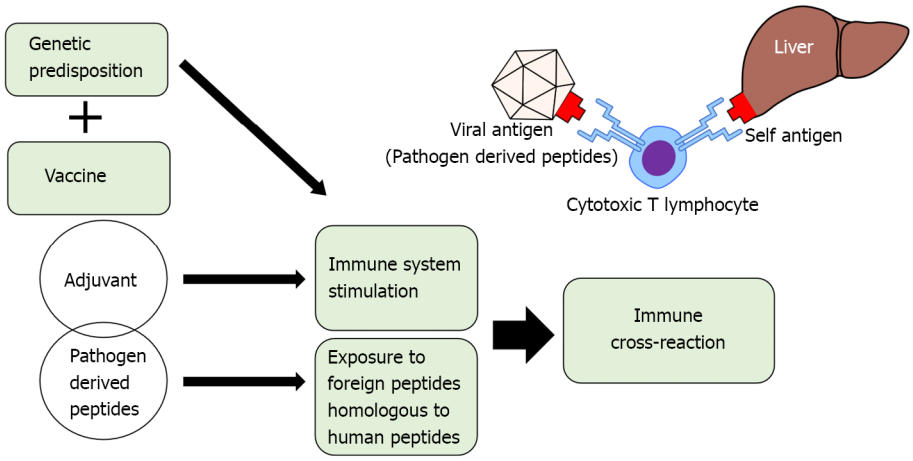
								inflammation		exchange	
Palla *et al*[100]	40/F	Sarcoidosis	BNT162b2	28 d after the 2nd dose	4 times upper limit of normal	NA	Increased	ANA: 1:640	Interface hepatitis with plasma cells infiltration	Compatible with AIH	Prednisolone (40 mg/d)	Improved
Mann *et al* [101]	61/F	Irritable bowel disease, cholecystectomy	BNT162b2	9 d after the 2nd dose	37/37	6.2	NA	ANA: negative	Scattered inflammatory cells consisting of lymphocyte and few eosinophils	Drug induced liver injury	Conservative treatment	Improved
Avci *et al*[102]	61/F	Hashimoto's disease, hypertension	BNT162b2	28 d after the 1st dose	455/913	11.8	Increased	ANA: 1:100, ASMA: 1:100	Interface hepatitis	Compatible with AIH	Prednisolone (40 mg/d) + azathioprine	Improved
Torrente *et al* [103]	46/F	Hypothyroidism, anemia	AZD1222	21 d after the 1st dose	241/353	Normal	Increased	ANA: 1:160	Lymphoplasmacytic portal infiltrate	Compatible with AIH	Prednisone (30 mg/d) + azathioprine	Improved
Ghorbani *et al* [104]	62/M	None	Corona Vac	3 d after the 2nd dose	722/435	8	NA	ANA: negative, ASMA: negative	Interface hepatitis, infiltration of lymphocytes and eosinophils in portal tract	Compatible with AIH	Ursodeoxycholic acid	Improved
Kang *et al*[105]	27/F	None	BNT162b2	7 d after the 2nd dose	1004/1478	8.6	Increased	ANA: 1:80	Interface hepatitis with a lymphoplasmacytic infiltrate, rosette, eosinophil	Typical for AIH	Prednisolone (40 mg/d)	Improved
Camacho-Domínguez *et al*[106]	79/M	None	AZD1222	15 d after the 1st dose	2003/1994	11.9	Increased	ANA: 1:80	Interface hepatitis with a lymphocytic infiltrate, eosinophil	Compatible with AIH	Prednisone (30 mg/d) + azathioprine	Improved
Shahrani *et al* [107]	59/F	Dyslipidemia	AZD1222	12 d after the 2nd dose	962/1178	7.5	Increased	NA	Lympho-plasmacellular portal infiltrate	Compatible with AIH	Prednisolone (40 mg/d)	Improved
Shahrani *et al* [107]	63/F	Ulcerative colitis, primary sclerosing cholangitis	AZD1222	14 d after the 1st dose	505/354	18.6	Increased	ANA: positive	Interface hepatitis	Compatible with AIH	Prednisolone (40 mg/d)	Improved
Shahrani *et al* [107]	72/F	None	BNT162b2	10 d after boostershot	1452/2280	1.7	Increased	ANA: negative, AMA: positive	Infiltration of lymphocytes and plasma cells in portal tract	Compatible with AIH	Prednisolone (40 mg/d)	Improved
Zin Tun *et al* [108]	47/M	None	mRNA-1273	3 d after the 1st dose; A few days after the 2nd dose	NA/1048	11.3	Increased	ANA: positive	Interface hepatitis with a lymphoplasmatic infiltrate, rosette, emperipolesis	Typical for AIH	Prednisolone (40 mg/d)	Improved
Suzuki *et al* [109]	80/F	Gastroesophageal reflux esophagitis	BNT162b2	10 d after the 2nd dose	995/974	3.5	Increased	ANA: 1:40	Lymphoplasmacytic infiltration in the portal area, interface hepatitis	Compatible with AIH	Prednisone (0.8 mg/kg/d)	Improved
Suzuki *et al* [109]	75/F	Dyslipidemia	BNT162b2	4 d after the 2nd dose	1085/820	17.7	Increased	ANA: 1:80	Lymphoplasmacytic infiltration in the portal area, interface hepatitis	Compatible with AIH	Prednisone (1.0 mg/kg/d)	Improved
Suzuki *et al* [109]	78/F	Primary biliary cholangitis	BNT162b2	7 d after the 1st dose	401/542	1.3	Increased	ANA: 1:80	Lymphoplasmacytic infiltration in the portal area, interface	Compatible with AIH	Prednisone (0.6 mg/kg/d)	Improved

	hepatitis

AIH: Autoimmune hepatitis; AMA: Anti-mitochondrial antibodies; ANA: Anti-nuclear antibodies; ASMA: Anti-smooth muscle antibodies; JAK: Janus kinase; NA: Not available; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; IgG: Immunoglobulin G.

its development. However, the association between vaccines and the development of autoimmune diseases is controversial and most studies related to this have been inconclusive[113].

Molecular mimicry and bystander activation have been hypothesized as possible mechanisms by which vaccines can trigger autoimmune reactions. In the antigen-specific mechanism of molecular mimicry, it is hypothesized that similarities between certain pathogenic elements contained in the vaccine and specific human proteins cause cross-reactions. It is believed that the injurious antibodies produced by this mechanism destroy human proteins and cause organ damage[114]. In support of this hypothesis, it has been reported that antibodies against the spike protein of SARS-CoV-2 have cross-reactivity against many human tissue antigens[115]. Although the target antigen of the autoimmune response in hepatocytes and specific autoantibodies in AIH have not yet been identified, Vojdani *et al* [115] reported that anti-SARS-CoV-2 protein antibodies cross-react with liver microsomal antigen, pyruvate dehydrogenase peptide E2, and mitochondrial M2 antigen[115]. It has also been shown that anti-SARS-CoV-2 spike protein antibodies cross-react with human tissue antigens, resulting in a marked increase in autoimmune markers such as ANA and AMA[116]. This suggests that COVID-19 vaccination might induce autoimmune reactions based on molecular mimicry in liver tissues, resulting in AIH-like liver injury (Figure 1). Bystander activation, on the other hand, is an antigen non-specific mechanism, in which self-antigens are released extracellularly by vaccination and are taken up by antigen-presenting cells. Then, autoreactive T cells are activated by type I interferon (IFN) and recognize the presented self-antigen, which is hypothesized to attack normal cells[117]. Bystander activation has also been proposed as one of the mechanisms of autoimmune disease development after vaccination. Furthermore, there is a rapid increase in type I IFN expression and oxidative stress coupled with effective anti-SARS-CoV-2 neutralizing antibody production after vaccination in healthy individuals[118]. Therefore, the side effects of COVID-19 vaccines are thought to be only a by-product of a transient burst of type I IFN generation with induction of an effective immune response[119]. It has also recently been hypothesized that the COVID-19 vaccine triggers autoimmune diseases *via* induction of age-associated B cells (ABCs) [120]. The number of ABCs, a rare subset of B cells that express CD11c and T-bet, increases with age in healthy individuals, and is increased early in patients with infectious diseases and autoimmune disorders[121]. In the pathogenesis of autoimmune diseases, ABCs are implicated in generating IgG, in increasing antigen presentation to T cells, and in germinal center formation. Moreover, ABCs are hyper-responsive to Toll-like receptor (TLR) 7 signaling, and are capable of generating autoreactive antibody-secreting plasmablasts. COVID-19 vaccines use TLR7/8 agonists as adjuvants, which might stimulate ABCs, leading to the triggering of post-vaccination autoimmune syndromes[122]. Activation of TLR7 can lead to the production of type I IFN, which is an important cytokine in the development of autoimmune disorders, such as rheumatic diseases and systemic lupus erythematosus[123]. It was previously shown in a mouse model that lipid nanoparticles, which are one of the potent adjuvants of mRNA vaccines, could trigger inflammatory responses. This is characterized by activation of diverse inflammatory pathways, massive neutrophil infiltration, and production of various inflammatory



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Figure 1 Schema of the process leading to the development of immune cross-reaction after vaccination.

cytokines, including the secretion of interleukin (IL)-1β and IL-6[11,124].

Hence, although several hypotheses have been considered for the mechanism of vaccine-induced autoimmune disease, the exact mechanism remains to be elucidated. In any event, only a very small percentage of vaccinated subjects subsequently developed autoimmune phenomena, suggesting a genetic predisposition to vaccine-induced autoimmune disorders. Further research into the direct associations between vaccines and autoimmune diseases, as well as the biological mechanisms behind them, is warranted.

RECOMMENDATION

Since COVID-19 is an infectious disease with a high burden of morbidity and mortality, and that has resulted in a global pandemic, vaccination against COVID-19 is our best strategy for its control. For this, highly effective and safe vaccines are desperately needed. Although various COVID-19 vaccines have been proven to be highly effective and have good safety profiles in healthy populations, data regarding the efficacy and safety of vaccination in special population groups is limited. Thus, we believe it is worthwhile to summarize the efficacy and safety of the COVID-19 vaccines in patients with CLD. Based on the evidence from real-world studies, this review shows that vaccination in patients with CLD is effective and safe, and should be strongly recommended.

As shown in Table 3, although a number of case reports of acute liver injury after COVID-19 vaccination have been reported, their frequency is extremely low. Thus, given the serious health sequalae from COVID-19 in patients with liver disease, the potential benefits of vaccination appear to outweigh the risk of vaccine-related liver injury. However, it is important to remember that most of the studies referred to in this review were conducted in the era before the emergence of new viral variants. Since new SARS-CoV-2 variants are still emerging all over the world, COVID-19 remains a global public health problem. In addition, since vaccines against the mutant viruses are still being developed, it will be necessary to continue evaluating the efficacy and safety of these new vaccines.

CONCLUSION

Given the increased rates of severity and mortality of COVID-19 in patients with CLD, especially those with cirrhosis, the importance of aggressive vaccination in the effective management of SARS-CoV-2 infection should be emphasized. However, there is insufficient evidence about the immunogenicity and safety of COVID-19 vaccines in patients with CLD. According to the accumulated real-world data on each vaccine, the safety of COVID-19 vaccines in patients with CLD appears to be comparable to that in healthy individuals. Regarding efficacy, the disease behavior of COVID-19 is known to be attenuated in vaccinated compared with unvaccinated patients, including in those with CLD+ADs- however, vaccine-induced immunity seems lower in CLD patients as compared with the general population. Since a rapid decrease in acquired antibodies has also been observed in this patient population, an effective booster shot is desirable, particularly in patients with cirrhosis, LT recipients, and those with HCC.

On the other hand, acute liver injury following COVID-19 vaccination has also been frequently reported. However, recent large cohort studies found no increased risk of liver injury after COVID-19 vaccines. Since acute liver injury after SARS-CoV-2 infection is much more common than after COVID-

19 vaccination, the benefits of vaccination might outweigh the risk of liver injury during this pandemic. The reported rare immune-mediated liver injury after COVID-19 vaccination is clinically and pathologically similar to AIH. Although the involvement of abnormalities in the immune system, including molecular mimicry, bystander activation, and induction of ABCs in the pathogenesis of this condition have been pointed out, the relationship between vaccination and acute liver injury is an issue that remains to be clarified in the future. Finally, clinicians should consider the possibility of AIH-like liver injury in patients who present with elevated liver enzymes following COVID-19 vaccination, and treat it with corticosteroids.

FOOTNOTES

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REVIEW

Mechanism and potential treatments for gastrointestinal dysfunction in patients with COVID-19

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Abstract

The global coronavirus disease 2019 (COVID-19) has become one of the biggest threats to the world since 2019. The respiratory and gastrointestinal tracts are the main targets for severe acute respiratory syndrome coronavirus 2 infection for they highly express angiotensin-converting enzyme-2 and transmembrane protease serine 2. In patients suffering from COVID-19, gastrointestinal symptoms have ranged from 12% to 61%. Anorexia, nausea and/or vomiting, diarrhea, and abdominal pain are considered to be the main gastrointestinal symptoms of COVID-19. It has been reported that the direct damage of intestinal mucosal epithelial cells, malnutrition, and intestinal flora disorders are involved in COVID-19. However, the underlying mechanisms remain unclear. Thus, in this study, we reviewed and discussed the correlated mechanisms that cause

gastrointestinal symptoms in order to help to develop the treatment strategy and build an appropriate guideline for medical workers.

Key Words: COVID-19; Angiotensin-converting enzyme-2; Transmembrane protease serine 2; Gastrointestinal symptom; Mechanism; Intestinal barrier permeability

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Core Tip: Gastrointestinal symptoms in coronavirus disease 2019 patients have ranged from 12% to 61%, which include anorexia, nausea and/or vomiting, diarrhea, abdominal pain, and so on. However, the underlying mechanisms remain unclear. This study reviewed and discussed the correlated mechanisms that cause gastrointestinal symptoms in order to help to develop the treatment strategy and build an appropriate guideline for medical workers.

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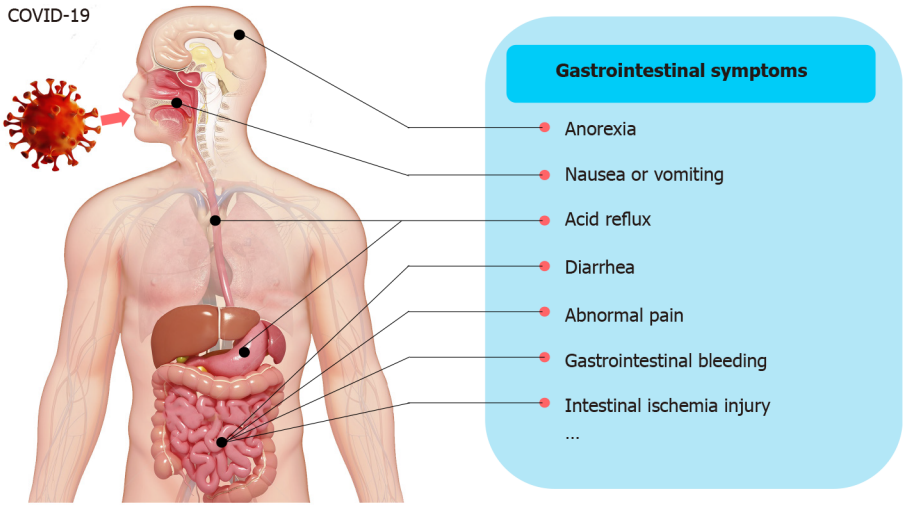
INTRODUCTION

Since 2019, coronavirus disease 2019 (COVID-19) has become one of the world's most serious threats. In the past, much attention has been given to the respiratory symptoms of patients, but the occurrence of extrapulmonary symptoms has been ignored. The occurrence of gastrointestinal symptoms has ranged from 12% to 61% in patients suffering from COVID-19[1-5], which may result in a longer duration of illness but not increased mortality[2,3]. In a recent meta-analysis from China, the main gastrointestinal symptoms in COVID-19 patients were reported to include anorexia (21%), nausea and/or vomiting (7%), diarrhea (9%), and abdominal pain (3%)[2]. Moreover, gastrointestinal bleeding was rarely observed[6]. A study from the United States reported a higher prevalence of gastrointestinal symptoms (anorexia, 34.8%; diarrhea, 33.7%; and nausea, 26.4%)[4] (Figure 1). Thus, diarrhea, nausea and/or vomiting, abdominal pain, and anorexia are considered to be the main gastrointestinal symptoms.

Currently, after struggling with the Omicron variant, China has found that a large number of COVID-19 patients, especially elderly patients in critical condition, are more likely to suffer from gastrointestinal dysfunction. Over 85% of patients showed symptoms such as intestinal barrier dysfunction, digestive and absorption dysfunction, or gastrointestinal motility dysfunction due to the direct damage the virus caused to the intestinal mucosal epithelial cells. In addition, malnutrition and intestinal flora disorders occurred next. Imaging studies show that COVID-19 patients with gastrointestinal symptoms presented thickening of the bowel wall, sometimes with hyperemia and thickening of the mesentery, and large bowel fluid[7]. However, the underlying mechanisms remain unclear. Thus, in this study, we reviewed and discussed the correlated mechanisms of gastrointestinal symptoms and damage in order to help build an appropriate guideline for medical workers.

PATHOPHYSIOLOGY

The pathophysiology of gastrointestinal damage in COVID-19 is probably multifactorial. The most important factor is due to the direct infection of the virus. High titers of viral RNA from COVID-19 have been isolated from fecal samples[8,9]. Live viral shedding of infectious virions in fecal matter has been reported even after the resolution of symptoms, which may be a potential source of transmission[10]. Angiotensin-converting enzyme-2 (ACE2), as the entry receptor for the causative coronavirus of COVID-19, is expressed in multiple extrapulmonary tissues, including gastrointestinal tissue[11]. A study based on single-cell sequencing also showed that ACE2 and transmembrane protease serine 2 (TMPRS2) are expressed in cholangiocytes, colonocytes, esophageal keratinocytes, gastrointestinal epithelial cells, and so on[12-14]. The expression profile of ACE2 in the digestive system is shown in Figure 2. Histopathological studies also indicated that gastrointestinal tissue is the target organ of COVID-19[15]. This finding indicates that direct viral-induced tissue damage is a plausible mechanism of COVID-19 injury. Here, however, we hold the idea that the expression of ACE2 is related to the virus entering the body, but the expression level of ACE2 does not appear to be directly proportional to the severity of the disease[16].



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Figure 1 Gastrointestinal symptoms involved in coronavirus disease 2019 infection. COVID-19: Coronavirus disease 2019.

In addition, responses following ACE2 activation are closely related to gastrointestinal side effects. ACE2 is also a key enzyme in the renin-angiotensin system (RAS)[17]. RAS dysregulation may exacerbate ion imbalance and inflammation, potentially affecting cellular metabolic status, microbial composition, and cell viability, leading to progressive bowel function and diarrhea[18]. Histopathological evidence also shows diffuse endothelial inflammation and mesenteric ischemia in the submucosal vessels of the small intestine in patients with COVID-19[19]. Furthermore, infiltrating plasma cells, lymphocytes, and interstitial edema have been found in the lamina propria of COVID-19 patients' stomachs, duodenums and rectums. Virus-induced cytokine storms, as well as inflammatory responses, may also contribute to enhanced permeability of the mucosal barrier, damaged enteric nervous system, altered intestinal flora[20], and gut-brain axis communication disorders and then form a vicious circle (Figure 3). The syndrome-correlated underlying mechanism is discussed in the following sections.

METHODOLOGY

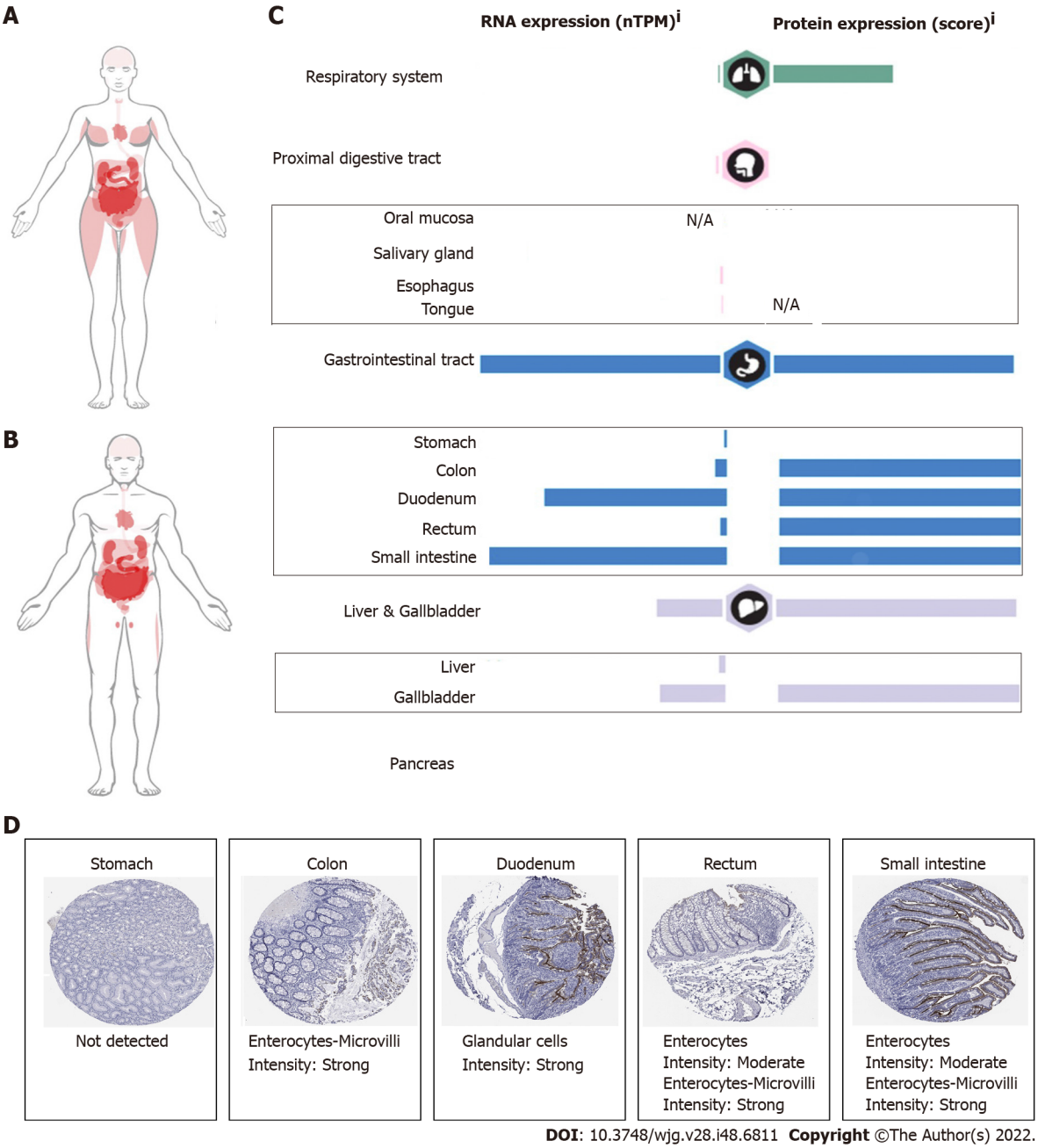
A literature review was conducted using a keyword search in PubMed from 2019 to 2022. Keyword search items included "COVID-19", "severe acute respiratory syndrome coronavirus 2", "gastrointestinal disorders", "nausea", "vomiting", "diarrhea", "ACE2", "abdominal pain", "anorexia", and "combinations thereof". The inclusion criteria included articles with randomized or blinded studies, case-control studies, descriptive research, and studies with objective outcomes. Exclusion criteria included articles of primary opinion papers with no reference to available data and industry-sponsored publications.

GASTROINTESTINAL SYMPTOMS AND THE UNDERLYING MECHANISMS

Nausea and/or vomiting

Nausea and/or vomiting is an early alerting symptom of a challenge (toxic food and chemicals, bacterial toxin, and virus) to the upper digestive tract, which can also be the early presenting symptom in COVID-19 patients[21]. The reported incidences of vomiting and nausea were 1.0%-12.5% and 1.0%-27.5%, respectively[4,22]. Some of the patients may eject aerosolized, virally contaminated vomit, which also occurs in patients infected with norovirus[23]. Importantly, nausea and vomiting can be the early presenting symptoms of COVID-19[21].

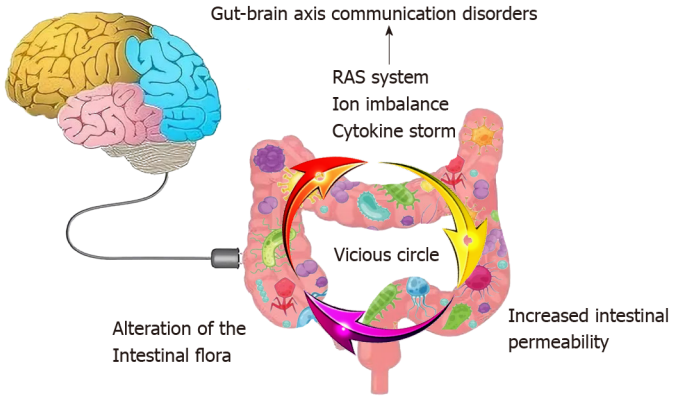
The virus enters the digestive tract with the air during swallowing and binds to ACE2 receptors, which are highly expressed in the airways and digestive tract. COVID-19 could increase the release of neuroactive agents from enteroendocrine cells and inflammatory mediations, which act by stimulating/sensitizing abdominal vagal afferent terminals and/or act on the area postrema in the dorsal medulla where the blood-brain and blood-cerebrospinal fluid barriers are relatively permeable [21]. The consequences of vagal afferent and area postrema activation induce nausea and vomiting by the projection of information to higher brain regions (nausea and anorexia) and vomiting by motor pathways in the ventral brainstem and spinal cord[21]. Some researchers also indicated that the



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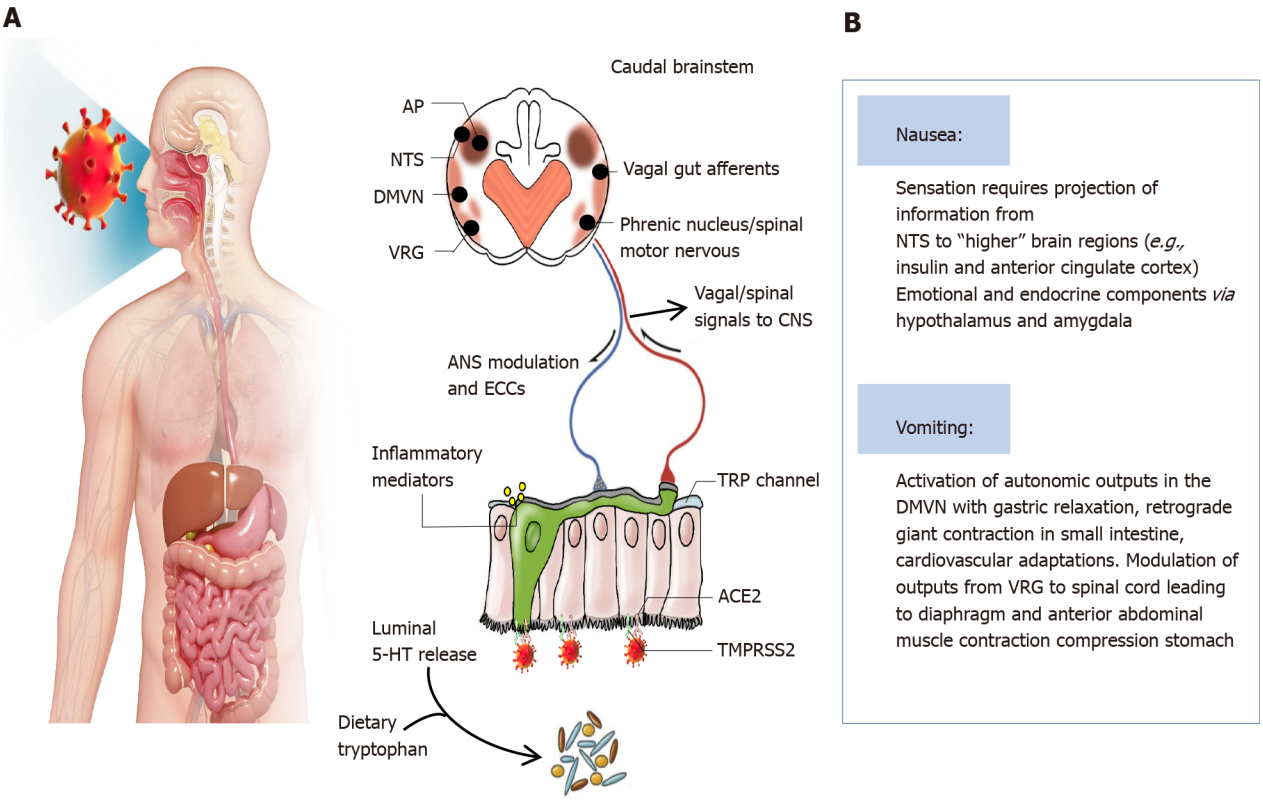
Figure 2 Angiotensin-converting enzyme 2 expression in human gastrointestinal tissues. A: Angiotensin-converting enzyme 2 (ACE) expression in females; B: ACE expression in males, darker colors indicate higher expression levels; C: ACE2 mRNA and protein expression in the respiratory system and digestive system; D: ACE2 positive cell types in the stomach, colon, duodenum, rectum, and small intestine. These data are summarized from the human protein atlas https://www.proteinatlas.org/. N/A: Not applicable.

interaction between transient receptor potential (TRP) channels and food intake might be associated with anorexia due to COVID-19[24,25]. Some TRP channels are broadly expressed in the gastrointestinal tract and play important roles in noxious irritants[26]. TRPV1 expression in esophageal sensory neurons, stomach-labeled vagal nodose neurons and colon-labeled afferent neurons has been well described[27-29]. A TRPV1 agonist, capsaicin, can evoke nausea[30]. Moreover, TRPV1 and TRPA1 are co-expressed in the esophagus, stomach, intestine, and colon[31-33]. TRPV1 can participate in appetite regulation by affecting hormones and gastrointestinal vagal afferent nerves[25]. *In vitro* activation of TRPA1 by allyl isothiocyanates can increase serotonin release, leading to the stimulation of vomiting [34]. These results suggest that TRP channel activation is involved in COVID-19-induced nausea and vomiting. However, whether inhibiting TRP channels can alleviate the gastrointestinal symptoms caused by COVID-19 needs further investigation. The underlying mechanisms are summarized in Figure 4.



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Figure 3 Main mechanisms involved in coronavirus disease 2019-induced gastrointestinal syndromes. Gut infection of coronavirus disease 2019 (COVID-19) results in cytokine storm, increased intestinal permeability and alteration of the intestinal flora, and forming a vicious circle, extending the recovery time. Moreover, COVID-19 binding to gastrointestinal angiotensin-converting enzyme 2 also leads to ion imbalance and activation of renin-angiotensin system. Abnormal enteric neurotransmitters may further lead to gut-brain axis communication disorders. RAS: Renin-angiotensin system.



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Figure 4 Potential mechanisms for coronavirus disease 2019-induced nausea and vomiting. A: The virus enters the body through the airways and digestive tract during swallowing and the potential mechanisms for coronavirus disease 2019 to induce nausea and vomiting (based on the evidence discussed in the text). The virus interacts with digestive tract epithelium leading to the release of neuroactive agents from enteroendocrine cells and inflammatory mediations, and these can act by stimulating or sensitizing abdominal vagal afferent terminals; B: The consequences of vagal afferent and area postrema activation induce nausea and vomiting by projection of information to nausea and anorexia-related regions, and vomiting by motor pathways in the ventral brainstem and spinal cord. 5-HT: 5-hydroxytryptamine; ACE2: Angiotensin-converting enzyme-2; AP: Area postrema; DMVN: Dorsal motor vagal nucleus; NTS: Nucleus tractus solitarius; TMPRSS2: Transmembrane protease serine 2; VRG: Ventral respiratory group of neurons; TRP: Transient receptor potential; ANS: Autonomic nervous system; ECCs: Enteroendocrine cells.

Diarrhea

Diarrhea is a frequent presenting symptom in patients suffering from COVID-19. In the clinical case analysis, the incidence of diarrhea is between 2% and 50%[35]. It may precede or trail respiratory symptoms. Although the specific mechanisms involved in COVID-19-related diarrhea are not entirely known, we hold the idea that the targeting of intestinal ACE2 by the virus, virus infection-induced

cytokine storms, increased intestinal barrier permeability, and even changes in microbiome are all considered to be the main causes of gastrointestinal symptoms. Moreover, hepatic and pancreatic injuries may also cause diarrhea. Antibiotic-induced iatrogenic diarrhea caused by the activation of *Clostridium spp.* should also be taken into consideration.

The direct effect of binding ACE2: ACE2 mRNA appears to increase with age and to display higher levels in patients taking ACE inhibitors. This may be one of the causes of gastrointestinal symptoms such as diarrhea being more common in elderly patients or those with hypertension. ACE2 controls the production of antimicrobial peptides and participates in the uptake of dietary amino acids[36], which promote the homeostasis of the gut flora. Additionally, ACE2 expression is positively correlated with the severity of colitis, suggesting that virus activity may lead to changes in the way certain enzymes function, making people more susceptible to developing diarrhea and intestinal inflammation[37].

Altered serotonin metabolism: Altered serotonin metabolism has been found in COVID-19 patients [38]. Serotonin, known as the mood neurotransmitter, is important in certain bodily processes, such as sleep, libido, and body temperature. Studies have reported that enterohemorrhagic *Escherichia coli* O157 can significantly reduce the expression of a group of genes that cause infection after exposure to serotonin[39,40]. In addition, a study in mice showed that increased serotonin levels in the gastrointestinal tract could reduce the ability of murine *Citrobacter* to infect the host and cause disease. Intervention with fluoxetine, a selective serotonin reuptake inhibitor, produced similar results[40]. Activation of intestinal serotonin receptors may also lead to diarrhea by modulating the enteric nervous system and intestinal motility[41,42]. The authors believe that COVID-19-related diarrhea is correlated with increased serotonin levels and that elevated serotonin levels may be a protective regulatory mechanism that accelerates the excretion of enteroviruses.

Changes in fecal calprotectin: Increased levels of fecal calprotectin expression have also been detected in COVID-19 patients[43,44]. Fecal calprotectin is a reliable fecal marker for the detection of inflammatory bowel disease and infectious colitis[45]. The calprotectin value in stool is elevated in patients with acute or bloody diarrhea[46]. Calprotectin is a calcium-containing protein derived from neutrophils and macrophages that serves as a marker of inflammatory cell activation[47]. Therefore, diarrhea and increased fecal calprotectin levels could be related to the immune activation and inflammatory responses caused by COVID-19.

Cytokine storms and their induced inflammatory cascade: Cytokine release syndrome caused by a dysregulated immune response is also one of the important factors causing multiple organ dysfunction, especially diarrhea, in patients with COVID-19. Severe COVID-19 manifests as acute respiratory distress syndrome (ARDS) with elevated plasma proinflammatory cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor α (TNF-α), C-X-C motif chemokine ligand 10, macrophage inflammatory protein 1 α, and chemokine (C-C motif) ligand 2, with low levels of interferon type I (IFN-I) in the early stage and elevated levels of IFN-I during the advanced stage of COVID-19[48]. Changes in these proinflammatory cytokines can also be found in clinical and experimental colitis[49,50], accompanied by increased intestinal permeability *via* activation of the inflammatory-related cascade. High levels of circulating cytokines and mediators of the toxic response, including IL-6, TNF-α, nitric oxide, and activity modulation of the calcium channel, have been described[51]. Toll-like receptors (TLRs) are important sensors that interact with COVID-19. Previous studies indicated that COVID-19 interacts with TLRs in the host cell membrane and increases gene 88 of the primary response to myeloid differentiation (MyD88), following active nuclear transcription factor-κB (NF-κB), promoting an inflammatory cascade [52], which in turn aggravates the inflammatory response and increases intestinal permeability. Additionally, severe COVID-19 individuals have been found to have significant levels of indicators for tight junction permeability as well as the translocation of bacterial and fungal products into the blood [53,54]. Thus, virus infection-induced cytokine storms and their induced inflammatory response may be other factors that cause diarrhea.

Increased intestinal barrier permeability and microbiome change: Accumulating evidence shows that the intestinal microbiome is broadly altered in COVID-19 patients, which may be followed by increased intestinal permeability. The incidence of sepsis and ARDS, two high-mortality risks in COVID-19, may be minimized by the intestinal microbiota[55]. Intestinal ACE2 functions as a chaperone for the amino acid transporter B0AT1. The B0AT1/ACE2 complex within the intestinal epithelium acts as a regulator of gut microbiota composition and function[56], which can also be considered a marker of inflammation and disease severity[57]. Changes in ACE2 by COVID-19 can impair intestinal uptake of certain dietary amino acids, such as tryptophan, which is involved in enteritis[58-60]. ACE2 knockout mice also exhibit microbiome dysbiosis[61-63]. Through shotgun sequencing of total DNA extracted from stool, researchers found that the gut microbial ecological network was markedly weakened and became sparse, which combined with a decrease in gut microbiome diversity[64], could reflect the disease's severity[65]. The infection causes intestinal microbiome disturbance and reduction, which may activate immune cells and provoke the release of inflammatory cytokines that increase systemic inflammation [66,67]. In addition, probiotics may help enhance the host immune system, improve the gut microbiome

and gut barrier function, and reduce COVID-19-related diarrhea[68].

More significantly, B0AT1 substrates such as tryptophan and glutamine operate as signals to reduce lymphoid proinflammatory cytokines, activate antimicrobial release peptides, and control mucosal autophagy as a defensive mechanism[69]. Downregulated intestinal ACE2-B0AT1 on the cellular surface leads to a series of downstream sequelae to promote leaky gut and dysbiosis of gut flora[69]. Gut microbiomes also play important roles in gut inflammatory regulation. Butyric acid from gut flora was reported to inhibit cytokine storms[27]. This indicates the disrupted composition of intestinal microbiota and impaired gut permeability, followed by the creation of a destructive cycle. In summary, we propose the important role of intestinal microbiota in preventing and decreasing COVID-19 complications. The underlying mechanism involved in COVID-19-correlated diarrhea is summarized in Figure 5.

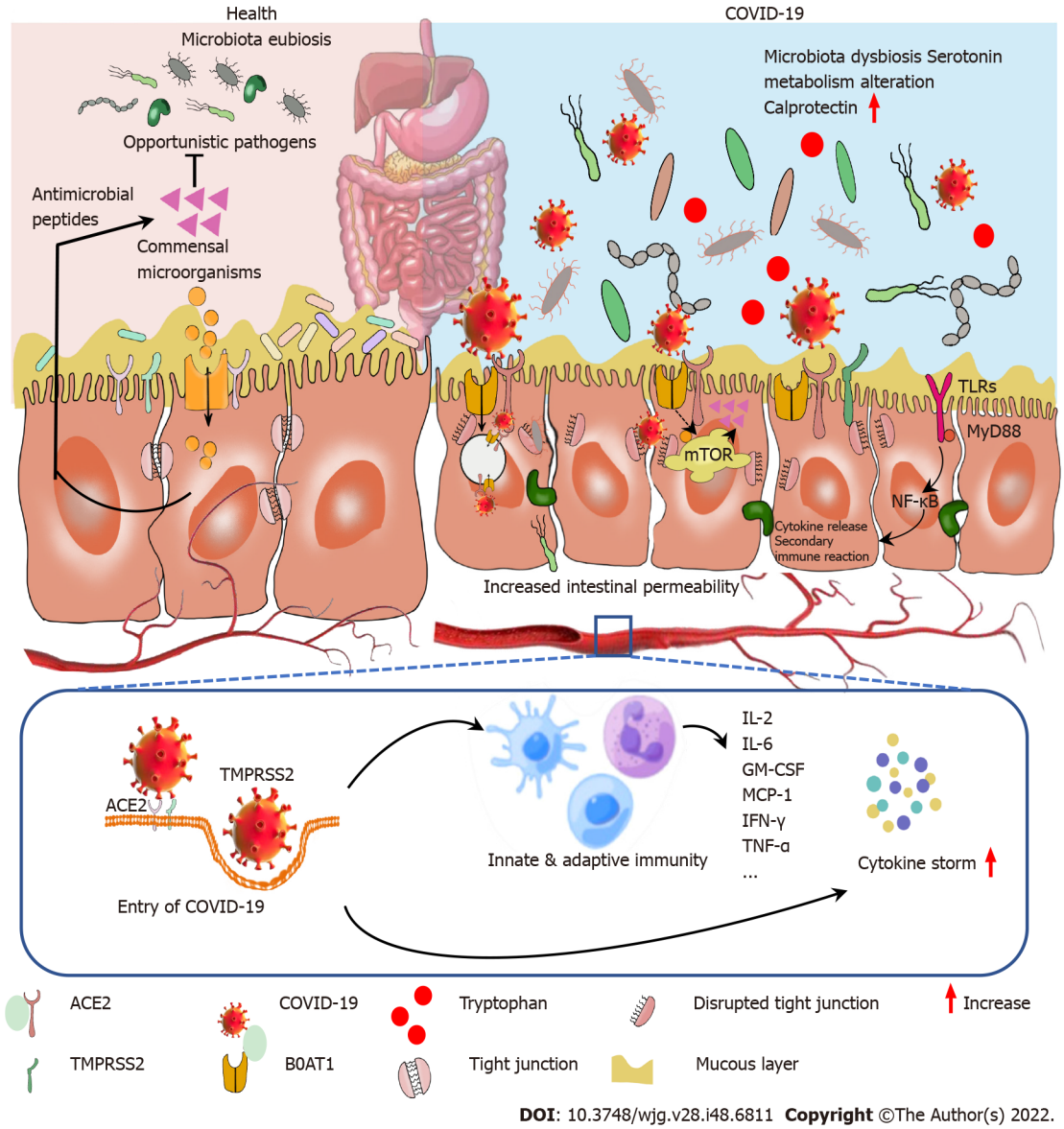
Abdominal pain

Abdominal pain is uncommon and extremely rare in patients with COVID-19. Clinical data indicate that abdominal pain is more common in intensive care unit (ICU) patients than in non-ICU patients[41]. The aggregative presence rate of RNA from COVID-19 in stool samples from COVID-19 patients was 54% [2]. In previous clinical research based on COVID-19 patients who underwent emergency abdominal surgery due to different conditions, the peritoneal samples from 5 patients were sufficient for reverse transcription polymerase chain reaction analysis, and no intraperitoneal viral RNA was observed in these 5 patients[70]. Although the number of cases is rare, we can speculate that abdominal pain is linked to gastrointestinal but not intraperitoneal viral infection. The possible mechanisms are summarized as follows.

Immune and inflammatory regulation: The most common cause of pain is the inflammation-induced release of many cytokines and chemokines. Cytokine storms are a potentially fatal immune disease characterized by high levels of activation of immune cells and excessive production of a large number of inflammatory cytokines and chemical mediators, and they have been reported to be associated with the exacerbation of a number of infectious diseases, including severe acute respiratory syndrome[71] and Middle East respiratory syndrome. They are also considered to be the main cause of disease severity and death in patients with COVID-19[72,73]. T cells play an important role in antiviral immunity. A rise in T cell activation and differentiation was found in early COVID-19-infected patients, resulting in immune rebalancing between IFN and NF-κB activity and restoration of cell homeostasis. Two major intracellular transduction antigen-activating signals, the phosphatidylinositol pathway and the MAP kinase-related pathway, are activated. However, the number of T cells is significantly decreased with increasing infective time in COVID-19 patients, accompanied by an increase in the T cell exhaustion marker programmed death 1[74]. The T cell count is negatively correlated with serum cytokine levels in patients with COVID-19[74]. Despite the lack of direct evidence for a relationship between T cell status and abdominal pain in COVID-19 patients, we hypothesized that abdominal pain is related to T cells. Moreover, COVID-19 can rapidly activate pathogenic Th1 cells to secrete proinflammatory cytokines, such as granulocyte-macrophage colony-stimulating factor and IL-6. Increased cytokines, chemokines, and other compounds can simultaneously cause a secondary pain response by activating pain-sensing neurons[75]. The COVID-19 cytokine storm is characterized by high expression of IL-6 and TNF-α[76]. Elevated IL-6 levels also increase mortality. Therefore, we hypothesized that the abdominal pain of COVID-19 patients may be associated with the high expression of IL-6 and TNF-α.

Eosinophils are circulating and tissue-resident white blood cells that have a powerful proinflammatory effect in many diseases. Recently, eosinophils have been shown to have a variety of other functions, including immunomodulatory and antiviral activity. Peripheral eosinophilia is hypothesized to play a protective role in COVID-19 patients[77]. In the United States, a review of administrative data compared the hospitalization rates, ventilator dependence, and death of patients with and without eosinophilic gastrointestinal disorders from an extensive central medical system. The results indicated that eosinophilic gastrointestinal disorder might provide a protective immune response[78]. The mechanism may be related to the upregulation of IL-13 in eosinophilic gastrointestinal disorder and the decreased expression of ACE2 and TMPRS2 on epithelial cells of eosinophilic gastrointestinal disorder patients. In addition, eosinophil-derived neurotoxins may exert direct antiviral effects[79]. Moreover, eosinophilia-related disorders such as eosinophilic gastroenteritis[80] and eosinophilic esophagitis[81] are often accompanied by symptoms of abdominal pain and vomiting, suggesting that abdominal pain in patients with COVID-19 may also be caused by increased eosinophils.

Regulation of the enteric and central nervous systems: Intestinal pain perception can be coregulated by the central and enteric nervous systems. A balance between neuronal excitatory and inhibitory signaling pathways maintains the physiological pain threshold in the intestine. Altered neurotransmitter levels may be closely linked to abdominal pain. A variety of receptors and their endogenous ligands are involved in pain signaling, including receptors responsible for nociceptive sensations [*e.g.*, 5-hydroxytryptamine (5-HT) receptors, TRP channels, IL receptors] and antinociceptive sensations (*e.g.*, opioid and cannabinoid receptors). In COVID-19 patients, intestinal inflammatory infiltration increases intestinal mucosal permeability, and the direct effect of viruses can aggravate dysbiosis and cause changes in tryptophan metabolism. Studies have shown that tryptophan is a precursor of 5-HT. 5-HT plays an



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Figure 5 Potential mechanism of cytokine storm and secondary pathogen infection resulting in diarrhea in patients with coronavirus disease 2019. After coronavirus disease 2019 (COVID-19) virus entry into the body, innate and adaptive immunity have been activated, followed by a cytokine storm. Gut microbiota is also disrupted by COVID-19 infection which potentially triggers cytokine storm and secondary pathogen infections. B0AT known as an angiotensin-converting enzyme-2 chaperone, mediates neutral amino acid uptake by luminal epithelial cells. B0AT substrates (tryptophan and glutamine) activate antimicrobial peptide release and promote tight junction formation, inhibit cytokine release and promote mucosal cell autophagy *via* mechanistic target of rapamycin signaling pathway. COVID-19 infection blocked this pathway, promoting opportunistic pathogen invasion, cytokine storm, activating toll-like receptors/nuclear factor-κB pathway and aggravated COVID-19. COVID-19: Coronavirus disease 2019; ACE2: Angiotensin-converting enzyme-2; TMPRSS2: Transmembrane protease serine 2; IL: Interleukin; GM-CSF: Granulocyte-macrophage colony-stimulating factor; MCP-1: Monocyte chemoattractant protein-1; IFN-γ: Interferon-γ; TNF: Tumor necrosis factor; mTOR: Mechanistic target of rapamycin; TLRs: Toll-like receptors; NF-κB: Nuclear factor-κB.

important role in gastrointestinal, nervous, and liver diseases. 5-HT acts on 5-HT receptors to initiate peristaltic and secretory reflexes in the viscera[82,83]. Research also indicated that 5-HT might be the key to exacerbating inflammatory bowel disease symptoms, including diarrhea and abdominal pain [84]. Intraperitoneal injection of 5-HT can significantly increase the expression of IL-1β and IL-6 and the activity of myeloperoxidase by activating 5-HT_3 and 5-HT_4 receptors in the colonic mucosa of mice with colitis and block the signal of pain relief[85]. Further studies have reported that elevated 5-HT can increase the expression of IL-6 and IL-8 and the production of monocyte chemoattractant protein-1, thereby leading to the initial event of intestinal inflammation[86]. However, the plasma 5-HT level is increased in hospitalized patients with COVID-19. The change in 5-HT in patients with COVID-19 may be an important cause of abdominal pain[87]. Therefore, we speculate that the abdominal pain in COVID-19 may be related to 5-HT. Moreover, regulating the level of 5-HT may be a therapeutic modality for the treatment of patients with abdominal pain due to COVID-19.

Ion channel: Pain signals are detected in response to harmful stimuli and release nerve impulses that encode pain. Many of these nociceptive neurons are equipped with a large number of specific ion channels that act as nociceptors. Stretching, inflammation, ischemia, pH, bacterial products, immune mediators, and neurotransmitters have all been implicated in visceral pain[88]. Studies have reported multiple electrolyte abnormalities in patients with COVID-19 infection[89]. COVID-19 infection is associated with decreased serum concentrations of sodium, potassium, magnesium, and calcium. Thus, we speculate that ion channels may play important roles in COVID-19-induced abdominal pain. As TRP channels are widely expressed in COVID-19-infected tissues, TRP channels and TRPML2 are also involved in the fusion of viral envelopes with endolysosomal membranes[90,91]. Thus, TRP channels may be valuable targets for disrupting the COVID-19 life cycle. A report indicated that TRP channels were involved in abdominal pain caused by COVID-19. TRPV1 and TRPA1 induce inflammation, increase sensory or vagal secretions, and cause pain[92]. It has also been suggested that afferent neuronal TRPV1 desensitization (*via* RTX) can reduce pain-related complications in COVID-19 patients [93]. Because TRP channels are widely expressed in the gastrointestinal tract, we speculate that the abdominal pain caused by COVID-19 is related to TRP channel activation.

Anorexia

Social pressure: Anorexia nervosa is an eating disorder characterized by restrictive eating and an intense fear of gaining weight. A study in 2020 evaluated the early effects of COVID-19 on patients with eating disorders and reported an increase in anxiety and alarming eating behaviors during the pandemic. This report shows that 69% of individuals had anorexia nervosa and experienced worries about their dietary schedules, while subjects with bulimia nervosa or binge eating disorders reported more episodes of binging[94]. This may be due to new living conditions, social distancing, self-isolation, changes in food access, daily habits, and so on; in addition, more difficult access to health care practitioners is also an essential factor leading to an increased incidence of anorexia[95].

Neuromodulation: Researchers have reported that neuroendocrine pathways are disrupted by miscommunication between brain-gut-adipose tissue in patients suffering from COVID-19[96]. Studies have shown that dopamine neurons in the ventral tegmental area of the midbrain and serotonin neurons in the dorsal raphe nucleus are involved in the regulation of motivational behaviors, including feeding[22,97], and increased serotonin levels have been observed in COVID-19 patients[38]. However, there is no direct evidence of whether it is related to anorexia nervosa. Changes in the microbiota-gut-brain axis from COVID-19 as well as gender differences may also be responsible for anorexia nervosa[98]. Moreover, changes in brain serotonin and tryptophan concentrations have been reported to be critical mechanisms in the regulation of eating behavior both in mice and humans[99]. Coincidentally, post-COVID-19 infection was also accompanied by changes in serotonin and tryptophan levels. We speculate that COVID-19-induced anorexia is at least partly correlated with increased serotonin and tryptophan levels. In addition, COVID-19 infection of nonneuronal cells can lead to anosmia and related odor perception impairment[100], which may be associated with the development and aggravation of anorexia[101,102].

Acid reflux

In a retrospective study of poor prognosis of gastrointestinal symptoms in patients with COVID-19, 12 (n = 1077, 1.1%) patients developed acid reflux[103], and the incidence of acid reflux was relatively low compared with other gastrointestinal symptoms. Generally, the main causes of gastric acid reflux include: (1) Relaxation of the lower esophageal sphincter; (2) Gastric and duodenal dysfunction leading to obstruction of gastric emptying; and (3) Esophageal mucosal barrier damage. In a clinical study, the prevalence of acid reflux was associated with poorer clinical outcomes in COVID-19, and the mechanism was related to damage to the upper esophageal sphincter[104]. We believe that increased serotonin levels and mucosal barrier damage caused by cytokine storms may also be risk factors for acid reflux. Antacid therapy is generally used, but some studies have noted that the use of proton pump inhibitors may increase the risk of achlorhydria-related intestinal infections in patients with COVID-19 [105], while histamine H_2 receptor antagonists do not increase this risk. Some studies have suggested that high-dose famotidine may be clinically beneficial to COVID-19 patients[106]. Therefore, the clinical use of histamine receptor antagonists may be more beneficial; however, due to low incidence and insufficient samples, there has been no systematic clinical evaluation of COVID-19 patients accompanied by acid reflux.

Gastrointestinal bleeding

A case-control study noted that the main causes of gastrointestinal bleeding in COVID-19 patients were peptic and rectal ulcers. Among the many potential predictors of upper gastrointestinal bleeding, a history of upper gastrointestinal bleeding was the only significant risk factor[107,108]. Gastrointestinal bleeding may be the direct impact of COVID-19 on gastrointestinal mucosa integrity. However, in a case of a COVID-19 patient who showed vomiting coffee crumbs and esophageal mucosal injury by esophagogastroscopy and duodenoscopy, there was no mucosal injury[107]. This may exist in other

mechanisms that have not been elucidated. Compared with patients in the initial presentation, most bleeding occurred during hospitalization. The results suggested that bleeding may be one of the treatment-related or secondary factors related to critical illness.

Intestinal ischemia injury

It has been reported in the literature that exposure to COVID-19 may lead to an increased risk of ischemia associated with extremity venous thrombosis, pulmonary embolism, and mesenteric ischemia [109,110]. The overall mortality in COVID-19 patients with gastrointestinal ischemia and imaging-diagnosed mesenteric ischemia was 38.7% and 40%, respectively[111]. Mesenteric ischemia can be a fatal clinical emergency with high mortality[112]. Existing studies also have shown that the incidence of intestinal obstruction and intestinal ischemia is positively correlated with elevated aminotransferase levels[113].

Other gastrointestinal symptoms

Abdominal distension and loss of taste also have been indicated in the previous investigation[114], however, the specific underlying mechanism needs to be further explored. Another important issue we should be concerned about is that some adverse gastrointestinal events in COVID-19 patients are related to medication[115]. An observation line cohort study among patients with moderate severity of COVID-19 pointed out that patients receiving hydroxychloroquine or chloroquine treatment may occur serious gastrointestinal adverse practices (diarrhea, nausea, abdominal pain, *etc*.) which is an important reason make the patients are forced to withdrawal[115].

CONCLUSION

Patients suffering from COVID-19 are often accompanied by various types of gastrointestinal symptoms. Gastrointestinal symptoms may be accompanied by or precede respiratory symptoms. This suggests that gastrointestinal symptoms may indicate the possibility of a new COVID-19 infection in the context of this unmanageable pandemic trend. During COVID-19 infection, the main mechanisms of gastrointestinal symptoms include the interaction between virus and gastrointestinal ACE2, inflammatory factor storm, intestinal mucosal barrier damage, and composition and metabolites of intestinal flora change. A vicious cycle is formed between the above factors in COVID-19 patients, which prolongs the recovery time. This also suggests that focusing on gut symptoms and alterations in gut microbes or their metabolites may be a beneficial option for coping with COVID-19. Adjunctive treatment with probiotics may help break this vicious cycle and help the patients recover. However, this review has some limitations. The assumptions in this review are mostly based on clinical observations. To clearly elucidate the mechanism of gastrointestinal symptoms caused by COVID-19 and find appropriate treatments still needs a lot of basic research.

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FOOTNOTES

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REVIEW

Clinical diagnosis and management of pancreatic cancer: Markers, molecular mechanisms, and treatment options

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Abstract

Pancreatic cancer (PC) is the third-leading cause of cancer deaths. The overall 5-year survival rate of PC is 9%, and this rate for metastatic PC is below 3%. However, the PC-induced death cases will increase about 2-fold by 2060. Many factors such as genetic and environmental factors and metabolic diseases can drive PC development and progression. The most common type of PC in the clinic is pancreatic ductal adenocarcinoma, comprising approximately 90% of PC cases. Multiple pathogenic processes including but not limited to inflammation, fibrosis, angiogenesis, epithelial-mesenchymal transition, and proliferation of cancer stem cells are involved in the initiation and progression of PC. Early diagnosis is essential for curable therapy, for which a combined panel of serum markers is very helpful. Although some mono or combined therapies have been approved by the United States Food and Drug Administration for PC treatment, current therapies have not shown promising outcomes. Fortunately, the development of novel immunotherapies, such as oncolytic viruses-mediated treatments and chimeric antigen receptor-T cells, combined with therapies such as neoadjuvant therapy plus surgery, and advanced delivery systems of immunotherapy will improve therapeutic outcomes and combat drug resistance in PC patients. Herein, the pathogenesis, molecular signaling pathways, diagnostic markers, prognosis, and potential treatments in completed, ongoing, and recruiting clinical trials for PC were reviewed.

Key Words: Pancreatic cancer; Pancreatic ductal adenocarcinoma; Molecular mechanisms; Diagnostic and prognostic markers; Treatment; Clinical trials

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Core Tip: Pancreatic cancer (PC) is the third-leading cause of cancer deaths. Pancreatic ductal adenocarcinoma is the most common type of PC in the clinic. Multiple pathogenic processes including inflammation, fibrosis, angiogenesis, epithelial-mesenchymal transition, and proliferation of cancer stem cells are involved in PC initiation and progression. Although some therapies have been approved for PC treatment, the overall 5-year survival rate is still very low. A combined panel of serum markers is very helpful for PC diagnosis. New treatments and more clinical trials are required to search for new potent therapeutic agents and to evaluate their efficacy in PC treatment.

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INTRODUCTION

Pancreatic cancer (PC) accounts for 7% of all cancer-related deaths[1]. The overall 5-year survival rate of PC is 9%, with only 3% for metastatic PC[2]. However, the number of PC-induced death cases will increase about 2-fold by 2060[3,4]. Many factors are involved in the development of PC[5-7], including genetic mutations, environmental factors, and metabolic diseases such as obesity and diabetes. The most commonly diagnosed PC in the clinic is pancreatic ductal adenocarcinoma (PDAC), which accounts for more than 90% of all PC cases[4]. The rest of the PC cases are pancreatic neuroendocrine neoplasms. Pancreatic neuroendocrine neoplasms originate from precursor cells in the pancreatic ductal epithelium with neuroendocrine differentiation, which can be divided into well-differentiated pancreatic neuroendocrine tumors and poorly differentiated pancreatic neuroendocrine carcinomas[8].

PC is a heterogenous and desmoplastic cancer. Genetic variants of tumor cells, immunosuppressive tumor microenvironment (TME), high metastatic rate, and limited therapeutic outcomes cause challenges for current therapies[9-11]. Early diagnosis of PC is critically important for longer survival outcomes. Serum biomarkers can be applied for PC diagnosis, including microRNAs[12] and cancer antigens such as carbohydrate antigen 19-9 (CA 19-9)[13]. In addition, some of these markers such as CA 19-9 can be applied to predict tumor recurrence and survival of PC patients[14,15].

Herein, this review first summarized the pathogenic factors and their associated molecular signaling pathways that are involved in PC development and progression. Then, diagnostic and prognostic markers were reviewed, especially serum biomarkers. Based on the pathogenic factors, corresponding treatments were discussed, and currently completed, ongoing, and recruiting clinical trials for PC treatment were summarized.

PATHOGENESIS OF PANCREATIC CANCER AND RELATIVE MOLECULAR MECHANISMS

The initiation and progression of PC are impacted by many factors, including chronic inflammation or pancreatitis, fibrosis, immunosuppressive TME, epithelial-mesenchymal transition (EMT), proliferation and differentiation of cancer stem cells, and alteration of gut microbiota. In this section, we discussed each of these factors in PC pathogenesis.

Inflammation

Inflammation is a key mediator for the initiation and progression of PC[16]. In TME during PC development, tumor growth accompanies the infiltration of innate and adaptive immune cells. For example, tumor-associated macrophages are one of the major immune cells in the TME, which have been shown to play an essential role in the initiation, progression, and metastasis of PC as well as chemotherapeutic resistance[17]. Tumor necrosis factor alpha-expressing macrophages are recruited by monocyte chemoattractive protein-1 or CCL2, which can induce the reprogramming of classical neoplastic cells into an aggressive phenotype *via* the bromodomain-containing protein 4-mediated signaling pathway[18]. In addition, other infiltrating immune cells including monocytes, myeloid-derived suppressor cells, natural killer cells, neutrophils, and $CD4^+$ and $CD8^+$ T cells interplay with cancer cells by secreting cytokines. Molecular signaling pathways such as nuclear factor-κB, reactive oxygen species, and toll-like receptors (TLRs) are involved in the inflammatory condition in the TME of

PC[16].

Both local and systemic inflammation contributes to PC development and progression. A clinical study showed that PDAC patients with systemic inflammation characterized by a neutrophil/lymphocyte ratio > 3.1 have a lower median overall survival compared to patients with a neutrophil/lymphocyte ratio < 3.1 in response to the treatment of anti-CD40 monoclonal antibody in combination with gemcitabine[19]. Obesity can induce systemic inflammation and contribute to cancer progression, including PDAC[20]. The chronic low-grade inflammation in white adipose tissues of obese patients plays an important role in PDAC progression[21]. Some important adipokines such as lipocalin 2, proinflammatory cytokines [*e.g.*, tumor necrosis factor alpha and interleukin (IL)-6], and chemokines (*e.g.*, monocyte chemoattractive protein-1 or CCL2) drive the progression of PDACs[21,22].

Chronic pancreatitis characterized by redness and swelling inflammation in the pancreas can be induced by factors such as heavy alcohol consumption[23], smoking, and gallstones[24]. It is a risk factor for the initiation of the progression of PDAC[25] or PC[26]. In the United States, 1.04% of patients with chronic pancreatitis were diagnosed with PDAC, which was higher than the rate in the control group (0.2%)[27]. Therefore, targeting inflammation is a therapeutic strategy for PC treatments.

Desmoplastic stroma

Chronic pancreatitis and PC are commonly associated with desmoplastic tissue proliferation, which is mainly caused by activated pancreatic stellate cells[28]. Fibrotic stroma is formed by fibroblasts and their secreted extracellular matrix proteins that contribute to cancer cell proliferation and invasion, an immunosuppressive environment, and therapeutic resistance[29]. Some molecules including epithelium-specific E-twenty six factor 3[30], galectin-1[28], β-catenin[31], and transforming growth factor-β1 (TGF-β1)[32] are essential drivers for pancreatic stellate cell activation in PC. In addition, activated pancreatic stellate cells can secrete many molecules, such as IL-6, TGF-β1, stromal cell-derived factor-1, hepatocyte growth factor, and galectin-1, to induce PC cell proliferation, migration, and chemotherapeutic resistance[33].

Cancer-associated stromal fibroblasts (CAFs) are a heterogenous cell population, which can secrete many factors to regulate inflammation, cancer development, progression, metastasis, recurrence, and drug resistance[34]. For instance, CAFs and tumor cells can crosstalk through extracellular vesicles (EVs). Annexin A6-enriched EVs secreted by CAFs can increase the aggressiveness of PDACs[35,36]. CD9 is an important component of these EVs[36]. In addition, the uptake of $CD9^{+}ANXA6^{+}$ EVs secreted by CAFs can activate the mitogen-activated protein kinase signaling pathway to promote PC cell migration and EMT[36].

Immunosuppressive TME

Overexpression of immune checkpoints, such as programmed cell death protein 1 (PD-1), lymphocyte-activation gene 3, and cytotoxic T-lymphocyte-associated antigen 4, and their ligands programmed death-ligand 1 (PD-L1) and PD-L2, CD80 and CD86 (cytotoxic T-lymphocyte-associated antigen 4), and major histocompatibility complex molecule II or major histocompatibility complex-II (lymphocyte-activation gene 3) mediate the immunosuppression in TME[37]. Therefore, targeting these molecular signaling axes provide novel therapeutic options. For example, PD-L1 is overexpressed by tumor cells and some immunosuppressive cells in PDAC, which can be targeted by PD-1-expressing chimeric antigen receptor (CAR) T cells to mediate anti-tumor activity by PD-1/PD-L1 interaction[38].

Additionally, other proteins, such as fibroblast activation protein, CD73, and inhibitor of DNA binding 1[39], can mediate the immunosuppressive environment in the TME. In a mouse PDAC model, elevated expression of CD73, a cell surface-localized ecto-5′-nucleotidase, is positively associated with the infiltration of myeloid-derived suppressor cells and expression of granulocyte-macrophage colony-stimulating factor, which causes suppression of interferon-gamma production in intratumoral T cells. The CD73-mediated suppressive effect on T cells can be abolished by genetic knockdown in PDAC cells [40]. In human PDAC cells, the elevated expression of CD73 causes cancer cell resistance to gemcitabine by activating the protein kinase B (AKT) signaling pathway[41].

EMT

EMT plays a pivotal role in PC progression and metastasis, which is defined by cell phenotypic transition from an epithelial to a mesenchymal state[42]. Dermokine genes regulate the oncogenesis of PC. Overexpression of dermokine-α can promote PC cell proliferation, EMT, migration, and invasion by regulating the phosphorylation of signal transducer and activator of transcription 3 (STAT3)[43,44]. Methylsterol monooxygenase 1 as a tumor suppressor can inhibit PC progression by suppressing the phosphoinositide 3-kinase-AKT-mammalian target of rapamycin signaling pathway and EMT[45]. EMT of PC stem cells also plays a critical role in PC initiation and progression, which is discussed in the following section.

Angiogenesis

Angiogenesis plays a key role in PC development, progression, and metastasis, which is commonly associated with the activation of proangiogenic and angiogenic molecules. For example, the epidermal

growth factor receptor is overexpressed in PC cells, which is associated with angiogenesis and cancer cell metastasis[46]. A high density of macrovessels, impaired integrity of microvessels, and poorly perfused vessels are the characterizations of vascularization in TME of PC[29]. Many proteins are involved in the angiogenesis of PC, including vascular endothelial growth factor (VEGF), platelet-derived growth factor, fibroblast growth factor, and their receptors such as VEGF receptors (VEGFR1-3)[29]. In inflammatory conditions, the expression of fibroblast growth factor 1 on PC cells is stimulated by inflammatory product prostaglandin E2, resulting in the proliferation of CAFs and an increased VEGFA expression to maintain angiogenesis[47].

Cancer stem cells

Cancer stem cells (CSCs) are a small part of the TME, where they play a vital role in chemotherapy resistance. Pancreatic CSCs express several surface markers such as CD24, CD44, CD133, C-X-C chemokine receptor type 4, tyrosine-protein kinase Met, epithelial cell adhesion molecule, and double-cortin like kinase 1 as well as intracellular markers such as aldehyde dehydrogenase 1 and RNA polymerase II-associated factor 1[48]. One study showed that miR-497 can resensitize pancreatic CSCs to gemcitabine treatment by inhibiting nuclear factor-κB expression[49]. Exosomes can horizontally transfer drug-resistant traits from gemcitabine-resistant pancreatic CSCs to gemcitabine-sensitive PC cells by delivering miR-210[50]. Small nucleolar RNAs such as SNORD35A play an important role in the proliferation, migration, invasion, and EMT of pancreatic CSCs through regulating the hepatocyte growth factor/tyrosine-protein kinase Met signaling pathway, which is a prognostic biomarker and therapeutic target for PC treatment[51]. In addition, CSCs are involved in drug resistance. For instance, a hypoxic niche can further activate AKT/Notch1 signaling pathway to enhance gemcitabine-induced stemness to cause chemoresistance[52].

Alteration of gut microbiota

Gut microbiomes, including bacteria, viruses, archaea, and fungal species, play important roles in energy digestion, synthesis of secondary bile acids, vitamins and proteins, and immune regulation. Most of the microbes reside (> 95%) within the gut and maintain intestinal homeostasis[53]. Dysbiosis of gut microbiota can regulate inflammation and immune response in the TME to promote cancer progression[54,55], including PC[56,57]. A preclinical study showed that gut microbiota can promote PDAC progression by regulating the infiltration and anti-cancer activity of natural killer cells in the TME[58]. Cohort studies in three different countries from Asia (Japan) and Europe (Spanish and German) showed that *Streptococcus* and *Veillonella spp* were significantly enriched and *Faecalibacterium prausnitzii* was depleted in gut microbial profiles of PDAC patients[59]. A comprehensive analysis of microbiota in several tumors including PC showed that most intratumor bacteria were present intracellularly in both cancer and immune cells[60].

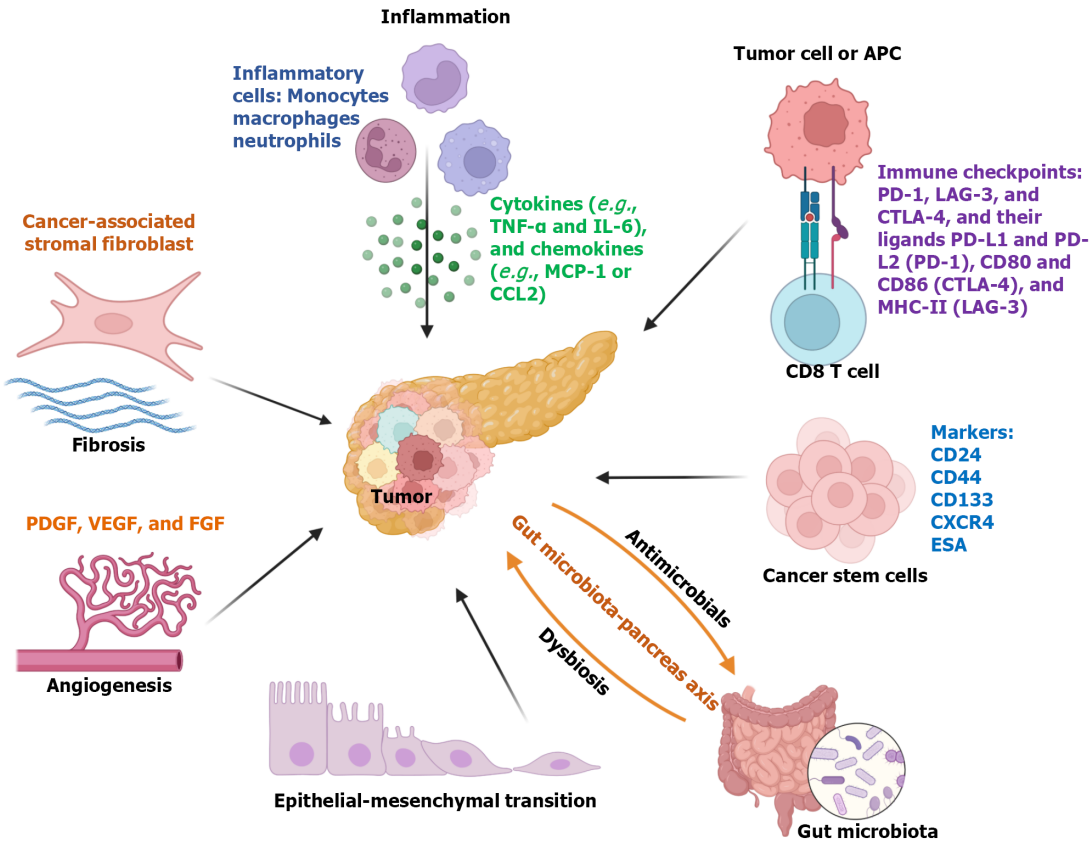
Recent studies also showed that intratumor bacteria were associated with the survival time of PDAC patients[61,62]. In addition, bacteria-mediated treatment (*e.g.*, a bacterium *Megasphaera sp. XA511*) can enhance the anti-tumor effect of anti-PD-1 treatment[61]. Another study also revealed that the alpha diversity of tumor microbiota was higher in long-term survival patients compared to that in short-term survival patients. Fecal microbiota transplantation from humans into mice can regulate tumor growth and anti-tumor immune response[62]. Moreover, pancreatic secretions (*e.g.*, antimicrobial peptides) influence the change of gut microbiota profiles, and the pancreas-bacteria interplay forms a gut-microbiota-pancreas axis[63].

Oral microbiota may also impact the development of PC. One study showed that the presence of oral *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* was positively associated with a high PC risk, whereas genus *Leptotrichia* (phylum Fusobacteria) was related to a low PC risk[64]. In addition, other oral bacterial species including *Clostridium difficile*, *Campylobacter jejuni*, *Escherichia coli*, *Enterococcus faecalis*, *Helicobacter pylori*, *Fusobacterium nucleatum*, *Vibrio cholera*, and *Porphyromonas gingivalis* have been reported to be associated with PC development, which may regulate anti-tumor immunity by signaling pathways such as the miR-21/phosphatase and tensin homolog axis[65].

Overall, multiple factors influence the initiation and progression of PC, which is summarized in Figure 1.

DIAGNOSTIC AND PROGNOSTIC MARKERS FOR PANCREATIC CANCER

The tumors in PC patients can be classified into resectable, borderline resectable, locally advanced, and metastatic tumors[66]. Early diagnosis ensures curable treatment by surgical resection[67]. An accurate diagnosis of PC can improve therapeutic outcomes. Although there is an advanced improvement in new diagnostic technologies, biopsy is still the gold standard for PC diagnosis[68]. Imaging methods including computed tomography, positron emission tomography, magnetic resonance imaging, and endoscopic ultrasound are commonly applied in PC diagnosis and staging[68,69]. In addition, several serum markers have promising diagnostic and prognostic values for PC.



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Figure 1 Many factors are involved in pancreatic cancer initiation and progression. Many factors contribute to pancreatic cancer initiation and progression, including inflammation, fibrosis, angiogenesis, dysbiosis of gut microbiota, cancer stem cells, and immune suppressive tumor microenvironment. APC: Antigen-presenting cells; CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4; CXCR: C-X-C chemokine receptor; ESA: Epithelial-specific antigen; FGF: Fibroblast growth factor; IL: Interleukin; LAG-3: Lymphocyte-activation gene 3; MCP-1: Monocyte chemoattractive protein-1; MHC: Major histocompatibility complex; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; PDGF: Platelet-derived growth factor; TNF-α: Tumor necrosis factor alpha; VEGF: Vascular endothelial growth factor. All cartoons in this figure were prepared using Biorender (https://biorender.com).

Serum markers for PC diagnosis

Serum markers including carcinoembryonic antigen and CAs can be applied in PC diagnosis and prognosis[70,71]. Currently, CA 19-9 is the most broadly used serum biomarker for PC diagnosis. A meta-analysis showed that the average sensitivity and specificity of CA 19-9 in PC diagnosis were 72% [95% confidence interval (CI): 71%-73%] and 86% (95%CI: 85%-86%), respectively, with an area under the curve (AUC) of 0.8474 (95%CI: 0.8272-0.8676)[72]. It has been reported that CA 19-9 serum levels are significantly associated with positive lymph nodes and positive margin status in patients with resectable PDAC, which is important for the decision of neoadjuvant treatments[73]. In addition, the preoperative levels of CA 19-9 are negatively associated with the overall survival, nodal involvement, and margin status positivity in resectable PC. However, some limitations impair the role of CA 19-9 in PDAC preoperative staging and management[74], including up to 50% of PDAC patients without CA 19-9 secretion.

In combination with other markers, the diagnostic value of CA 19-9 can be amplified or increased. For example, a combination of CA19-9 with serum mucin 5AC, a heavily glycosylated protein of the mucin family, improves both sensitivity (73.8%) and specificity (88.6%) as well as the AUC (0.894; 95%CI: 0.844-0.943) for PC diagnosis in patients, better than the values of each individual marker[75]. There are many other serum markers that can be used for PC or PDAC diagnosis, including macrophage inhibitory cytokine-1[76], keratin 8[77], protein induced by vitamin K absence II[78], and gremlin 1 (GREM1)[79] (Table 1). Meanwhile, combining different markers in a panel could increase the values of sensitivity, specificity, and AUC. For example, a panel including CA 19-9, factor VIII, fibrinogen, albumin, and alkaline phosphatase increase the AUC value to 0.95 (95%CI: 0.89-0.99) when compared to 0.80 (0.71-0.88) for CA 19-9 alone in distinguishing PDAC from intraductal papillary mucinous neoplasm, a benign tumor[80]. Another study also showed that a panel of four biomarkers including S100 calcium-binding protein A2, S100 calcium-binding protein A4, CA 125, and CA 19-9 increased AUC to 0.913[81].

Table 1 Diagnostic markers of pancreatic cancer

Cancer type	Markers	Expression	Sensitivity	Specificity	AUC	Ref.
PC	CA 19-9	Serum	72.0%	86%.0	0.8474	[72]
PC	MUC5AC + CA19-9	Serum	73.8%	88.6%	0.8940	[75]
PC	MIC-1	Serum	80.0%	85.0%	0.8945	[76]
PDAC	Keratin 8	Serum	80.0%	85.0%	0.8945	[77]
PDAC	PIVKA-II	Serum	78.7%	90.7%	0.9000	[78]
PDAC	GREM1	Serum	Unknown	Unknown	0.7180	[79]

AUC: Area under the curve; CA19-9: Carbohydrate antigen 19-9; GREM1: Gremlin 1; MIC-1: Macrophage inhibitory cytokine-1; MUC5AC: Mucin 5AC; PC: Pancreatic cancer; PDAC: Pancreatic ductal adenocarcinoma; PIVKA-II: Protein induced by vitamin k absence II.

MicroRNAs are small non-coding RNAs with about 22 nucleotides, which regulate the expression of their target mRNAs through degradation or translational repression. Serum expression profiles of microRNAs in PC patients are significantly changed compared to healthy controls. Among them, a panel model including miR-125a-3p, miR-5100, and miR-642b-3p showed the most promising value for PC diagnosis with an AUC of 0.95, sensitivity of 0.98, and specificity of 0.97[82]. Another study also showed that serum microRNAs such as miR-25-3p, miR-19a/b-3p, miR-192-5p, miR-223-3p, and let-7b-5p were upregulated in PC patients and can be used as diagnostic markers as a panel[83].

Multi-omics profiling studies can provide new markers for PC diagnosis. Proteomic analysis of EVs derived from co-cultured epithelial and stromal cells in the condition mimicking TME showed that kinesin family member 5B and secreted frizzled related protein 2 had promising values as early PC biomarkers[84]. PancRISK score evaluated by three urine markers, including lymphatic vessel endothelial hyaluronan receptor 1, regenerating family member 1 beta, and trefoil factor 1, showed reasonable sensitivity and specificity for PDAC detection compared to CA 19-9[85]. Another study also showed that a panel of three urine markers with CA 19-9 had pre-diagnostic values before PDAC diagnosis, with a sensitivity of 72% at 90% specificity up to 1 year and 60% sensitivity with 80% specificity up to 2 years[86].

Bioinformatics analysis showed that gremlin 1, a bone morphogenetic protein signaling regulator, was overexpressed in PDAC and predicted a poorer prognosis for patients with PDAC[79]. In addition, serum gremlin 1 is increased in PDAC patients compared to healthy controls and has a diagnostic value. In combination with CA 19-9, the AUC value increases from 0.718 to 0.914[79].

Prognostic markers of pancreatic cancer

One study displayed that PC patients with low lymphocyte-C-reactive protein ratio have significantly low recurrence-free survival and overall survival values[87]. Another study showed that the systemic immune-inflammation index, which is calculated using the absolute platelet, neutrophil, and lymphocyte counts [systemic immune-inflammation index = platelet × (neutrophil/lymphocyte)], can be used as an independent negative prognostic marker of overall survival of PDAC patients receiving neoadjuvant therapy[88]. A meta-analysis showed that mucin 4 (hazard ratio = 2.04, 95%CI: 1.21-3.45) and mucin 16 (hazard ratio = 2.10, 95%CI: 1.31-3.37) had predictive values for the prognosis of PC patients[89]. The expression of aquaporin-5, a water channel protein, is increased in pancreatic adenocarcinoma (PAAD), which is positively associated with the infiltration of different immune cells (*e.g.*, macrophages) in tumors and poor prognosis of PAAD patients[90]. A bioinformatics study showed that overexpression of matrix metallopeptidase 14 and collagen XII alpha 1 is significantly related to the poor prognosis of PAAD patients[91]. Analysis of RNA sequencing data from the Gene Expression Omnibus and the Cancer Genome Atlas databases gives us some conclusion that some biomarkers such as transmembrane protein 170B (TMEM170B) can be applied to predict cancer progression[92]. Overall, improvement in bioinformatics and new technologies accelerates the development of new diagnostic and prognostic markers for PC, which will result in good outcomes for PC therapy.

TREATMENT OPTIONS

Surgical resection is a curable therapy for patients with early stages of PC and good health conditions [66,68]. There are some Food and Drug Administration (FDA)-approved drugs for PC treatment (Table 2), including belzutifan[93,94], erlotinib hydrochloride[95,96], everolimus[97,98], fluorouracil, also known as 5-fluorouracil[99,100], gemcitabine hydrochloride[101,102], irinotecan hydrochloride liposome[103,104], mitomycin[105,106], olaparib[107,108], paclitaxel albumin-stabilized nanoparticle

Table 2 Food and Drug Administration-approved treatments for pancreatic cancer patients

Drug names	Conditions	Targets	Ref.
Belzutifan	Pancreatic neuroendocrine tumors	An inhibitor of hypoxia-inducible factor-2α	[93, 94]
Erlotinib hydrochloride	Gemcitabine hydrochloride-treated PC, not removable with surgery, with metastasis or local progression	An EGFR inhibitor	[95, 96]
Everolimus	Progressive pancreatic neuroendocrine tumors, not removable with surgery, with metastasis or local advance	A mammalian target of rapamycin inhibitor	[97, 98]
Fluorouracil, also called 5-FU	Pancreatic cancer	An anti-metabolite drug with multiple functions such as inhibition of cellular thymidylate synthase to prevent DNA replication and inhibit RNA synthesis	[99, 100]
Gemcitabine hydrochloride	PC with metastasis, local advance, or fluorouracil treatment	An antimetabolite drug and an inhibitor of DNA synthesis	[101, 102]
Irinotecan hydrochloride liposome	Metastatic PC or gemcitabine hydrochloride-treated PC with precision	An inhibitor of topoisomerase I	[103, 104]
Mitomycin	Pancreatic adenocarcinoma with local advance or metastasis to other parts of the body, which has no approvement with other types of treatment	An inhibitor of DNA synthesis and thioredoxin reductase	[105, 106]
Olaparib	Metastatic PC after first-line therapy with platinum chemotherapy and with certain germline mutations in the breast cancer 1 or BRCA2 gene	A poly ADP-ribose polymerase inhibitor	[107, 108]
Paclitaxel albumin-stabilized nanoparticle formulation	PC with metastasis	It prevents cell mitosis and inhibits the growth of cancer cells	[109, 110]
Sunitinib malate	Progressive neuroendocrine tumors that are not removable with surgery, with metastasis to other parts of the body or local advance	An antiangiogenic tyrosine kinase inhibitor	[111, 112]

EGFR: Epidermal growth factor receptor; PC: Pancreatic cancer.

formulation[109,110], and sunitinib malate[111,112]. In addition, combined treatments including leucovorin calcium (folinic acid) + fluorouracil + irinotecan hydrochloride + oxaliplatin, gemcitabine hydrochloride + cisplatin, gemcitabine hydrochloride + oxaliplatin, and oxaliplatin + fluorouracil + leucovorin calcium (folinic acid) have been also approved by the United States FDA for PC treatment [113-115].

In this section, we discussed some treatments targeting the driving factors of PC, including immune checkpoint inhibitors, antifibrosis, anti-inflammation, anti-angiogenesis, growth factor inhibitors, anti-cancer peptides, alteration of gut microbiota, T cell therapy, and oncolytic viruses as well as combined therapies.

Immune checkpoint inhibitors

Immunotherapy by targeting immune checkpoints such as PD-1 and PD-L1 has achieved big success in the treatment of many different tumors[116,117]. Currently, pembrolizumab (anti-PD-1) is the only FDA-approved immune checkpoint inhibitor for the treatment of patients who have advanced PDACs with mismatch repair deficiency or microsatellite instability high[118]. There are many ongoing clinical trials for the evaluation of synergistic effects of ipilimumab or tremelimumab (anti-cytotoxic T-lymphocyte-associated antigen 4 antibody), nivolumab (anti-PD-1 antibody), and durvalumab (anti-PD-L1 antibody) with other chemotherapy, vaccines, or radiotherapy[119].

Antifibrotic treatments

CAFs are one of the most abundant stromal cells in PDAC and contribute to cancer progression and chemoresistance[120]. Therefore, reshaping the fibrotic stroma is a strategy to treat PC. The integrin-mediated signaling pathway plays a critical role in remodeling and induction of pancreatic tissue stiffness during PC development and progression, promoting chemoresistance. The phosphorylation of tyrosine397 in focal adhesion kinase (FAK) of CAFs is significantly increased compared to that in fibroblasts of the normal pancreas. Therefore, inhibiting FAK activity can dramatically suppress CAF migration and extracellular matrix deposition[120]. Meanwhile, FAK inhibition can also resensitize PDAC cells to chemotherapy[121]. Some tyrosine kinase inhibitors such as cabozantinib, pazopanib, lenvatinib, and surufatinib are under clinical evaluation for the treatment of pancreatic neuroendocrine tumors[122]. A study in a murine PC model also showed that stromal hyaluronan degradation by PEGylated recombinant human hyaluronidase in combination with FAK inhibitor could improve anti-

PD-1 antibody efficacy on the survival of PDAC-bearing mice by increasing T cell infiltration and efficacy[123].

Anti-inflammatory treatments

The anti-inflammatory drug aspirin can inhibit cell proliferation of PC cell lines by suppressing cyclin D1 expression to induce G_0/G_1 cell cycle arrest. Aspirin can also inactivate the glycogen synthase kinase-3β signaling pathway and regulate the expression of microRNAs in PC cells[124]. A phase I trial (https://clinicaltrials.gov, registration number NCT03207724) showed that treatment with bermekimab (anti-IL-1α antibody) can decrease inflammatory cytokines and endothelial growth factor, which is associated with an increase in healthy gut microbiota *Akkermansia* compared to the baseline[125]. A phase 3 clinical trial (NCT02923921) showed that adding pegilodecakin (PEG, a pegylated recombinant human IL-10) to folinic acid, fluorouracil, and oxaliplatin increased the expression of total IL-18, interferon-gamma, and granzyme B and decreased TGF-β in patients with post-gemcitabine metastatic PDACs[126].

Anti-angiogenesis treatments

The expression of mitofusin-2 in PC tissues is significantly decreased, which is negatively associated with VEGFA expression. A molecular study showed that overexpression of mitofusin-2 could inhibit the expression of VEGFA, VEGFR2, angiopoietin-1 gene, and tissue inhibitor of metalloproteinase 1 in human umbilical vein endothelial cells[127]. Another study showed that escin, a pentacyclic triterpenoid isolated from the horse chestnut, can inhibit angiogenesis by suppressing the expression of IL-8 and VEGF in PC cells through the blockade of nuclear factor-κB[128]. Treatment with apatinib, a small molecule targeting VEGFR2, can inhibit the proliferation, migration, and invasion of PC cells (ASPC-1 and PANC-1 cells) and the growth of their xenografted tumors by inhibiting cancer cell growth and angiogenesis *via* suppression of the phosphorylation of VEGFR2, AKT, and ERK1/2[129].

Anti-growth factors and growth factor receptor inhibitors

Growth factors play essential roles in PC cell survival, progression, and drug resistance[130]. For example, TGF-β contributes to PDAC progression by inducing N-glycomic changes in SMA-related and MAD-related protein 4-deficient PDAC cell line PaTu-8955S cells[131].

Anti-cancer peptides

Anti-cancer peptides are short peptides with direct and indirect anti-cancer properties. Anti-cancer peptides can be classified into natural and synthetic peptides. For example, human cathelicidin peptide LL-37 can suppress PC growth *in vitro* and *in vivo* by activating the mammalian target of rapamycin signaling pathway to suppress autophagy of PC cells and induce reactive oxygen species production to cause DNA damage and cell cycle arrest[132]. KS-58, a derivative of KRpep-2d that is an artificial cyclic peptide that can selectively inhibit K-Ras (G12D), can suppress the human PC cell line PANC-1 proliferation *in vitro*. In addition, KS-58 also displays anti-tumor activity in subcutaneous and orthotropic PANC-1 cell xenografted tumors, which shows a synergistic effect with gemcitabine[133].

Alteration of gut microbiome

Patients with high levels of antibodies against a pathogenic periodontal bacterium *Porphyomonas gigivalis* have a double risk of developing PC compared to subjects with low levels of antibodies. In contrast, individuals who have high levels of antibodies against commensal oral bacteria reduce the risk of PC development[134]. The microbiome in the cancerous pancreas can induce immune tolerance by causing macrophage-mediated suppression of T cell functions through TLR signaling pathways (*e.g.*, TLR2 and TLR5)[135]. Depletion of gut microbiota by antibiotics can inhibit tumor progression and metastasis *via* upregulation of interferon-gamma-producing T cells and downregulation of IL-10 and IL-17A-producing T cells[136]. Therefore, the alteration of microbial components in the gut and mouth, as well as the TME, can effectively inhibit PC progression.

CAR-engineered T cell therapy

CAR-engineered T cell (CAR-T) therapy shows potential for many tumors. To date, six CAR-T products have been approved by the United States FDA for the treatment of hematopoietic cancers, such as B-cell acute lymphoblastic leukemia (tisagenlecleucel), mantle cell lymphoma (brexucabtagene autoleucel), and multiple myeloma (idecabtagene vicleucel)[137]. However, current clinical trials of CAR-T therapy in PC patients have not shown significance in the improvement of survival and other outcomes[138]. CAF-derived extracellular matrix proteins, enzymes, and growth factors impact the infiltration and efficacy of CAR-T. Enhancing the expression of chemokine ligands such as chemokine (C-C motif) ligand 19 (CCL19) can increase the infiltration of the CAR-T to promote their anti-PDAC efficacy[139].

Oncolytic viruses

Virus-mediated delivery of cytokines and shRNAs shows promising effects in PC treatment. Oncolytic viruses are designed to directly target tumor cells or to activate anti-tumor immune responses.

However, treatment of oncolytic viruses alone is not sufficient to eliminate PC to date[140]. For example, oncolytic adenoviruses loading CD55-ST13 (suppression of tumorigenicity 13)-tumor necrosis factor-related apoptosis-inducing ligand can significantly inhibit but not delete tumor development in a murine xenografted PDAC tumor model by inducing cancer cell apoptosis[141].

Combined therapies

The combined therapy of MEK inhibitor (trametinib) and STAT3 inhibitor (ruxolitinib) inhibits the phenotype of proinflammatory and myofibroblastic IL6$^+$CXCL1$^+$LRRC15$^+$CAFs and increases mesenchymal stem cell-like Ly6a$^+$Cd34$^+$CAFs in a murine model detected by single-cell RNA sequencing[142]. The CAF phenotype change is associated with M2-to-M1 reprogramming of tumor-associated macrophages s and effector CD8$^+$ T cell infiltration. In addition, treatment of MEK and STAT3 inhibitors together with a PD-1 inhibitor (nivolumab) shows clinical benefit in patients with chemotherapy-refractory metastatic PDACs[142]. In contrast, a pharmacodynamic separation treatment for erlotinib plus gemcitabine can improve their treatment efficacy, especially for patients with detected plasma Kirsten rat sarcoma virus mutation[143].

Four-week intraperitoneal injection of gemcitabine together with oral administration of probiotic cocktails (*Lactobacillus paracasei* GMNL-133 and *Lactobacillus reuteri* GMNL-89) can inhibit pancreatic intraepithelial neoplasia formation and suppress serum levels of aspartate aminotransferase and alanine aminotransferase and the expression of vimentin and Ki-67 in pancreatic sections[144].

CLINICAL TRIALS

Some treatments are under clinical investigation, see Table 3 (https://clinicaltrials.gov, accessed on September 6, 2022). These treatments include galunisertib plus gemcitabine[145], Janus kinase 1/2 inhibitor ruxolitinib[146], adoptive transfer of T cells[147], gemcitabine and trastuzumab plus erlotinib [148], erlotinib plus gemcitabine[149], pegilodecakin plus folinic acid, fluorouracil, and oxaliplatin[126], liposomal irinotecan plus 5-fluorouracil/leucovorin[150], bevacizumab[151], and sunitinib[152].

In addition, more than 900 studies in the world are recruiting or enrolling by invitation, and some have a 'not yet recruiting' status for evaluating PC treatments (https://clinicaltrials.gov, accessed on September 17, 2022). A graphic map shows the number of studies at different locations (Figure 2), and some examples are listed in Table 4.

FUTURE PERSPECTIVES

A synergistic treatment is a good option for killing drug-resistant PC cells. For example, astaxanthin can resensitize human PC cells to gemcitabine by activating the hypoxia-inducible factor 1α/STAT3 signaling pathway to promote gemcitabine-induced cell apoptosis and inhibit gemcitabine-induced EMT of PC cells[153]. Guadecitabine, an effective inhibitor of DNA methyltransferase 1, has the potential to resensitize PDAC cells to chemotherapy and immune checkpoint blockade therapy (*e.g.*, anti-PD-L1)[154,155].

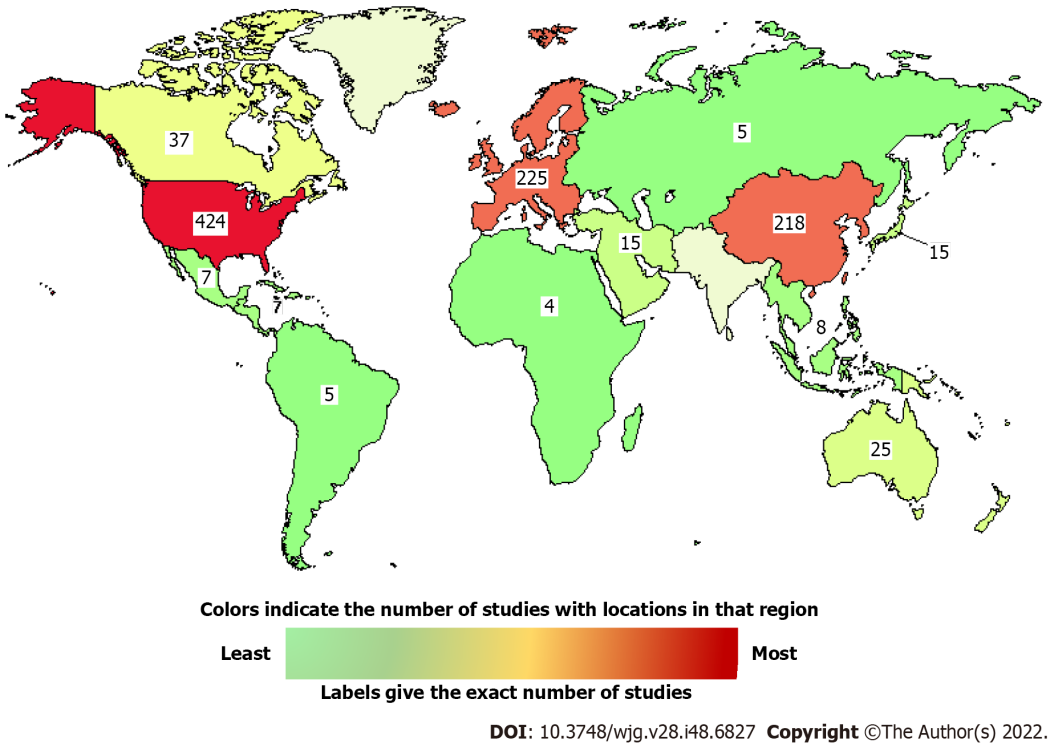
Neoadjuvant therapy has been applied in clinical trials for patients with resectable PDACs, which include neoadjuvant chemotherapy, neoadjuvant radiotherapy, and neoadjuvant chemoradiotherapy [156]. The results from three randomized controlled trials with a total of 130 patients (56 receiving neoadjuvant therapy and 74 in the control group) indicated that neoadjuvant therapy (chemotherapy or chemoradiation + surgery followed by adjuvant therapy) increased the disease-free survival time compared to upfront surgery followed by adjuvant therapy[157]. Another single-center long-term study also showed that PDAC patients with treatment of neoadjuvant therapy, consisting of folinic acid + fluorouracil + irinotecan + oxaliplatin, single gemcitabine, or combined with cisplatin, nab-paclitaxel, or capecitabine with or without radiation had longer median disease-specific survival and disease-free survival than those receiving treatment with upfront surgery[158]. The benefit of neoadjuvant therapy could be a stage-dependent manner. A retrospective cohort study showed that neoadjuvant therapy was positively associated with an improved survival benefit compared with conventional upfront surgery, especially in clinical stage III PC after propensity score matching within each stage[159]. Overall, it can benefit surgical treatment.

Delivery systems can be applied to enhance the efficacy of anti-cancer treatments. For example, arginine glycine peptide-human serum albumin-mediated drug nanoparticles show tumor-targeting effects and increase the cytotoxicity of gemcitabine and curcumin[160]. Administration of VG161, the first recombinant oncolytic herpes simplex virus type 1 that delivers multiple synergistic antitumor immunomodulatory factors, can systematically activate both innate and adaptive immunity and improve the anti-tumor function of the tumor immune microenvironment[161]. Another study showed that using gold nanoparticles could enhance the intracellular delivery of oncolytic adenoviruses into PC cell lines[162]. Radiofrequency hyperthermia also can enhance the local delivery of oncolytic immuno-

Table 3 Completed clinical trials with results for pancreatic cancer treatment

Trial number	Phase	Treatment	Condition
NCT01373164	1b/2	Galunisertib, a TGF-β receptor inhibitor, or placebo plus gemcitabine	Unresectable PC
NCT01423604	2	Ruxolitinib, a Janus kinase 1/2 inhibitor or placebo plus capecitabine	PC
NCT00965718	2	Activated T lymphocyte (*ex vivo*-expanded, CIK cells cultured with anti-CD3 monoclonal antibody and IL-2)	PC
NCT01204372	2	Gemcitabine, trastuzumab plus erlotinib	Metastatic PC
CONKO-005	3	Erlotinib (inhibits the intracellular phosphorylation of tyrosine kinase associated with the EGFR) or placebo plus gemcitabine	Primarily resectable PDAC after R0 resection
NCT02923921	3	Pegilodecakin (a pegylated recombinant human IL-10) plus folinic acid, fluorouracil, and oxaliplatin	Metastatic PDAC
NCT01494506	3	Liposomal irinotecan (it prevents the religation of the DNA strand by binding to the topoisomerase I-DNA complex.) or placebo plus 5-FU/LV	Metastatic PC
NCT01214720	3	Bevacizumab that acts by selectively binding circulating VEGF, thereby inhibiting the binding of VEGF to its cell surface receptors plus chemotherapy	Metastatic PC
NCT00428597	3	Sunitinib, an inhibitor of multiple receptor tyrosine kinases	Metastatic pancreatic neuroendocrine tumors
NCT01525550	4		

5-FU/LV: 5-Fluorouracil/leucovorin; CIK: Cytokine-induced killer; EGFR: Epidermal growth factor receptor; IL: Interleukin; PC: Pancreatic cancer; PDAC: Pancreatic ductal adenocarcinoma; TGF-β: Transforming growth factor beta; VEGF: Vascular endothelial growth factor.



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Figure 2 Recruiting studies for pancreatic cancer at different locations in the world. The studies are in Africa (4), South America (5), Europe (225), the Middle East (15), the United States (424), Canada (37), Mexico (7), Pacifica (25), Japan (15), East Asia (218), North Asia (5), and Southeast Asia (8).

virotherapy for PAAD *in vitro* and *in vivo*[163].

Furthermore, radiofrequency ablation may be applied to treat patients with PC who are unfit for surgery and includes endoscopic ultrasound-guided radiofrequency ablation[164] and endoluminal biliary radiofrequency ablation[165].

Table 4 Ongoing or recruiting clinical trials for pancreatic cancer treatment

Trial number	Phase	Treatments	Conditions
NCT03192462	1 or 2	Intravenous infusions of TAA-specific cytotoxic T lymphocytes	Pancreas cancer with metastatic, locally advanced unresectable, or resectable disease
NCT04637698	1 or 2	Oncolytic viral therapy (Type 2 Herpes simplex virus) expressing GM-CSF	Pancreatic cancer
NCT04247165	1 or 2	Dual checkpoint inhibition (nivolumab and ipilimumab) in combination with gemcitabine and nab-paclitaxel followed by immune-chemoradiation	Borderline resectable, locally advanced, or metastatic pancreatic cancer
NCT05141149	1 or 2	Anti-PAUF monoclonal antibody PBP1510 or in combination with gemcitabine	Advanced/metastatic pancreatic cancer
NCT04825288	1 or 2	Anti-IL-1α true human antibody XB2001 or in combination with ONIVYDE + leucovorin + 5-FU chemotherapy	Advanced pancreatic cancer
NCT03662412	1 or 2	Sirolimus, a selective inhibitor of mTOR	Advanced pancreatic cancer
NCT05131776	2 or 3	Concurrent EUS-guided intratumor injection of P-32 microparticles (OncoSil)	Locally advanced pancreatic carcinoma
NCT03941093	3	Neoadjuvant treatment with pamrevlumab or placebo in combination with either gemcitabine plus nab-paclitaxel or FOLFIRINOX	Locally advanced pancreatic cancer
NCT05529940	3	FOLFIRINOX	Resectable pancreatic cancer
NCT04969731	3	Adjuvant Immuncell-LC (Cytokine-induced killer cells) therapy combined with gemcitabine	Resectable pancreatic cancer
NCT04025840	4	Perioperative epidural block and/or dexamethasone	Resectable pancreatic cancer
NCT04217096	4	Paclitaxel liposome plus S-1, an oral anticancer drug that consists of tegafur, gimeracil, and potassium oteracil in a molar ratio of 1.0:0.4:1.0	Advanced metastatic pancreatic cancer as the first-line therapy

5-FU: 5-Fluorouracil; EUS; Endoscopic ultrasound; FOLFIRINOX: Folinic acid + fluorouracil + irinotecan + oxaliplatin; IL: Interleukin; GM-CSF: Granulocyte macrophage colony-stimulating factor; mTOR: Mammalian target of rapamycin; PAUF: Pancreatic adenocarcinoma up-regulated factor; TAA: Tumor-associated antigen.

CONCLUSION

PDAC is the most common type of PC in the clinic. Multiple factors induce PC development and progression, including but not limited to inflammation, fibrosis, angiogenesis, EMT, and proliferation of CSCs. A combined panel of serum markers is very helpful for PC diagnosis, which is essential for curable therapy. Although several mono or combined therapies have been approved by the FDA for PC treatment, the overall 5-year survival rate is still not promising. The development of novel immunotherapies such as oncolytic viruses-mediated treatments and CAR-T, combined therapies (neoadjuvant therapy plus surgery), and advanced delivery systems of immunotherapy will improve therapeutic outcomes and combat drug resistance in PC patients. More clinical trials are required to evaluate the efficacy of existing treatments and to find new potent therapies.

FOOTNOTES

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REVIEW

Bile acids and microbes in metabolic disease

Dhiraj Kumar Sah, Archana Arjunan, Sun Young Park, Young Do Jung

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Abstract

Bile acids (BAs) serve as physiological detergents that enable the intestinal absorption and transportation of nutrients, lipids and vitamins. BAs are primarily produced by humans to catabolize cholesterol and play crucial roles in gut metabolism, microbiota habitat regulation and cell signaling. BA-activated nuclear receptors regulate the enterohepatic circulation of BAs which play a role in energy, lipid, glucose, and drug metabolism. The gut microbiota plays an essential role in the biotransformation of BAs and regulates BAs composition and metabolism. Therefore, altered gut microbial and BAs activity can affect human metabolism and thus result in the alteration of metabolic pathways and the occurrence of metabolic diseases/syndromes, such as diabetes mellitus, obesity/hypercholesterolemia, and cardiovascular diseases. BAs and their metabolites are used to treat altered gut microbiota and metabolic diseases. This review explores the increasing body of evidence that links alterations of gut microbial activity and BAs with the pathogenesis of metabolic diseases. Moreover, we summarize existing research on gut microbes and BAs in relation to intracellular pathways pertinent to metabolic disorders. Finally, we discuss how therapeutic interventions using BAs can facilitate microbiome functioning and ease metabolic diseases.

Key Words: Bile acids; Metabolic diseases; Gut microbe; Diabetic mellitus; Obesity; Hypercholesterolemia

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Core Tip: Bile acids (BAs) in enterohepatic circulation regulate metabolism through interorgan communication between the gut and liver microbiota. BAs secreted from the liver contribute to glucose and lipid metabolism. Disruption of the BA-gut microbiome link contributes to the occurrence of metabolic diseases, such as obesity, type 2 diabetic mellitus, and dyslipidemia. BAs and their metabolites can be used as potential therapeutics for treating metabolic diseases.

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INTRODUCTION

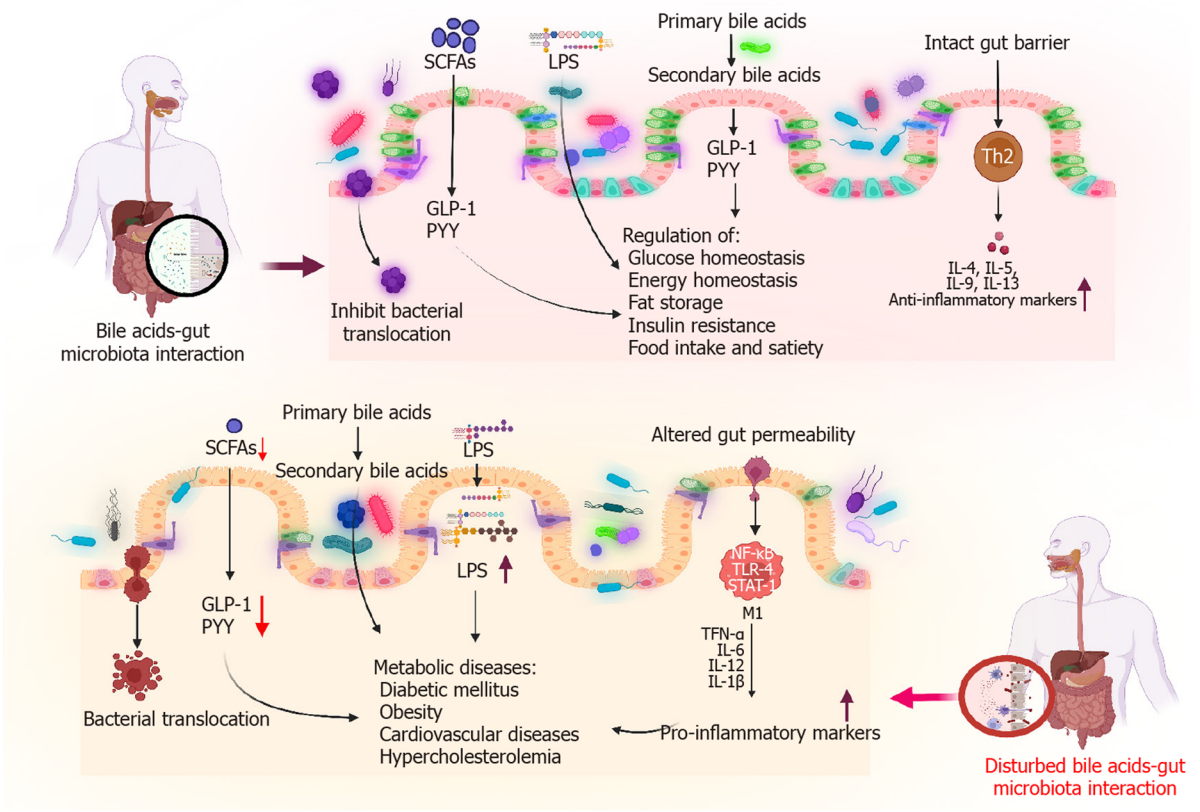
Bile acids (BAs) are unique amphipathic molecules that are primarily produced in the liver. They function as physiological detergents to facilitate bile flow and promote the transportation of nutrients, vitamins, and lipids *via* intestinal absorption[1]. Hepatic BA production accounts for a significant portion of the total cholesterol turnover in humans[2]. The principal constituents of bile are BAs, bilirubin, cholesterol, and phospholipids. BAs are mainly classified into primary and secondary types. Primary BAs (PBAs) include cholic acid (CA) and chenodeoxycholic acid (CDCA). Their corresponding secondary BAs are deoxycholic acid (DCA) and lithocholic acid (LCA), which are produced by microbial enzymes in the colon *via* deconjugation and 7α-dehydroxylation and are the most ubiquitous BAs in humans[3]. PBAs are formed by cholesterol in pericentral hepatocytes through a series of staged processes that are catalyzed by metabolic enzymes, particularly cytochrome P450 enzymes[4].

BAs synthesis is predominantly mediated by classic and alternative pathways in the liver. In the classic pathway, the rate-limiting enzyme CYP7A1 in the endoplasmic reticulum converts cholesterol into 7α-hydroxycholesterol (HOC). The intermediate 7α-hydroxy-4 cholesterin-3-one (C4) is converted by the sterol 12′α-hydroxylase (CYP8B1) to 7′α, 12′α-dihydroxy-4-cholesterin-3-one, which results in the production of CA. Without the 12α-hydroxylation of CYP8B1, C4 is ultimately transformed into CDCA. Both CA and CDCA syntheses use the mitochondrial enzyme CYP27A1 to catalyze the oxidation of the steroid side chains. In the alternative pathway, cholesterol is transformed by CYP27A1 into 27-HOC, which is in turn transformed into CDCA. Bacterial 7-dehydroxylase eliminates a hydroxyl group at C-7 in the large intestine, which converts CA into DCA and CDCA into LCA. The secondary BAs hyocholic acid, murideoxycholic acid, α-muricholic acid (ω-MCA), hyodeoxycholic acid (HDCA), and ursodeoxycholic acid (UDCA) are produced by CYP3A1 and epimerases from CDCA. The majority of LCA and ω-MCA are eliminated *via* feces[5].

In addition to their involvement in the absorption of dietary lipids and cholesterol homeostasis, BAs play a versatile signaling role. Many signaling pathways can be activated by BAs. These include a wide range of metabolic pathways, such as those involved in glucose, lipid, drug, and energy metabolism[6]. During BAs metabolism, cholesterol is converted into BAs in the liver and is further metabolized by the gut microbiota. Moreover, dense populations of microorganisms inhabit the gut, making it one of the most complex ecosystems for health. For the past two decades, research has focused on the influence of the gut microbiome on health. BAs deconjugation occurs in the small intestine and is mediated by bile salt hydrolase (BSH)-active bacteria, resulting in the maintenance of normal circulating levels of deconjugated BAs and cholesterol. Through these bioconversions, BAs modulate diverse metabolic pathways in the host through signaling mediated by nuclear farnesoid X receptors (FXRs) and G-protein-coupled membrane receptors (GPCRs). Furthermore, BAs can influence the gut microbial composition both directly and indirectly by activating innate immune responses. Consequently, the host metabolism is affected by altered signaling *via* BA receptors (BARs) induced by microbial modification and by altered microbiota composition[7]. Therefore, the gut microbiota must be maintained for normal metabolic function and homeostasis. Altered gut microbiota composition may be related to metabolic diseases, such as diabetes mellitus (DM) and obesity[8]. Altered BAs synthesis and function are also associated with metabolic diseases. This review mainly focuses on the relationship between gut microbiota and BAs in metabolic diseases, emphasizing on the BA-mediated reversal of metabolic diseases (Figure 1).

ROLE OF BILE ACIDS-GUT MICROBIOME INTERACTION IN METABOLIC REGULATION

The term microbiome refers to the entire genome of the gut microbiota, which is two orders of magnitude larger than the human nuclear genome. Humans inherit the vaginal microbiome of their mothers at the time of birth. Eventually, a mutualistic relationship between this microbiota and the host



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Figure 1 Graphical abstract. GLP-1: Glucagon-like peptide-1; LPS: Lipopolysaccharide; PYY: Peptide YY; SCFAs: Short-chain fatty acids.

is developed[9]. The human gastrointestinal system is colonized by numerous microorganisms, collectively known as the gut microbiota; these include bacteria, viruses, archaea, fungi, and protozoa. The human gut microbiota contains up to 100 trillion microorganisms[10]. It plays an integral role in maintaining host health as it not only helps derive nutrients from food but also builds various metabolites that can regulate host metabolism[11]. One of these metabolites, BA, is produced in the liver through cholesterol and is additionally metabolized by the gut microbiota into secondary BAs[12]. A key physiological function of the gut microbiota is the modification of BAs composition. In addition to secondary BAs, different BAs species are produced in humans by the gut microbiota[13]. In the gut, branched-chain amino acids, short-chain fatty acids (SCFAs), indole, succinate, and imidazole are metabolites produced by gut microbes during anaerobic fermentation. These metabolites serve as key signaling components in the BA-gut microbe signaling pathways[14]. Various microbial genera produce these metabolites; these include *Akkermansia*, *Bacteroides*, *Clostridium*, *Coprococcus*, *Eubacterium*, *Faecalibacterium*, *Fusobacterium*, *Lactobacillus*, *Prevotella*, *Propionibacterium*, *Ruminococcus*, *Roseburia,* and *Streptococcus*[15]. The BAs composition is shaped by gut microbes that exhibit certain enzymatic activities, *e.g.*, BSH or 7-dehydroxylation activity mediated by BA-inducible enzymatic reactions. BAs exert their effects by activating a class of receptors known as BARs. This receptor family comprises nuclear receptors, such as FXRs, vitamin D receptors, pregnane X receptor, and GPCRs (including GPBAR1)[13]. The gut microbiota modulates fibroblast growth factor 15 (FGF15) signaling through an FXR-dependent mechanism[16]. Recent research has linked gut microbe metabolism to the size of the BAs pool.

In germ-free (GF) and conventionally raised mice, the gut microbiota could not only regulate secondary BAs metabolism but also inhibit hepatic BA synthesis by suppressing FXR inhibition in the ileum[16]. Moreover, BAs can affect the gut bacterial composition by directly and indirectly activating genes associated with innate immunity in the small intestine[7]. Therefore, bacteria-induced changes in BAs may result in altered signaling of BARs and affect host metabolism. BAs and the gut microbiota can interact in various ways, and interruptions in these physiological interactions can cause several diseases. The composition of the intestinal microbiota and/or the intraluminal metabolome may be the cause or consequence of various disorders; however, their association remains unknown. Various recent studies have reported the association of dyslipidemia, insulin resistance (IR), and DM with the dysregulation of BAs metabolism and alteration of the gut microbiota. This review mainly focuses on altered BA-gut microbiome interactions in metabolic diseases.

METABOLIC DISEASES CAUSED BY DISRUPTION OF BILE ACIDS-GUT MICROBIOME INTERACTIONS

Research on BA has significantly enriched our understanding of BAs synthesis and metabolic syndrome over the last two decades. BAs play a crucial role in regulating glucose, lipid, and energy metabolism. Several metabolic diseases, including type 2 DM (T2DM), obesity, and nonalcoholic fatty liver disease (NAFLD), result from disrupted BA homeostasis[17]. The gut microbiota is a "metabolic organ" that regulates host metabolism[18]. Gut microbes, including *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterobacter*, and *Lactobacillus* species, play an important role in the synthesis, modification, and signaling of BAs[15]. The gut microbiota has recently been reported to play a role in obesity, in addition to other widely acknowledged major causes, which include an increased caloric intake and decreased energy expenditure. These factors are also linked to T2DM, metabolic syndrome, and CADs[19]. Diverse mechanisms have been proposed by which gut microbes can modulate metabolic diseases. Disrupted BA-gut microbiome interactions can cause metabolic disease (Table 1 and Figure 2).

OBESITY

The global prevalence of various chronic diseases is increasing; obesity is the main cause and has been a serious concern for decades[20]. Obesity is linked to T2DM, NAFLD, hypertension, CAD, and cancer [21]. The prevalence of obesity is influenced by genetic and environmental factors, such as diet, culture, and socioeconomic status[22]. There is mounting evidence that the intestinal microbiota is inextricably related to general health, including obesity risk. Obesity-related metabolic diseases are defined by unique changes in the diversity and function of the human gut microbiome[23]. The human gut is home to trillions of microbes, which break down otherwise indigestible foods[24]. A study revealed that transferring the gut microbiota from healthy mice to GF recipients could increase body fat without a significant increase in food consumption and suggested that the composition of gut microbial communities could affect how much energy is derived from food[25]. In particular, the gut–brain axis indirectly affects commensal organisms, intestinal permeability, motility, and secretion and modifies the levels of plasma peptides, particularly glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), by releasing signaling molecules into the gut lumen[26].

In humans, the gut microbiota has been linked to body weight and weight loss following a lifestyle change. Gram-negative opportunistic pathogens in the gut may play a significant role in obesity[27]. The gut microbiota of ectomorphs has more *Bacteroidetes* species, whereas that of obese individuals has more *Firmicutes* species, particularly *Clostridium* clusters[23]. Thus, the bacterial composition could enhance the capacity of the host to absorb energy from their diet and retain it in adipose tissues[28]. In lean as well as obese pregnant subjects, an increase in *Bacteroides*, *Staphylococcus*[29], and *Bifidobacterium* species was found to increase high-density lipoprotein cholesterol (HDL-C) and folic acid levels and reduce triglyceride (TG) levels[22]. On the other hand, the abundance of *Akkermansia muciniphila* (*A. muciniphila*), a mucoprotein-degrading bacterium present in the mucus layer[30], was negatively correlated with body weight[22]. A decrease in the abundance of *A. muciniphila* was noted in obese and diabetic mice[31]. Feeding high-fat diets with viable *A. muciniphila* can hinder the development of metabolic disorders, such as obesity, low-grade inflammation, and metabolic endotoxemia[32]. A metatranscriptomic analysis revealed that mice receiving the microbiome of obese twins had higher expression levels of microbial genes associated with detoxification and oxidative stress, amino acid metabolism, cobalamin biosynthesis, and the pentose phosphate pathway[13].

BA metabolism is altered in obese and diabetic individuals[33]. Patti *et al*[34] reported that patients who underwent Roux-en-Y gastric bypass (RYGB) had improved glucose and fat metabolism. This finding was attributed to the activation of GPCRs and subsequent stimulation of GPBAR1 (TGR5, a membrane-bound BAR) and increase in deiodinase (a type II thyroid hormone) levels. Although recent research has revealed a link between the gut microbiota and obesity, the precise molecular pathways remain unknown. In particular, the role of distinct gut microbial species and their metabolites in the regulation of obesity-related lipid metabolism and formation of the obese phenotype remains unknown. Mechanisms linking the gut microbiota to obesity are being revealed through a collaborative approach of translation-focused human and animal studies. Increasing evidence indicates that the gut microbiota mediates the effects of diet on host metabolism[35]. In BA metabolism, TGR5 signaling is regulated by the microbiota by generating agonists[36], whereas FXR signaling is regulated by metabolizing antagonists[3]. Both TGR5 and FXR have a significant influence on metabolism, and an altered microbiota may impact host physiology by modifying the signals transmitted through these receptors. The ability to metabolize TauroMCA, a naturally occurring FXR antagonist, is required for the microbiota to induce obesity, steatosis, and impaired glucose and insulin tolerance. An altered microbiota is responsible for these effects[37]. Taken together, these results indicate that targeting BAs, which function as microbiome-produced molecular regulators of energy homeostasis, can offer a substantial opportunity for treating obesity.

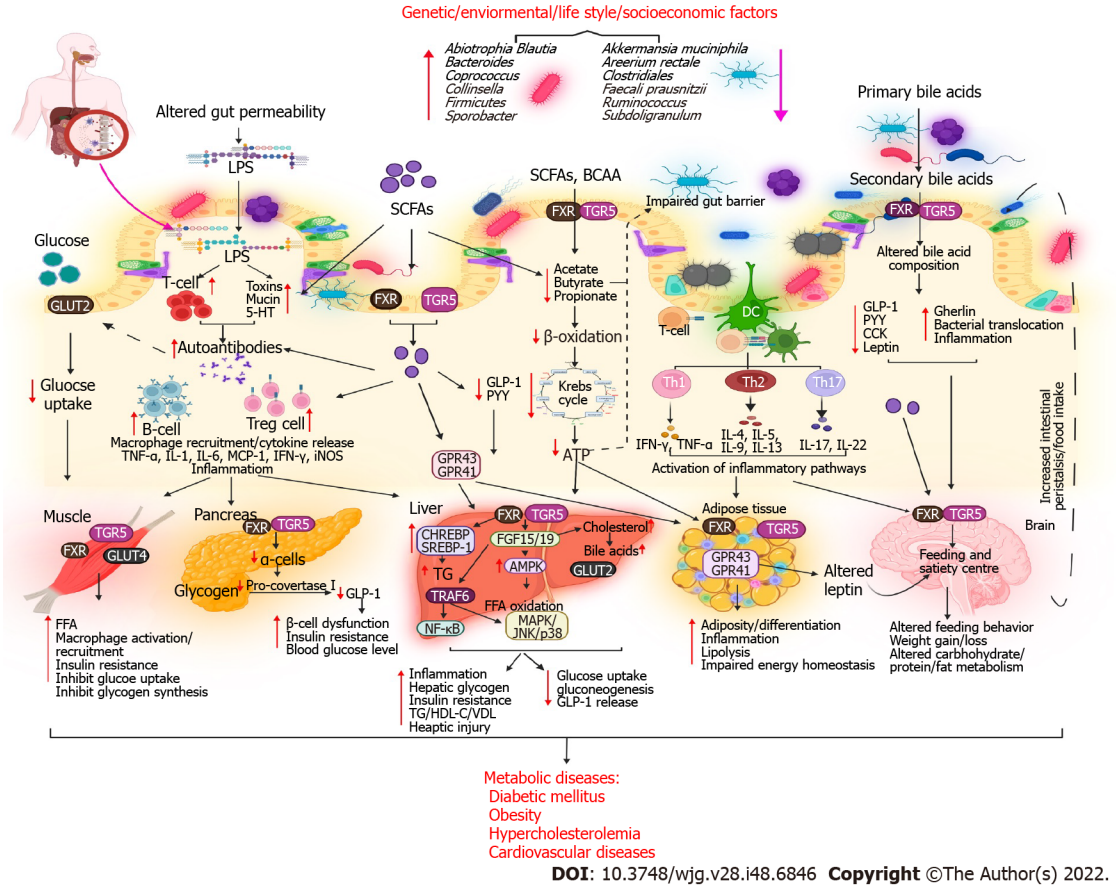
Table 1 Metabolic diseases caused by altered bile acid–gut microbiome interactions

No.	Model	Findings	Ref.
1	A T1DM clinical study	The abundance of *Alistipes shahii*, *Asaccharobacter celatus*, *Blautia obeum*, *Coprococcus eutectic*, *Coprobacillus cateniforms*, *Clostridium symbiosum*, and *Eggerthella lenta* significantly increased in adolescents with T1DM. Compared with healthy adolescents, the biosynthesis of vitamins, amino acids, electron carriers, and enzyme cofactors was downregulated, whereas fermentation pathways were upregulated in adolescents with T1D	[150]
2	An HFD-fed obese mouse model	Non-12-OH BA levels were higher in HF-OR mice. The levels of non-12-OH BASs, such as UDCA, CDCA, and LCA, decreased in HF-OP mice and were linked to changed gut flora. The abundance of *C. scindens* were reduced in HF-OP mice and positively correlated with UDCA and LCA. The administration of *C. scindens* to animals increased the levels of hepatic non-12-OH BAs and serum 7-hydroxy-4-cholesterin-3-one (C4). Changes in BA composition in HF-OP mice were associated with considerably lower GLP-1 expression levels in the ileum and PGC1 and UCP1 expression levels in brown adipose tissues	[18]
3	Patients with GDM and germ-free mice	The abundance of *Bacteroides and Akkermansia* decreased and that of *Faecalibacterium* increased with hyperglycemia	[151]
4	Women with GDM: A clinical study	The relative abundance of *Streptococcus*, *Faecalibacterium*, *Veillonella*, *Prevotella*, *Haemophilus*, and *Actinomyces* significantly increased with an increase in FBG levels and hyperlipidemia	[51]
5	A combination of BAs with dietary lard feeding in C57BL/6N mice	Impaired glucose tolerance; lower fasting insulin levels; lower counts of enteroendocrine cells; fatty liver; and elevated levels of hepatic TGs, cholesteryl esters, and monounsaturated fatty acids were noted. The relative abundance of *Lachnospiraceae* decreased and that of *Desulfovibrionaceae*, *Clostridium lactatifermentans*, and *Flintibacter butyricus* increased	[152]
6	A T2DM clinical study	Postprandial total BAs levels increased with an increase in the meal fat content and peaked after 1-2 h. Unconjugated and glycine-conjugated forms of DCA, CA, and UDCA were altered and FGF-19 levels were reduced in participants with T2DM	[153]
7	HFD-fed C57BL/6J mice with *Enterobacter cloacae* B29	Obesity and IR were induced	[45]
8	A T2DM clinical study	BAs increased twofold, and more hydrophobicity and higher 12α-hydroxy/non-12α-hydroxy BAs ratios were linked with lower insulin sensitivity and higher plasma TG levels	[154]
9	C57BL/6J ob/ob mice, lean ob/$^{+}$, and HFD-fed mice	The abundance of *A. muciniphila* decreased in mice who were obese and had T2DM	[32]
10	A clinical study	The postprandial total bile acid response decreased in obese participants	[155]
11	Pregnancy with obesity: A clinical study	The abundance of *Bifidobacterium* and *Bacteroides* decreased and that of *Staphylococcus,* Enterobacteriaceae, and *E. coli* increased in overweight pregnant women compared with that in normal-weight pregnant women. The abundance of *E. coli* was higher in women with excessive weight gain than in those with normal weight gain during pregnancy. *Bifidobacterium* and *A. muciniphila* showed an opposite trend. The abundance of total bacteria, *Staphylococcus*, *Bacteroides*, *Bifidobacterium*, Enterobacteriaceae, and *E. coli* increased and that of *Bifidobacterium* decreased	[22]
12	ApoA-1-knockout mice, HFD-fed mice, and wild-type mice	Gut barrier-protecting *Bifidobacterium* species were absent, and impaired glucose tolerance was significantly increased	[27]
13	Zucker rats (obese/lean)	Increased numbers of *Halomonas* and *Sphingomonas* species, plasma LDL and VLDL levels, and reduced urinary hippurate and creatinine levels were noted in obese rats	[156]
14	Overweight pregnant women: A clinical study	Increased numbers of *Bacteroides* and *Staphylococcus* species were noted in obese pregnant women	[29]
15	HFD-fed mice	The abundance of intestinal gram-negative and gram-positive bacteria and *Bifidobacterium* species significantly decreased and endotoxemia significantly increased	[146]
16	C57BL/6J ob/ob mice, lean ob/$^{+}$, and wild-type mice	A 50% reduction was noted in the abundance of *Bacteroidetes*, and an increase was noted in the abundance of *Firmicutes*	[157]

ApoA-1: Apolipoprotein A-1; *A. muciniphila*: *Akkermansia muciniphila*; BAs: Bile acids; BASs: Bile acid sequestrants; C4: 7α-hydroxy-4-cholesten-3-one; CA: Cholic acid; CDCA: Chenodeoxycholic acid; *C. scindens*: *Clostridium scindens*; DCA: Deoxycholic acid; *E. coli*: *Escherichia coli*; FGF-19: Fibroblast growth factor 19; FBG: Fasting blood glucose; GDM: Gestational diabetes mellitus; GLP-1: Glucagon-like peptide-1; HFD: High-fat diet; LCA: Lithocholic acid; LDL: Low-density lipoprotein; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; TGs: Triglycerides; UDCA: Ursodeoxycholic acid; VLDL: Very-low-density lipoprotein.

DM

The prevalence of T2DM is increasing worldwide. By 2030, the incidence of T2DM is expected to be 360 million worldwide, with the estimated population being 8.5 billion[38]. BAs are involved in the alteration of glucose metabolism associated with obesity and T2DM. By stimulating GLP1 synthesis in



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Figure 2 Metabolic diseases resulting from altered bile acid–gut microbiome interactions. The health and gut microbiota of individuals are affected by genetic, environmental, lifestyle, and socioeconomic factors. Due to altered gut microbial composition, the permeability of the gut barrier is impaired, facilitating pathogen invasion in the intestinal lumen *via* receptor-mediated pathways. Increased levels of lipopolysaccharide and short-chain fatty acids trigger the immune system, result in the production of autoantibodies (B cells and Treg cells), and cause the infiltration of macrophages, which release toxins and lead to inflammation and metabolic endotoxemia. Conversely, the release of glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and ghrelin is decreased, altering carbohydrate, protein, and fat metabolism. When glucose uptake is decreased in the intestine, free fatty acid synthesis is increased, macrophages are activated, and muscles become insulin resistant. When circulating autoantibodies enter the pancreas, they destroy α and β cells, thereby reducing insulin secretion and enhancing insulin sensitivity. A decrease in FGF15/19, CHREBP, SREBP-1, and TRAF6 expression levels in the liver activates NF-κB/MAPK inflammatory pathways, resulting in liver dysfunction and altered fat metabolism in the liver. Proinflammatory markers and reduced GLP-1, PYY, and ghrelin levels affect adipose tissue function, leading to increased adiposity and impaired energy metabolism. In the brain, altered levels of GLP-1, PYY, ghrelin, and leptin affect feeding and satiety centers. These changes lead to the development of metabolic diseases. FFA: Free fatty acid; GLP-1: Glucagon-like peptide-1; SCFAs: Short-chain fatty acids; LPS: Lipopolysaccharide; PYY: Peptide YY; CCK: Cholecystokinin; FXR: Farnesoid X receptor; CCK: Cholecystokinin; TG: Triglycerides; HDL-C: High-density lipoprotein cholesterol; VLDL: Very-low-density lipoprotein.

the small bowel and colon, BAs contribute to carbohydrate and fat metabolism. In addition to inducing IR and T2DM, BAs exhibit an insulin-sensitizing effect[33]. They control glucose homeostasis by directly acting on FXR and TGR5 in the liver, intestine, and pancreas and by indirectly stimulating FXR-dependent intestinal FGF15/19 production[39]. Both FXR and TGR5 are abundant in pancreatic b cells, where they favorably control insulin production and glucose-induced insulin secretion. TGR5 activation in pancreatic α cells promotes the expression of proconvertase-1, which shifts the synthesis of glucagon to GLP-1, thereby enhancing β-cell density and functioning in a paracrine manner. In patients with DM, BAs may promote FXR activation in L cells in the ileum. In an animal model of obesity, Cipriani *et al*[40] found that 6E-CDCA activated FXR, reversed IR, and restored lipid metabolism. Moreover, the microbiota could downregulate FXR with the maximum efficiency by converting PBAs into secondary BAs[16].

External factors, such as diet, can alter the gut microbiota and cause dysregulation and secretory changes in intestinal microbial metabolites, triggering a series of possible mechanisms that lead to DM and insulin sensitivity[41]. In a metagenome-wide association study involving 345 Chinese participants with DM, gut microbial dysbiosis caused by opportunistic pathogens was moderate. Moreover, the reduction of butyrate-producing bacteria was associated with sulfate reduction and oxidative stress resistance[42]. Complex interactions between the immune system and gut microbiome have also been linked to both T1DM and T2DM. Aggarwal *et al*[43] reported that a combination of antidiabetic and antibiotic treatments reversed IR, hyperglycemia, and dyslipidemia and normalized blood glucose utilization in iNOS$^{-/-}$ mice. Duodenal-jejunal bypass liner (DJBL) replacement in obese patients with

T2DM was found to increase unconjugated BA levels in a clinical study[44]. Fei *et al*[45] reported a high percentage of endobacteria (35%), pathogenic bacteria that produce endotoxin, in the gut microbiome of obese participants with hypertension, DM, and other severe metabolic complications. Patients with T2DM are particularly deficient in butyrate-producing microbes, such as *Clostridiales* species, *Ruminococcus* species, *Subdoligranulum* species, *Areerium rectangle*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, and *R. inulinivorans*, and exhibit a high abundance of specific genera, such as *Abiotrophia*, *Blautia*, *Coprococcus*, *Collinsella*, *Parasutterella*, *Peptostreptococcus*, and *Sporobacter*[15].

Moreover, Lambeth *et al*[46] demonstrated that *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Pseudonocardiaceae*, *Verrucomicrobia*, and *Colorado* species were significantly more prevalent in the pre-DM stage, whereas *Enterobacteriaceae* and *Collinsella* species were significantly more prevalent in patients with T2DM. Similarly, Larsen *et al*[47] reported a significant decrease in the prevalence of phylum *Firmicutes*, class Betaproteobacteria, and genus *Clostridium* in patients with T2DM. The *Bacteroidetes*: *Firmicutes* ratio and the *Bacteroides-Prevotella* group: *C. coccoides-E. rectale* group ratio showed a positive correlation and significantly increased the plasma glucose levels. During pregnancy, the abundance of the beneficial species *R. intestinalis* and *F. prausnitzii* decreases, whereas that of Proteobacteria and Actinobacteria phyla increases[48]. If these compositions are altered, pregnant women may experience an increase in adipose mass, blood sugar levels, IR, and circulating proinflammatory cytokines, resulting in gestational DM (GDM)[49]. In patients with GDM, obesity, and T2DM, the relative abundance of SCFA-producing bacteria belonging to the genera *Faecalibacterium*, *Rubinococcus*, *Roseburia*, *Coprococcus*, *Akkermansia*, *Phascolarctobacterium*, and *Eubacterium* was found to decrease[50]. Moreover, Liu *et al*[51] demonstrated increased hyperlipidemia and fasting blood glucose (FBG) levels and increased relative abundance of *Streptococcus*, *Faecalibacterium*, *Veillonella*, *Prevotella*, *Haemophilus*, and *Actinomyces* species in patients with GDM.

THERAPEUTIC INTERVENTIONS TARGETING BILE ACIDS-GUT MICROBIOME INTERACTIONS TO ALLEVIATE METABOLIC DISEASES

BAs play an important role in signaling and metabolism, reigniting interest in these molecules as potential therapeutic targets. Studies have revealed that drugs used to treat metabolic diseases can alter the gut microbial environment. Similarly, antidiabetic medications may alter the composition of the gut microbiota, plasma, and fecal BAs, which may improve metabolic health. Notably, patients with T2DM had better glycemic control when taking medications for preventing BAs absorption from the small intestine or limiting enterohepatic circulation. Hence, experimental and clinical studies have focused on the therapeutic applications of BAs in metabolic diseases. Furthermore, microbiota targeting could open novel research avenues. Table 2 and Figure 3 depict BAs and their metabolites used for treating metabolic diseases.

BAS METABOLITES ALLEVIATE METABOLIC DISEASES

BA sequestrants

For several years, BA sequestrants (BASs) have been utilized as therapeutics for patients with dyslipidemia and T2DM[52]. The BA-binding properties of BASs reduce the amount of BAs in enterohepatic circulation, thereby accelerating the conversion of cholesterol to BAs[53]. The effects of BASs on hyperglycemia have been demonstrated in both animal and clinical models of T2DM[54]. Furthermore, animal studies have revealed that BAs and BASs influence energy expenditure. A BAS molecule, also known as a resin, is a large, nonabsorbable polymeric molecule that binds negatively charged bile salts to the intestinal lining[55]. This promotes BA excretion through the feces by diverting the acids from the enterohepatic cycle. Consequently, BA synthesis increases and low-density lipoprotein (LDLR) receptors are upregulated. BASs can lower blood glucose as well as cholesterol levels, which may be beneficial for treating T2DM[54].

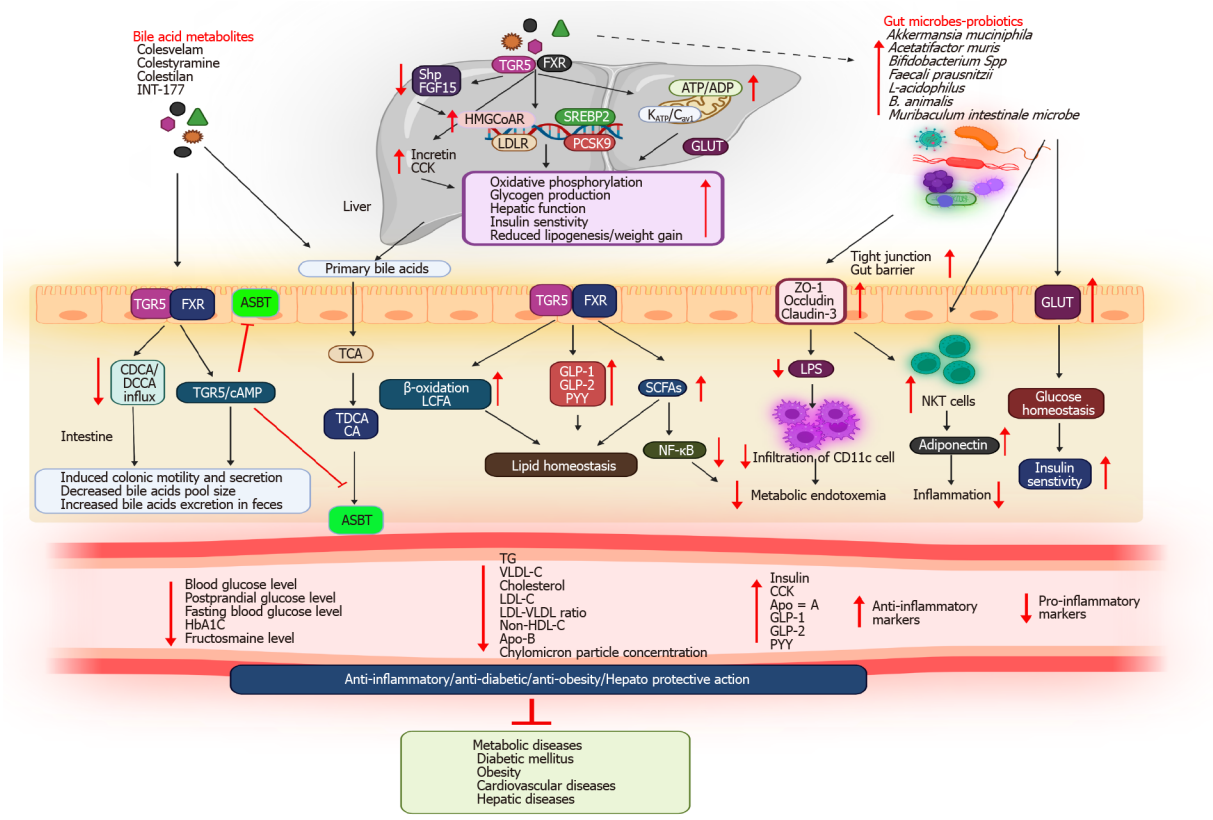
BASs can exert their metabolic effects beyond cholesterol-lowering effects through several mechanisms, including GLP-1, the FXR-small heterodimer partner-liver X receptor pathway, and TGR5 [56]. BAS reduces intestinal FXR activity by trapping BAs in the lumen, resulting in decreased expression levels of ileal *Shp* and *Fgf15*[57]. The resulting decrease in the hepatic accessibility of BA and FGF15/19 leads to the deactivation of hepatic FXR and the CYP7A1-mediated conversion of cholesterol to BA, reducing LDL cholesterol (LDL-C) levels. Consequently, lipogenesis is attenuated by FXR. BAS raises plasma TG levels and accumulates hepatic lipids, while lowering LDL-C levels[58]. However, the exact mechanism by which BASs exert their metabolic effects beyond cholesterol-lowering effects remains unknown. Rectal administration of taurocholic acid (TCA) was found to increase GLP-1 and PYY production in obese participants and those with T2DM[59]. Similarly, CDCA and colesevelam increased glycogen and GLP-1 levels and delayed stomach emptying in patients with T2DM[60].

Table 2 Drugs that target bile acids and gut microbes to alleviate metabolic diseases

No.	Drugs	Model	Findings	Ref.
1	A combination of GLP-1 and DMR	An insulin-dependent T2DM clinical study	Combined treatment allowed the discontinuation of insulin treatment in 69% of patients, increased postprandial unconjugated bile acid responses, induced an overall increase in the secondary bile acid response, induced an increase in the 12α-hydroxy: non-12α-hydroxy BA ratio, and improved the microbiome response	[158]
2	Colesevelam	Germ-free C57BL/6 mice with obesity, NAFLD, and NASH	Reduced body and liver weight gain were noted in microbiome-humanized mice, in addition to the amelioration of hepatic inflammation, steatosis, fibrosis, and IR. Colesevelam increased *de novo* bile acid synthesis and reduced the hepatic cholesterol content in microbiome-humanized mice, induced the expression of the antimicrobial genes *Reg3g* and *Reg3b* in the distal small intestine, and reduced plasma LPS levels	[159]
3	Vancomycin	iNOS$^{-/-}$ mice	Metabolic disturbances, dyslipidemia, and insulin resistance in iNOS$^{-/-}$ mice were improved by the vancomycin-mediated reduction of gram-positive bacteria	[160]
4	Sevelamer	Western diet-fed C57BL/6J mice with NASH	Interruption of intestinal reabsorption and reduction of circulating bile acid levels were noted. Microbiota complexity in the cecum was reversed by increasing the abundance of *Lactobacillus* and decreasing the abundance of *Desulfovibrio*. Hepatic injury was reversed, and the progression of NASH, including steatosis, inflammation, and fibrosis, was inhibited	[161]
5	Sevelamer	CDHF-fed C57BL/6J mice	Hepatic steatosis, macrophage infiltration, and pericellular fibrosis were prevented in CDHF-fed mice. The portal levels of total bile acid were reduced, and hepatic and intestinal FXR activation was inhibited. The α-diversity was decreased, and decreases in *Lactobacillaceae* and *Clostridiaceae* populations and increases in *Desulfovibrionaceae* and *Enterobacteriaceae* populations were prevented in CDHF-fed mice. Intestinal tight junction proteins were restored and portal LPS levels were reduced, resulting in the suppression of the hepatic toll-like receptor 4 signaling pathway	[162]
6	*B. animalis* 01	A T2DM rat model	Treatment with *B. animalis* 01 improved OGTT, HOMA-IR, and lipid profiles; reduced hepatic tissue injury; increased glycogen levels; improved antioxidant levels; and modulated the expression of genes involved in hepatic glucose metabolism and the IRS/PI3K/AKT pathway. Moreover, it positively regulated the hepatic Keap1/Nrf2 pathway	[141]
7	*A. muciniphila*	Overweight/obese insulin-resistant volunteers	*A. muciniphila* improved insulin sensitivity; reduced insulinemia and plasma total cholesterol levels; and slightly reduced body weight, fat mass, and hip circumference. Three months after supplementation with *A. muciniphila*, liver dysfunction and inflammatory blood marker levels decreased without affecting the gut microbiome structure	[132]
8	*Bacteroides* transplantation	A clinical study on children with diabetes/germ-free NOD mice	Compared with germ-free NOD mice, the onset of diabetes was markedly delayed in all bacteriome-humanized participants	[163]
9	*A. muciniphila*	C57BL/6 mice/HFD-fed mice	*A. muciniphila* treatment reversed HFD-induced fat mass gain, metabolic endotoxemia, adipose tissue inflammation, and IR. *A. muciniphila* supplementation increased the intestinal levels of endocannabinoids that control inflammation, the gut barrier, and gut peptide secretion	[32]
10	Acarbose	A clinical study	The ratio of primary: secondary BAs and plasma levels of unconjugated BAs were increased. The relative abundance of *Lactobacillus* and *Bifidobacterium* species in the gut microbiota was increased and *Bacteroides* species were depleted in participants with T2DM	[164]
11	Metformin	A clinical study	Metformin-altered microbiota improved glucose tolerance, and a significant negative correlation was noted between unconjugated BAs and HbA1c levels	[165]
12	*Lactobacillus acidophilus*, *L. casei*, and *Bifidobacterium bifidum* for 6 wk	GDM: A clinical study	Significant reductions were noted in fasting plasma glucose, serum insulin, serum triglyceride, and VLDL cholesterol levels and a significant increase was noted in the quantitative insulin sensitivity check index in women with GDM	[119]
13	Probiotics	Cherry Valley Pekin ducks	The LXRα and CYP7α1 enzymatic activity increased and TG and TC concentrations decreased	[123]
14	Probiotics (*Lactobacillus salivarius* UCC118)	GDM: A clinical study	The body weight, FBG, and IR index significantly decreased and insulin sensitivity index increased in women with GDM	[166]
15	Probiotics (*Lactobacillus salivarius* UCC118)	Obese pregnant women: A clinical study	Significant alteration was noted in the BMI	[167]
16	Probiotic *Lactobacillus sporogenes*	Third-trimester pregnancy: a Clinical study	A significant decrease was noted in serum insulin levels and HOMA-IR, and a significant difference was noted in HOMA-B	[168]
17	Probiotics (VSL#3)	C57J/B6 male mice/HFD-fed mice	Probiotic supplementation reduced the body weight IR; modulated the gut microbe composition; and increased GLP-1 release, glucose tolerance, SCFA levels, and butyrate levels	[121]
18	Probiotics	Pregnant women: A	A significant reduction was noted in serum total LDL, HDL cholesterol, serum TG, and	[122]

		clinical study	serum TC levels	
19	Fecal microbiota transplantation	Male Caucasian obese participants	Improvement in peripheral and hepatic insulin sensitivity was noted, along with an increase in butyrate-producing intestinal microbiota	[126]
20	Probiotics	Obese (ob/ob) mice	An increase in the abundance of *Bifidobacterium* species reduced metabolic endotoxemia and inflammation. Intestinal permeability was lowered by altering GLP-2 levels	[147]
21	Probiotics (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12; diet/probiotics)	First-trimester pregnancy: A clinical study	Reduced blood glucose and insulin levels, improved glucose tolerance, and the highest quantitative insulin sensitivity check index were noted	[169]

A. muciniphila: *Akkermansia muciniphila*; BAs: Bile acids; *B. animalis*: *Bifidobacterium animalis*; BMI: Body mass index; CDHF: Choline-deficient high-fat diet; DMR: Duodenal mucosal resurfacing; FBG: Fasting blood glucose; FXR: Farnesoid X receptor; GDM: Gestational diabetes mellitus; GLP-1: Glucagon-like peptide-1; HFD: High-fat diet; HbA1c: Hemoglobin A1C; HDL: High-density lipoprotein; HOMA: Homeostatic model assessment; IR: Insulin resistance; *L. casei*: *Lactobacillus casei*; LDL: Low-density lipoprotein; LPS: Lipopolysaccharide; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; SCFAs: Short-chain fatty acids; T2DM: Type 2 diabetes mellitus; TC: Total cholesterol; TGs: Triglycerides; VLDL: Very-low-density lipoprotein.



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Figure 3 Mechanism by which bile acid metabolites and gut microbes alleviate metabolic diseases. Bile acid metabolites, *e.g.*, bile acid sequestrants (BASs), enter the liver *via* receptor-mediated pathways. As a result of their effects, mitochondrial oxidative phosphorylation is stimulated; *HMGCoAR*, *LDLR*, and *SREBP2* gene expression is induced; incretin and cholecystokinin levels are increased; and the abundance of intestinal bacteria is increased, reducing the levels of triglyceride (TG), very-low-density lipoprotein (VLDL), and cholesterol in the blood. By increasing the expression of ZO-1, occludin, and claudin-3 in the gut lumen, probiotics facilitate tight junction proteins, preventing macrophage infiltration and metabolic endotoxemia. In contrast, BAS improves colonic motility; increases glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and ghrelin secretion; and regulates carbohydrate, fat, and energy metabolism, thereby reducing blood glucose, TG, cholesterol, and VLDL levels and improving insulin sensitivity and liver function. Because of their anti-inflammatory, antidiabetic, and antiobesity properties, BASs and gut microbes alleviate metabolic diseases. CDCA: Chenodeoxycholic acid; TDCA: Taurodeoxycholic acid; GLP-1: Glucagon-like peptide-1; SCFAs: Short-chain fatty acids; LPS: Lipopolysaccharide; PYY: Peptide YY; FXR: Farnesoid X receptor; CCK: Cholecystokinin; TG: Triglycerides; LDLR: Low-density lipoprotein; NKT: natural killer T.

Colestyramine

Cholestyramine is a polystyrene-based polymer that has been crosslinked with divinylbenzene and functionalized to quaternary ammonium units to produce a robust anion exchange resin and increase the secretion of the pancreatic exocrine hormone cholecystokinin (CCK)[61]. A study revealed that cholestyramine administration increases the expression levels of genes encoding HMGCoAR, LDLR,

PCSK9, and SREBP2[62]. Cholestyramine stimulates hepatic BA synthesis from cholesterol, which activates SREBP2 by inhibiting BA absorption from the intestine. LDLR increases the transport of cholesterol from the plasma when SREBP2 is expressed. The upregulation of HMGCoAR compensates for the reduction in LDL-C levels in the plasma. In addition to activating SREBP2, PCSK9 gets upregulated, thereby degrading LDLR. By modulating PCSK9, cholestyramine-induced increases in LDLR expression can be modulated[58]. In addition to treatments using cholestyramine and inhibitors of ileal BA uptake, treatments aimed at reducing PCSK9 expression would be beneficial for reducing the enterohepatic circulation of BAs[63].

Similarly, several clinical and experimental models have revealed that cholestyramine improves BA-gut microbiome interactions, thereby facilitating glucose and fat metabolism. In clinical models of primary biliary cholangitis (PBC), two SCFA-producing *Lachnospiraceae* species were found to be enriched in the microbiome of the superior remission group after cholestyramine treatment. SCFAs derived from dietary fibers are produced by the gut microbiota, and SCFA signaling has anti-inflammatory, antiobesity, and antidiabetic properties[52]. This denotes the favorable effects of cholestyramine in treating PBC by enhancing BA-gut microbiome interactions[64]. Newman *et al*[57] reported that cholestyramine reduced hyperglycemia by increasing the ileal expression of glucagon through an increase in the prevalence of *Acetatifactor Muris* and *Muribaculum intestinal*. In another study, cholestyramine-treated ZDF rats showed reduced glycosylated hemoglobin A1c (HbA1c) levels, serum glucose levels, and FXR activation and increased PYY levels, GLP-1 Levels, and insulin release[65].

Colesevelam

Colesevelam hydrochloride (HCl) is a polyallylamine that has been crosslinked with epichlorohydrin and alkylated with (6-bromohexyl)-trimethylammonium bromide and 1-bromodecane[66]. In clinical and animal studies on T2DM, obesity, and hyperlipidemia, colesevelam reduced blood glucose[67], FBG [68], mediator complex subunit 1, miR-182[69], HbA1c[70], hepatic TG, total LDL[71], very-low-density lipoprotein (VLDL), chylomicron particle[72], LDL-C[73], non-HDL-C, ApoB, TGR5/GLP-1-dependent glycogenolysis, FXR-dependent cholesterol, cytochrome P450, Cyp7a1[74], FGF-19[75], BA reabsorption [76], high-sensitivity C-reactive protein[77], and fructosamine levels[78-80] and increased glycolysis, postmeal glucose tolerance, insulin levels[81], splanchnic sequestration of meal-derived glucose[82], GLP-1/GIP levels[83], total HDL particle levels, miR-96/182/183 expression levels, β-cell function [as revealed by homeostatic model assessment (HOMA)][56], BA synthesis, ApoA-1 levels[54], and CCK levels[84]. As a molecularly engineered, second-generation BA sequestrant, colesevelam has been recommended for reducing LDL-C in patients with primary hypercholesterolemia by inhibiting b-hydroxymethylglutaryl coenzyme A reductase[85]. Colesevelam enhances glycemic control in patients with T2DM[86]. When metformin-based, sulfonylurea-based therapy fails to completely control T2DM, colesevelam can improve glycemic and lipid indices[54]. Moreover, colesevelam significantly alters BA metabolism. A non-absorbable complex of colesevelam in the gastrointestinal tract can stimulate the excretion of BAs through feces and their removal from enterohepatic circulation. Therefore, colesevelam treatment may reduce the total BA pool size[75]. Colesevelam reduces the influx of CDCA and DCA, two of the most potent FXR ligands, into ileal enterocytes. Therefore, plasma levels of FGF19 are likely to decrease when FXR is less activated[75].

Colestimide

Colestimide, an anion exchange resin, lowers serum cholesterol levels by binding to BAs in the intestinal tract[87]. Although colestimide is used to treat hyperlipidemia in Japanese patients, the mechanism by which it lowers blood glucose levels remains poorly understood[88]. CA reduces blood glucose levels and facilitates energy metabolism through the type 2 iodothyronine deiodinase (D2) enzyme. Various clinical and experimental studies have revealed that colestimide treatment reduced blood glucose, FBG, postprandial blood glucose, HbA1c, IR, and serum LDL-C levels and increased serum 1,5-AG and postprandial plasma GLP-1 Levels in patients with T2DM[89-91]. Another study revealed that colestimide altered BA composition and CA ratios, thereby reducing blood glucose levels *via* the TGR5-Camp-D2 pathway[92]. Similarly, elobixibat induced colonic motility and secretion by inhibiting an ileal BA transporter in a highly selective manner[93], reduced the LDL-C levels and LDL-C: HDL-C ratio, and increased the circulating GLP1 levels in a clinical study on dyslipidemia[94]. Colestilan is also a BAS that could reduce body weight and HbA1c, FBG, LDL-C, and total cholesterol levels and increase fecal lipid excretion in patients with T2DM[95].

Receptor-mediated therapeutics

Since BAs were initially considered lipid solubilizers, they have evolved into complex metabolic integrators. BAs can modulate their energy expenditure through the stimulation of TGR5- and FXR-mediated signaling pathways[36]. The metabolism-related protein TGR5 may be a novel promising target for treating metabolic disorders associated with obesity. Recently, TGR5 expression has been reported in enteroendocrine L cells, including STC-1 cells, which secrete GLP-1 upon calorie intake[96]. In preclinical studies, INT-177 (a semisynthetic BA derivative) and nonsteroidal TGR5 agonists promoted glucose homeostasis[97]. The activation of TGR5 by BAs reduced diet-induced obesity by

increasing energy expenditure in brown adipose tissues and muscles[97]. Moreover, TGR5-mediated release of GLP-1 modulated the ATP/ADP ratio and oxidative phosphorylation in the mitochondria by activating the K_{ATP}/C_{av} channels. Thus, the TGR-5-mediated pathway is therapeutically beneficial, considering that incretin-based therapies are effective in treating DM[98]. Moreover, FXR activation has not yet been associated with significant weight loss[99]. FXR activation reduces hepatic glucose and fatty acid outputs by increasing glycogen production and decreasing lipogenesis and VLDL production, thereby increasing insulin sensitivity[99]. Similarly, 6E-CDCA was found to reduce blood glucose, insulin, TG, and plasma cholesterol levels and fatty acid synthesis and facilitate FXR activation in Zucker (fa/fa) obese rats with liver steatosis[40]. Moreover, tauroUDCA increased muscular and hepatic insulin signaling by phosphorylating the insulin receptor substrate Tyr and Akt at Ser473 in obese participants[100]. In summary, TGFR5 agonists activate the TGR5 signaling pathway by increasing mitochondrial function and enteroendocrine cell function, ultimately leading to increased incretin release. This has various metabolic effects, including reduction of weight gain and hepatic steatosis, improvement of liver function, and maintenance of insulin sensitivity and glucose homeostasis.

BAs metabolites and bariatric surgery

CDCA (3α,7α-dihydroxy-5β-cholan-24-oic acid) is a PBAs produced in the liver from cholesterol. CDCA is a potent inhibitor of CYP7A1, the enzyme responsible for BA synthesis. In addition to suppressing cholesterol synthesis, CDCA may inhibit HMGCoA reductase[101]. Mantovani *et al*[102] reported decreased plasma levels of CA and TCA but significantly increased plasma levels of TCDCA, TDCA, HDCA, GDCA, GLCA, and DCA in patients with T2DM. Moreover, Cariou *et al*[103] reported that plasma levels of CDCA, CA, and DCA were negatively correlated with insulin sensitivity in patients with T2DM. CDCA may inhibit high-fat diet (HFD)induced obesity and hyperglycemia through the activation of TGR5 and inhibition of PPARγ transcriptional activity[104]. Another study revealed that CDCA increased GLP-1 and glucagon secretion and delayed gastric emptying by activating GPBAR1 in patients with T2DM[60]. The activation of FXR and TGR5 through CDCA and DCA mimicked and suppressed SPX promoter activity induced by CDCA and DCA. SPX promoter activity was significantly increased by adenylate cyclase (AC)/cAMP activators and reduced by CDCA, DCA, and PKA pathway inhibitors. Through FXR- and TGR5-mediated AC/cAMP/PKA and MAPK cascades, CDCA and DCA could promote SPX expression at the hepatic level[105].

Obeticholic acid (OCA, 6E-CDCA) is a semisynthetic BAs with a 30-fold higher potency than that of CDCA for activating FXR. OCA-mediated inhibition of BAs synthesis increased the abundance of *Firmicutes* species and reduced nonalcoholic steatohepatitis in humans[106]. UDCA is commonly used for treating liver dysfunction. UDCA treatment reduced hyperinsulinemia and fasting hyperglycemia in a mouse model of T2DM with hepatic steatosis[107]. Moreover, Osorio *et al*[108] reported that UDCA inhibited sodium–glucose co-transporter overexpression, thereby reducing oxidative stress in mice with streptozotocin (STZ)-induced DM. A recent meta-analysis revealed that UDCA significantly reduced fasting plasma glucose, HbA1c, and insulin levels, indicating a positive impact on glucose homeostasis [109]. Another clinical trial demonstrated that UDCA treatment reduced HbA1c levels and increased early-phase GLP-1 secretion[110].

Bariatric surgery is effective in treating obesity, DM, and related complications. However, this surgery is not the only factor responsible for treating obesity. Bariatric surgery alters gut microbiota profiles and induces gut microbes to synthesize SCFAs. Gut microbes are crucial for improving the outcomes of bariatric surgery. Gut microbes are also important for reducing weight and lowering adverse events after bariatric surgery. Therefore, prebiotics, probiotics, and postbiotics are recommended for patients who have undergone bariatric surgery in order to improve their clinical outcomes[111]. Bariatric surgery causes changes in the gut microbiota because of a malabsorptive status and changes in BA metabolism, gastric pH, and hormone metabolism[112]. It may also change the levels of hormones, such as leptin and ghrelin. Changes in hormones have been reported as a result of energy metabolism and the microbiota. Prebiotics modulate the intestinal microbiota and reduce the levels of ghrelin in the blood; however, the relationship between the two is not fully understood[113]. Similarly, postsurgical microbiomes were more different from lean microbiomes than obese microbiomes, whereas postsurgical microbiomes were less different from lean microbiomes than obese microbiomes. Body mass index loss following bariatric surgery could be predicted based on the presurgical microbiome. After surgery, the relative abundance of Proteobacteria and Fusobacteria increased, whereas that of *Firmicutes* decreased[114]. On the other hand, in patients with mild obesity, RYGB is an effective treatment option. It can also improve the metabolic and inflammatory status. Lau *et al*[115] reported that RYGB altered 29 rich bacterial genera in the gut microbiota of patients with T2DM. To better understand the weight-independent antidiabetic mechanisms of RYGB, researchers have developed DJB surgery. Han *et al*[116] demonstrated that DJB increased intraduodenal BAs levels and upregulated duodenal SIRT1 expression in rats with HFD- and STZ-induced DM. Patients with T2DM reported significant and long-lasting glycemic improvements after undergoing duodenal mucosal resurfacing, an endoscopic technique that involves circumferential hydrothermal ablation and subsequent regeneration of the duodenal mucosa[117].

Gut microbe-mediated alleviation of metabolic diseases

Studies have revealed that obesity alters microbial composition and nature[23,28]. The development of metabolic illnesses, such as obesity and T2DM, has recently been linked to the gut microbiota. Increasing attention has been paid to altering the gut microbiota for treating metabolic diseases. Numerous microbial compositions (probiotics, symbiotics, and antibiotics) have been used to treat illnesses. Probiotics are live microorganisms that have positive effects on host health when administered in adequate concentrations[118].

PROBIOTICS

The use of probiotic bacteria as prophylactics and therapeutics is receiving attention because of the potential effects of gut microbes in lowering IR and lipid levels. Increasing evidence suggests using probiotics to prevent metabolic diseases[119]. Probiotics are live microbes that provide the host with health benefits when administered in optimal concentrations[120]. Probiotic administration may lower TG and VLDL cholesterol (VLDL-C) levels by inhibiting the NF-κB pathway and the gut microbiota-SCFA-hormone axis[121]. Moreover, a substantial decrease in lipid levels was noted in healthy pregnant women without GDM after the administration of a two-strain probiotic containing *L. acidophilus* LA5 and *B. animalis* BB12 for 9 wk[122]. Probiotics can also increase the β-oxidation of long-chain fatty acids in muscle and liver tissues, modifying the energy pathways for fatty acid oxidation, lowering the formation of new TGs, and eventually reducing serum TG and VLDL-C levels[123]. Furthermore, probiotic consumption can increase the number of natural killer T cells in the liver[124], reduce inflammatory signaling, increase adiponectin levels, reduce inflammation, and prevent GLUT4 inhibition to improve glucose homeostasis. Probiotic dosages can also trigger enteroendocrine L cells to release GLP-1, thereby improving glucose metabolism, reducing glucotoxicity, and improving insulin sensitivity in the target tissue[125].

Recently, there have been many discussions on fecal microbiota transplantation. Vrieze *et al*[126] reported that participants with metabolic syndrome showed enhanced peripheral and hepatic insulin sensitivity in response to modest intestinal transfusions of fecal microbiota from allogenic lean donors, together with an upsurge of the gut microbiome. Various microbes are used as probiotics. Our review mainly focuses on *A. muciniphila* and *Bifidobacterium* species, which are closely associated with metabolic diseases.

A. muciniphila

In recent years, *A. muciniphila*, a commensal bacterium found in the intestine, has attracted increasing interest because of its health-promoting effects[127]. Interestingly, various clinical disorders in humans, including obesity, T2DM[128], inflammatory bowel disorder, hypertension, and liver disease, decrease the abundance of *A. muciniphila*[129]. Animal studies have demonstrated that *A. muciniphila* can alleviate obesity and related illnesses, such as steatosis, gut permeability, glucose intolerance, and IR[130,131]. Moreover, in one study, animals treated with live *A. muciniphila* did not exhibit IR or inflammatory cell (CD11c) infiltration in adipose tissues, which are crucial for the development of obesity, because of low inflammation[32]. *A. muciniphila* and *F. prausnitzii* can protect against the development of T2DM[132]. By activating tight junction proteins (occludin, claudin-3, and ZO-1) and preventing the accumulation of lipopolysaccharides (LPSs) and occurrence of metabolic endotoxemia, *A. muciniphila* can restore the thickness of the mucus layer[127]. Furthermore, *A. muciniphila* exhibits antibacterial and anti-inflammatory effects when administered endogenously and influences the endogenous synthesis of GLP-1 and GLP-2[133]. Notably, all these findings have now received backing from different firms and have been used for treating various disorders, including metabolic diseases, such as DM[134], obesity[135], atherosclerosis, hepatic inflammation, and hypercholesterolemia[136].

Bifidobacterium species

Probiotics, which are a component of the gut microbiome, successfully regulate the intestinal microbiota and have potential antidiabetic applications[137]. *Bifidobacterium*, one of the most significant probiotics found in the mammalian gut, exhibits positive effects on health[138]. Numerous studies have demonstrated that *Bifidobacterium* species improved insulin sensitivity in patients with T2DM[139,140]. In HFD-fed rats with T2DM, the administration of *B. animalis* 01 reduced food and water intake, blood glucose levels, HbA1c levels, and hepatic injuries and increased the antioxidant status, HOMA-IR, and lipid levels by affecting the IRS/PI3K/AKT and Keap1/Nrf2 signaling pathways[141]. Similarly, Le *et al*[142] reported that STZ-induced C57BL/6J mice treated with *Bifidobacterium* species exhibited significantly reduced blood glucose levels and significantly increased IR, IRS1, Akt/PKB, IKKα, and IκB α levels. Moreover, increased extracellular signal-regulated kinase 2 and adiponectin expression levels and decreased macrophage chemoattractant protein-1 and interleukin-6 expression levels were noted following the administration of *Bifidobacterium* species. Furthermore, in obese and DM models, treatment with *B. animalis* subsp. *lactis* GCL2505 reduced visceral fat accumulation, increased GLP-1 and acetate levels, and enhanced glucose tolerance[143].

Bifidobacterium, one of the most significant gut bacteria, diminishes intestinal endotoxin concentrations and enhances mucosal barrier function[144]. Recently, HFD-induced models verified an increase in intestinal inflammation. By lowering the levels of metabolic endotoxins and reducing intestinal inflammation, *Bifidobacterium* species may benefit patients with metabolic syndrome[145]. In an HFD-fed mouse model, *Bifidobacterium* species dramatically improved glucose-induced insulin secretion, increased glucose tolerance, and reduced endotoxemia and proinflammatory cytokine levels [146] by altering GLP-2 levels[147]. Thus, by lowering metabolic endotoxin levels and intestinal inflammation and increasing the expression level of intestinal Reg I, a growth factor regulator[148], *Bifidobacterium* supplementation could alleviate HFD-induced metabolic syndrome. Specific strategies for altering the gut microbiota in favor of *Bifidobacterium* species may be beneficial for mitigating the effect of HFD on the occurrence of metabolic syndrome.

In summary, *A. muciniphila* and *Bifidobacterium* species are highly viable and proliferative probiotics that can alleviate metabolic syndrome through increased glucose tolerance and reduced visceral fat accumulation by altering the overall bacterial composition of the gut microbiota. Moreover, they can increase the levels of SCFAs, which can activate several signaling pathways, including the AKT/PKB/IRS/ERK/Nrf2 pathways.

LIMITATIONS AND FUTURE PERSPECTIVES

Research on the synthesis of BAs and the pathogenesis of liver diseases and metabolic diseases has made significant progress in the last two decades. BAs exert several metabolic functions, and their physicochemical properties can affect their metabolic activities. Gut microbes can be modified by various factors, such as age, diseases, diet, and drugs. BAs play a significant role in regulating gut microbes. Moreover, the size of the BA pool has been shown to be affected by microbial metabolism in the intestines; however, most of these studies have been conducted on experimental animals. Therefore, further research is warranted to identify novel therapeutic targets for maintaining human intestinal health. Importantly, while increasing experimental evidence is available, clinical research on the importance of the human microbiota in relation to rodent metabolic functions is still in its inception. For example, BAS is not recommended for individuals who have a bowel obstruction or are pregnant. Cholestyramine and colestipol are classified as pregnancy category C, while colesevelam HCl is classified as pregnancy category B[149].

Clinical research has mainly been epidemiological in nature and has therefore failed to determine whether modifications in the intestinal microbiota play a molecular role in metabolic diseases. A better understanding of these aspects is required to determine whether BA-gut microbiota axes can promote human health and how these pathways can be used to design novel therapeutic interventions for metabolic diseases, such as obesity, DM, and hyperlipidemia, and CADs using BAs and its metabolites, probiotics, and microbial transplantation.

CONCLUSION

The major objective of this review was to assess the functional implications of gut microbes and BAs for metabolic diseases. In the past, the gut microbiota was considered a bystander in the intestinal tract. The role of these microbes in supporting intestinal function has become more widely recognized in recent years. BAs and the gut microbiota interact in a mutually beneficial manner. When the gut microbiota is disturbed in metabolic illnesses, inflammation occurs and the gut barrier is compromised. Modulating receptor-mediated transport, energy balance, gut permeability, and serum LPSs can impact BAs metabolism. The gut microbiota composition and the specific mechanisms in which the gut microbiota and BAs interact to alter the metabolism and functioning of the host-gut barrier remain somewhat unclear. Understanding the significance of the BAs-gut microbiota relationship in metabolic health could lead to revolutionary advances in the treatment of metabolic illnesses in the future.

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MINIREVIEWS

Recent advances in the management of autoimmune pancreatitis in the era of artificial intelligence

Sahar Mack, Yves Flattet, Philippe Bichard, Jean Louis Frossard

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Abstract

Autoimmune pancreatitis (AIP) is a type of immune-mediated pancreatitis subdivided into two subtypes, type 1 and type 2 AIP. Furthermore, type 1 AIP is considered to be the pancreatic manifestation of the immunoglobulin G4 (IgG4)-related disease. Nowadays, AIP is increasingly researched and recognized, although its diagnosis represents a challenge for several reasons: False positive ultrasound-guided cytological samples for a neoplastic process, difficult to interpret levels of IgG4, the absence of biological markers to diagnose type 2 AIP, and the challenging clinical identification of atypical forms. Furthermore, 60% and 78% of type 1 and type 2 AIP, respectively, are retrospectively diagnosed on surgical specimens of resected pancreas for suspected cancer. As distinguishing AIP from pancreatic ductal adenocarcinoma can be challenging, obtaining a definitive diagnosis can therefore prove difficult, since endoscopic ultrasound fine-needle aspiration or biopsy of the pancreas are suboptimal. This paper focuses on recent innovations in the management of AIP with regard to the use of artificial intelligence, new serum markers, and new therapeutic approaches, while it also outlines the current management recommendations. A better knowledge of AIP can reduce the recourse to surgery and avoid its overuse, although such an approach requires close collaboration between gastroenterologists, surgeons and radiologists. Better knowledge on AIP and IgG4-related disease remains necessary to diagnose and manage patients.

Key Words: Autoimmune pancreatitis; Pancreatic ductal adenocarcinoma; Immunoglobulin G4-related disease; Prednisone; Rituximab; Artificial intelligence; Plasmablasts

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Core Tip: The diagnosis of autoimmune pancreatitis (AIP) is challenging. Indeed, 60% and 78% of type 1 and type 2 AIP, respectively, are retrospectively evaluated on surgical specimens of resected pancreas for suspected cancer. Obtaining a definitive diagnosis can thus prove difficult, since endoscopic ultrasound fine-needle aspiration or biopsy of the pancreas are suboptimal. This paper focuses on recent innovations in the management of AIP using artificial intelligence, new serum markers, and new therapeutic approaches and outlines the current recommendations. Improved knowledge of AIP can reduce the recourse to surgery, although this requires collaboration between gastroenterologists, surgeons and radiologists.

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a type of chronic fibro-inflammatory response in immune-mediated pancreatitis[1,2]. Histological examination reveals diffuse lymphoplasmacytic infiltration associated with extensive storiform fibrosis, acinar atrophy, and obliterative venulitis[2,3]. Radiological imaging shows ductal stenosis and an enlarged pancreas or pancreatic mass resembling pancreatic ductal adenocarcinoma (PDAC)[3]. The distinction between these two entities is sometimes difficult and has clear therapeutic implications. Indeed, AIP has a good response to steroids, which constitutes an important diagnostic criterion[4,5].

In the last decade, the prevalence of AIP has increased worldwide due to the better description and recognition of the disease[6-8]. In the majority of the studies conducted in Asian countries, its prevalence more than doubled between 2011 and 2016. In Japan, for example, the prevalence was estimated at 10.1 per 100000 inhabitants in 2016 with an annual incidence of 3.1 per 100000 inhabitants [7]. The prevalence in Europe seems to be less than 1 per 100000 inhabitants (0.29/100000), or 9% of patients with non-alcoholic acute pancreatitis[8], although these numbers are most certainly underestimated due to the lack of diagnoses and the occurrence of paucisymptomatic cases that do not require treatment[8].

Two AIP subtypes, AIP-1 and AIP-2, present different clinical profiles such as mean age at disease onset, male/female ratio, geographical distribution, as well as histological and immunological features (Table 1)[9,10]. AIP-1, the most prevalent type in Asia, is a systemic disease with the possible involvement of other organs, higher immunoglobulin G4 (IgG4) in blood, IgG4 positive infiltrates, as well as increased autoantibody levels in blood. AIP-1 primarily affects men aged over 50 years and is currently considered the pancreatic manifestation of the IgG4-related disease[11]. AIP-2 corresponds to the idiopathic duct-centric pancreatitis, which can be identified by pathognomonic histological features known as granulocyte epithelial lesions[9,12]. This subgroup is more common in Europe and affects younger patients with an equivalent male/female ratio. AIP-2 often occurs with isolated cases of pancreatitis without other organ involvement, although it is associated with chronic inflammatory bowel disease in 20%-30% of cases[6,13]. The physiopathological mechanisms of AIP are poorly understood and multiple immunological pathways have been proposed. The aim of this paper is not to describe these different mechanisms.

ESTABLISHING A DIAGNOSIS

Several diagnostic criteria have been proposed for AIP based on its clinical, biological, radiological and histological presentation in addition to treatment response: Diagnostic criteria of the Japanese (2002, 2006)[14], Korean (2007), Asian (2008) and Italian Societies of Gastroenterology (2003, 2009), as well as the Mannheim (2009) and HISORt criteria (2009)[15]. With the improved detection of AIP-2 and IgG4-related disease, a group of international experts published new reference criteria known as the International Consensus Diagnostic Criteria (ICDC) in 2011 with five main diagnostic criteria categorized according to two levels of evidence (Tables 2 and 3)[10]. New Japanese diagnostic criteria (JPS2011 followed by JPS2018) were subsequently published[16,17]. Unlike the ICDC criteria, the JPS2011 criteria provided the following clarifications: (1) Differentiation between diffuse, segmental and focal types in the classification; (2) Blood IgG4 used as the only biological marker; (3) Sclerosing cholangitis, sclerosing sialadenitis and retroperitoneal fibrosis classified as other organ involvement; (4) No level of evidence given for other organ involvement or serological criteria (IgG4); and (5) The optional use of steroids only after excluding pancreatic cancer by fine-needle aspiration (FNA)[16]. In 2018, the JPS2018 added

Table 1 Characteristics of the two subtypes of autoimmune pancreatitis

Characteristic	AIP-1	AIP-2
Male/female ratio	3/1	1/1
Mean age	65 yr	40 yr
Geographical distribution	Asia > Europe and United States	Europe and United States > Asia
Clinical presentation	Jaundice 60%-80%. Acute pancreatitis 15%. Weight loss 65%	Acute pancreatitis 80%. Jaundice < 30%
Biological presentation	IgG4 > 1.35 g/L (sensitivity 70%, specificity 93%). IgG4 > 2.7 g/L (sensitivity 53%, specificity 99%). Lipase < 3xN. Cholestasis: > 80% of cases. Diabetes: 65% of cases. Insulin-dependent diabetes: 20% of cases. Exocrine pancreatic insufficiency: 40% of cases	Unspecific. Lipase > 3xN. Rare endocrine and exocrine pancreatic insufficiency
Histological criteria	Lymphoplasmacytic infiltration without neutrophils. Storiform fibrosis. Obliterative venulitis. IgG4 plasma cells > 10 in a high-power field	Destruction of inter- and intralobular ducts by neutrophils (granulocytic epithelial lesions). Few or no IgG4 plasma cells
Relapse rate after corticosteroid therapy	> 30%	< 15%

AIP: Autoimmune pancreatitis; IgG: Immunoglobulin G.

Table 2 Summary table of the International Consensus Diagnostic Criteria for autoimmune pancreatitis-1[10]

ICDC	Level 1	Level 2
P: Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement (rim-like enhancement)	Indeterminate: Segmental or focal enlargement with delayed enhancement
D: Ductal imaging	Single long stricture (> 1/3 length of MPD) or multiple stricture without marked upstream dilatation	Segmental or focal narrowing without marked upstream dilatation (< 5 mm)
S: Serology	IgG4 > 2x upper limit of normal value (> 2.70 g/L)	IgG4 rate: 1-2x upper limit of normal value
OOI: Other organ involvement	Histology of extra-pancreatic organ (3/4)	Histology of extra-pancreatic organ must show both: (1) Periductal lympho-plasmacytic infiltration without granulocyte epithelial lesions; and (2) > 10 cells/HPF of IgG4 positive cells
	Typical radiological evidence: (1) Stenosis of intrahepatic bile duct or proximal and distal common bile duct; and (2) Retroperitoneal fibrosis	Physical or radiological evidence (1/2): (1) Symmetrically enlarged salivary/lachrymal glands; and (2) Radiological renal involvement
H: Pancreatic histology	3/4 criteria	2/4 criteria
Periductal lymphoplasmacytic infiltration without granulocyte epithelial lesions		
Obliterative phlebitis		
Storiform fibrosis		
> 10 cells/HPF of IgG4 positive cells		
Rt: Corticosteroid response	Rapid (≤ 2 wk) radiologically demonstrable resolution or marked improvement in pancreatic/extrapancreatic manifestation	

HPF: High power field; ICDC: International Consensus Diagnostic Criteria; IgG: Immunoglobulin G; MPD: Main pancreatic duct.

two new factors to its diagnostic criteria: The use of magnetic resonance imaging (MRI) for radiological diagnosis, primarily using magnetic resonance cholangiopancreatography, and the use of endoscopic ultrasound (EUS)-FNA in order to exclude a neoplastic process by histology[17].

The main limitation of this diagnostic algorithm concerns AIP-2 patients with normal IgG4 levels and disease limited to the pancreas[18]. Indeed, 50% of AIP-1 patients present with other organ involvement [19], which facilitates the diagnosis. If specific clinical, morphological, or biological evidence confirms the AIP diagnosis, no further investigation is necessary. Nevertheless, in the presence of a focal mass or

Table 3 Summary table of the International Consensus Diagnostic Criteria for autoimmune pancreatitis-2[10]

ICDC	Level 1	Level 2
P: Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement (rim-like enhancement)	Indeterminate: Segmental or focal enlargement with delayed enhancement
D: Ductal imaging	Single long stricture (> 1/3 length of MPD) or multiple stricture without marked upstream dilatation	Segmental or focal narrowing without marked upstream dilatation (< 5 mm)
OOI: Other organ involvement		Clinically diagnosed inflammatory bowel disease
H: Pancreatic histology	Both of the following: (1) Granulocytic infiltration of duct wall with or without granulocytic acinar inflammation; and (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells	Both of the following: (1) Granulocytic and lymphoplasmacytic acinar infiltration; and (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells
Rt: Corticosteroid response	Rapid (≤ 2 wk) radiologically demonstrable resolution or marked improvement in manifestations	

HPF: High power field; ICDC: International Consensus Diagnostic Criteria; IgG: Immunoglobulin G; MPD: Main pancreatic duct.

diffuse pancreatic enlargement without associated autoimmune disease or specific biological and morphological features, a biopsy is necessary for histological analysis. The effectiveness and feasibility of obtaining pancreatic samples by EUS-FNA or biopsy (EUS-FNB) are still the subject of debate. Indeed, the primary aim of EUS-FNA and EUS-FNB is to collect pancreatic tissue so as to exclude a malignant process and thus contribute to the AIP diagnosis. The ICDC therefore recommends the use of biopsy tissue (trucut biopsy). However, given that this procedure is not feasible in all healthcare establishments, it is not compulsory in the diagnostic algorithm, although it is an important diagnostic criteria of the JPS2018 classification[17]. In the last decade, the proportion of pancreatic samples obtained by EUS has significantly increased from 48% in 2007 to 86% in 2016 in Japan[7]. Several studies nevertheless report the difficulty in diagnosing AIP with EUS-FNA (sensitivity of 43%-60%) and obtaining a sufficient amount of fibrotic tissue[11-16], which explains the shift toward EUS-FNB[20,21]. A Japanese study on 44 AIP patients obtained an adequate histological sample in 93% of cases, leading to a confirmed diagnosis of AIP in 43% of cases, a diagnosis of idiopathic chronic pancreatitis (CP) in 50% of cases, and no false positives for pancreatic cancer[22].

Laboratory tests

The serological diagnostic criteria corresponds to IgG4 levels at the upper limit of normal between 135 and 140 mg/dL[23]. It is generally accepted that IgG4 levels twice the normal limit are a valid criteria for AIP, although this can also occur in 10% of PDAC. Moreover, elevated IgG4 levels that are more than twice the normal value are associated with recurrence and exocrine pancreatic insufficiency in IgG4-related disease[24]. Nonetheless, some AIP-1 cases do not present elevated blood IgG4 or IgG4-positive cells on histology[25].

The efficacy of monoclonal anti-CD20 antibodies in AIP highlights the possible involvement of B cells in the pathogenesis of this disease[26,27]. Two types of B cells have been investigated in IgG4-related disease: Regulatory B cells and plasmablasts. Derived from the B cell lineage, plasmablasts are characterized as $CD27^{+}CD38^{+}$, which situates them between B cells and plasmocytes. Diagnostic tools such as the quantification of circulating plasmablasts in serum have already been shown to contribute to the diagnosis of AIP in patients with autoimmune disease[10]. In a retrospective study on 37 patients with IgG4-related disease, all patients showed high levels of plasmablasts, while only 64% had high IgG4 serum[28].

Imaging

When investigating pancreatic lesions, several types of imaging are necessary, as no single imaging technique can provide a definitive diagnosis of AIP. The most typical feature is a global enlargement of the pancreatic gland associated with the loss of lobulations, giving it a sausage-like appearance[29]. The capsule-like rim sign, which can also be seen with other procedures, is a relatively distinctive feature of AIP in computed tomography (CT). This sign is defined by a band-like structure around all or part of the pancreas. It is characterized by a lower absorption than the pancreatic parenchyma of the lesion during the pancreatic parenchymal phase and shows a delayed enhancement pattern with dynamic CT. Other elements have been described such as decreased peripheral enhancement causing a peripheral halo or ring, involution of the pancreatic tail, enhancement of the thickened bile duct wall resembling a cocoon, stenosis of the Wirsung duct without upstream dilation, and focal hyperdense pseudotumors. MRI shows a loss of T1 signal intensity and the T2 hyperintensity of the parenchyma correlated with an inflammation of the gland. In terms of ducts, stenosis of the Wirsung duct without upstream dilation can be observed, even in the focal pseudotumors[29]. A capsule-like rim reflecting the strong fibrosis of the peripancreatic lesions can be observed on T2-weighted images as a low signal and is highly specific

to AIP. EUS findings in AIP can be hypoechoic with scattered high-echo spots in the enlarged area in some cases show a diffuse or localized lesion of the parenchyma and irregularities in the main pancreatic duct such as bile duct wall thickening, or produce a duct-penetrating sign[30]. Further use of Positron-Emission-Tomography-Fluorodeoxyglucose can be useful in detecting other organs involved in AIP.

Artificial intelligence

The use of artificial intelligence in the medical domain has expanded rapidly in recent years. Artificial intelligence is a mathematical technique that automates the learning and recognition of data patterns. Diagnostic techniques such as digestive EUC (DEUS) can interact with this interface. A database was developed in Rochester using DEUS images of normal pancreas (NP) and pancreas of patients with AIP, PDAC, and CP with the aim to develop a convolutional neural network, a type of network with artificial neurons that recognize and classify images [convolutional neural network (CNN)] able to distinguish between these entities. For every patient in each cohort, all available still images and recorded video assets were identified and extracted. Images and videos obtained from both the radial and curvilinear echoendoscopes were included. Potentially confounding image features and patient identifying information were removed during image processing. Liver images, images with marks or annotations, and images in which calcification was visible were excluded. Using data from the training and validation subsets, various candidate CNN architectures, optimizers, and configurations were implemented, trained, and evaluated to determine an effective design for the EUS-CNN. Occlusion heatmaps were then generated and used to assess the features identified by the CNN model to differentiate all conditions (AIP, PDAC, CP, and NP). In a cohort of 585 patients (146 AIP, 292 PDAC, 72 CP, and 73 NP) with 1174461 extracted images, the CNN was 99% sensitive and 98% specific to differentiate AIP from NP, 95% sensitive and 71% specific to differentiate AIP from CP, 90% sensitive and 93% specific to differentiate AIP from PDAC, and 90% sensitive and 85% specific to differentiate AIP from all other pancreatic diseases[31]. Other groups have used this technology to discriminate portal venous CT images with the aim to differentiate between AIP and PDAC[32].

PANCREATIC CANCER AS A DIFFERENTIAL DIAGNOSIS

As the main important differential diagnosis of AIP is pancreatic cancer, it is important to recognize any differences in the clinical, radiological, and histological features[3,6]. Clinically, AIP patients present with mild abdominal pain such as discomfort, rarely with weight loss, and fluctuant jaundice that tends to respond positively to steroid therapy. On the other hand, PDAC patients present severe, persistent, and progressive abdominal pain with weight loss and progressive jaundice. Extrapancreatic manifestations are more frequent in AIP, whereas PDAC is more localized in the pancreatic gland and induces lower bile duct stenosis, presenting metastatic lesions and direct invasion in some cases. Biologically, IgG4 is elevated in AIP patients, although elevated levels have also been reported in a few cases of PDAC[33]. By contrast, elevated carbohydrate antigen 19-9 is rarely seen in AIP. Radiologically, smooth margins and capsule-like rims in the body and tail region that represent severe fibrotic changes are seen in the CT and MRI of patients with AIP. Amelioration of swelling after steroid treatment is a characteristic of AIP, whereas PDAC patients do not or rarely present an improvement. Duct dilatation should raise the suspicion of PDAC. Using contrast-enhanced CT, AIP is characterized by homogenous delayed enhancement of the gland that indicates the diffuse loss of parenchymal volume and severe fibrosis, whereas heterogenous enhancement that represents necrosis or bleeding in the tumor can be seen in PDAC. Using EUS, AIP is characterized by a duct penetrating sign as well as a diffuse homogenous hypoechoic pattern and linear or reticular hyperechoic inclusions that reflect interlobular fibrosis. In PDAC, EUS findings show a localized hypoechoic mass and a double duct sign, often accompanied by lymph node swelling or vascular invasion. Histological patterns of AIP are characterized by periductal lymphoplasmacytic infiltration, storiform fibrosis, and obstructive phlebitis. Immunohistological identification of carcinoma cells is observed in PDAC, and inflammatory reactions can be commonly observed.

TREATMENT

Approximately 10%-25% of patients spontaneously improve and do not require specific treatment or intervention. Since no triggers for AIP have been identified to date, no lifestyle modifications have been proposed. Nevertheless, according to the 2017 recommendations, untreated patients with active AIP should receive treatment with the exception of those with a steroid contraindication[34]. The treatment of choice and the standard treatment at present is corticosteroid therapy. There are currently no standard therapeutic protocols regarding the indications for corticosteroid therapy, its duration, posology, monitoring measures, and maintenance therapy. In Asia, the initial dose of prescribed oral

prednisone is 0.6 mg/kg/d for 2 to 4 wk, followed by a single maintenance dose of 7.5 mg/d for 6 mo to 3 years. In the United States and Europe, the dose is 40 mg/d for 4 wk followed by a recommended reduction of 5 mg per week following symptom improvement; a single maintenance dose of 5-7.5 mg/d is recommended for 12 wk to 6 mo. A smaller dose of 30 mg/d can be given to diabetic patients[35]. An alternative administration with two courses of methylprednisolone 500 mg for 3 d with a 4-d interval can be useful to induce remission in refractory cases[34].

The aim of treatment is to improve symptoms, prevent fibrosis development within the affected organs, and improve endocrine and exocrine pancreatic insufficiency. Corticosteroid therapy has an effectiveness of around 90%, with a recurrence rate of 30%-50% after reducing treatment. The rate of recurrence is higher in AIP-1 (31%-37.5%) than in AIP-2 (9%-15.9%)[36,37]. Treatment evaluation by imaging and biological analysis is recommended within 1-2 wk of induction.

Three treatment options exist in the case of recurrence. The first approach is to maintain long-term low-dose corticosteroids (7.5 mg/d for 1-3 years), while the second is to use immunomodulator therapy such as azathioprine (2 mg/kg/d for 1-3 years)[38], methotrexate, or mycophenolate mofetil. A new therapeutic approach was proposed with rituximab, a monoclonal anti-CD20 antibody, and it seems to be a promising treatment, notably in IgG4-related disease[34,39].

Diverse studies comparing immunomodulators with corticosteroids alone did not show its superiority in terms of efficacy[34,40]. For patients who are resistant or intolerant of steroids and immunomodulators, rituximab is the only possible therapeutic alternative to induce remission. Rituximab can be used as first-line treatment for patients with a high risk of recurrence. Proximal duct involvement, young age, and higher alkaline phosphatase at initial presentation are high risk factors of recurrence after first-line treatment[41]. Moreover, for these patients with a significantly higher chance of recurrence, an induction and maintenance phase (375 mg/m^2 1x/wk every 2-3 mo for 2 years) would be significantly more effective than an induction phase alone (375 mg/m^2 per week for 4 wk or two injections of 1000 mg at 15 d interval)[41].

CONCLUSION

To conclude, the diagnosis of AIP remains challenging for clinicians as it must rapidly be distinguished from PDAC. The available diagnostic tools such as EUC are currently evolving, and the use of artificial intelligence could lead to the development of new approaches, allowing for a more precise diagnosis of AIP and a better differentiation of the disease from pancreatic cancer. The use of rituximab in the treatment algorithm in case of recurrence has already been proven, and it should be proposed as first-line treatment for patients with risk factors for recurrence. The optimal dose and treatment duration are yet to be defined.

FOOTNOTES

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MINIREVIEWS

Molecular mechanisms implicated in SARS-CoV-2 liver tropism

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Hepatic involvement is common in SARS-CoV-2-infected individuals. It is currently accepted that the direct and indirect hepatic effects of SARS-CoV-2 infection play a significant role in COVID-19. In individuals with pre-existing infectious and non-infectious liver disease, who are at a remarkably higher risk of developing severe COVID-19 and death, this pathology is most medically relevant. This review emphasizes the current pathways regarded as contributing to the gastrointestinal and hepatic ailments linked to COVID-19-infected patients due to an imbalanced interaction among the liver, systemic inflammation, disrupted coagulation, and the lung.

Key Words: SARS-CoV-2; Viral hepatitis; Non-infectious liver disease; Hyperinflammation; Coronavirus disease 2019

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Core Tip: Clinical manifestations of coronavirus disease 2019 (COVID-19) may be triggered by the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in the liver. SARS-CoV-2 genomic RNA and its replicative intermediates were found in liver tissues. SARS-CoV-2 causes direct cholangiocyte damage. Systemic inflammation due to COVID-19 correlated with the degree of acute liver injury as revealed by the rise in aspartate aminotransferase levels. SARS-CoV-2 infection increased the risk of morbidity and mortality in patients with a history of advanced liver disease.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Globally, as of 5 August 2022, there have been 579119505 confirmed cases of COVID-19, including 6457101 deaths, as reported by the World Health Organization[1].

Coronaviruses are enveloped with crown-shaped spike glycoprotein, positive-sense single-stranded RNA viruses that include three genera: alphacoronavirus, betacoronavirus, and gammacoronavirus, mainly related to respiratory infections. SARS-CoV-2 employs receptor recognition mechanisms comparable to those used by preceding virulent coronaviruses such as SARS-CoV, responsible for the SARS epidemic of 2003[2-5].

In addition to common respiratory symptoms, COVID-19 patients also present with gastrointestinal symptoms comprising diarrhea, nausea, and vomiting[6]. Anal swabs from COVID-19 patients test positive for genomic SARS-CoV-2 RNA, and the virus can be isolated from stool samples[7,8]. However, the possibility of fecal-oral transmission cannot be completely ruled out[9,10].

The entry of the virus into target cells is mediated by the coronavirus spike protein (S) that engages angiotensin-converting enzyme 2 (ACE2) expressed in multiple cell types allowing SARS-CoV-2 to infect different organs such as the nasopharynx, lungs, lymph nodes, kidney, stomach, small intestine, spleen, brain, and liver leading to multiple organ damage[11]. Cell entry also requires the transmembrane serine protease 2 or other proteases[12]. The binding efficiency of the virus to ACE2 is a key determinant of transmissibility[13]. Different reports have revealed that SARS-CoV-2 has higher binding affinity to ACE2 than the previous SARS-CoV. This finding may in part explain the increased transmissibility of SARS-CoV-2, organ tropism, and ultimately multi-organ damage and mortality[14-16]. The mechanisms that could be involved in the multi-organ injury due to infection with SARS-CoV-2 comprise the damage of endothelial cells, dysregulation of the immune response, and an imbalance in the renin-angiotensin-aldosterone system (RAAS). The relative significance of these mechanisms in the pathophysiology of COVID-19 is at present not completely known. Although some of these mechanisms comprising ACE2-mediated viral entry and tissue injury with misbalance of the RAAS may be specific to COVID-19, immune pathogenesis produced by systemic delivery of cytokines and impaired microcirculatory function can also take place due to viral sepsis[17].

Hepatic involvement is common in SARS-CoV-2-infected patients. In cases with severe COVID-19 and to a lesser extent in mild/moderate COVID-19, some authors have reported a significant increase in alanine aminotransferase and aspartate aminotransferase (AST) indicating abnormal liver function[18]. Likewise, albumin decreased while alkaline phosphatase, gamma-glutamyl transferase (GGT), C-reactive protein (CRP), procalcitonin, ferritin, lactate dehydrogenase (LDH) as well as bilirubin levels were also significantly higher in severe than in other cases[19-21]. Patients without a history of liver illness were found to have abnormal liver test results[20]. These data suggests a direct connection between SARS-CoV-2 infection and digestive tract impairment. This review emphasizes the pathways currently regarded as contributing to the gastrointestinal and hepatic ailments linked to COVID-19.

To recognize the relevant literature, we employed a search and screening strategy. This process consisted of an extensive search of the online scientific database on the PubMed webpage using the most frequent synonyms to detect all possibly pertinent studies. In the following steps, references were analyzed to eliminate papers that were not relevant, and the remaining references were organized into categories for additional evaluation.

MOLECULAR MECHANISMS IMPLICATED IN SARS-COV-2 LIVER TROPISM DETECTION OF SARS-COV-2 IN LIVER TISSUE

The liver coordinates an essential role in the host-microbe defense by assembling the portal and systemic circulation. Changes in the liver such as ductular fibrosis, hepatic steatosis, cholestasis, acute liver necrosis, and central vein thrombosis with concomitant lymphocytic infiltrate were detected in autopsies from COVID-19 deceased patients[22,23].

In line with previous reports, ACE2 is mostly expressed in cholangiocytes and to a lesser extent in hepatocytes. Accordingly, formalin-embedded liver tissues contain the SARS-CoV-2 RNA genome[24]. Other studies have stated that 2 of 3 autopsy livers carry the infectious virus, and 31 of 45 postmortem liver tissues contain the SARS-CoV-2 RNA genome[25]. Such studies that demonstrate the presence of SARS-CoV-2 RNA and proteins in liver cells are significant, as these may be found in the vascular

lumen of blood vessels feeding the liver but also in portal vein endothelial cells, suggesting that the virus can also invade the liver through the circulation[26]. Postmortem liver biopsies have shown the presence of typical coronavirus particles in the cytoplasm of hepatocytes by electron microscopy[27], while hepatic parenchymal cells have also shown the presence of SARS-CoV-2 RNA[25]. Furthermore, the viral nucleocapsid protein has been detected in hepatic stem cells, hepatocytes, and cholangiocytes [28]. The presence of the SARS-CoV-2 RNA genome and its replicative intermediates in liver tissues has also been reported[28]. Most importantly, the nucleocapsid and the spike protein have been found in the liver 6 mo after recovery from COVID-19[29].

There is some proof that COVID-19 may be triggered by the presence of SARS-CoV-2 infection in the liver. However, other histological examinations revealed non-detectable viral particles in the liver and signs of significant hepatic damage[23,30]. This supports the notion that both direct and indirect pathways contribute to liver damage in the context of SARS-CoV-2 infection; more investigation is required to assess the importance of each pathway.

VIRAL EFFECT ON THE LIVER

The concept that SARS-CoV-2 can reach the liver cells through the ACE2 receptor is supported by the fact that ACE2 is present in liver and bile duct cells[11]. Furthermore, recent research found that 59.7% of cholangiocytes and 2.6% of hepatocytes express ACE2. Likely, SARS-CoV-2 might infect cholangiocytes and cause liver damage since the amount of ACE2 found in cholangiocytes is similar to that reported in type 2 alveolar cells of the lung[31]. SARS-CoV-2-associated liver damage may be related to the presence of ACE2 receptors in cholangiocytes rather than hepatocytes[32]. Moreover, given the rich supply of blood to the liver from the small bowel, the SARS-CoV-2 virus can use the gut-liver way across the liver reticular system to reach the liver[33,34]. Taking into account that ACE2 was regarded as an interferon-inducible gene in human epithelial cells from respiratory tissues, the hepatocyte permissiveness for SARS-CoV-2 might be also modified when the viral receptor expression is increased after liver injury[35], but it would be because of the shortened isoform of ACE2, identified as deltaACE2, instead of the functional viral receptor[36].

In homeostasis, the bile acid released by hepatocytes into bile ducts is transported by cholangiocytes. The tight junction between cholangiocytes conserves the barrier function of the bile ductal epithelial cells, allowing bile acid collection and excretion. Besides, cholangiocytes play an important function in liver renewal and immune response[37]. Thus, the disruption of the cholangiocyte function can induce hepatobiliary injury. Previous reports have demonstrated that SARS-CoV-2 infection impairs the barrier through the modulation of tight junctions[38]. This effect could be attributed to the direct viral cytopathic effect on cholangiocytes. Likewise, SARS-CoV-2 infection induces the expression of apoptotic factors, including cluster of differentiation 40 (CD40), caspase recruitment domain family member 8, and serine/threonine kinase 4 in cholangiocytes[38]. Thus, SARS-CoV-2 causes direct cholangiocyte damage with subsequent liver homeostasis disruption in COVID-19 patients.

Liver biopsies from SARS-CoV-2 infected patients have revealed fatty degeneration, cellular infiltration, hepatocellular necrosis, increased ballooned hepatocytes, and mitotic cells. These findings are in line with the idea that liver damage in COVID-19 patients is caused by an indirect effect as a result of direct viral cholangiocyte damage and subsequent bile acid accumulation. However, it remains uncertain whether hepatic involvement during SARS-CoV-2 infection reveals direct cytopathic effects of the virus, ischemia and hypoxic reperfusion-related injury, exacerbated immune response responsible for the systemic inflammatory response syndrome, or a combination of mechanisms that have not been completely elucidated until now[39].

EFFECT OF SYSTEMIC HYPERINFLAMMATION IN THE LIVER

SARS-CoV-2 infection is related with an acute phase response characterized by the secretion of very high levels of proinflammatory cytokines in conjunction with CRP and ferritin[40]. The mechanisms involved in this "cytokine storm" are not completely elucidated. The proinflammatory response appears to be associated with the ability of the SARS-CoV-2 spike protein to activate C-type lectins and 20 family member 2 on innate immune cells[41]. A transcriptome analysis of 284 samples from 196 SARS-CoV-2-infected patients revealed that diverse peripheral immune alterations are associated with clinical characteristics comprising severity and disease phase of COVID-19. The increased expression and release of S100A8/A9 during inflammation exerts a critical role in controlling the inflammatory reaction by inducing leukocyte recruitment and stimulating cytokine release. These are calcium-binding proteins constitutively expressed as a heterodimer in neutrophils and monocytes, and they are the most important platelet-derived activators of endothelial cells. Additionally, SARS-CoV-2 RNA was detected in different epithelial and immune cells, followed by transcription alterations within virus-positive cells [42].

One of the mechanisms that encourage platelet adhesion to inflamed vascular endothelium is the early adhesive events that stimulate platelets to "roll" along endothelium. Among them is the interaction between the main platelet membrane receptor, GP1b (CD42)-1X-V, and von Willebrand factor secreted by endothelial cells. This phenomenon is strengthened by interactions between upregulated CD62P expression, which is present in both cell types and its counter receptor, P selectin glycoprotein ligand-1, which is also present in both cell types, although weakly in platelets. In a similar manner, the SARS-CoV-2 spike protein bound to the CD42b receptor to activate platelets *via* two different signaling pathways and stimulated platelet-monocyte interaction by engaging P selectin glycoprotein ligand-1 (PGSL-1) and CD40 ligand (CD40L)/CD40, which causes monocytes to produce proinflammatory cytokines. These findings demonstrate the correlation between hypercoagulation, monocyte activation, and a cytokine storm in patients severely affected.

The contact between the spike protein of SARS-CoV-2 and CD42 activates platelets and stimulates platelet-monocyte interactions through CD40L/CD40 and P-selectin/PGSL-1, contributing to hypercytokine secretion by monocytes[43]. Additionally, systemic inflammation is evidenced by a complement activation induced by the interferon (IFN)-Janus Kinase 1/2 (JAK1/2)-signal transducer and activator of transcription 1 (STAT1) signaling pathway[44].

The significance of the IFN pathways has been revealed in postmortem analyses of deceased patients due to severe COVID-19, where the livers displayed a significant induction of type I and II IFN responses and their related-JAK-STAT signaling pathways[25].

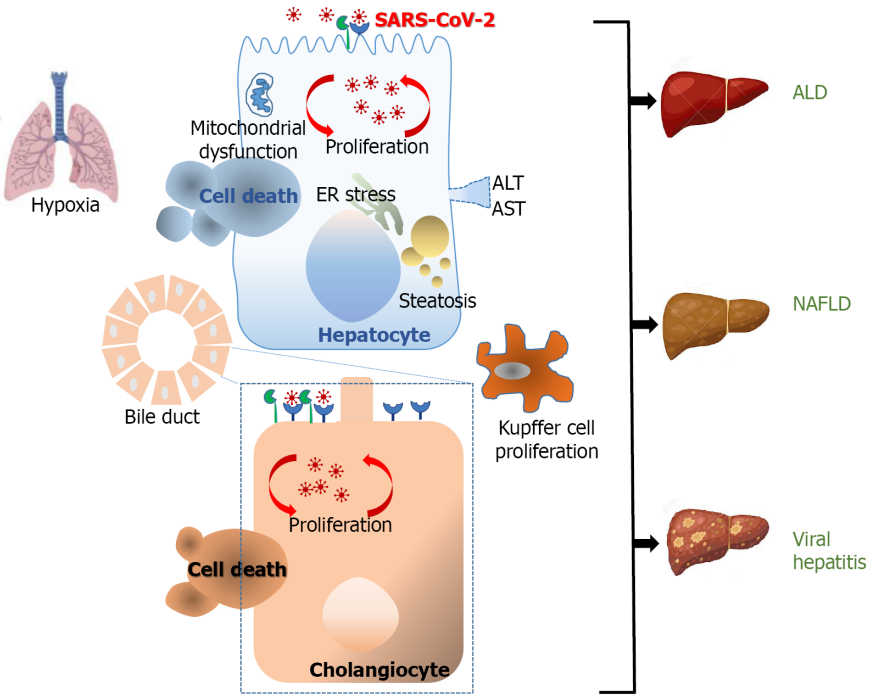
Moreover, the inflammation and cytokine stimulation observed in SARS-CoV-2 disease can contribute to endothelial injury and vascular damage, revealed by hypercoagulation, and arterial and venous embolism with the activation of immune cells and platelets, leading to clot formation[45,46]. High levels of CRP, which are indicative of acute liver injury, are correlated with the degree of systemic inflammation[47,48]. Increased levels of high-sensitive CRP, interleukin 6 (IL-6), and ferritin in COVID-19 patients are indicative of systemic inflammation, which correlates with the degree of acute liver injury as determined by the rise in AST levels[49]. Through the downregulation of hepatobiliary uptake and deficiencies in the excretory systems, the massive systemic inflammation contributes to hepatocellular cholestasis in the late stages of the disease[50]. Thus, it can be conclusively stated that SARS-CoV-2 infection is accompanied by a "cytokine storm" with a high proinflammatory cytokine profile that causes hepatic injury. It is obvious that systemic inflammation may contribute to acute liver damage, but SARS-CoV-2 infection cannot be ruled out directly. This theory is supported by the observation that liver damage appears early in the course of infection, as indicated by an increase in AST levels.

SARS-COV-2 INFECTION IN PATIENTS WITH PREVIOUS LIVER DISEASE

Since the onset of the COVID-19 pandemic, an increased risk of morbidity and mortality was observed among SARS-CoV-2-infected patients with a history of advanced liver disease[51]. Data obtained from multicenter studies have revealed a higher possibility of hospitalization and risk of death compared to patients without chronic liver disease[52]. To date, the mechanisms linking both pathologies are unknown. However, it has been proposed that the prothrombotic alteration caused by COVID-19 upsets the delicate homeostatic balance of cirrhotic patients, leading to venous microthrombosis and parenchymal dysfunction along with subsequent respiratory failures[53] (Figure 1).

VIRAL HEPATITIS

Patients with viral hepatitis are more likely to experience liver damage, according to data from previous SARS-CoV infections. The severity of liver disease and a worse prognosis are associated with SARS-CoV-2 superinfection in patients with chronic hepatitis B virus (HBV) infection. According to a study of 105 patients with both chronic HBV infection and SARS-CoV-2, the risk of complications such as acute chronic liver failure, acute cardiac injury, and shock was higher in co-infected patients than in patients who only had the SARS-CoV-2 virus[54]. Consequently, patients with liver damage had a higher mortality rate (28.6%) than patients without liver damage (3.3%), and the treatment of COVID-19 in co-infected individuals had a significant negative impact on liver function[54]. A higher risk of abnormal liver function was found in inactive HBV carriers in a retrospective study that included 133 hospitalized patients with confirmed COVID-19, 116 of whom tested negative for serum hepatitis B antigen, and 17 were HBV inactive carriers. Hepatocytes and the immune response, as shown by the production of IL-6, D-dimer, and LDH are involved in the liver damage observed in SARS-CoV-2/HBV[55]. Moreover, chronic HBV infection-induced immune dysfunction may be a key factor in the development of COVID-19. Due to persistent viral antigens, studies have shown that chronic HBV infection is linked to the depletion of virus-specific $CD4^+$ and $CD8^+$ T cells[56]. Interleukin (IL-2) and tumor necrosis factor alpha (TNF-α) release are particularly hampered by HBV-specific exhausted T lymphocytes, resulting in progressively diminished antiviral function[57]. IL-2, IL-6, and TNF-α are among the proinflammatory



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Figure 1 Mechanisms of pathological injury upon severe acute respiratory syndrome coronavirus 2 infection. The major cause of mortality in coronavirus disease 2019 (COVID-19) is largely caused by lung damage with the increase of acute respiratory distress syndrome (ARDS). Liver damage or liver dysfunction has been linked with the general severity of COVID-19 infection and serves as a prognostic factor for ARDS progress. The scale of liver injury may range from direct severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral antigens, inflammatory progressions, hypoxemia, the antiviral treatments that induced hepatic damage and the existence of previous liver diseases. Angiotensin-converting enzyme 2 was mostly expressed in cholangiocytes and to a lesser extent in hepatocytes. These findings are in line with the viral presence reported in cholangiocytes and hepatocytes and the direct cytopathic effects observed. SARS-CoV-2 infection of hepatocytes and the indirect effects of "cytokine storm" induce a significant increase in alanine aminotransferase and aspartate aminotransferase indicating abnormal liver function. This leads to endoplasmic reticulum stress, steatosis, and finally to hepatocyte cell death. Consequently, Kupffer cell activation appears to be commonly funded in livers. The synergism between SARS-CoV-2 and chronic viral hepatitis B and C has also been suggested. Additionally, the superimposed cytokine storm caused by SARS-CoV-2 in patients with alcohol-associated liver disease, and non-alcoholic fatty liver disease (NALDF) displayed a higher risk of severe COVID-19. ALD: Alcohol-associated liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

cytokines overproduced as a result of the excessive immunological response to SARS-CoV-2 infection (cytokine storm), which is to our knowledge a significant factor associated with disease severity and mortality[58]. In this situation, it is conceivable that immunosuppression and depletion of HBV-specific T lymphocytes might prevent an excessive immunological response to SARS-CoV-2 and lessen the cytokine storm, resulting in milder illness. Although HBV reactivation is a potential side result of SARS-CoV-2 infection, the overall risk is minimal. Reactivation is primarily described as an abrupt and quick rise in HBV DNA levels in individuals with a history of detectable HBV DNA or recurrence of HBV DNA viremia in those with previous undetectable viral DNA[59]. Immunosuppressive therapy such as IL-6 receptor antagonists, IL-1 receptor antagonist, and high-dose corticosteroids are frequently used to treat HBV reactivation[60]. These treatments may be utilized in severe COVID-19 patients to manage the cytokine storm and to lessen the immune-mediated multiorgan damage. The impaired balance between the host's immune system and viral replication is the main cause of the progression to HBV reactivation after infection with SARS-CoV-2. The dosage of glucocorticoids or immunosuppressive medications is a major risk factor for the reactivation of HBV during the treatment of COVID-19, together with the host baseline virological markers[61].

The COVID-19 pandemic may delay the global commitment to eradicate HBV infection for several years because of the decrease in chronic hepatitis B prevention, diagnosis, and treatment. According to estimates, during the COVID-19 pandemic, half of low- and middle-income families were unable to access healthcare facilities for the diagnosis, clinical evaluation, and treatment of HBV. This was primarily due to travel restrictions, job and income losses, and patients' fear of contracting SARS-CoV-2 [62]. Only 18% of the patients with hepatocellular carcinoma (HCC) and 32% of those with decompensated cirrhosis had continuity of care, while 23% of the health centers postponed HBV infection therapy initiation[63].

Regarding hepatitis C virus (HCV), some studies have revealed that corticosteroid-treated individuals might experience significant viral reactivation, which is mostly caused by immunosuppression[64-66]. Steroid therapy can lead to HCV reactivation through two different mechanisms: first, they increase the capacity of the virus to replicate by upregulating two essential HCV entry factors:

occludin and scavenger receptor class B type I; and second, they do so by inhibiting the immune response against the virus, including T lymphocytes and plasmacytoid dendritic cells[67-69]. In individuals with persistent HCV infection, corticosteroid therapy can aggravate the course. Evidence suggests that HCV viremia rises in response to corticosteroids and falls back to normal levels in response to their cessation[70]. Therefore, it is best to avoid using corticosteroids in individuals with HCV infection[71].

However, the synergism between SARS-CoV-2 and chronic viral HBV and HCV has not been clearly elucidated. Hence, more widespread studies are required to evaluate the use of this therapy.

COVID-19 IN NON-INFECTIOUS CHRONIC LIVER DISEASE PATIENTS

Currently, decompensated liver disease and HCC cause 2 million deaths annually as a result of liver cirrhosis, which affects 112 million people globally[72]. High COVID-19 death rates in cirrhotic patients have been found in numerous recent reports[73,74]. The baseline Child-Pugh score also showed to be substantially correlated with death. The most frequent cause of mortality in COVID-19 patients is lung damage. Liver dysfunction is a possible cause of persistent lung damage. Indeed, liver failure plays a significant role in individuals with bacterial chest sepsis[75]. Cirrhosis and SARS-CoV-2 may have a fatal synergy because of alterations in the immune system caused by viral infection and coagulation. Dysregulation of pulmonary dynamics has been attributed to a number of factors, including ascites or deteriorating encephalopathy, immunological dysfunction in viral infection, a rise in the burden of venous thromboembolic illness, and concurrent lung disease. According to Marjot *et al*[52], mortality rates in patients with cirrhosis was 32% compared to 8% in those without it, while the mortality rate rose in connection with the Child-Pugh class [A (19%), B (35%), and C (51%)] in patients with cirrhosis. The principal cause of decease was respiratory failure (71%).

Alcohol-associated liver disease and COVID-19

There is very little effect of COVID-19 on patients with alcoholic hepatitis or alcoholic liver disease[74,76]. However, research on cirrhotic patients has revealed that, like other cirrhotic patients, those with alcohol-related cirrhosis have higher mortality rates[74,76]. Chronic kidney disease, obesity, and diabetes are common comorbidities among patients with alcohol-related cirrhosis, leading to higher risks of COVID-19 complications[77]. In a study involving 867 patients with COVID-19 and chronic liver disease, Kim *et al*[78] found that alcohol-associated liver disease (ALD) was independently associated with a higher risk of poor survival and a higher COVID-19 mortality rate. ALD is connected to the inflammatory state prompted by danger-associated molecular patterns which trigger the secretion of pro-inflammatory cytokines by particular immune cells[74,79]. It was hypothesized that the superimposed cytokine storm produced by SARS-CoV-2 in patients with ALD could exacerbate the heightened inflammatory process, leading to worse outcomes[80].

Non-alcoholic fatty liver disease

Diabetes, hypertension, cardiovascular disease, and obesity are well-known risk factors for severe COVID-19[81]. These metabolic comorbidities are closely related to non-alcoholic fatty liver disease (NAFLD).

Wide-ranging effects of the course of COVID-19 make distinguishing the independent effect on NAFLD a challenge. The difficulty is raised by a number of confounding cofactors, reverse causality from steatosis caused by SARS-CoV-2, as well as population heterogeneity and the diagnostic conditions studied. Consequently, results from clinical studies have been ambiguous. In a retrospective study of 202 SARS-CoV-2 infected patients, 35% were individuals with NAFLD. When compared to SARS-CoV-2 infected patients without NAFLD, patients with NAFLD displayed a higher risk of severe COVID-19, as evidenced by a higher probability of liver abnormalities in the hospitalized patient and long-term viral shedding[33].

Seventy-one consecutive COVID-19 patients from a different case-control study were divided into groups based on whether or not they had NAFLD. After reviewing all medical records, including demographic, clinical, and laboratory data, this study concluded that NAFLD poses a significant risk for developing severe COVID-19[82]. Patients with NAFLD were more probable to be admitted to the intensive care unit, according to a retrospective multicenter study with a cohort of hospitalized adults with COVID-19[83]. These results were supported by two thorough systematic reviews, as well as a meta-analysis[84,85].

On the other hand, NAFLD was not linked to severe COVID-19, as shown by a study conducted by Mushtaq *et al*[86] in a Middle Eastern cohort. The authors revealed that gene variants associated with NAFLD (transmembrane 6 superfamily 2 [TM6SF2], patatin-like phospholipase domain-containing protein 3 [PNPLA3], glucokinase regulator, and membrane-bound O-acyltransferase domain-containing protein 7 [MBOAT7]) and the severity of COVID-19 disease (TM6SF2, PNPLA3, and MBOAT7) is associated with genetic variation as a mechanism to establish a genetic risk score[87]. Additionally, other studies have concluded that some PNPLA3 allelic variants may act protectively by lowering the

risk of COVID-19 mortality[88]. Finally, a study that used a Mendelian randomization approach to investigate the correlations between COVID-19 severity and NAFLD found scant evidence supporting such a relationship[89].

COVID-19 AND HCC

At the beginning of the SARS-CoV-2 pandemic, serious measures were taken to preserve cancer patients from morbidity and mortality by restraining hospital presence and submitting anti-cancer therapy. The European Association for the Study of the liver recommended postponing treatments and evaluating the possibility of gradually removing anti-cancer immunological therapy[90].

In the context of rapidly escalating viral transmission, a series of precautionary principles were dictated. These measures rested on the hypothesis that cancer and active anti-cancer therapy have an unfavorable effect on the consequences of SARS-CoV-2 infection. It is widely known that cancer patients are commonly immunosuppressed as a consequence of chemotherapy. Therefore, different studies indicated that patients with subjacent cancer were at major risk of acquiring SARS-CoV-2 infection and severe outcomes could eventually evolve[91,92].

Recently, a multicenter retrospective revision including 250 non-vaccinated patients with liver cancer and COVID-19 infection showed that the mortality rate was 12.96% in patients with a diagnosis of HCC simultaneously with SARS-CoV-2 infection, and 20.25% in individuals with HCC history[74].

However, the prevalence of SARS-CoV-2 infection did not display a real rise in patients with chronic liver disease or in HCC patients. Nevertheless, HCC cirrhotic patients with COVID-19 may have a worse prognosis than the general population regarding severe disease, complications of SARS-CoV-2 infection, and mortality. Hereafter, the significance of applying actions to decrease the possibility of infection in these patients[93].

COVID-19 AMONG LIVER TRANSPLANT RECIPIENTS

Managing liver transplantation in the curse of SARS-CoV-2 pandemic was difficult as numerous hospitals had to essentially stop or drastically scale back their transplantation operations owing to a sudden drop in donor numbers and were forced to convert several care facilities into COVID-19 units. Due to the limited information available and the necessity of continuing immunosuppressive medication in these patients, the medical staff faced difficult challenges to manage post-liver transplant receivers in the course of COVID-19 pandemic while patients were at risk for a more severe COVID-19 infection and possible continued viral shedding. Qin *et al*[94] described the first instance of SARS-CoV-2 infection in a patient with hepatocellular cancer who had undergone liver transplantation, and discovered a higher viral load with an increasing immunosuppressive dosage. Immunosuppressive medications had no effect on the frequency of COVID-19 severity, according to Bhoori *et al*[95]. Early studies from Italy claimed that transplant patients experienced low death rates of less than 5%[96]. However, later assessments revealed that liver recipients and other solid organ transplants experienced mortality rates of over 25%[97,98]. Recently, findings from a prospective European trial comprising 57 liver transplant patients with proven SARS-CoV-2 infection and 19 transplant facilities were released. These results are consistent with is consistent with the projected mortality rate because patients with severe COVID-19 infection had overall and in-hospital case fatality rates of 12% and 17%, respectively. Five of the seven patients who passed away had a cancer history at the time of their deaths[99]. The evidence currently available does not support the idea that transplantation or certain immunosuppressive therapies have a significant impact on the likelihood of disease severity. Nevertheless, patients with underlying malignancies may need special care[97]. A number of COVID-19 vaccines have lately approved and have demonstrated effectiveness in healthy individuals. However, careful assessment and immunization of immunocompetent individuals are still required due to the potential immunological imbalance brought on by their illness or immunosuppressive medication. According to Boyarsky *et al*[100], solid organ transplant recipients who were fully vaccinated with the mRNA vaccine showed an appropriate humoral response, while the subpar response was linked to the use of antimetabolite immunosuppression.

CONCLUSION

It is widely acknowledged that the effects of SARS-CoV-2 infection on the liver have played a significant role during the COVID-19 pandemic. In patients with pre-existing infectious and non-infectious liver disease, who are at an extra high risk of developing severe COVID-19 and death, this feature is most medically relevant. This review aims to provide an overview of the current research on the molecular mediators responsible for inflammatory liver injury during SARS-CoV-2 infection. To fully comprehend

the pathogenic pathways that cause clinical deterioration of patients with COVID-19 due to an imbalanced interaction between the liver, systemic inflammation, disrupted coagulation, and the lung, further research should be conducted.

FOOTNOTES

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MINIREVIEWS

Current status of novel biologics and small molecule drugs in the individualized treatment of inflammatory bowel disease

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Abstract

Treatment strategies for inflammatory bowel disease (IBD) are rapidly evolving with the development of biologics and small molecule drugs (SMDs). However, these drugs are not guaranteed to be effective in all patients, and a "ceiling effect" of biologic monotherapy may occur. This issue highlights an unmet need for optimizing the use of biologics and predicting therapeutic responses. Thus, the development of new drugs with novel mechanisms of action is urgently needed for patients with primary nonresponse and secondary loss of response to conventional biologics and SMDs. In addition, combining different biologics or SMDs has been proposed as a novel strategy to enhance treatment efficacy in IBD, which theoretically has multidimensional anti-inflammatory potential. Based on the current evidence available for IBD, dual targeted therapy may be a promising strategy for refractory IBD patients who have failed in multiple biologic treatments or who have extraintestinal manifestation. Additionally, identifying the subgroup of IBD patients who are responding to biological combination therapies is also equally important in stable disease remission. In this review, we summarize the newly developed biologics and SMDs and the current status of biologics/SMDs to highlight the development of individualized treatment in IBD.

Key Words: Inflammatory bowel diseases; Biologic; Dual targeted therapy; Therapeutic drug monitoring; Bispecific antibodies

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Core Tip: The emergence of biologics and small molecules has significantly changed the therapies used for inflammatory bowel disease (IBD). However, the efficacy of these drugs is not satisfactory for every patient, which indicates an unmet need for optimizing the use of biologics/small molecules and for predicting therapeutic responses. Here, we describe the current status of novel biologics and small molecules and new treatment strategies to combat IBD by using more than one biologic.

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INTRODUCTION

The inflammatory bowel diseases (IBD) ulcerative colitis (UC) and Crohn's disease (CD) are progressive inflammatory diseases with the gastrointestinal tract being the major site of inflammation. Patients require lifelong medical therapy in the context of the complicated aetiology of IBD[1]. Encouragingly, the advent of biologics and small molecule drugs (SMDs) has fundamentally changed patient prognoses and improved their quality of life. Strong evidence has indicated that early treatment with these drugs might lead to more favorable outcomes, such as deeper inflammation control and longer steroid-free remission[2]. Despite the optimization of biological therapies, the proportion of patients who exhibit primary nonresponse and secondary loss of response to biologics remains high, and approximately only 40% of patients who respond to biologic therapies maintain clinical remission in one year[3]. This highlights a potential "ceiling effect" of biological monotherapy and an unmet need for optimizing the use of biologics and for predicting therapeutic responses. Thus, patients need not only new drugs but also optimized treatment strategies. In the last decade, increasing numbers of new biologics and SMDs have been developed for IBD treatment[4], and a novel therapy combining different biologics and/or SMDs targeting multiple inflammatory signalling pathways, which is called dual targeted therapy (DTT), has begun to emerge in recent years[5]. However, whether DTT is superior to monotherapy in achieving the new target of long-term deep healing is uncertain. Additionally, DTT might only work in a selected subgroup of IBD patients, and indiscriminate use of DTT is expensive, ineffective, and unsafe [6]. Thus, in the era of biologics, it is important to identify eligible patients and treat them with individualized therapy. In this review, we describe newly emerging drugs and advanced strategies to provide insight for optimizing the current treatments for IBD in the context of individualized medicine.

EMERGING BIOLOGICS AND SMDS IN IBD

Currently, the goal of IBD treatment is not only to maintain clinical remission but also to achieve transmural healing to prevent further structural damage. Therefore, biologics and/or SMDs are recommended for patients with moderate to severe IBD. To date, the approved biological and small molecule therapies for IBD consist of the following anti-tumor necrosis factor (TNF) agents [infliximab (IFX), adalimumab (ADA), certolizumab (CZP), golimumab (GOL)], anti-adhesion agents [vedolizumab (VDZ), natalizumab (NAT)], anti-interleukin (IL)-12/23 agents [ustekinumab (UST)], and Janus kinase (JAK) inhibitors (tofacitinib). However, the current biological monotherapies are efficacious only in a certain proportion of patients. For example, only 30%-50% of active patients can achieve clinical or mucosal remission after biological inducing therapy. Besides, the rates of long-term corticosteroid-free remission are even lower and are less than 30%[7]. Thus, new drug development is rapidly advancing to meet the needs of patients with primary nonresponse, loss of response or intolerance to conventional biologics and SMDs (Table 1).

Anti-TNF agents

Anti-TNF agents were the first class of biologics to be approved for IBD treatment, and since then, they have tremendously changed IBD management. However, even in patients who respond to anti-TNF agents, the scope of anti-TNF use is limited due to systemic effects, such as infection and immunosuppression[8]. In addition, immunogenicity is another complex problem in anti-TNF-based treatment. Although some randomized controlled trials (RCTs) have shown that adding immunomodulators (IMs), such as the thiopurines azathioprine and 6-mercaptopurine, may reduce the immunogenicity of anti-TNF agents, and then improve the efficacy of anti-TNF therapy, only a minority of patients will benefit from this strategy[9,10]. Gut-selective anti-TNF agents might overcome these defects. An oral anti-TNF agent is currently in development. Since the antibodies comprising this therapy are derived from cow

Table 1 Summary of emerging biologics and small molecule drugs in inflammatory bowel disease treatments

Drug class	Agent	Target	Route	IBD type	Ref.
Anti-TNF	AVX470	Anti-TNF	Oral	UC	[11]
Anti-IL-23	Risankizumab	IL-23/p19 subunit	IV/SC	CD/UC	[17]
	Brazikumab	IL-23/p19 subunit	IV/SC	CD/UC	[15]
	Mirikizumab	IL-23/p19 subunit	IV/SC	CD/UC	[18]
	Guselkumab	IL-23/p19 subunit	IV/SC	CD/UC	[16]
Anti-lymphocyte trafficking	Etrolizumab	α4β7 and αEβ7 integrins	SC	CD/UC	[22]
	AJM300	α4 integrin	Oral	UC	[23]
	Ontamalimab	MAdCAM	SC	CD/UC	[38]
S1P receptor modulators	Ozanimod	S1PR1 and S1PR5	Oral	CD/UC	[24]
	Etrasimod	S1PR1, S1PR4 and S1PR5	Oral	CD/UC	[39]
JAK inhibitor	Filgotinib	JAK1	Oral	CD/UC	[27]
	Upadacitinib	JAK1	Oral	CD/UC	[28]
PDE4 inhibitor	Apremilast	PDE4	Oral	CD/UC	[30]

Anti-TNF: Anti-tumour necrosis factor; CD: Crohn's disease; IBD: Inflammatory bowel disease; IL-23: Interleukin-23; IV: Intravenous; MAdCAM: Mucosal addressin cell adhesion molecule-1; PDE4: Phosphodiesterase 4; SC: Subcutaneous; SMDs: Small molecule drugs; SIP: Sphingosine-1-phosphate; UC: Ulcerative colitis.

colostrum, this agent can act on the small intestine and colon in a delayed-release manner. A preclinical study that assessed the efficacy of AVX-470 showed a higher clinical response rate in the treatment group at week 4 than in the control group (25.9% *vs* 11.1%)[11]. Additionally, serious systemic side effects and formation of anti-drug antibodies were not observed. The current new oral formulation of anti-TNF agents might bring gut specificity to anti-TNF treatments. However, many more clinical studies are needed to confirm the efficacy of this novel formulation.

Anti-IL-12/23 agents

IL-12/23 signalling pathways are the key in regulating the differentiation and maturation of Th17 cells, which results in intestinal inflammation in IBD[12]. The conventional anti-IL-12/23 agent, UST, prevents activation of the IL-12/23 signalling pathway by targeting the shared subunit of cytokine p40 of IL-23 and IL-12[13,14]. At present, several monoclonal antibodies are in development that targets other subunits of IL-12/23.

Briakinumab is a human monoclonal antibody that acts specifically against the p19 subunit of IL-23 and exerts no effect on IL-12. In a clinical phase II study, 119 CD patients who failed anti-TNF therapy received brazikumab or placebo randomly at the beginning of the trial and 4 wk later. A higher clinical response rate was observed in brazikumab-treated patients than for those in the placebo group (49% *vs* 27%, $P = 0.01$)[15]. Guselkumab is another anti-p19 human mAb that was assessed in a phase II study in 250 patients with moderate-to-severe CD. Patients in all guselkumab groups treated with different doses exhibited a significant reduction in inflammatory activity at week 12. In addition, more patients in guselkumab treatments achieved clinical response [200 mg: 54%, 600 mg: 65%, 1200 mg: 50% *vs* 15.7% placebo ($P < 0.001$, respectively)] and safety events were similar between the groups[16]. Risankizumab, another anti-p19 monoclonal antibody, resulted in a 31% remission rate in treated CD patients in a phase III study, which was much higher than that in the control group (15%)[17]. Similarly, another anti-p19 antibody, mirikizumab, seemed to be effective in inducing remission in patients with moderate to severe UC[18]. Inhibition of the IL-12/23 signalling pathway is a promising therapeutic option for IBD, especially if the safety of the new anti-IL-12/23 agents targeting the p19 subunit can be confirmed.

Anti-lymphocyte trafficking agents

Inhibition of immune cell migration to inflamed tissue has emerged as a novel therapeutic mechanism for IBD[19]. VDZ is the most commonly used antiadhesion agent with a selective blocking effect of the α 4β7 integrin in the intestine[20]. In addition to this agent, etrolizumab is a newly developed monoclonal antibody that targets the β7 subunit of the α4β7 and αEβ7 integrins. In a study that included 1081 patients with moderate to severe UC, the rate of remission induction in the etrolizumab group was 18.5% compared with only 6.3% in the placebo group[21]. Etrolizumab was also reported to be effective in CD patients[22]. In addition, inhibition of the integrin-α4 subunit might also be useful for inflam-

mation control in IBD. AJM300 is a small molecule inhibitor of the α4 subunit of integrin that led to disease remission rate of 63% compared with a 26% remission rate in the placebo group among 102 UC patients in a randomized controlled study[23]. Another mechanism that limits immune cell migration is the inhibition of the sphingosine-1-phosphate receptor (S1PR). The S1P signalling network is mediated by 5 S1P G-protein coupled receptors (S1PR1-5). Ozanimod is a new class of S1PR modulators that shows activity against S1PR1 and S1PR5. In the TOUCHSTONE study, ozanimod therapy showed excellent efficacy in remission induction and maintenance in moderate to severe UC, and mucosal healing was better (34% with ozanimod *vs* 12% with placebo)[24]. Anti-lymphocyte migration might be an attractive therapeutic strategy in some situations, and these drugs may be promising and powerful in IBD management.

JAK inhibitors

The JAK family comprises important intracellular signalling molecules consisting of 3 subtypes (*e.g.*, JAK1, JAK2, and JAK3). Tofacitinib is the only SMD targeting JAK1 and JAK3 for moderate to severe UC that is approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA)[25]. However, some studies have revealed an association between tofacitinib and systemic side effects, such as malignancies, cardiovascular events, and venous thromboembolism, in patients with rheumatoid arthritis[26]. Thus, more selective JAK inhibitors are needed for IBD. Currently, filgotinib is a selective JAK1 inhibitor that has shown promising effects in the induction of disease remission in CD patients in the phase II FITZROY study[27]. More patients treated with filgotinib achieved endoscopic response, remission and healing compared with those who received placebo (47% *vs* 23%, $P = 0.0077$). However, data from that study showed that patients in the filgotinib group experienced more serious adverse events (9% *vs* 4%) and more serious infections (3% *vs* 0%) than those in the placebo group. Upadacitinib is another oral selective JAK1 inhibitor. The CELEST trial recently assessed upadacitinib in patients with moderate to-severe CD. At week 16, clinical remission was notable in the 6 mg group (upadacitinib 27% *vs* 11% placebo, $P < 0.1$). However, at week 52, patients in the upadacitinib groups had a higher incidence of serious infections. In addition, patients treated with 12 or 24 mg twice daily had increased serum lipids[28]. Generally, these new selective JAK inhibitors provide a promising prospects in IBD treatments, but their safety profiles should not be ignored.

Phosphodiesterase 4 inhibitors

Phosphodiesterase 4 (PDE4) is involved in intracellular cAMP transformation and activation of the nuclear transcription factor kappaB and promotes inflammation in the intestine[29]. Thus, PDE4 inhibition may reduce cytokine release syndrome. A phase II RCT assessed the efficacy of the PDE4 inhibitor apremilast in 170 adult UC patients. The results showed a higher clinical remission rate in patients treated with apremilast *vs* placebo[30]. In addition, significant decreases in inflammatory markers, such as C-reactive protein (CRP) and faecal calprotectin, were observed in this study.

Taken together, although the understanding of the pathogenesis of IBD is rapidly evolving and increasing numbers of new biologics and SMDs that have been developed, none of these drugs is effective in all patients. Thus, there is increasing interest in the therapeutic potential of the combination of biologics and/or SMDs with different mechanisms of action in patients with refractory IBD.

THE CURRENT STATUS OF BIOLOGICAL COMBINATIONS IN IBD

The immune response in IBD is multifaceted and accompanied by multiple activated inflammatory pathways in the intestinal mucosa. Single-targeted therapy consisting of biological monotherapy blocks only one inflammatory pathway, which is inadequate to control inflammation completely. Combinations of biologics with different mechanisms may have synergistic effects and contribute to the control of refractory IBD[3,31]. Currently, an emerging strategy, DTT, which is a combination of two biologics or a biologic and tofacitinib has been applied in patients with refractory disease. However, most studies on DTT are case reports and case series, and therefore, we could not summarize data to provide a comprehensive understanding of experiences with this strategy. From the limited evidence available, we briefly discuss the current paradigms of DTT in treating patients with refractory IBD who have failed multiple biologics.

As a prominent anti-TNF biologic, IFX was the first biologic agent used in IBD and has achieved great success[20]. In the last decade, several RCTs have demonstrated the efficacy of IFX combined with immunosuppressive agents[9,32,33]. However, approximately one-third of IBD patients exhibit no response to anti-TNF biologics, and another third need to switch to different agents within one year due to the secondary loss of response[20,34]. With the permission of the FDA and EMA, VDZ has become the first choice among second-line biologics for moderate to severe CD and UC patients who have experienced failure with conventional medications or anti-TNF agents[31]. Thus, in clinical practice, anti-TNF + VDZ is the most common combination paradigm used in DTT. A meta-analysis consisting of 30 studies of dual biologics or SMDs in IBD management revealed that the proportion of anti-TNF + VDZ algorithms ranked first among the various DTT paradigms and accounted for 48% of all

algorithms used. The combination of UST + VDZ was the second most popular paradigm and accounted for 19%. The clinical response rates and endoscopic response rates were comparable for different DTT groups in this meta-analysis[35]. Yang *et al*[36] reported that the rates of endoscopic improvement [reduction of simple endoscopic score for CD (SES-CD) > 50%] for anti-TNF + VDZ and anti-TNF + UST were both 33%. Additionally, VDZ + UST had the highest rates of endoscopic improvement (63%) compared with other combinations, but all DTT paradigms had similar efficacy in terms of endoscopic remission (SES-CD < 3). To date, the broadest experience with IBD patients treated with various DTTs is reported by a retrospective study[37]. Fifty patients with IBD [31 CD, 18 UC and 1 IBD-unclassified (IBD-U)] were included in this study. VDZ + UST was the most used combination paradigm (25/50), followed by VDZ + ADA (3/50), VDZ + GOL (2/50), and VDZ + CZP (2/50). Notably, 20 patients received tofacitinib combined with biologic treatment, but no specific data on this subgroup were provided in this report. The results from this study showed that CRP levels were significantly reduced from baseline (2.35 mg/dL *vs* 5.00 mg/dL, $P = 0.002$), 56% (18/32) of patients treated with dual biologic therapy maintained clinical remission after 3 mo, and that 11 of 32 patients were still in endoscopic remission after 8 mo[37]. Currently, growing numbers of newer biologics and SMDs are included in the candidate pools for DTTs, including anti-IL-23 agents such as mirikizumab, risankizumab, brazikumab and guselkumab, anti-integrin agents such as etrolizumab and ontamalimab [38], new SMDs such as PDE-4 inhibitors, as well as IL-6 inhibitors and S1PR agonists[4,39,40].

Although the use of DTTs that address different targets is increasingly applied to treat patients with refractory CD or UC, no strong evidence has shown that DTT might be effective in all patients. An early RCT included 79 patients with active CD who failed to respond to IFX treatment and did not report a statistically significant difference in efficacy between the IFX + NAT group and the IFX + placebo group [41]. Another observational study conducted on 16 paediatric patients with refractory IBD (7 CD, 9 UC, 1 IBD-U) showed that 75% achieved steroid-free clinical remission 6 mo after DTT but that 19% of patients discontinued DTT treatment because of inflammation control failure[42]. Additionally, some low-quality evidence suggested that DTT is more effective in CD patients with a penetrating phenotype [36,43]. Among the patients enrolled in a study conducted by Kwapisz *et al*[43], the median disease duration was 12.5 years, 86.7% (13/15) had penetrating disease, and 3.8 types of biologics were ineffective for these patients. Despite the disease severity, more than half of patients exhibited improved clinical symptoms and had less steroid use after DTT[43]. However, it is still unclear which combinations of biologics work best in specific IBD subgroups. Understanding of the pathophysiology of IBD and identifying prognostic biomarkers may significantly optimize DTT therapy.

WHAT CAN WE DO TO IMPROVE THE RESPONSE TO DTT

Heterogeneity among patients is one of the main features of IBD and is reflected by different disease behaviors and responses to therapeutics[44]. Although remarkable progress has been achieved in the development of new agents with novel mechanisms of action assisted by advanced management strategies, the current treatment pattern for IBD still relies on clinical symptoms and endoscopy examinations[45]. In addition, as mentioned above, many drugs are effective only in selected patients with IBD, and even DTT strategies cannot guarantee a response in all patients. Thus, the identification of patients who can benefit from DTT is urgent so that individualized treatment with biological agents can be provided.

Therapeutic drug monitoring enhanced the response to DTT

DTT is mainly used as an add-on therapy for patients who exhibit a partial response to monotherapy or who relapse during maintenance therapy. A major problem associated with failure of biological therapy is a loss of response, and therapeutic drug monitoring (TDM) may be a useful auxiliary tool in the management of patients treated with DTT[6,46].

TDM was originally suggested as a way to monitor the response to monotherapy and is divided into two categories: Proactive TDM (performed regularly to target an appropriate drug trough concentration) and reactive TDM (performed upon loss of response)[46]. Strong evidence indicates that TDM implementation is associated with higher rates of clinical remission, better endoscopic mucosal healing, and lower rates of secondary loss of response to biologics[47,48]. To date, TDM has achieved great success in optimizing the management of a combined biologics approach in patients treated with anti-TNF agents. The most convincing evidence comes from the management of secondary failures for IFX and ADA. The personalized anti-TNF therapy in CD study (PANTS), which included 1610 anti-TNF-naive patients with exposure to IFX or ADA, demonstrated that monitoring drug concentrations helps greatly in predicting therapeutic responses. The results showed that the only risk factor associated with primary nonresponse was low drug concentrations at week 14 [IFX: Odds ratio (OR) = 0.35, 95% confidence interval (CI): 0.20-0.62, $P = 0.00038$; ADA: OR = 0.13, 95%CI: 0.06-0.28, $P < 0.0001$] and that ideal drug concentrations at week 14 (7 mg/L for IFX and 12 mg/L for ADA) were a strong predictor for clinical remission at week 54. With the guidance of proactive TDM, dose intensification of initial biologics or combinations with IM (thiopurine or methotrexate) therapy improved outcomes of patients

with suboptimal drug concentrations at week 14[47]. Additionally, an expert consensus statement by Cheifetz *et al*[49] recommended a proactive TDM strategy during remission induction with anti-TNF agents and at least once during maintenance.

Reactive TDM could help distinguish patients who need to switch or combine with another class of biologic due to anti-drug antibodies (immunogenicity) from those who might benefit from dose escalation of monotherapy[50]. Actually, identifying patients with pharmacokinetic failure in biologics therapy is extremely useful in the guidance of biologics regimens, especially in DTT. Inadequate trough concentrations of drugs can not only lead to an insufficient efficacy of biologics, but patients may also become insensitive to the mechanism of action. For example, it was recently found that anti-TNF resistance in CD patients may be related to increased numbers of CD4+ T cells that overexpress the IL-23 receptor. Thus, it is possible that combinations with IL-23 inhibitors may help restore the sensitivity to the mechanism of action of anti-TNFs in such patients[51].

At present, there is little research focusing on the role of TDM for biologics other than anti-TNF agents, such as VDZ (anti-α4β7 integrin) or UST (anti-IL-12/23)[52]. Although the relationship between drug concentrations and clinical outcomes has been demonstrated, the value and cost-effectiveness of TDM in optimizing these biologic therapies are uncertain, and all the information given regarding TDM is derived from studies performed in patients treated with monotherapy. Therefore, relevant guidelines about TDM implementation in DTT have not been recommended by any academic association. Overall, TDM has great value in optimizing biologics therapy and providing individualized treatment for IBD but still has very significant problems and challenges in clinical practice.

Biomarkers help predict responses to biological therapies

IBD treatments are a long-term process, and disease monitoring is essential once treatment has started. A growing number of studies have put great effort into identifying prognostic and predictive biomarkers[53,54]. To date, various biomarkers have been proposed as clinical predictors of the response to biologics, including serological and faecal proteins, cytokines, proteomic-related and microbiome-related factors as well as metabolomic and genetic factors[55].

Serum and faecal markers have been widely applied in evaluating the efficacy of biologics. Serum CRP and fecal calprotectin, as inflammatory markers, have been shown helpful in response detection of anti-TNF agents. Although fecal markers are more sensitive than serum markers, such as CRP, in monitoring intestinal inflammation, there is no solid evidence demonstrating the association between fecal biomarkers and the response to anti-TNF agents[56,57]. Some proteins in the intestinal mucosa can also play a predictive role in response to biologics, such as Piwi-like protein 1, MYCBP associated and testis expressed 1, regulators of G-protein signaling 13 and Dachsous 2. Elevated expressions of these cytokines or proteins are beneficial for achieving a stable response to anti-TNF therapy[58,59].

Exploration of the genetic factors that predict the responses to anti-TNF therapy has also made great progress. The genetic polymorphisms in TNFRSF1A (rs4149570), IL-6 (rs10499563), IL-1β (rs4848306), toll-like receptors 2 (TLR2) (rs3804099), and TLR4 (rs5030728) are associated with the response to anti-TNF agents[60]. In addition, an observational study including 1240 European patients with CD found that the human leukocyte antigen-DQA1*05 mutation increased the risk of developing anti-TNF antibodies[61]. Single-cell sequencing revealed that some activated cells [*e.g.*, macrophages, immunoglobulin G (IgG) cells, T cells, and dendritic cells] in patients with failure to receive anti-TNF therapy are dysfunctional with genetic variation[62].

Many studies have suggested that the composition of the gut microbiota is related to the response to therapies. In the STORI study, Rajca *et al*[63] found that lower levels of *Faecalibacterium prausnitzii* (*F. prausnitzii*) were associated with early recurrence of CD after IFX withdrawal. Additionally, higher abundances of *F. prausnitzii* were associated with better responses to anti-TNF treatments[64,65]. Nevertheless, this relationship could not be confirmed in other studies[66], and when we examine the effects of other bacteria, studies with conflicting results are common[65,67]. To date, the microbiome has not been shown to be a predictive indicator of the response to biologics due to the very high heterogeneity in different individuals.

Metabolomics is a novel method that can quantify small metabolite sugars, such as lipids and amino acids, and thus offers a promising opportunity to identify candidate markers. Through metabolomic analysis, Nikolaus *et al*[68] found that the serum tryptophan levels increased in IBD patients who responded to IFX therapy but were unchanged in patients who did not respond to IFX or VDZ therapy. In addition, another study including 76 CD patients found that responders and non-responders have distinctive patterns of bile acids derived from feces, serum, and urine. By combining these representative markers, the responses to anti-TNFs may be predicted[69]. The biomarkers mentioned above could assist only with the accuracy of disease monitoring and response to biologics in IBD, and more studies are needed to identify a gold standard in the DTT strategy of IBD.

BISPECIFIC ANTIBODIES: THE NEXT GENERATION OF DTT

It has been proven that targeting multiple inflammatory signaling pathways by combining different

biologics has better outcomes for IBD patients than monotherapies[41,70]. However, the doses used in the DTT strategy are based on those for individual therapies, which might have unfavorable benefit-risk ratios, such as placing patients at greater risk of serious infections or malignancies[6,35]. The advent of bispecific antibodies (BsAbs) may provide new insights to help avoid some of these problems in DTT.

BsAbs are antibody formats that can bind to two different antigens or two different epitopes of the same antigen. Broadly, they can be classified as a special type of DTT. To date, BsAbs are divided into two major structural classes: IgG-like BsAbs carrying an Fc domain and non-IgG-like formats, which rely entirely on their antigen-binding capacity to exert therapeutic effects[71]. BsAbs can function by: (1) Impacting specific cell types by targeting multiple receptors; (2) Activating novel signaling *via* receptor colocalization or hyper crosslinking; and (3) Destroying pathogenic T cells through redirection[72]. Currently, different BsAbs are in different stages of clinical trials, and three BsAbs have been approved for clinical practice globally, namely, catumaxomab (for malignant ascites), blinatumomab (for leukemia) and emicizumab (for hemophilia)[71].

Although there are currently no BsAbs approved for IBD patients in clinical practice, several promising BsAbs are under investigation. For instance, BsAb drugs targeting both TNF and IL-23 are in the preclinical stage for autoimmune diseases, including IBD[72]. These BsAbs showed synergistic efficacy in alleviating colitis in a CD40-induced colitis model compared with anti-TNF and anti-IL-23 agents alone. Another ongoing phase I trial is investigating the effects of APVO210 in treating UC. These BsAbs are composed of an anti-CD86-IL-10 fusion protein and selectively deliver IL10 to CD68+ antigen-presenting cells, in which they have been demonstrated to induce a tolerogenic phenotype to relieve inflammation[73].

The unique mechanism of BsAbs provides an opportunity to target multiple molecular pathways with a single therapeutic agent. With careful dose adjustments, IBD patients can achieve maximal benefits from BsAbs and also good benefit-risk ratio[72]. However, this therapeutic approach is not without defects. On the one hand, the formulation of the two antibody binding domains of BsAbs is fixed, so it is impossible to change the single administration dose of different monoclonal antibodies according to patient needs, as we usually do in traditional dual biologic combination paradigms[74,75]. On the other hand, immunogenicity is an ever-present concern during the development of biological medication in IBD, especially in BsAbs. The large antibody complexes on the surface of BsAbs could act as "danger signals" that induce immunogenicity and eventually lead to loss of response[76].

CONCLUSION

Medical treatment patterns for IBD are rapidly evolving with the increased understanding of disease pathogenesis and development of new drugs that target various pathways. This review describes novel biological agents and SMDs that are in development and highlights the current status of DTT strategies in IBD management. Although drugs and therapeutic strategies that can cure all patients have not yet emerged, the efficacy of DTT in inducing and maintaining disease remission has been dramatically improved by taking advantage of new biological combination paradigms, modern TDM strategies, and novel predictive biomarkers. In future work, the identification of biomarkers that can predict subsets of patients and a more profound comprehension of the immunological landscape with IBD would help to enable more specific individualized medicine.

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MINIREVIEWS

Confusion and prospects for carcinogenesis of gastric adenoma and dysplasia: What is the correct answer currently?

Shinichi Kinami, Sohsuke Yamada, Hiroyuki Takamura

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Abstract

There are differences in the diagnoses of superficial gastric lesions between Japan and other countries. In Japan, superficial gastric lesions are classified as adenoma or cancer. Conversely, outside Japan, the same lesion is classified as low-grade dysplasia (LGD), high-grade dysplasia, or invasive neoplasia. Gastric carcinogenesis occurs mostly *de novo*, and the adenoma-carcinoma sequence does not appear to be the main pathway of carcinogenesis. Superficial gastric tumors can be roughly divided into the *APC* mutation type and the *TP53* mutation type, which are mutually exclusive. *APC*-type tumors have low malignancy and develop into LGD, whereas *TP53*-type tumors have high malignancy and are considered cancerous even if small. For lesions diagnosed as category 3 or 4 in the Vienna classification, it is desirable to perform complete *en bloc* resection by endoscopic submucosal dissection followed by staging. If there is lymphovascular or submucosal invasion after mucosal resection, additional surgical treatment of gastrectomy with lymph node dissection is required. In such cases, function-preserving curative gastrectomy guided by sentinel lymph node biopsy may be a good alternative.

Key Words: Gastric adenoma; Low-grade dysplasia; High-grade dysplasia; Intramucosal carcinoma; Submucosal carcinoma; Endoscopic submucosal dissection

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Core Tip: Gastric carcinogenesis occurs mostly *de novo*. Superficial gastric tumors can be roughly divided into the *APC* mutation type and the *TP53* mutation type, which are mutually exclusive. *APC*-type tumors have low malignancy and develop into low-grade dysplasia, whereas TP53-type tumors have high malignancy and are considered cancerous even if they are small. For lesions diagnosed as category 3 or 4 in the Vienna classification system, endoscopic submucosal dissection and staging should be performed. If the tumor is diagnosed with lymphovascular or submucosal invasion, additional surgical treatment of gastrectomy with lymph node dissection is required.

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INTRODUCTION

Gastric cancer is one of the most common cancers worldwide. However, the incidence of gastric cancer declined in many Western countries during the 20th century. Japan was one of the countries with a high incidence of gastric cancer, but the incidence is also decreasing. This fact proves that *Helicobacter pylori* (*H. pylori*) infection is deeply involved in the development of gastric cancer[1]. In Japan, the water supply and sewerage systems were completed in the 1960s, and the *H. pylori* infection rate has decreased among the generations born subsequently[2,3]. Most patients with gastric cancer in Japan are elderly, and the incidence of gastric cancer among age groups with low *H. pylori* infection rates is low. Besides *H. pylori*, many factors are known to be involved in gastric carcinogenesis. These include salt intake, smoking, exposure to N-nitroso compounds, and Epstein-Barr virus infection[4-7]. However, the molecular mechanisms leading to gastric carcinogenesis are not well understood.

In contrast, the molecular mechanism leading to colorectal cancer has been clarified to some extent. Colorectal cancer is one of the most common cancers worldwide. Many colorectal cancers are thought to develop from adenomas and serrated polyps through the adenoma-carcinoma sequence. The molecular mechanism of colorectal carcinogenesis has long been a subject of interest and has been well-studied, with genetic and epigenetic changes in oncogenes and tumor suppressor genes identified in considerable detail.

There are various reasons for this difference in the understanding of the molecular mechanisms between gastric carcinogenesis and colorectal carcinogenesis. The most important is that gastric carcinogenesis is often of the *de novo* type and does not necessarily follow the adenoma-carcinoma sequence, making it difficult to examine the genetic changes from benign lesions to carcinoma in a sequential manner. Another reason is that the diagnostic criteria for gastric adenomas are vague and differ between countries in the East and West.

In this article, we describe the issues surrounding gastric adenomas, the molecular mechanisms of carcinogenesis that have been identified to date, and future perspectives.

DIAGNOSTIC CRITERIA FOR GASTRIC ADENOMA

It has long been known that some benign superficial gastric lesions are difficult to distinguish from adenocarcinoma. They are conventionally called atypical epithelial lesions or IIa-subtype[8,9]. These cases were organized and given the diagnostic name "gastric adenoma[10]" approximately during the time the World Health Organization (WHO) histological classification of gastric cancer was established in the 1970s. In Japan, superficial gastric lesions are classified into adenoma and cancer and a treatment policy is adopted: The cancer is resected, small adenomas are followed up, and large adenomas are regarded as early gastric cancer (EGC) and treated by mucosal resection. In Japan, gastric adenomas are classified mainly according to glandular structure, with occasional reference to immunohistochemical mucin staining. Recently, foveolar-type gastric adenomas with a raspberry-like appearance in *H. pylori*-negative cases have become a contentious issue[11,12]. Conversely, outside Japan, dysplasia is used to describe lesions that are difficult to distinguish from benign to malignant. Dysplasia is defined as a histologically probable neoplastic lesion without evidence of invasive growth within the specimen. Intraepithelial neoplasia is a synonymous condition. Dysplasia is classified into low-grade dysplasia (LGD) and high-grade dysplasia (HGD) according to the degree of cellular atypia[13]. Gastric adenoma exists outside Japan but mainly refers to a protruding tumor.

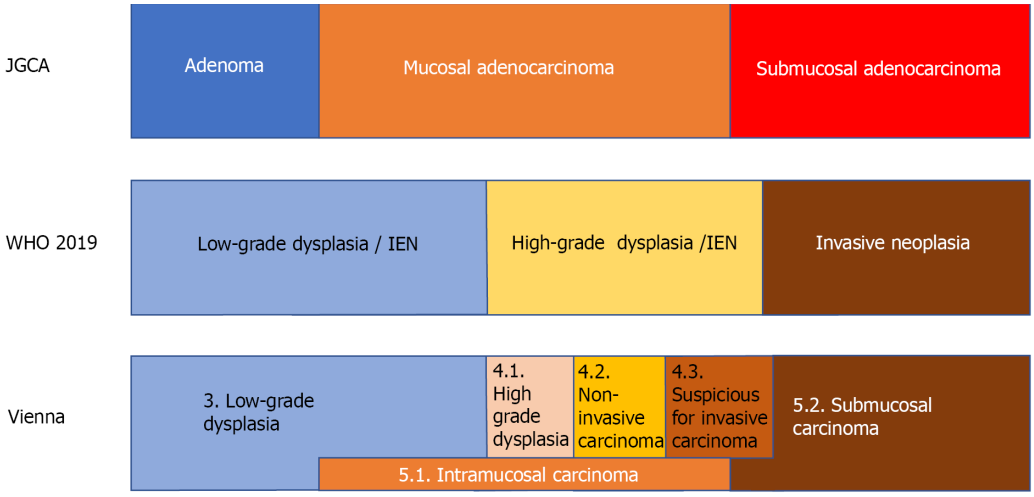
Therefore, there are differences in the diagnoses of superficial gastric lesions between Japan and other countries. Table 1 also shows the classification of gastric lesions according to the WHO classification, Vienna classification proposed at the worldwide pathologists' consensus meeting[14], and revised

Table 1 The classifications of gastric adenoma of the Japanese Gastric Cancer Association and gastric superficial lesions of the WHO classification and the Vienna classification

Classification	Code	Diagnosis	Subtype	Subtype 2
JGCA[16]		Gastric adenoma	Intestinal type	
			Gastric type	Pyloric gland type
				Foveolar type
WHO 2019[13]	8148/0	Glandular intraepithelial neoplasia, low grade		
	8148/2	Glandular intraepithelial neoplasia, high grade		
	8213/0	Serrated dysplasia, low grade		
	8213/2	Serrated dysplasia, high grade	Intestinal-type dysplasia	
			Foveolar-type (gastric type) dysplasia	
			Gastric pit/crypt dysplasia	
	8144/0	Intestinal-type adenoma, low grade		
	8114/2	Intestinal-type adenoma, low grade	Sporadic intestinal-type gastric adenoma	
			Syndromic intestinal-type gastric adenoma	
	8210/0	Adenomatous polyp, low-grade dysplasia		
	8210/2	Adenomatous polyp, high-grade dysplasia		
Vienna[14]	3	Non-invasive low-grade neoplasia	Low-grade adenoma/dysplasia	
	4	Non-invasive high-grade neoplasia		
	4.1		High-grade adenoma/dysplasia	
	4.2		Non-invasive carcinoma (carcinoma *in situ*)	
	4.3		Suspicion of invasive carcinoma	
	5	Invasive neoplasia		
	5.1		Intramucosal carcinoma	
	5.2		Submucosal carcinoma or beyond	
Revised Vienna[15]	3	Mucosal low-grade neoplasia	Low-grade adenoma/dysplasia	
	4	Mucosal high-grade neoplasia		
	4.1		High-grade adenoma/dysplasia	
	4.2		Non-invasive carcinoma (carcinoma *in situ*)	
	4.3		Suspicious for invasive carcinoma	
	4.4		Intramucosal carcinoma	
	5	Submucosal invasion by carcinoma		

JGCA: Japanese Gastric Cancer Association.

Vienna classification[15]. Figure 1 shows the relationship between the diagnosis of gastric lesions and the Japanese classification of gastric carcinoma[16], WHO classification, and Vienna classification. The diagnosis of adenomas in Japan is probably the most limited. It is difficult to determine the correct classification system because they all have advantages and disadvantages, and there are also differences in the frequency of encountering lesions and treatment strategies. In Japan, where the prevalence of gastric cancer is high, clinicians perform numerous endoscopic screenings. They often find EGCs, and targeted biopsy is frequently used for definitive diagnosis. Pathologists and clinicians must determine benign or malignant lesions from biopsies and determine cancer based on cellular and structural atypia. As the presence or absence of submucosal invasion is not required under the Japanese classification,



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Figure 1 The relationship of the diagnosis of superficial gastric lesions between the Japanese classification of gastric carcinoma by the Japanese Gastric Cancer Association, the WHO classification, and the Vienna classification. In Japan, gastric cancer is diagnosed based on cellular and structural atypia. On the other hand, outside Japan, dysplasia is used to describe lesions that are histologically probable neoplastic lesions without evidence of invasive growth. Intraepithelial neoplasia is a synonymous condition. Therefore, all mucosal and some submucosal cancers diagnosed by the Japanese Gastric Cancer Association criteria are diagnosed as dysplasia outside Japan. The original Vienna classification is the answer to this discrepancy by setting non-invasive carcinoma and intramucosal carcinoma. IEN: Intraepithelial neoplasia; JGCA: Japanese Gastric Cancer Association.

cancer can be diagnosed without performing complete resection and determining the presence or absence of submucosal invasion. However, intramucosal carcinoma is also considered a cancer, although this is not accepted by some pathologists in Western countries. Conversely, according to criteria other than those of the Japanese Gastric Cancer Association (JGCA), it is difficult to obtain tissue from the submucosal layer with endoscopic biopsy; therefore, pathological judgment using biopsy material can only be performed in dysplasia, making it difficult to diagnose cancer using biopsy. Pathologists cannot determine gastric cancer without complete resection of the lesion, and intramucosal cancer is not defined as cancer. Although intramucosal carcinoma has a good prognosis and rarely metastasizes, lymph node metastasis still occurs in 2% of cases[17]; if left untreated, it can progress to submucosal and advanced cancer[18]. Therefore, intramucosal cancer should still be considered life-threatening. In Japan, adenomas are regarded as "benign lesions"; therefore, even among Japanese pathologists, it is difficult to distinguish high-grade intestinal-type adenomas and foveolar-type adenomas from cancer, and discrepancies in diagnosis sometimes occur.

DOES GASTRIC ADENOMA BECOME CANCER?

Many colorectal cancers are thought to develop from adenomas and serrated polyps. Do gastric adenomas become cancers, similar to colorectal cancer?

Some gastric lesions might be cancerous after mucosal resection, even if the preoperative diagnosis is adenoma using targeted biopsy. The frequency of such cases varies in the literature; however, there are reports of a reasonably high rate; therefore, caution should be exercised[19]. It is sometimes difficult to distinguish between high-grade intestinal-type adenomas and very well-differentiated tubular adenocarcinomas, and sampling errors may occur in targeted biopsies[19]. In contrast, adenocarcinoma in adenoma, unlike colorectal cancer, is rarely observed in low-grade intestinal-type adenoma, and it is rare for low-grade adenoma of Vienna classification category 3 to become malignant[20]. In addition, gastric minute carcinomas without adenomatous components are common. Gastric carcinogenesis is mostly *de novo*, and the adenoma-carcinoma sequence does not appear to be the main pathway of carcinogenesis.

The Cancer Genome Atlas system classifies gastric cancer into four categories based on molecular biological characteristics[21]. A summary of this classification system is presented in Table 2. The molecular biological features revealed here help in the consideration of treatment strategies for advanced gastric cancer; however, this system does not provide insight into genetic alterations in the early stages of carcinogenesis.

The pathway for the accumulation of gene mutations leading to gastric carcinogenesis is not as clear as that in colorectal cancer. This may be mainly due to the lack of a clear adenoma-carcinoma sequence in the stomach and the discrepancy in the diagnostic criteria for gastric adenoma and intramucosal carcinoma between countries in the East and West.

Table 2 Brief summary of The Cancer Genome Atlas classification

Type	Chromosomal instability	EBV	Microsatellite instability	Genomically stable
Percentage	50%	9%	21%	20%
Profile of patients		Male prevalence	Elderly age	Younger age
Location	GEJ, cardia	Corpus or fundus	Antrum	Distal location
Lauren type	Intestinal		Intestinal	Poorly cohesive
Other pathological feature	DNA aneuploidy	Carcinoma with lymphoid stroma		
Prognosis			Favorable	Worst
Genetic features	*TP53* mutation	Extensive DNA promoter methylation	*MLH1* promoter hypermethylation	Low copy number alterations and mutational burden
	Amplification of *TKR*	*CDKN2A* promoter hypermethylation	High mutational burden	*ARID1*, *RHOA*, *CDH1* mutations
		PIK3CA, *ARID1A*, *BCOR* mutations		*CLDN18-ARHGAP26* fusion in 15%

TCGA: The Cancer Genome Atlas; EBV: Eptein-Barr virus; GEJ: Esophagogastric junction.

GENETIC MUTATION AND CANCERIZATION OF GASTRIC ADENOMA AND DYSPLASIA

Recent advances in genetic analysis have provided insights into gene mutations in adenomas and dysplasia, and the pathway to carcinogenesis in adenomas and dysplasia is becoming more clear.

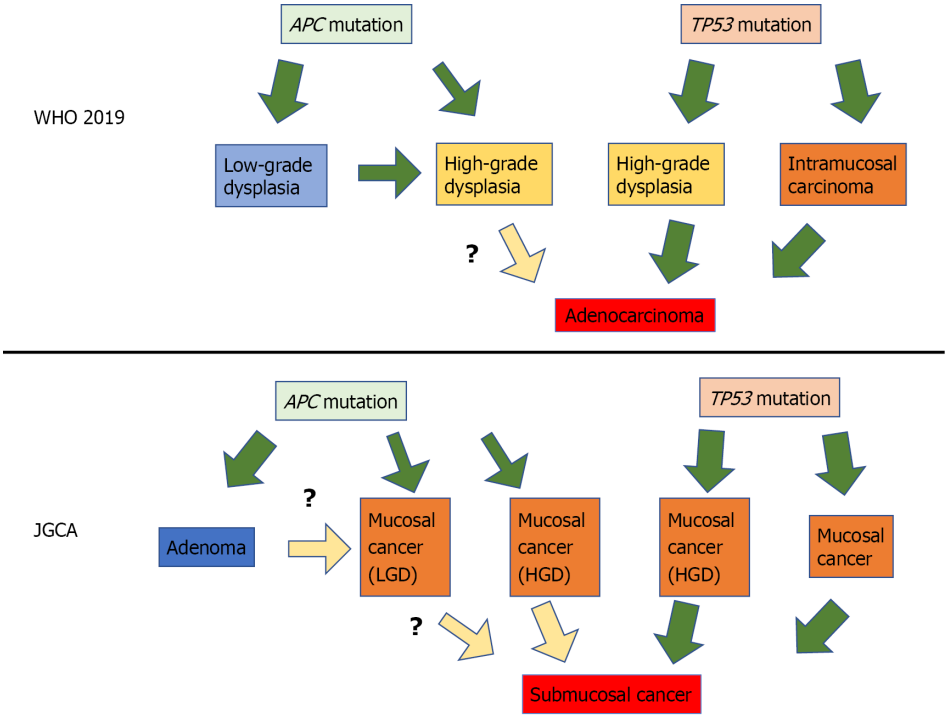
Fassan *et al*[22] investigated the mutational status of HGD and EGC using high-throughput mutation profiling. Mutations in *APC*, *ATM*, *FGFR3*, *PIK3CA*, *RB1*, *STK11*, and *TP53* were confirmed in both HGD and EGC. Lim *et al*[23] examined the mutation profiles of LGD using whole-exome sequencing and confirmed that *APC* mutations occur in LGD. Lee *et al*[24] examined *APC* mutations in adenomas, dysplasias, and adenocarcinomas. They found that *APC* mutations play an essential role in the pathogenesis of adenoma and dysplasia but have a limited role in the progression to adenocarcinoma. Rokutan *et al*[25] investigated the mutational status of LGD, HGD, and intramucosal carcinoma using targeted deep DNA sequencing. They found that *APC* mutations and *TP53* mutations were highly prevalent in these lesions and were the initial mutations in the tumors. *TP53* mutations were also found in microscopic intramucosal carcinomas of 1 mm and 3 mm. *APC* mutations were found in all the LGDs examined. In contrast, no *TP53* mutations were detected in the LGD group. *APC* mutations and *TP53* mutations are frequently observed in patients with HGD, but they are mutually exclusive.

Based on these results, superficial gastric tumors can be roughly divided into the *APC* mutation type and the *TP53* mutation type. *APC*-type tumors have low malignancy and develop into LGD, whereas *TP53*-type tumors have high malignancy and are judged as cancerous even if they are small[25]. It is still unclear whether *APC*-type LGD progresses into HGD or whether *APC*-type HGD progresses into cancer. In contrast, it is reasonable to treat *TP53*-type HGD as cancer. This finding is illustrated in Figure 2; it also shows the translation of this to the JGCA criteria. Many Japanese gastric cancer specialists believe that all mucosal cancers progress from submucosal to advanced. However, some mucosal cancers may not progress to submucosal cancer, although they may progress laterally.

HOW SHOULD GASTRIC TUMORS BE TREATED?

Superficial gastric tumors are often observed in *H. pylori*-positive stomachs under numerous gastroscopies. There is still no consensus regarding the treatment of these tumors.

In Japan, EGC is often detected, and endoscopic submucosal dissection (ESD) is frequently performed; therefore, most superficial gastric tumors, including gastric adenomas, are resected by ESD [19]. The treatment policy is the same in China and South Korea, where there are many *H. pylori*-positive individuals. In contrast, in Western countries, the treatment of dysplasia is not always standardized due to the small number of *H. pylori*-positive patients, low number of gastroscopies performed, and lack of widespread use of ESD. In the Vienna classification[14,15], a target biopsy diagnosis is set from category 1 to 5, with category 1 being negative for neoplasia and should undergo no treatment, category 2 is indefinite for neoplasia and should undergo repeat biopsy, and category 5 is indicated for surgical resection. The problem is the treatment of categories 3 and 4. The revised Vienna classification[15] recommends endoscopic resection or follow-up for category 3 and endoscopic or surgical local resection



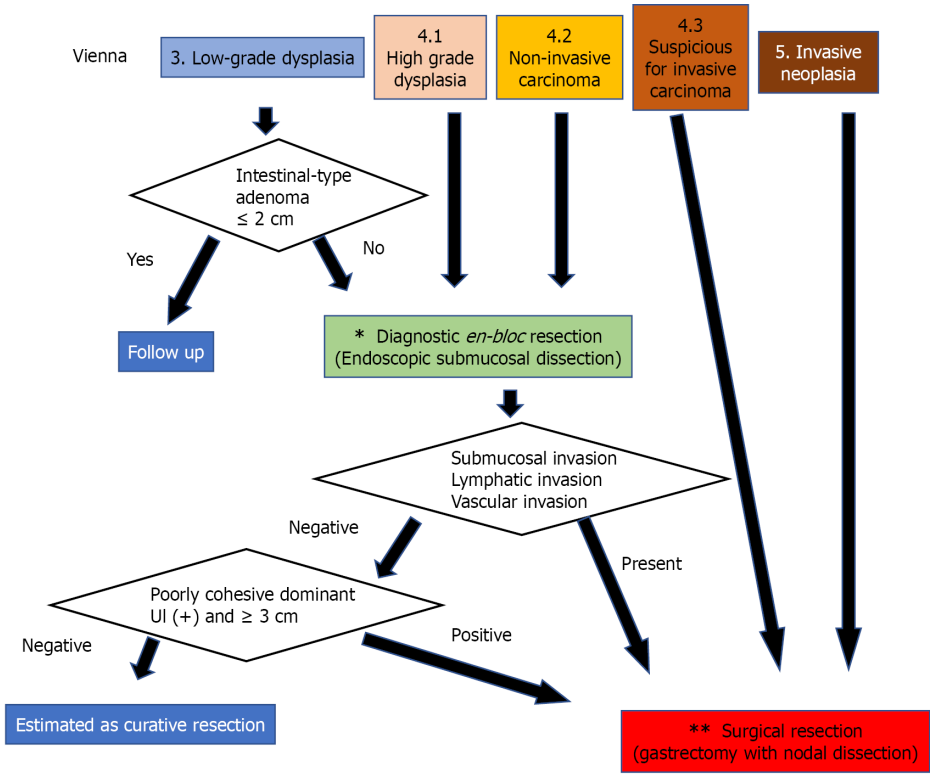
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Figure 2 The diagram assuming the relationship between gene mutations and gastric carcinogenesis. Superficial gastric tumors can be roughly divided into two types by specific gene mutations: The *APC* mutation type and the *TP53* mutation type. *APC*-type tumors have low malignancy and develop into low-grade dysplasia, whereas *TP53*-type tumors have high malignancy and are considered cancerous even if small. JGCA: Japanese Gastric Cancer Association; HGD: High-grade dysplasia; LGD: Low-grade dysplasia.

for category 4. The 2012 MAPS guideline[26] states that "patients with endoscopically visible HGD or carcinoma should undergo staging and adequate management". According to this, category 3 should be followed up, and category 4 should undergo excision. In contrast, the 2019 MAPS II guideline[27] states that "patients with an endoscopically visible lesion harboring LGD, HGD, or carcinoma should undergo staging and treatment". Due to the uncertainty of biopsy diagnosis[28,29], it is assumed that LGD would be upgraded to HGD or adenocarcinoma after resection. Therefore, treatment is also required for LGD [27], and category 3 is targeted for diagnostic treatment. However, staging and treatment methods have not been described. Considering the invasiveness of surgical resection, it is desirable to perform complete *en bloc* resection by ESD first[30] and then perform staging. Subsequent treatment should follow the Japanese Gastric Cancer Treatment Guidelines[31]. If the tumor is a well to moderately differentiated mucosal cancer with no lymphovascular invasion, treatment is completed, and if lymphovascular invasion or submucosal invasion is found, additional surgical treatment of gastrectomy with lymph node dissection is required.

Conversely, do we need ESD for all category 3 cases? Endoscopic resection of colorectal adenomas reduces the incidence of colorectal cancer, which provides evidence that the adenoma-carcinoma sequence is an essential pathway for colorectal carcinogenesis. In contrast, low-grade intestinal adenomas, which are rarely associated with adenocarcinomas, are unlikely to become cancerous even if left untreated. Upgrading to HGD or adenocarcinoma has been reported to be less than 10% after follow-up for adenoma and LGD[20,32], and the possibility of regression with *H. pylori* eradication therapy has also been reported[32]. For these reasons, category 3 adenomas can be safely treated with observation; if the adenoma meets the intestinal type in the JGCA criteria and is less than 2 cm in size, resection may not be necessary. However, a case of gastric-type adenoma that was adenocarcinoma in adenoma with submucosal invasion has been reported[33], and follow-up of gastric-type adenoma may not always be safe. In addition, the safety of observing LGDs that fall into mucosal cancer in the JGCA criteria is not guaranteed. In the future, further understanding of the relationship between genetic mutations in LGD and the natural history of lesions will provide profiles for safe follow-up of category 3 Lesions. Category 3 patients with *APC* mutations may be observed. However, at this time, category 3 adenomas, other than intestinal-type adenomas, seem to have no choice but to undergo complete diagnostic resection with ESD.

The results are summarized in Figure 3. Since category 3 and 4 Lesions are highly likely to be mucosal adenocarcinomas according to the JGCA criteria, complete *en bloc* resection of the mucosal layer is desirable even for diagnostic purposes, and ESD is appropriate. However, ESD is a complicated procedure. Surgical mucosal resection and laparoscopic intragastric surgery may also be acceptable in



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Figure 3 The strategy for diagnosis, staging, and treatment of gastric dysplasia and cancer according to the Vienna classification. Since category 3 and 4 Lesions are highly likely to be mucosal adenocarcinomas according to the Japanese Gastric Cancer Association (JGCA) criteria, complete *en bloc* resection of the mucosal layer is desirable for diagnosis and initial treatment. However, a small part of category 3, such as a small intestinal-type adenoma judged by the JCGA criteria, can be followed up. In contrast, category 5 corresponds to submucosal adenocarcinoma according to the JGCA criteria; therefore, curative surgery is necessary. Category 4.3 was also treated surgically. The asterisk (*): For *en bloc* mucosal resection, endoscopic submucosal dissection is appropriate; however, laparoscopic intragastric surgery may also be acceptable in cases where there is no skilled endoscopist. The two asterisks (**): Gastrectomy with lymph node dissection up to $D1^+$ is recommended for surgical treatment. However, since the possibility of lymph node metastasis is only 15%-20% even for such lesions, function-preserving curative gastrectomy guided by sentinel lymph node biopsy can be performed by a specialist.

cases where there is no skilled endoscopist[34]. In contrast, category 5 corresponds to submucosal adenocarcinoma in the JGCA criteria; therefore, gastrectomy with lymph node dissection is necessary [17,30]. Category 4.3. also has a high possibility of developing similar lesions; thus, surgery should be performed from the beginning. In addition, since the possibility of lymph node metastasis is only 15%-20% even for such lesions, not only gastrectomy with nodal dissection up to $D1^+$ but also function-preserving curative gastrectomy guided by sentinel lymph node biopsy may be a good indication[17].

CONCLUSION

Gastric carcinogenesis occurs mostly *de novo*, and the adenoma-carcinoma sequence does not appear to be the main pathway of carcinogenesis. Superficial gastric tumors can be roughly divided into the *APC* mutation type and the *TP53* mutation type, which are mutually exclusive. For lesions diagnosed as category 3 or 4 in the Vienna classification, it is desirable to perform ESD for accurate diagnosis and staging. If there is lymphovascular or submucosal invasion, additional surgical treatment of gastrectomy with lymph node dissection is required.

FOOTNOTES

Author contributions: Kinami S was responsible for the scientific conception of the study and writing of the manuscript; Yamada S and Takamura H contributed to the drafting, editing, and critical revision of the manuscript; all authors approved the final version of the manuscript.

Conflict-of-interest statement: The authors declare no conflict of interest related to the publication of this study.

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MINIREVIEWS

Nuclear factor erythroid 2-related factor 2-mediated signaling and metabolic associated fatty liver disease

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Abstract

Oxidative stress is a key driver in the development and progression of several diseases, including metabolic associated fatty liver disease (MAFLD). This condition includes a wide spectrum of pathological injuries, extending from simple steatosis to inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma. Excessive buildup of lipids in the liver is strictly related to oxidative stress in MAFLD, progressing to liver fibrosis and cirrhosis. The nuclear factor erythroid 2-related factor 2 (NRF2) is a master regulator of redox homeostasis. NRF2 plays an important role for cellular protection by inducing the expression of genes related to antioxidant, anti-inflammatory, and cytoprotective response. Consistent evidence demonstrates that NRF2 is involved in every step of MAFLD development, from simple steatosis to inflammation, advanced fibrosis, and initiation/progression of hepatocellular carcinoma. NRF2 activators regulate lipid metabolism and oxidative stress alleviating the fatty liver disease by inducing the expression of cytoprotective genes. Thus, modulating NRF2 activation is crucial not only in understanding specific mechanisms underlying MAFLD progression but also to characterize effective therapeutic strategies. This review outlined the current knowledge on the effects of NRF2 pathway, modulators, and mechanisms involved in the therapeutic implications of liver steatosis, inflammation, and fibrosis in MAFLD.

Key Words: Nonalcoholic fatty liver disease; Metabolic-associated fatty liver disease; Nuclear factor erythroid 2-related factor 2; Oxidative stress; Antioxidants; Liver injury

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Core Tip: This updated literature review contributes to the role of nuclear factor erythroid 2-related factor 2 in combating inflammation, oxidative stress, steatosis, and fibrosis in metabolic associated fatty liver disease. There are several reviews that elucidated the advantages of nuclear factor erythroid 2-related factor 2 in human diseases, but this is the first review reporting the broad range of nuclear factor erythroid 2-related factor 2 modulators and their therapeutic implications in metabolic associated fatty liver disease.

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INTRODUCTION

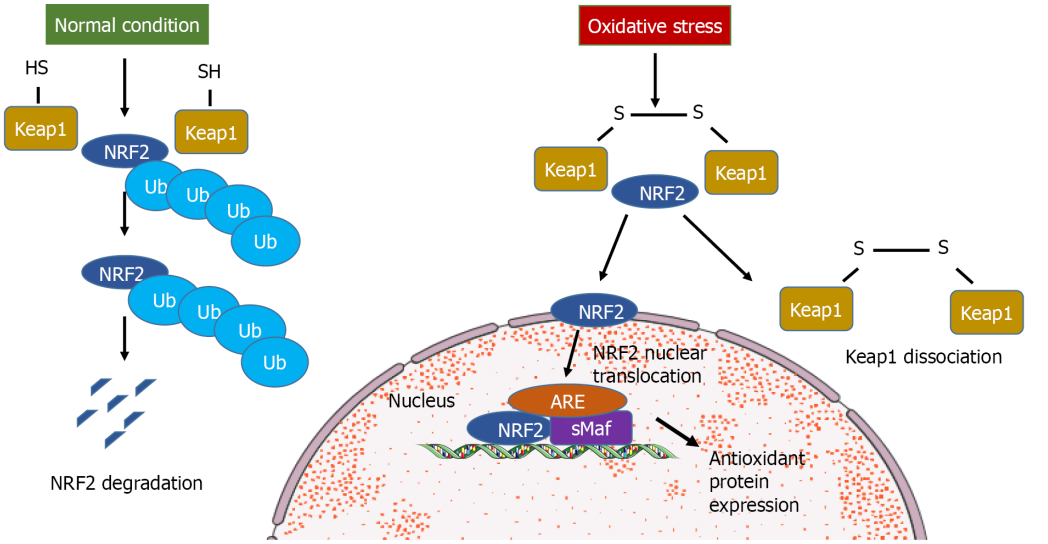
Nonalcoholic fatty liver disease is the most frequent chronic liver disease, affecting about 25% of the global population. Due to the reappraisal in its nomenclature, a group of experts changed the terminology from nonalcoholic fatty liver disease to metabolic-associated fatty liver disease (MAFLD), strengthening the link of this disease to metabolic alterations[1]. MAFLD is defined as a condition where hepatic fat accumulation exceeds 5% of the liver weight without alcohol consumption (< 30 g per day). It covers a wide spectrum of pathological conditions, extending from simple steatosis (deposit of fat in hepatocytes) to nonalcoholic steatohepatitis (characterized by the presence of 5% hepatic steatosis and inflammation with hepatocellular damage, with or without fibrosis), cirrhosis, and ultimately leading to hepatocellular carcinoma[2]. MAFLD is emerging with the prevalence of type 2 diabetes mellitus, obesity, and metabolic syndrome[3]. Of note, patients with MAFLD, and particularly with nonalcoholic steatohepatitis, exhibit an increased liver-related mortality rate and higher incidence of cardiovascular-related morbidity and mortality[2].

MAFLD is the hepatic expression of metabolic syndrome, but its pathogenesis is still not clearly known. Insulin resistance (IR) seems to play a key role in the initiation and progression of the disease from simple fatty liver to advanced forms[4]. MAFLD pathogenesis is complex and multifactorial. The first theory was based on a two-hit hypothesis, where the first hit is liver steatosis, which is due to increased hepatic lipogenesis and reduced free fatty acid degradation caused by IR. This alteration is followed by the second hit of oxidative stress, which induces hepatocyte inflammation and cell death[5, 6]. However, this simplistic theory has been recently replaced by the multiple hit hypothesis, where many factors including systemic and hepatic IR, intestinal microbiota, genetic predisposition, and oxidative stress act simultaneously resulting in a cascade of detrimental effects such as hepatic inflammation, free radical production from gut and adipose tissue, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and hepatocyte apoptosis[7]. Among all the contributing factors of MAFLD, oxidative stress plays a major role. Oxidative stress promotes inflammation by activating Kupffer cells and stimulating the release of proinflammatory cytokines, directly leading to lipid, protein, and DNA/RNA damage. Nuclear factor erythroid 2-related factor 2 (NRF2) is the most important transcription factor in preserving redox homeostasis in the cell and counteracting oxidative or electrophilic stress by producing antioxidant and cytoprotective enzymes such as heme oxygenase 1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and those involved in glutathione (GSH) metabolism[8].

Thus, due to its antioxidative and detoxicant properties, it is currently accepted that NRF2 plays a pivotal role and has been recognized as a potential target to prevent the pathological spectrum of MAFLD. Even though the beneficial role of NRF2 in human diseases has been the topic of several recent reviews, the broad range of NRF2 modulators and their therapeutic implications in MAFLD were not completely summarized in recent literature. In this review, we described the current knowledge on the effects of NRF2-dependent mechanisms involved in the therapeutic implications of liver steatosis, inflammation, and fibrosis in MAFLD.

NRF2 PATHWAY

NRF2 belongs to the basic leucine zipper transcription factors in the Cap "n" Collar subfamily including seven functional domains, Nrf2-ECH homology (Neh) 1 to Neh7[9]. Neh2 is important for interaction between NRF2 and Kelch-like ECH-associated protein 1 (Keap1), a negative modulator of NRF2[10]. Keap1 is a substrate for Cullin based E3 ubiquitin ligase. During homeostatic conditions, Keap1 targets NRF2 that is localized in cytoplasm, causing its polyubiquitination and degradation. The binding and regulation of NRF2 by Keap1 has been defined as the "hinge and latch model"[11]. During oxidative stress, hyperactive cysteine residues of Keap1 undergo thiol modification and NRF2 is dissociated from Keap1, preventing ubiquitination and proteasomal degradation (Figure 1). The newly generated NRF2



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Figure 1 Kelch-like ECH-associated protein 1-dependent nuclear factor-erythroid 2-related factor 2 signaling. During oxidative stress, nuclear factor-erythroid 2-related factor 2 (NRF2) detaches from kelch-like ECH-associated protein 1 (Keap1) and translocates to the nucleus to bind the target genes. In normal conditions, NRF2 is ubiquitinylated and undergoes degradation. ARE: Antioxidant responsive element; SMaf: Small musculoaponeurotic fibrosarcoma oncogene homologue; Ub: Ubiquitin.

escaped from Keap1 control translocates to the nucleus and heterodimerizes with the Maf proteins, promoting the expression of antioxidant response element genes like *HO-1*, superoxide dismutase (*SOD*), catalase, glutathione-S-transferase, glutathione reductase, glutathione peroxidase (*GSH-Px*), *NQO1*, *etc*[12].

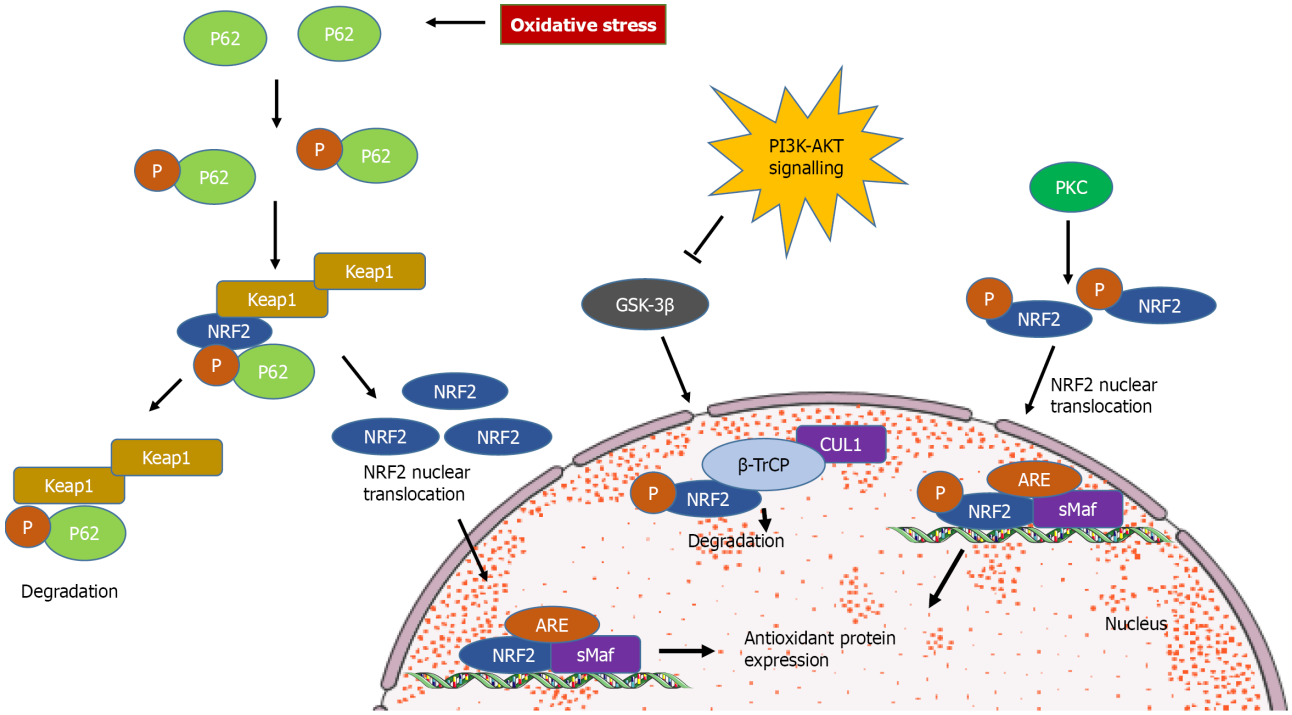
Of note, emerging evidence revealed Keap1-independent novel mechanisms of NRF2 regulation. The phosphatidylinositol 3'-kinase/protein kinase B pathway is protective against oxidative stress and is able to activate NRF2 signaling[13]. Phosphatidylinositol 3'-kinase-protein kinase B-NRF2 signaling pathway involves the glycogen synthase kinase-3β as a key mediator. Glycogen synthase kinase 3β can phosphorylate the NRF2 domain Neh6, containing serine residues that can be recognized by the β-transducin repeats-containing protein. β-transducin repeats-containing protein is a substrate receptor for ubiquitin ligase complex, which targets NRF2 for ubiquitination and proteasomal degradation[14,15]. During autophagy, NRF2 is stabilized by the binding of p62 (autophagy substrate) to Keap1 at the NRF2 binding site, resulting in the transcriptional activation of NRF2-target genes[16,17]. In addition, oxidative stress-induced protein kinase C phosphorylates Neh2 at serine and threonine residue on Ser40, dissociating the Keap1 homodimer and transferring NRF2 to the nucleus, thus binding to the antioxidant response element-mediated cytoprotective genes[18] (Figure 2).

NRF2 IN THE PATHOGENESIS OF MAFLD

MAFLD is the most widespread chronic liver condition worldwide, potentially leading to end stage disease, which requires liver transplantation[19,20]. MAFLD is a lipotoxic disease characterized by both structural and functional mitochondria abnormalities and oxidative stress. Impairment in mitochondrial electron transport chain causes excessive production of reactive oxygen and nitrogen species (ROS and RNS)[21]. ROS and RNS play a crucial role in cellular signaling, proliferation and differentiation, metabolism, and immune defense mechanisms. Besides mitochondria, ROS and RNS are continuously produced by the ER and peroxisomes as byproducts during their normal physiological processes. Oxidative stress is described as the imbalance between production of ROS/RNS and antioxidant systems[22]. Oxidative stress is intrinsically linked to the pathogenesis of MAFLD, and NRF2 has been found to be a key regulator to protect against the hepatocellular injury. Since MAFLD development and progression are characterized by alterations of redox balance, NRF2 is involved in every stage of disease, from simple steatosis to inflammation, advanced fibrosis, and initiation/progression of hepatocellular carcinoma[8].

NRF2 and liver steatosis

Accumulation of lipids in hepatocytes is the first step characterizing MAFLD development. This process is the result of increased fatty acid uptake/synthesis and decreased fatty acid oxidation/removal[23]. Fatty acid oxidation in peroxisomes produces H_2O_2, which in turn decreases the expression of enzymes



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Figure 2 Kelch-like ECH-associated protein 1-independent nuclear factor-erythroid 2 signaling. During oxidative stress, selective autophagy substrate p62 competes with nuclear factor-erythroid 2 (NRF2) to bind with kelch-like ECH-associated protein 1 (Keap1). As a consequence, NRF2 dissociates from Keap1 and translocates to the nucleus to induce target genes. Glycogen synthase kinase 3β (GSK-3β) phosphorylates the NRF2 subunit Nrf-ECH homology (Neh) 6, leading to degradation by β-transducin repeats containing protein (β-TrCP). Phosphatidylinositol 3'-kinase-protein kinase B (AKT) signaling could inhibit GSK-3β. Protein kinase C phosphorylates Ser40 in Neh2, inducing NRF2 translocation to the nucleus. ARE: Antioxidant responsive element; SMaf: Small musculoaponeurotic fibrosarcoma oncogene homologue.

involved in fatty acid oxidation as carnitine palmitoyltransferase 1A and acyl-CoA oxidase through their regulatory factor peroxisome proliferator activated receptor alpha. Besides, H_2O_2 promotes lipid accumulation by upregulating the expression of sterol regulatory element-binding protein-1c (SREBP-1c), which further activates fatty acid synthase, and stearoyl coenzyme-A desaturase 1, contributing to MAFLD pathogenesis[24]. In addition, ER-stress activates SREBP-1c and increases the expression of hepatic very-low density lipoprotein receptor, leading to deposition of triglycerides (TG)[12,24].

NRF2 is a key player in maintaining cellular homeostasis, suppressing MAFLD promotion and progression. A microarray analysis of mouse hepatic gene expression revealed that pharmacologic and genetic activation of *NRF2* suppresses key enzymes involved in lipid synthesis and reduces hepatic lipid storage. NRF2$^{-/-}$ mice fed a high-fat diet (HFD) are more prone to develop steatosis and oxidative stress than wild-type mice[25]. Consistent to this, NRF2-knockout mice fed a methionine- and choline-deficient (MCD) diet developed a severe form of micro- and macrovesicular steatosis and neutrophil recruitment compared to wild-type mice[26-28]. Studies on hepatic protein expression in NRF2-null and wild-type mice found two major groups of NRF2-modulated proteins. One group of proteins in NRF2 wild-type animals was implicated in phase II drug metabolism and antioxidant defense, while the other group of proteins in NRF2-null animals was involved in lipid and fatty acid synthesis and metabolism [29]. Another study in NRF2-null 8-wk old mice revealed a higher expression of SREBP-1c and fatty acid synthase than wild-type mice[30]. Nonetheless, NRF2 has little effect on hepatic fatty acid metabolism in 12-25 wk old mice[31,32].

In addition, flavonoid glycoside scutellarin ameliorates MAFLD pathogenesis by reducing blood lipid levels and enhances antioxidant capacity by activating peroxisome proliferator-activated receptor gamma (PPAR-γ) and its cofactor-1α as well as NRF2-dependent enzymes HO-1 and glutathione-S-transferase. Moreover, scutellarin suppresses nuclear factor κ B (NF-κB) and Keap1 mitigating MAFLD [33]. Another study revealed that scutellarin contains breviscapine as its active component, possibly exerting its antioxidant effects through phosphatidylinositol 3'-kinase/protein kinase B activation and subsequent enhancement of NRF2 nuclear translocation, increasing the expression of HO-1 and NQO1. Thus, breviscapine could be used in MAFLD and hyperlipidemia due to its potential therapeutic effects [34].

In addition, the food-derived compound apigenin is a modulator of PPAR-γ, which attenuates the NRF2-associated antioxidative response and hepatocyte lipid metabolism in MAFLD[35]. The specific deletion of NRF2 in mice diminished the signs of MAFLD induced by HFD, decreasing the accumulation of TGs. Hepatic NRF2 deficiency dampens the expression of PPAR-γ, suggesting that the NRF2-

dependent expression of PPAR-γ is critical in initiation and progression of MAFLD[36].

Liver X receptors are a family of nuclear receptors implicated in the modulation of lipid homeostasis. Directly or *via* SREBP-1c, liver X receptor α triggers the expression of lipogenic genes involved in the uptake and synthesis of fatty acids, TGs, cholesterol, and phospholipids. Treatment with the NRF2 activator sulforaphane suppresses T0901317-induced lipogenesis, promoting deacetylation of farnesoid X receptor (FXR) by competitive binding of p300, a protein necessary for the acetylation of FXR. The FXRE chromatin immunoprecipitation assay confirmed that NRF2 may complex with p300 and, as a result, dissociate from the FXR complex[37-39]. Moreover, NRF2 activator inhibits SREBP-1c and lipogenic genes by promoting deacetylation of FXR and inducing small heterodimer partner, which accounts for the repression of liver X receptor α-dependent gene transcription, protecting the liver from excessive fat accumulation[40].

NRF2 and liver inflammation

NRF2 is further involved in the regulation of pro- and anti-inflammatory mediators. NRF2 is known for its anti-inflammatory effects as it inhibits the expression of proinflammatory cytokines like interleukin-6 (IL-6), tumor necrosis factor, and inducible nitric oxide synthase. Moreover, NRF2-dependent antioxidant genes, such as *HO-1*, *NQO1*, and glutamate cysteine ligase catalytic and modifier subunits, inhibit the transcription of proinflammatory mediators by blocking NF-κB activation[41-43]. Of note, NRF2 also triggers the NLR family pyrin domain containing 3 inflammasome, which cleaves caspase-1 and initiates the processing of pro-IL-1β to mature IL-1β[44]. NLR family pyrin domain containing 3-dependent production of proinflammatory response can be inhibited by activation of NRF2 through dimethyl fumarate in alcoholic liver disease[45], and 4-acetylantroquinonol B in mice fed with a methionine- and choline-deficient diet[46] inducing the expression of NQO1, which inhibits the ROS/RNS-dependent priming.

NRF2-KO mice fed the methionine- and choline-deficient diet lose the antioxidant and detoxification enzymes and show an increase in steatosis, inflammation, oxidative stress, lipid peroxidation, and fibrinogenesis[26,28]. In line with these results, feeding the NRF2-KO mice with the HFD yielded significantly greater amounts of lipids and inflammation compared to wild-type mice. NRF2-KO mice fed a diet containing 4% soyabean oil and 16% lard for 12 wk exhibited massive lipid accumulation, inflammation, oxidative stress, and iron accumulation when compared to their wild-type counterparts [47]. NRF2-KO mice fed a diet containing 45 kcal% fat (0.02% cholesterol) for 24 wk displayed a higher MAFLD activity score compared to wild-type animals. In HFD-fed NRF2-KO mice, livers scored higher for steatosis, ballooning, inflammation, and fibrosis when compared to $Nrf2^{+/+}$ mice. The biochemical characterization studies of such mice revealed higher expression of sterol regulatory element binding transcription factor 1 and 2 and carbohydrate response element binding protein also known as MLX-interacting protein-like in HFD-fed NRF2-KO mice, suggesting exaggerated lipogenic transcription[48]. In another study, NRF2-KO mice fed a high-fat plus 30% fructose in drinking water exhibited a higher MAFLD score than wildtype. Moreover, these NRF2-KO mice overexpress lipogenic transcription factor sterol regulatory element binding transcription factor 1, fatty acid synthase, stearoyl coenzyme-A desaturase 1, and CD36 and exhibited higher proinflammatory factors as NF-κB p65 and p50 subunits [49].

In another investigation, NRF2-KO mice fed a chow diet were subjected to scanty inflammation with minimal increases in *IL-1β*, *Cox2*, and *Nos2* mRNA[26,28]. This is due to the compromised expression of zonula occludens-1 and claudin-1, which are responsible for the translocation of lipopolysaccharides from the gut microbiota to the liver through the portal vein. In addition, the phagocytic ability of Kupffer cells is diminished in NRF2-KO due to lower expression of the macrophage receptor with collagenous structure that restricts TLR4 signaling and boosts the inflammatory response on exposure to lipopolysaccharide[50].

NRF2 and liver fibrosis

Liver fibrosis is a reversible wound healing response and degenerative condition caused by extensive deposition of extracellular matrix proteins like collagen fibrils[51]. Mechanisms underlying liver fibrosis include the activation of both hepatic stellate cells and Kupffer cells, resulting in functional and biological alterations[52]. Oxidative stress is a serious process involved in liver damage, and the activation of the Keap1/NRF2 pathway plays a protective role in liver fibrosis[12]. NRF2 activation triggers the reverse IR and attenuates liver fibrosis by inhibiting hepatic steatosis. These noticeable effects during NRF2 activation are due to the disruption of JAK2/STAT3 signaling and higher expression of suppressor of cytokine signaling 3[53]. Moreover, administration of fibroblast growth factor 1 variants carrying substitutions of heparin-binding sites in 9-mo-old mice inhibited activity and expression of lipogenic genes, improving both steatohepatitis and fibrosis[54].

CCl_4-induced hepatic fibrosis is accompanied by elevated serum transaminases, alkaline phosphatase, and bilirubin, decreased albumin, and increased proinflammatory cytokines. In addition, CCl_4-intoxicated rats display an increase in NF-κB, p65, malondialdehyde and a decrease in antioxidants. Bone marrow-derived mesenchymal stem cells show favorable effects in ameliorating the hepatic effects of CCl_4 through NRF2/HO-1 signaling, suppressing liver fibrosis, inflammation, and oxidative stress [55].

A major bioactive extract from the plant *Schisandra chinesis*, known as Schisandrin B, exerts anti-inflammatory, anti-tumor, antioxidative, and hepatoprotective properties. Schisandrin B effectively improves liver function and decreases collagen deposition in the CCl_4-induced liver fibrosis in rats through the modulation of NRF2-antioxidant response element and transforming growth factor-β /Smad signaling pathways[56]. Tanshinol, a water-soluble compound isolated from *Salvia miltiorrhiza Bunge*, is known to exert a variety of biological effects, including anti-fibrotic effects. Rats with CCl4-induced liver fibrosis treated intraperitoneally with tanshinol show lower serum levels of aspartate aminotransferase, alanine aminotransferase, and total bilirubin, as well as circulating hyaluronic acid, laminin, type IV collagen, and procollagen III peptide as compared to controls. Tanshinol is also able to suppress the expression of inflammatory cytokines such as transforming growth factor-β, tumor necrosis factor, Cox2, IL-1β, and IL-6 through regulation of the NF-κB pathway. In addition, tanshinol treatment is able to regulate the NRF2/HO-1 signaling pathway increasing SOD and GSH-Px and decreasing malondialdehyde levels. In this regard, tanshinol exerts protective effects on CCl4-induced liver fibrosis by activating the NRF2 pathway[57].

Asiatic acid (AA), a bioactive compound extracted from *Centella asiatica*, is known to have anti-inflammatory, antioxidative, and hepatoprotective properties[19-22]. Fan *et al*[34] showed that treatment with AA in the CCl_4-induced liver fibrosis dramatically ameliorates oxidative stress, inflammation, and fibrosis in rats. The nuclear NRF2 levels were increased after AA treatment, and the NRF2-dependent proteins like HO-1, NQO-1, and Glutamate cysteine ligase catalytic subunit were significantly increased to counteract oxidative stress. Furthermore, AA inhibited the NF-κB/IkBα and JAK1/STAT3 signaling pathway to suppress the activation of hepatic stellate cells and the production of inflammatory markers, suggesting that AA could be used for the treatment of liver fibrosis[58]. Another water soluble compound, salvianolic acid A, extracted from a traditional Chinese herb *Radix Salvia miltiorrhiza*, was found to have anti-fibrotic effects. salvianolic acid A is able to modulate the NRF2/HO-1, NF-κB/IkBα, p38 MAPK, and JAK1/STAT3 signaling pathways, and to ameliorate the CCl4-induced liver fibrosis, improve morphology and attenuate collagen deposition in the fibrotic liver. Besides, salvianolic acid A is able to increase the levels of SOD and GSH-Px and decrease the malondialdehyde levels, indicating the effectiveness in preventing liver fibrosis by inhibiting inflammation and oxidative stress[59].

Pharmacological stimulation of NRF2 by acetylenic tricyclic bis (cyano enone) TBE-31 reverses IR in wild-type mice, decreases liver steatosis by increasing hepatic fatty acid oxidation and reducing ER stress, and lessens markers of oxidative stress, apoptosis, and fibrosis. Of note, histology studies showed that TBE-31 decreases the fibrosis score and MAFLD activity score[59]. In another study, NRF2 activator NK-252 (1-(5-(furan-2-yl)-1,3,4-oxadiazol-2-yl)-3-(pyridin-2-ylmethyl)urea) significantly reduced markers of fibrosis like COL1A1, TIMP-1, and transforming growth factor-β in rats, suggesting that this compound could be used as a therapeutic agent to reverse liver fibrosis. In addition, NK-252 attenuated the serum aspartate aminotransferase and alanine aminotransferase levels in male Fischer rats and upregulates *NQO1* gene expression[60].

THERAPEUTIC IMPLICATIONS OF NRF2 IN MAFLD

Currently, there is no medicine that can treat MAFLD, but some therapeutic agents are useful in managing the problems associated with the disease (Table 1). Thus, it is necessary to develop and test drugs for the prevention and treatment of MAFLD, and it is conceivable that NRF2-activating compounds can attenuate MAFLD progression. Plant-derived compounds including resveratrol, curcumin, quercetin, and synthetic molecules like oltipraz and pirfenidone could be used to prevent oxidative stress by modulating the NRF2 pathway[12,21].

Flavonoids represent a class of bioactive antioxidants extracted from vegetables, plants, and fruits known to exhibit therapeutic properties in MAFLD. The flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside activates NRF2 and improves the ratio of GSH/glutathione disulfide and increases the expression of HO-1 and GSH-Px3[61,62]. The flavonoid scutellarin (4′,5,6-trihydroxy flavonoid-7-glucuronide) increases NRF2 protein in C57BL/6J mice, increases the expression of HO-1, glutathione-S-transferase, and NQO1, and inhibits both NF-κB and Keap1[33]. Furthermore, 7,8-dihydroxyflavone upregulates NRF2 activity to counteract alcohol-induced and HFD-induced liver toxicity[63]. Apigenin (4′,5,7-trihydroxyflavone), a flavonoid derived from fruits, inhibits lipid peroxidation and exerts protective effects against hepatic steatosis. Moreover, apigenin increases the activities of SOD, CAT, and GSH-Px [35,64].

Gastrodin is a water-soluble extract of *Gastrodia elata* BI that exerts antioxidative activity and improves lipid metabolism in MAFLD mice by promoting NRF2 nuclear translocation[65]. Clusterin, a glycoprotein extracted from ram rete testis fluid, improves steatosis and hepatitis induced by methionine and choline-deficient diet by triggering NRF2 and HO-1 expression[66]. Osteocalcin treatment improves hepatic TG accumulation, promotes NRF2 nuclear translocation, and inhibits phosphorylation of c-Jun N-terminal kinase pathway[67].

In addition, compounds like scutellarin containing breviscapine, hesperitin, apigenin, scoparone, Schisandrin B, tanshinol, and AA and other tabulated compounds are known to exert antioxidative and

Table 1 Modulators of nuclear factor erythroid 2-related factor 2 pathway in metabolic associated fatty liver disease

Compound name	Species	Diet/duration	Treatment	Key findings	Reference
MonoHER	Female C57BL/6J mice ($Ldlr^{-/-}$)	High fat and high cholesterol/13 wk	Administered daily subcutaneously at a dosage of 500 mg/kg of body weight (25 μL/g of body weight)	NRF2 activation, ↑GSH/GSSG ratio, ↑HO-1, GSH-Px	[62]
Scutellarin	Male C57BL/6 mice, hepaG2 cells	High fat/10 wk	Administration of 12.5, 25.0, and 50.0 mg/kg per day	↑PPARγ, PGC-1α, NRF2, HO-1, NQO1, Keap1, NF-κB	[33]
	Sprague-Dawley rats	High fat/12 wk	Administered orally 50, 100, and 300 mg/kg/d	NRF2, HO-1, NQO1; PI3K/AKT activation	[34]
Apigenin	Male C57BL/6J mice	High fat/16 wk	Injected intraperitonially 30 mg/kg daily for 3 wk	NRF2 activation; PPARγ inhibition; SOD, CAT, GSH-Px	[35]
7,8-dihydroxyflavone	Male wistar rats	High fat, ethanol/12 wk	Administered intraperitonially at 5 mg/kg/d for 4 wk	Amelioration of liver architecture, vescicular changes, infiltration; restored serum biomarkers like AST, ALT, and TC; ↑NRF2; ↓NF-κB	[63]
Resveratrol	Male C57BL/6 mice	High fat/16 wk	Supplemented with 0.4% resveratrol in HFD for 16 wk	Attenuated liver steatosis; ↑ NRF2 activation; attenuated HFD induced methylation of NRF2 promoter; ↓oxidative stress	[68]
Quecertin	HepG2 cells	-	Treated with quecertin at 5-50 μM concentrations for 0, 10, 30, 60, 120, 240, and 1080 min	↑GSH, GSH-Px, GCS; p38-MAPK is involved in NRF2 modulation; ↓oxidative stress	[69]
Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione)	Male C57BL/6 mice	High fat and high fructose/8 wk	Administered orally 50 and 100 mg/kg/d for 4 wk	↑CYP3A, CYP7A; regulation of NRF2/FXR/LXRα pathway; ↓ SREBP-1C, FAS	[70]
	Male Sprague-Dawley rats	High fat/6 wk	Administered orally 50 mg/kg daily for 6 wk	↓Steatosis and inflammation; ↓ Serum aminotransferases, lipids, and insulin resistance; ↓ TNF, IL-6, MDA; ↑NRF2, GSH, HO-1, SOD	[71]
Oltipraz	Male Fischer 344 rats	Choline-deficient L-amino acid–defined/10 wk	Administered orally at 60 mg/kg/d for 9 wk	↑NRF2 activation; antifibrotic and anti-inflammatory; ↓AST and ALT; ↑*NQO1* gene expression	[61]
GSTD	HL-7702 cells, male C57BL/6J, male Sprague-Dawley rats	Oleic acid (OA)/24 h, high fat/10 wk; high fat and high cholesterol/10 wk	Cells were treated with GSTD for 24 h, administered orally at 10, 20, 50 mg/kg per day for 10 wk, administered orally at 20, 50 mg/kg per day for 10 wk	↑NRF2, HO-1, SOD; activate AMPK/NRF2; ↓proinflammatory response, and hepatic steatosis; ↓MDA, ROS	[65]
NK-252 1-(5-(furan-2-yl)-1,3,4-oxadiazol-2-yl)-3-(pyridin-2-ylmethyl)urea)	Male Fischer 344 rats	Choline-deficient L-amino acid–defined/10 wk	Administered orally at 20, 60 mg/kg/d for 9 wk	Attenuated histological abnormalities; ↑antifibrotic effects; ↓TGF-β1, collagen α1; NRF2 activation; ↑NQO1 expression	[61]
Clusterin	Male hCLU-tg mice	MCD/3 wk	Generated hepatocyte-specific clusterin overexpression transgenic mice and fed with MCD diet	↓Hepatic TGs; less infiltration of macrophages; ↓TNF; ↑NRF2 activation and mRNA of *HO-1*	[66]
Osteocalcin	Male C57/BL6J mice	High fat/12 wk	Injected intraperitonially at concentration 3 ng/μL/d for 12 wk	↓Hepatic TG accumulation; ↑ NRF2 activation; ↑CAT, SOD, GSH-Px; ↓JNK activation	[67]
Orlistat	Male Sprague-Dawley rats	High fat/12 wk	Administered at 10 mg/kg/d for 12 wk	↑NRF2 activation; protection against insulin resistance, hyperlipidemia, oxidative stress, and liver injury	[72]
Garcinia Cambogia	Male C57BL/6N mice	High fat/8 wk	Administered 200, 400 mg/kg/d for 8 wk	↑NRF2 activation; ↓ROS production; suppressed lipogenic factors C/EBPα and	[73]

				PPARγ; suppressed apoptosis by normalizing Bcl-2/BAX ratio and PARP cleavage	
HTT	Male Sprague-Dawley rats, 3T3-L1 murine embryo fibroblast cells	High fat/4 wk, 3T3-L1 cells treated with FBS/DMEM for 8 d	Administered orally HTT at 350, 700, and 1400 mg/kg/d, 3T3-L1 cells treated with HTT at 500 μg/mL for 24 h or 48 h	↑NRF2-HO-1 activation, antioxidant activities; HTT inhibited liver weight gain; reduced lipid profile; improved liver function; HTT promoted lipolysis and increased antioxidant activities in 3T3-L1 cells	[74]
Hesperitin	HepG2 cells, male wistar rats	OA/24 h, high fat/16 wk	Treated cells at 0.25, 0.50, 1.00, 2.50, 5.00, and 10.00 μM; administered 100 mg/kg in 0.5% CMC-Na	Alleviated hepatotoxicity and oxidative stress by increasing SOD, GSH-Px, GCLC, and HO-1; ↑NRF2 activation; suppressed OA induced inflammation; reduced TC, TGs, and LDLC in a dose-dependent manner	[75]
Glucoraphanin	Male C57BL/6JSlc mice	High fat/14 wk	Administered 0.3% glucoraphanin orally for 14 wk	Decrease in weight gain; improved insulin resistance; reduced hepatic steatosis and oxidative stress; decrease in circulating LPS; ↑NRF2 activation; ↑energy expenditure and; UCP1 protein expression	[76]
Scutellaria baicalensis extract	Male KK-A^{y} mice	1% Orotic acid and 33% sugar/7 d	Supplemented with diet for 7 d	Diminished increase in liver weight; attenuated hepatic steatosis; ↑NRF2 expression; suppress *SREBP-1c* gene and protein expression	[77]
Ginkgolide B	Male C57/BL6 ApoE$^{-/-}$mice, HepG2 cells	High fat/5 wk, 100 μM palmitic acid (PA) and 200 μM OA/24 h	Administered orally at 20, 30, and 1.3 mg/kg/d; treated cells at dosages 0, 1, 2, 4, 8, 16, and 32 μg/mL	NRF2 activation; inhibition of oxidative stress and lipid peroxidation through NRF2 pathway; increase in HO-1 and GSH-Px4	[78]
Scoparone	Male C57BL/6 J mice, AML2 and RAW264.7 cells	MCD/4 wk; AML12/300 μM PA and RAW264.7/10 μM/ Chloroquine	Administered daily intraperitonially for 4 wk at 20, 40, and 80 mg/kg; AML12 and RAW264.7 cells were pretreated with scoparone for 2 h	Ameliorated hepatic inflammation; improved hepatic autophagy; suppressed inflammation by inhibiting ROS/P38/NRF2 axis and PI3K/AKT/mTOR pathway	[79]
DA	Male C57BL/6J mice, HL7702 cells	High fat/12 wk, 0.6 mM OA/24 h	Administered by gavage at 10 and 20 mg/kg/d for 9 wk; treated with 2.5, 5.0, and 10.0 μM DA	Ameliorated liver ferroptosis in mice and cells; improved oxidative stress and lipid peroxidation *in vivo*; ↑NRF2-HO-1 expression; ↑GSH, GSH-Px4	[80]
Silibinin	Male C57BL/6 mice, NCTC-1469 cells	MCD/6 wk, OA plus PA/24 h	Administered by gavage at 10 and 20 mg/kg/d for 6 wk, 0.25 mM/L PA and 0.5 mM/L OA/24 h	Prevented CFLAR-JNK pathway; ↑β-oxidation and efflux of fatty acids; ↑ expression of CAT, GSH, GSH-Px, and HO-1; ↓expression of CYP2E1 and CYP4A; ↑NRF2 activation	[81]
Chicoric acid	Male C57BL/6 mice	High fat/9 wk	Administered by gavage at 15 and 30 mg/kg/d for 9 wk	Attenuated hyperglycemia, dyslipidemia, and systemic inflammation; alleviated hepatic lipid accumulation and oxidative stress; suppressed hepatic inflammation and NF-κB pathway; ↑NRF2/Keap1 activation; improved gut microbiota	[82]
Carbon monoxide releasing molecule-A1	Male C57BL/6J mice	High fat/16 wk	Administered intraperitonially 2 mg/kg/d for 7 wk	↑NRF2/ARE activation; improved lipid homeostasis; ↑ ATP production; improved mitochondrial biogenesis; ameliorated oxidative stress	[83]

NRF2: Nuclear factor erythroid 2-related factor 2; GSH: Reduced glutathione; GSSG: Oxidized glutathione; HO-1: Heme oxygenase-1; GSH-Px: Glutathione peroxidase; PPAR-γ: Peroxisome proliferator-activated receptor-γ; PGC-1α: Proliferator-activated receptor gamma coactivator-1α; NQO1: NAD(P)H quinone oxidoreductase 1; DA: Dehydroabietic acid; PA: Palmitic acid; NF-κB: Nuclear factor κ B; PI3K: Phosphatidylinositol 3′-kinase; SOD: Superoxide dismutase; CAT: Catalase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TC: Total cholesterol; HFD: High-fat diet; GCS: Glutamylcysteine-synthetase; MAPK: Mitogen-activated protein kinase; CYP3A: Cytochrome P450, family 3, subfamily A, CYP7A Cytochrome P450, family 7, subfamily A; FXR: Farnesoid-X-receptor; LXRα: Liver X receptor α; SREBP-1C: Sterol regulatory element-binding protein-1c; FAS: Fatty acid synthase; TNF: Tumor necrosis factor; IL-6: Interleukin-6; MDA: Malondialdehyde; AMPK: AMP kinase; ROS: Reactive oxygen species; TGF-β1: Transforming growth factor-β1; TG: Triglycerides; JNK: c-Jun N-terminal kinase; C/EBPα: CCAAT/enhancer binding protein α; Bcl-2: B-Cell Leukemia/Lymphoma 2; BAX: BCL2 associated X protein; PARP: Poly-ADP ribose polymerase; HTT: Hedansanqi Tiaozhi Tang; GCLC: Glutamate cysteine ligase catalytic; LDLC: Low density lipoprotein cholesterol; LPS: Lipopolysaccharide; UCP1: Uncoupling protein 1; GSH-Px4: Glutathione peroxidase 4; mTOR: Mammalian target of rapamycin; CFLAR: CASP8 And FADD like apoptosis regulator; CYP2E1: Cytochrome P450 family 2 subfamily E member 1; CYP4A: Cytochrome P450 family 4 subfamily A; ARE: Antioxidant response element; AKT: Protein kinase B; MCD: Methionine- and choline-deficient; GSTD: Gastrodin; Keap1: Kelch-like ECH-associated protein 1.

hepatoprotective activity by modulating the NRF2 pathway.

CONCLUSION

Oxidative stress can be a potent inducer of inflammation and fibrosis in the spectrum of chronic liver diseases. Among them, MAFLD is the most widespread chronic liver condition worldwide. The transcription factor NRF2 has gained importance in recent years as a possible therapeutic target for the treatment of liver diseases. The expression of antioxidant protective genes through the NRF2 pathway counteracts oxidative stress and prevents progression of liver damage in MAFLD. The different antioxidative molecules modulating the NRF2 pathway have exerted beneficial effects in ameliorating liver damage. Currently, there is no efficient treatment to counteract the complex pathophysiology of liver diseases. Thus, compounds having antioxidative properties could be useful candidates for the treatment of liver diseases by modulating the NRF2 signaling pathway. NRF2 activators could improve and prevent the advanced stages of MAFLD such as liver fibrosis and liver cirrhosis. Natural plant-derived and synthetic NRF2 activators require further experimental validation to be promoted as efficient therapeutic agents. Some drugs have entered clinical trials, and further attempts are ongoing to find NRF2 inducers with high bioavailability, safety, and specificity.

FOOTNOTES

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MINIREVIEWS

Current and future perspectives on acute-on-chronic liver failure: Challenges of transplantation, machine perfusion, and beyond

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Abstract

Acute-on-chronic liver failure (ACLF) is a syndrome that occurs in patients with chronic liver disease and is characterized by acute decompensation, organ failure and high short-term mortality. Partially due to the lack of universal diagnostic criteria, the actual ACLF prevalence remains unclear; nevertheless, it is expected to be a highly prevalent condition worldwide. Earlier transplantation is an effective protective measure for selected ACLF patients. Besides liver transplantation, diagnosing and treating precipitant events and providing supportive treatment for organ failures are currently the cornerstone of ACLF therapy. Although new clinical specific therapies have been researched, more studies are necessary to assess safety and efficacy. Therefore, future ACLF management strategies must consider measures to improve access to liver transplantation because the time window for this life-saving therapy is frequently narrow. Thus, an urgent and global discussion about allocation and prioritization for transplantation in critically ill ACLF patients is needed because there is evidence suggesting that the current model may not portray their waitlist mortality. In addition, while donor organ quality is meant to be a prognostic factor in the ACLF setting, recent evidence suggests that machine perfusion of the liver may be a safe tool to improve the donor organ pool and expedite liver transplantation in this scenario.

Key Words: Acute-on-chronic liver failure; Liver cirrhosis; Liver transplantation; Machine perfusion; Hypothermic oxygenated machine perfusion

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Core Tip: Acute-on-chronic liver failure (ACLF) is characterized by high short-term mortality. Although new clinical specific therapies have been researched, more studies are necessary to assess safety and efficacy. Conversely, earlier transplantation is effective for selected patients. Therefore, future ACLF management strategies must consider measures to improve access to liver transplantation. Discussions about donor organ allocation and recipient prioritization are necessary because there is evidence suggesting the current model may not portray the waitlist mortality of these patients. In this scenario, machine perfusion of the liver may prove to be a safe tool to improve the donor organ pool.

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INTRODUCTION

Despite the heterogeneity in the diagnosis criteria, consensually, acute-on-chronic liver failure (ACLF) is a condition that occurs in patients with chronic liver disease developing multi-organ failure in the presence of one or more hepatic or extrahepatic precipitant events. In addition, it is associated with high 28-d mortality[1-4]. Several international consortiums proposed different definitions of ACLF, reflecting their own types of underlying liver disease and precipitant events. The Asian Pacific Association for the Study of the Liver (APASL), the European Association for the Study of the Liver (EASL), formed by the Chronic Liver Failure (CLIF) Consortium and the North American Consortium for the Study of End-Stage Liver Disease (NACSELD) are the most widely accepted[1,5,6].

Because of the lack of universal diagnostic criteria and study design limitations, the actual ACLF prevalence remains unclear. However, it is expected to be a highly prevalent condition worldwide. For example, in the CANONIC study (EASL-CLIF Acute-on-Chronic Liver Failure in Cirrhosis study), a prospective evaluation of 1343 patients admitted for acutely decompensated cirrhosis, 22.6% met the EASL-ACLF criteria for ACLF, and 8.3% developed the condition during hospitalization. In another study using APASL criteria, 12% of complicated cirrhotic patients were diagnosed with ACLF[1,7]. Recently, a large meta-analysis of global epidemiological data found about 35% of ACLF in patients admitted due to acutely decompensated cirrhosis using EASL-ACLF criteria, with a higher prevalence in South Asia, reaching 65%[8]. Notably, the overall mortality in ACLF is about 32% and increases in parallel with the number of organ failure (OF). The 28-d mortality rate for ACLF - grades 1 to 3 - ranges from 20% to 80%, 49% to 77%, and 13% to 86% for EASL-CLIF Consortium, NACSELD, and APASL-ACLF, respectively[4,9].

In recent years, several studies have investigated the pathophysiology, prognosis, and treatment options for ACLF. Although the benefit of timing liver transplantation (LT) in ACLF is undeniable, issues still exist with assessing the mortality risk of patients on the waiting list to avoid futile LT. In addition, another urgent and global discussion is about allocation and prioritization for LT in critically ill ACLF patients, as the time window for transplantation is frequently narrow[2,10,11].

This review provides an overview of ACLF management, focusing on the current challenges of LT in this scenario and future perspectives, including machine perfusion of the liver.

DIAGNOSTIC CRITERIA FOR ACLF

The natural history of cirrhosis is characterized by a long asymptomatic phase called compensated cirrhosis. The increase of portal pressure above the hepatic venous pressure gradient of 10 mmHg plays a central role in the transition to decompensated cirrhosis. Acute decompensation (AD) is defined by the new development of ascites, hepatic encephalopathy, hepatorenal syndrome, variceal bleeding, or infection[1,9].

In the PREDICT study (PREDICTing ACLF), a prospective and observational analysis of 1071 patients hospitalized with AD, 218 (20%) developed ACLF in 90 d (3-mo mortality rate of 53.7%), called pre-ACLF. Two hundred and thirty-three patients (22%) required frequent hospitalization unrelated to ACLF, called unstable decompensated cirrhosis (3-mo mortality rate of 21.0%), and 620 (58%) reached a state called stable decompensated cirrhosis (1-year mortality rate of 9.5%). These three clinical courses revealed by the PREDICT Study group originated from data of the CANONIC study, which created the EASL-CLIF definition of ACLF - probably the most widely accepted definition used to date[1,12]. Therefore, cirrhotic patients with pre-ACLF should be promptly identified due to the high risk of death in the short term.

THE EASL-CLIF CONSORTIUM DEFINITION OF ACLF

The EASL-CLIF definition considers ACLF in cirrhotic patients with or without prior episodes of decompensation and one or more OF. The CANONIC study enrolled 1343 patients from 29 centers in 12 European countries. Chronic hepatitis C virus and alcohol were the most frequent underlying causes of chronic liver diseases. The OF definitions were adapted from the Sequential Organ Failure Assessment (SOFA) score and called the CLIF-SOFA score. The CLIF-SOFA score includes subscores ranging from 0 to 4 for each of the six components (liver, kidney, brain, coagulation, circulation, and lungs). Aggregated scores range from 0 to 24 and provide information on overall severity[13].

OF was defined by liver failure (level of serum bilirubin) or extrahepatic failure. The latter includes kidney failure (defined by serum creatinine), cerebral (by grade of encephalopathy according to West-Haven classification), coagulation (by the international normalized ratio [INR]), circulatory (by blood pressure or need of vasopressors), and respiratory failure (by partial pressure of oxygen [PaO_2]/fraction of inspired oxygen [FiO_2] or oxygen saturation [SpO_2]/FiO_2) (Table 1). The most prevalent OF was kidney (43.6%), followed by coagulation (27.7%) and cerebral (24.1%). At presentation, the prevalence of ACLF grades 1, 2, and 3 were 49%, 35%, and 16%, respectively. The mortality in 28 d without LT was 32.8% in the seminal study.

THE APASL ACLF RESEARCH CONSORTIUM DEFINITION

This definition was based on an expert opinion on ACLF from the APASL, published in 2009 with updates in 2014 and 2019, using data from 1402 and 3300 patients, respectively[5,14,15]. This definition considers patients with compensated cirrhosis (diagnosed or not) and non-cirrhotic liver disease patients with a first episode of acute liver deterioration due to an insult directed to the liver. This reflects most of the patient population seen in Asia. In Asia, there is mainly hepatitis B virus reactivation or superinfection with hepatitis viruses A, D, or E. Patients with extrahepatic precipitants and those with kidney, circulatory or respiratory failures are excluded, meaning the liver dysfunction is the basis of the APASL definition. Extrahepatic OF may subsequently develop but are not needed for diagnostic criteria. The acute hepatic insult can be manifested by jaundice (serum bilirubin ≥ 5 mg/dL), coagulopathy (INR ≥ 1.5) and complications within 4 wk such as ascites, encephalopathy, or both. With these criteria, there is an estimated 25% to 37% 30 d-mortality[16].

THE NACSELD DEFINITION OF ACLF

ACLF was defined as the development of two or more OF (maximum of four) in patients with AD cirrhosis with or without prior episodes of decompensation. They used a prospective multicenter Canadian and American cohort of 507 patients with non-elective hospitalization in 18 centers. The OF criteria were: Kidney if dialysis is required; brain if encephalopathy grades 3 or 4 as West Haven classification occurs; respiratory if mechanical ventilation is needed; circulatory if vasopressor support is required or a reduction in systolic blood pressure by 40 mmHg from baseline despite adequate fluid resuscitation. The most prevalent OF was cerebral (36%), followed by circulatory (16%), kidney (13%) and respiratory (9%)[6]. Lately, these criteria were validated in a cohort of 2675 patients with or without infections, and mortality was higher in patients with infections, whatever the number of OF[17].

PRECIPITANTS FACTORS FOR ACLF AND PROGNOSIS

This section will discuss the most frequent precipitants for ACLF, its pathophysiology, and prognosis; nevertheless, a more thorough review of these subjects is outside the scope of this manuscript. Regardless of the ACLF definition employed, the precipitant factors can be recognized in only about 50% of patients with ACLF[9]. In 2021, the EASL-CLIF Consortium published a second paper derived from the PREDICT study reporting the precipitant factors which could influence the clinical course and the prognosis of ACLF patients. The most prevalent, more than 96% of cases, were bacterial infections (documented) and severe alcoholic hepatitis, whereas gastrointestinal bleeding with shock and toxic encephalopathy were rare. Although not so common, other precipitant factors like drug-induced liver injury, surgery, viral hepatitis, and ischemia can also be considered[18]. Mezzano *et al*[8] in a recent review and meta-analysis using ACLF-EASL-CLIF criteria in patients from Europe, East/South Asia and North/South America, showed that bacterial infections (35%), followed by gastrointestinal bleeding (22%) and acute alcohol consumption (19%) were the most frequent triggers to ACLF, with kidney dysfunction being the most common organ failure (49%). Although it is crucial to identify precipitant factors for ACLF, a recent study reported that they could not be detected in up to one-third of patients [12,19].

Table 1 European Association for the Study of the Liver Chronic Liver Failure Consortium acute-on-chronic liver failure grades

ACLF grades	Number of organ failures
1	Single organ kidney failure
	Single liver, coagulation, circulatory or lung failure with creatine levels ranging from 1.5 mg/dL to 1.9 mg/dL or encephalopathy grade 1 or 2, or both
	Single brain failure with creatinine ranging from 1.5 mg/dL to 1.9 mg/dL
2	2 organ failures
3	3 or more organ failures

ACLF: Acute-on-chronic liver failure.

Data from the CANONIC cohort showed that ACLF is a dynamic syndrome and may evolve to resolution, improvement or worsening in a short period[1]. ACLF grade 1 could be reversible in most patients (54.0%), while 21.0% remain stable in grade 1, and 24.5% progress to a higher grade. The clinical course after 3-7 d from diagnosis of ACLF may be a better predictor of outcome than its initial severity. Indeed, patients with grade 3 ACLF 3-7 d after diagnosis showed the worst prognosis[20,21]. This was called the opportunity window, wherein LT could reach the best results.

In severely ill patients, the prognosis differed according to the number of OF. For example, three OF had lower 28-d transplant-free mortality than those with four OF, 53.0% *vs* > 90.0%, respectively[20,21]. Indeed, the number of OF - according to the EASL-CLIF definition - along with age and white cell count compose the EASL-CLIF ACLF score. This predictive tool developed by the CANONIC study group proved superior to older models)Acute Physiology and Chronic Health Evaluation [APACHE] II, Child-Turcotte-Pugh, Model for End-stage Liver Disease [MELD]) to predict ACLF patients' mortality [22]. Accordingly, a recent study demonstrated that patients with an EASL-CLIF ACLF score of greater than 70 had 90% mortality at 90 d, regardless of care setting[23]. Another study reported a 28-d mortality rate of 100% for patients with a score greater than 70 at 48 h post-intensive care unit (ICU) admission[24].

Nevertheless, importantly, a 90-d and a 1-year post-LT survival rate of 90% and 81%, respectively, were reported for ACLF patients with 5 to 6 OF[25]. Conversely, without LT, survival rates dropped dramatically[25]. Therefore, although application and reassessment of the EASL-CLIF ACLF score may prevent prolongation of futile therapy, especially after a short trial of ICU stay, withdrawal of care must only be considered if the patient is not a LT candidate.

Recent evidence suggests systemic inflammation is the key to AD and ACLF disease progression[3,10,26]. Briefly, in patients with sepsis as the precipitant event, the inflammatory response is triggered by the recognition of pathogen-associated molecular patterns by pattern recognition receptors. The inflammatory response is then exacerbated, resulting in organ damage, cell death and release of damage-associated molecular patterns, which could aggravate and accelerate OF development in the ACLF setting. In addition, cirrhotic patients have portal hypertension, secondary intestinal congestion, and splanchnic endothelial dysfunction. These features can enhance gut permeability and facilitate bacterial translocation, driving local and systemic inflammation. Traditionally, inflammatory response causes organ dysfunction and stimulation of nitric oxide production, worsening pre-existing circulatory collapse and activation of immune cells. Another mechanism involved is mitochondrial metabolic impairment, resulting in metabolic disorder and cellular dysfunction with a preferential allocation of circulant nutrients to innate immune cells due to high metabolic demands. Secondarily, this process decreased mitochondrial energy production and enhanced organ dysfunction. So, inflammation and immunoparesis are thus key features of ACLF[3,26].

CURRENT MANAGEMENT OF ACLF

Besides LT, diagnosing and treating precipitant events and providing supportive treatment for OF are currently the cornerstone of ACLF therapy.

TREATMENT OF PRECIPITANT EVENTS

Infections: antimicrobial therapy should commence as quickly as possible based on the suspected site involved, existing culture results and local antimicrobial sensitivity patterns. Empirical broad-spectrum antimicrobials should be promptly initiated and deescalated once the results are available. Empirical

antifungals should be considered in patients without clinical improvements within 48-72 h. In addition, antifungal therapy may also be used in ACLF patients with multiple risk factors, such as corticosteroid use, prolonged antimicrobial therapy, long-term central venous access devices, parenteral nutrition, renal replacement therapy, sarcopenia and malnutrition[4,17,21]. Recent studies also recommended avoiding proton pump inhibitors unless there is a clear indication (like stress-ulcer prophylaxis) because they increase the risk of infection, mainly due to *Clostridioides*. Nonselective beta-blockers, when tolerated, and rifaximin may be beneficial by reducing bacterial translocation and intestinal dysbiosis.

Alcoholic hepatitis: although corticosteroids are indicated in patients with alcoholic hepatitis, especially when Maddrey's discriminant function score is higher than 32, the response negatively correlates with the number of OF in ACLF. The risk of new infections is one of the most critical factors in the decision-making process for steroid therapy[4,21]. The response to steroids should be assessed with the Lille score on day 7.

Acute viral hepatitis or reactivation: potent nucleotide or nucleoside analogues should be started in the event of hepatitis B infection or reactivation.

Surgical procedures: surgery of any type in patients with cirrhosis is associated with a significant risk of OF and ACLF. Consequently, it must be carefully considered. For example, open abdominal non-liver surgery, high preoperative cardiovascular risk, or hepatic venous pressure gradient greater than 16 mmHg were frequently associated with ACLF. Recently, a new score, the VOCAL PENN score, demonstrates an excellent ability to predict 30-d mortality when surgery is needed in patients with ACLF[18,27].

SUPPORTIVE THERAPY

ACLF patients frequently require admission to an ICU for advanced OF support and assistance from a multidisciplinary team.

Hemodynamic: Early goal-directed therapy using intravenous fluid resuscitation, preferably with crystalloids, must target mean arterial pressure > 65 mmHg. If vasopressors are required, norepinephrine is the first option, and a low dose of vasopressin can be necessary. Next, terlipressin or epinephrine can be added, though they are no longer the second option. Finally, intravenous hydrocortisone can be indicated in refractory septic shock, whereas no long-term survival benefit exists [28].

Acute kidney injury (AKI): It is essential to eliminate or avoid nephrotoxic drugs. The assessment of AKI severity using the modified Kidney Disease Improving Global Outcomes (KDIGO) criteria established the use of albumin in patients with stages 2-3 and albumin plus terlipressin or norepinephrine in type-1 hepatorenal syndrome. In non-responders, it should be necessary to start renal replacement therapy, mainly in patients with LT perspectives[29,30].

Lungs and respiratory failure: The airway should be protected in West-Haven grade 3 or 4 hepatic encephalopathy patients with elective intubation. Patients should be sedated with short-acting agents such as propofol. Benzodiazepines should be avoided. Hypoxemia (PaO_2 < 80 mmHg) should be prevented, and paracentesis is clinically indicated in case of tense ascites[28].

Gastrointestinal: Consider the use of stress-ulcer prophylactic drugs. As soon as possible, initiate oral or enteral feedings[18].

Coagulation: Consider prophylaxis for deep-vein thrombosis in the absence of severe coagulopathy. However, avoid correction of INR alterations with fresh frozen plasma. Instead, assessing the risk of bleeding and thrombosis in ACLF patients should be done with viscoelastic testing (rotational thromboelastography or rotational thromboelastometry), which must also guide correction when needed or before invasive procedures[31,32].

NEW PERSPECTIVES FOR ACLF MANAGEMENT

The use of albumin has been well recommended to prevent AKI and renal failure in spontaneous bacterial peritonitis, besides preventing post-paracentesis circulatory dysfunction[18,26,33,34]. Recent data have highlighted the non-oncotic properties of albumin as homeostatic effects, antioxidants, immunomodulation, endothelial stabilization, and toxic binding metabolites, including bile acids. Three studies recently evaluated the routine outpatient administration of intravenous albumin. One of them, the ANSWER trial, which included outpatients in an early stage of liver disease, showed improvement in mortality and reduction in cirrhosis-related complications, mainly when the albumin level was maintained above 4 g/dL[35]. However, further studies are necessary to indicate albumin infusion use routinely[18,28].

Various artificial and bioartificial extracorporeal liver support systems have been attempted to treat ACLF. However, artificial liver support such as molecular adsorbent recirculating system and fractionated plasma separation and adsorption system (Prometheus) have failed to show any survival benefit in this setting. Some bioartificial liver supports with a source of cells, traditionally human or

porcine hepatocytes, are under investigation, but the clinical benefit is still unclear[18,21].

While the use of plasma exchange has been shown to improve survival in acute liver failure, the actual effect in the ACLF scenario is unknown. Some studies in Asia using selected patients with ACLF showed improvement in 30 and 90-d survival in non-transplanted patients. Still, randomized trials are needed on the duration and amount of plasma exchange required[36].

One potential therapy for ACLF is the administration of granulocyte colony-stimulating factor (G-CSF). Several trials, mainly in patients with hepatitis B from Asia and India, have studied the efficacy of G-CSF and showed an increased leukocyte and neutrophil count, reduced severity of the disease and a protective effect on the development of sepsis, hepatorenal syndrome and hepatic encephalopathy[3]. However, trials in Western cohorts did not demonstrate survival benefits, CLIF OF scores modification or recurrence of infections. Therefore, to date, G-CSF cannot be recommended as part of routine treatment for ACLF[3,18,33,37].

Another promising therapy is mesenchymal stem cell transplantation, which can be a bridge to stabilization in patients with ACLF until LT. However, if the concept is interesting in theory, the studies were made with a few patients, and consequently, many questions remain open[38].

In addition to treatment for hypercholesterolemia, statins may have a role in ACLF therapy because of their potential hepatoprotective and anti-inflammatory properties. Currently, two clinical trials are underway to address the benefits and safety of statin (simvastatin and atorvastatin) in cirrhotic patients [26,39,40].

The gut microbiota plays an important role in complications associated with cirrhosis, with specific intestinal microbiomes being associated with adverse outcomes. The changes in the gut microbiome parallel the disease stages reaching their peak in ACLF. ACLF patients were shown to present an increase of *Enterococcus* and *Peptostreptococcus* sp and a reduction of some autochthonous bacteria[41]. Individual microbiome signatures could possibly identify ACLF patients and their prognosis, leading to more personalized treatment, a topic under investigation in the MICROB-PREDICT study (https://microb-predict.eu/). Manipulation of the gut microbiome using fecal microbiota transplantation may positively impact the course of cirrhosis, as shown in patients with severe alcoholic hepatitis. In addition, one novel-engineered carbon bead (Carbalive™) designed to absorb toxins from the gut and prevent translocation is under investigation[26]. Whereas promising, more studies are necessary to assess the clinical significance of gut microbiome manipulation in managing ACLF.

PRIORITISATION CRITERIA FOR LIVER TRANSPLANTATION

Although the MELD has enhanced equity in organ allocation in LT, there is evidence suggesting it may not portray the waitlist mortality of ACLF patients. This is in accordance with distinct pathological mechanisms in decompensated cirrhosis and ACLF[42].

Sundaram *et al*[43], in a large retrospective study, reported that irrespective of the MELD-Na score, ACLF-3 patients had a worse prognosis (ACLF grades refer to the EASL-CLIF classification hereafter unless contrarily stated). They found that 43.8% of patients with ACLF-3 and MELD-Na < 25 died or were removed from the waitlist at 28 d, having the worst prognosis among ACLF groups. This is probably associated with the more frequent occurrence of extrahepatic failures, which, although not fully captured by the MELD score, result in a high mortality rate. In addition, the authors report that LT within 30 d of listing was the only significant independent protective factor for 1-year patient survival after transplantation. More importantly, in this study, yet ACLF-3 patients had a greater mortality risk, they presented a similar probability of being transplanted than non-ACLF-3 patients with similar MELD scores[44].

In the CANONIC study, Jalan *et al*[22] developed an organ function scoring system (CLIF Consortium Organ Failure score, CLIF-C OFs) to diagnose ACLF and the prognostic EASL-CLIF ACLF score. The latter score discussed previously revealed significantly higher mortality 28-d predictive accuracy than the MELD and MELD-Na[22].

Allocation systems must privilege ACLF patients once so far earlier transplantation is the cornerstone of their successful management. The question remains whether ACLF-3 patients must be prioritized or whether a new scoring system that depicts better OF must be implemented for these cases[44-46].

DONOR CHARACTERISTICS AND OUTCOMES AFTER TRANSPLANTATION

Donor organ selection in the ACLF scenario can be a real conundrum for transplant teams to solve. This is because there is evidence that donor characteristics may be associated with a poor outcome after transplantation[25,47,48], which adds complexity to the decision of whether to accept or not a donor organ offer. This aspect is troubling, considering that strict donor organ selection may even postpone further transplantation to this threatened population, especially in regions with frequent high MELD recipients.

A recent retrospective analysis from the United Network for Organ Sharing (UNOS) involving 50552 transplanted ACLF patients reported the donor risk index (DRI) above 1.7 as an independent risk factor for mortality within 1 year after LT (hazard ratio [HR] 1.22; 95% confidence interval [CI] 1.09-1.35)[47]. On the other hand, independently of the MELD score, they described LT within 30 d of the ACLF diagnosis as a predictor of improved 1-year survival after transplantation[47]. Other authors also reported similar results[48,49]. These findings together pressurize even more transplant teams, which face the dilemma of whether to proceed with a high-risk donor or wait for an ideal donor which might not come on time.

It is ideally argued that the benefit of transplantation to this population surpasses the potential negative impact of a suboptimal graft when comparing this risk with a 1-year survival probability without transplantation. This is especially true for ACLF-3 patients, whose reported 1-year survival probability with and without transplantation is 83.9% and 7.9%, respectively[50,51]. Nevertheless, the scarce literature available also reports extra caution during donor organ selection for ACLF-3 patients in real life. For example, in the UNOS database retrospective study, ACLF-3 patients received organs from younger donors (mean age 38.7 years) with the cause of death predominantly related to head trauma (38.0%) and a small percentage of organs from high-risk donors with DRI ≥ 1.7 (22.9%)[47].

Kitajima *et al*[52] using the Organ Procurement and Transplantation Network and the UNOS registry, recently analyzed 17300 transplanted ACLF patients between 2002 and 2019. They grouped the patients by eras, Era 1 (2002-2007, n = 4,032), Era 2 (2008-2013, n = 6,130), and Era 3 (2014-2019, n = 7,138). Donor characteristics were classified according to the DRI (DRI < 1.2, 1.2-1.6, 1.6-2.0, and > 2.0). They have shown a significant improvement in overall patient survival and transplant outcomes throughout eras. However, although donors with DRI > 2.0 were associated with a lower risk of patient death in Eras 2 and 3 than Era 1 in ACLF-1 and 2, this was not confirmed for ACLF-3[52]. Therefore, the authors advise the need for particular caution for high-risk donors (DRI > 2.0) for ACLF-3 patients.

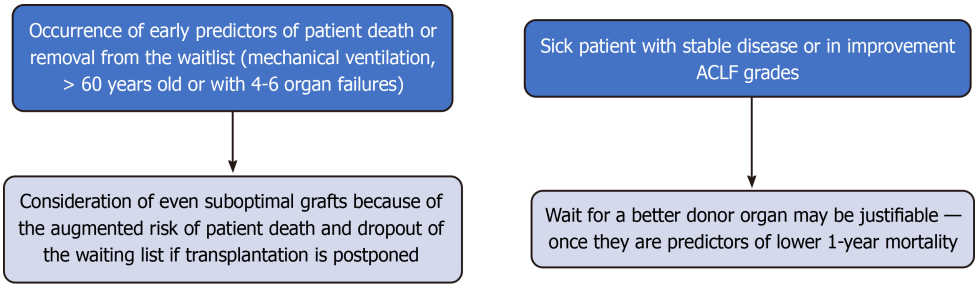
Comparatively, ACLF grades 1 and 2 are associated with lower mortality than ACLF grade 3. The reported 28-d and 90-d mortality rates for ACLF grades 1 and 2 are 25.8% and 28.6%, and 41.1% and 65.4%, respectively[1]. Therefore, more judicious donor organ selection could be applied, mainly when disease progression is evaluated concomitantly. Whereas data regarding the impact of donor organ selection on postoperative outcomes are scarcer for this population, the results of the study mentioned above support a wider acceptance of suboptimal grafts in ACLF-1 and 2 patients[52]. Nevertheless, the quality of evidence (retrospective study) must be considered before drawing definitive conclusions.

In the setting of donor organ shortage, another question to consider is the allocation of an ideal organ to a very sick recipient who may not survive the procedure. Again, the coexistence of additional risk factors and the real-time change in OF may guide the decision to accept a non-ideal donor organ offer rather than wait (Figure 1). Accordingly, there is evidence suggesting that in ACLF-3 patients, a better survival rate can be achieved if the transplant occurs after organ failure recovery[48].

Indeed, the timing for LT in ACLF patients is critical. In a recent study involving specifically ACLF-3 patients, authors investigated the optimal timing for transplantation and the impact of extended criteria donor (ECD) organs (defined exclusively by the DRI ≥ 1.7)[49]. They analyzed three variables to define the groups of patients (age ≤ 60 or > 60 years, 3 OF or more, and hepatic or extrahepatic ACLF-3). Through two-way sensitivity analyses, they found that overall survival is optimized by earlier transplantation, especially among candidates > 60-years-old or with 4-6 OF[49]. These findings are in accordance with the proposal mentioned above and reinforce the need to consider early transplantation, even with suitable suboptimal grafts, to this population of ACLF patients.

To date, just the DRI was evaluated as a donor parameter within ACLF studies. The DRI is a quantitative score developed to predict the risk of graft failure[53]. It identified seven donor characteristics associated with graft failure, donor age, donation after circulatory death (DCD), split/partial grafts, race, height, and cause of brain death[53]. Yet, the DRI has known limitations which must be considered in this analysis. First, the DRI does not account for steatosis, a known risk factor for postoperative graft dysfunction. Second, cold ischemia time cannot be anticipated in all cases, especially in challenging logistical scenarios, such as in countries with long territorial extensions and complex surgical cases. Consequently, a more thorough evaluation of the impact of donor features within ACLF studies is needed.

Living-donor liver transplantation (LDLT) could expand the donor organ pool and expedite transplantation in ACLF patients. Whilst studies in countries where deceased donors are scarce for cultural reasons demonstrated good LDLT postoperative outcomes[54,55], concerns regarding the prognosis of the sickest patients leading to stringent patient selection criteria hinder the applicability of this option thus far. In a retrospective analysis of 60 patients with EASL-CLIF grade 1 and 2 ACLF, LDLT transplanted patients exhibited a 1-year survival rate of 92% *vs* 11% in those who did not undergo transplantation[55]. In another retrospective study involving 218 ACLF patients, employing strict selection criteria -no high vasopressors or respiratory failure- for LDLT transplantation, the 1-year postoperative patient survival was 92.9% for EASL-CLIF grade 1, 85.4% for grade 2, and 75.6% for grade 3[54]. Despite suggesting the benefit of LDLD in this setting, the justifiable caution patient selection may have biased the conclusions. In addition, right lobe LDLT is most often required, leading to a right hepatectomy in the donor, which increases their morbidity and mortality.



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Figure 1 Suggested flowchart on when to accept a non-ideal donor organ offer for transplantation in the acute-on-chronic liver failure setting. In this scenario, the coexistence of additional risk factors and the real-time change in organ failures are key components in deciding to proceed with transplantation. ACLF: Acute-on-chronic liver failure.

While nowadays, consensually amongst experts, ECD organs must be considered in ACLF patients due to the transplant benefit, further studies detailing the real impact of donor characteristics on posttransplant patient survival are awaited. This is particularly valid for ACLF-3 patients, in which the limited literature available urges caution before proceeding with a high-risk donor. Thus far, this general concept is more theoretical than practical and originates from the need to provide timely transplantation to these patients. Therefore, this is a warranted subject of deeper investigation for future studies.

WHAT ROLE DOES MACHINE PERFUSION OF THE LIVER MAY PLAY IN THE ACLF SETTING

Machine perfusion of the liver (MPL) is currently a hot topic in LT. It has gained growing attention from the transplant community with the expansion of the ECD population and, therefore, the need to prevent the frequent ischemia-reperfusion injury (IRI)-ECD postoperative-related complications. IRI is an intrinsic consequence of solid organ transplantation and the basis of major postoperative complications. Although ECD organs are highly vulnerable to IRI, surpassing the protective capacity of traditional static cold storage (SCS) preservation solutions and, consequently, associated with higher morbidity and mortality rates after transplantation, their increased utilization is needed to attend to the rising number of patients on the waiting list. Thus far, the two most studied modalities of MPL in LT are *ex situ* hypothermic and normothermic machine perfusion.

The hypothermic oxygenated machine perfusion (HOPE) of the liver was shown to enhance mitochondrial respiratory function[56]. The optimized mitochondrial respiratory chain and oxidative phosphorylation system increase cellular energy production -replenishing the exhausted stores of adenosine triphosphate-, avoid the reverse flow of electrons with the production of reactive oxygen species, and prevent the activation of the inflammatory cascade with subsequent tissue damage[56].

The normothermic machine perfusion of the liver (NMP) allows the recovery of the full metabolism of the organ at 37 °C. Consequently, it requires an oxygen carrier to attend to the cellular metabolic demand. NMP permits the assessment of parameters that traditionally indicate appropriate liver function such as bile production, vascular flow, lactate metabolism, glucose metabolism, and hepatocellular injury such as transaminases released into the perfusate[57]. In addition, NMP enables prolonged organ preservation and, potentially, *ex situ* organ treatments[57,58].

So far, most clinical trials on MPL in LT were intended to demonstrate the safety and feasibility of the technique and were centered in European countries. Arguably, the sickest patients with very high morbidity and mortality risk were not included in these studies and were not even on the waiting list in many of these countries. Therefore, regional divergence and particularities amongst geographical areas, such as mean MELD on the waiting list and territorial extensions, must also be considered.

Hurdles to timely access of ACLF patients to LT and their disadvantage in receiving a donor organ offer in the MELD allocation system were presented herein. In addition, concerns about accepting ECD organs to these sick patients were discussed beforehand. Consequently, none of the MP clinical trials has encompassed ACLF patients thus far. Nevertheless, hypothetically, MPL can be even more advantageous for this population.

Studies suggest that MP may recondition ECD organs before transplantation, preventing further deterioration or even improving their quality. Although DCD LT has not been reported for ACLF patients, clinical trials described similar results for patients transplanted with DCD organs treated with HOPE and those transplanted with low-risk donors after brain death (DBD)[59]. Furthermore, in a randomized clinical trial, hypothermic machine perfusion reduced the occurrence of postreperfusion syndrome and early allograft dysfunction (EAD) after DCD LT. These factors contribute to the early

recovery of the sickest patients after the procedure[60]. Accordingly, concerning the applicability of the technique in ECD DBD LT, a multicenter randomized clinical trial recently reported that, compared to SCS, HOPE led to a significant reduction in 90-d complications with a shorter hospital stay and a trend toward a reduced rate of EAD[61].

Prolonged organ preservation and assessment of organ viability are two critical features related to NMP which may benefit ACLF transplantation. Whereas it could not find a difference in graft survival or patient survival compared to SCS, the first randomized clinical trial on NMP demonstrated that it could safely extend the organ preservation time[62]. The median total preservation time was close to 12 h for NMP-preserved livers. Driven mainly by the difference in peak aspartate transaminase, NMP also reduced the occurrence of EAD[62]. The VITTAL clinical trial (NCT02740608), from Birmingham, United Kingdom, transplanted twenty-two donor livers discarded by all United Kingdom centers of 31 meeting specific high-risk criteria based on the lactate clearance to levels ≤ 2.5 mmol/L within 4 h on NMP with 100% 90-d patient and graft survival[63]. Nevertheless, applying the Birmingham criteria, NMP could not prevent non-anastomotic biliary strictures in DCD livers, and 4 (18%) patients needed re-transplantation[63].

Our group recently reported for the first time the successful transplantation of an ACLF patient using an ECD DBD liver graft treated with HOPE[64]. The autoimmune hepatitis-related cirrhosis ACLF-2 patient (liver and coagulation failure) with a MELD-Na score of 42 was offered an ECD DBD organ with a DRI of 2.79 after 8 d from hospital admission (well above the previously identified threshold of 1.7). HOPE started after 06 h and 19 min of cold ischemia time and lasted 5 h and 19 min. The flavine mononucleotide was measured in the perfusate to assess the viability of the organ and revealed a low value after 30 min of perfusion (3097 A.U.) - permissive for transplantation in any recipient. During transplantation, the reperfusion was uneventful. Postoperatively, the graft recovered well, without EAD, according to the Olthoff criteria, and the patient developed AKI KDIGO stage 3 with complete recovery after 1 wk[64].

While the case suggests the feasibility and safety of employing MP within this setting, more conclusive evidence to prove the benefit of the technique is still needed. So far, because of the scarce existing literature, the evidence of the impact of ECD transplantation in ACLF grades 1 and 2 is still anecdotal. In addition, there are no reports on the application of MPL in ACLF-3 patients, those with greater risk and the subject of more concerns in the literature. Earlier transplantation was shown to improve overall survival in ACLF-3 patients > 60-years-old or with 4-6 OF[49]. This population and those with the coexistence of additional risk factors and worsening organ failures may be the target population for future MPL studies.

Yet the evidence is very limited currently, MPL may play a game-changing role in ACLF transplantation. First, it can expedite LT because it allows more liberal acceptance of ECD organs based on the properties of the technique. The proven capacity of MPL to recondition ECD livers reassures surgeons in their decision to accept an ECD organ. This effect is amplified with the application of biomarkers for organ viability assessment during perfusion. Second, rescuing discarded high-risk organs or prolonging the preservation of organs compromised by logistics *via* MPL may increase the donor organ pool, which may also help tackle the shortage of donor organs for transplantation. This is especially important in countries with frequent high MELD score patients and long cold ischemia time, which may need to adapt their organ preservations systems.

CONCLUSION

Although new clinical specific therapies have been researched for ACLF management, earlier transplantation -within the frequently narrow opportunity window- is a proven effective therapy for selected ACLF patients. Thus far, other options encompass diagnosing and treating precipitant events and supportive treatment for organ failures. Therefore, current and future perspectives on ACLF management must envisage improved access to LT. Accordingly, discussions about allocation and prioritization for transplantation in critically ill ACLF patients are awaited because there is evidence suggesting the current model may not portray their waitlist mortality. Furthermore, whereas donor organ quality is meant to be a prognostic factor in the ACLF setting, recent evidence suggests that MPL may be a safe tool to improve the donor organ pool and expedite access to this life-saving procedure.

FOOTNOTES

Author contributions: Boteon YL, Della Guardia B, and Boteon APCS designed this study; Boteon YL, Della Guardia B, Boteon APCS, Matielo CE, and Felga G performed the literature review and analysis; Boteon YL, Della Guardia B, and Boteon APCS drafted the manuscript; Boteon YL, Della Guardia B, Boteon APCS, Matielo CE, and Felga G reviewed the manuscript critically; All authors contributed to editing and approving the final manuscript version.

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ORIGINAL ARTICLE

Basic Study

Bladder-colon chronic cross-sensitization involves neuro-glial pathways in male mice

Karim Atmani, Fabien Wuestenberghs, Maximilien Baron, Illona Bouleté, Charlène Guérin, Wafa Bahlouli, David Vaudry, Jean Claude do Rego, Jean-Nicolas Cornu, Anne-Marie Leroi, Moïse Coëffier, Mathieu Meleine, Guillaume Gourcerol

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Abstract

BACKGROUND

Irritable bowel syndrome and bladder pain syndrome often overlap and are both characterized by visceral hypersensitivity. Since pelvic organs share common sensory pathways, it is likely that those syndromes involve a cross-sensitization of

the bladder and the colon. The precise pathophysiology remains poorly understood.

AIM

To develop a model of chronic bladder-colon cross-sensitization and to investigate the mechanisms involved.

METHODS

Chronic cross-organ visceral sensitization was obtained in C57BL/6 mice using ultrasound-guided intravesical injections of acetic acid under brief isoflurane anesthesia. Colorectal sensitivity was assessed in conscious mice by measuring intracolonic pressure during isobaric colorectal distensions. Myeloperoxidase, used as a marker of colorectal inflammation, was measured in the colon, and colorectal permeability was measured using chambers. c-Fos protein expression, used as a marker of neuronal activation, was assessed in the spinal cord (L6-S1 level) using immunohistochemistry. Green fluorescent protein on the fractalkine receptor-positive mice were used to identify and count microglia cells in the L6-S1 dorsal horn of the spinal cord. The expression of NK1 receptors and MAPK-p38 were quantified in the spinal cord using western blot.

RESULTS

Visceral hypersensitivity to colorectal distension was observed after the intravesical injection of acetic acid *vs* saline ($P < 0.0001$). This effect started 1 h post-injection and lasted up to 7 d post-injection. No increased permeability or inflammation was shown in the bladder or colon 7 d post-injection. Visceral hypersensitivity was associated with the increased expression of c-Fos protein in the spinal cord ($P < 0.0001$). In green fluorescent protein on the fractalkine receptor-positive mice, intravesical acetic acid injection resulted in an increased number of microglia cells in the L6-S1 dorsal horn of the spinal cord ($P < 0.0001$). NK1 receptor and MAPK-p38 levels were increased in the spinal cord up to 7 d after injection ($P = 0.007$ and 0.023 respectively). Colorectal sensitization was prevented by intrathecal or intracerebroventricular injections of minocycline, a microglia inhibitor, by intracerebroventricular injection of CP-99994 dihydrochloride, a NK1 antagonist, and by intracerebroventricular injection of SB203580, a MAPK-p38 inhibitor.

CONCLUSION

We describe a new model of cross-organ visceral sensitization between the bladder and the colon in mice. Intravesical injections of acetic acid induced a long-lasting colorectal hypersensitivity to distension, mediated by neuroglial interactions, MAPK-p38 phosphorylation and the NK1 receptor.

Key Words: Cross-organ sensitization; MAPK-p38; Microglia; NK1 receptor; Pain; Visceral hypersensitivity

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Core Tip: A model of chronic cross-organ visceral sensitization in mice was developed using ultrasound-guided intravesical injections of acetic acid. Visceral hypersensitivity to colorectal distension was observed as early as 1 h post-injection and lasted up to 7 d. Visceral hypersensitivity was associated with an increased expression of c-Fos protein in the spinal cord. The NK1 receptor and MAPK-p38 levels were upregulated in the spinal cord 7 d post-injection. Colorectal sensitization was prevented by intrathecal or intracerebroventricular injections of minocycline, a microglia inhibitor, by intracerebroventricular injection of CP-99994, a NK1 antagonist, and by intracerebroventricular injection of SB203580, a MAPK-p38 inhibitor.

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INTRODUCTION

Irritable bowel syndrome (IBS) and bladder pain syndrome (BPS) are two functional disorders that affect the gastrointestinal tract and the urinary tract, respectively[1,2]. Their prevalence in the general

population is 4.6%[3] and 4.2%[4], respectively, and recent studies have shown a strong overlap between both syndromes[1,2]. Indeed, BPS is found in 40%-60% of IBS patients[5], and IBS is observed in 25.4%-38.6% of BPS patients[1,2]. Both syndromes are characterized by visceral mechanical hypersensitivity at the urinary tract level for BPS and at the intestinal level for IBS[6,7]. The involvement of several mechanisms has been suggested in the onset and/or the maintenance of visceral hyperalgesia, including urothelial and/or intestinal epithelial permeability, mucosal immune activation and altered brain-gut interaction[8].

Despite increasing knowledge of the pathophysiology of IBS and BPS, limited mechanistic data is available in the context of BPS-IBS overlap. Based on the fact that pelvic organs share common sensory pathways, a few studies have offered evidence that cross-sensitization between the bladder and the colon may explain sensitization of both organs[9]. This may involve primary extrinsic afferents or central sensitization, both at the spinal and supraspinal levels[10]. All these studies involved acute sensitization models, often in anesthetized animals[11]; therefore, there is not yet any data regarding the chronicization of pelvic organ cross-sensitization.

The role of spinal glia has recently been highlighted in the sensitization of the bladder or colon[12] and even in cross-organ sensitization between both organs[13]. The role of spinal glia, however, has never been demonstrated in the maintenance of such sensitization using models of bladder-colon cross-sensitization induced chronic visceral hyperalgesia. The aims of our study were therefore to develop a model of chronic cross-organ sensitization between the bladder and the colon and to investigate the mechanisms involved in the development and persistence of this cross-sensitization.

MATERIALS AND METHODS

Ethics

The experiments were carried out in accordance with the ethical guidelines of the International Association for the Study of Pain[14] and in accordance with the guidelines of the French Ministry of Agriculture and Fisheries (Decree No. 874848). Our protocol was approved by the local Ethics Committee for Animal Experiments (CENOMEXA No: N/02-01-13/02/01-16).

Animals

Adult male wild type C57Bl/6 mice (Janvier Laboratories, Le Genest-Saint-Isle, France) and transgenic mice expressing the green fluorescent protein on the fractalkine receptor of microglial cells (Inserm Laboratory U1239, Dr. David Vaudry Team, Mont Saint Aignan, France[15]) were 8 wk old on the day of the experiment (weight range: 22-26 g). The animals were randomized by their weight in several cages, with five mice per cage, housed in a 12 h light/dark cycle in an animal housing facility free of specific pathogenic organisms and maintained at room temperature (22 ± 2 °C). The animals received a standard diet (RM1 diet; SDS, Witham, Essex, United Kingdom). Drinking water and food were available *ad libitum*. Each manipulation or experiment took place after at least 1 wk of acclimatization to the housing conditions. All animals were euthanized by cervical dislocation after anesthesia with ketamine (100 mg/kg, Imalgene 1000; Merial, Lyon, France) and xylazine (10 mg/kg, Rompun® 2%; Bayer, Berlin, Germany) administered intraperitoneally before tissue collection.

Study design

The animal protocol was designed to minimize pain or discomfort to the animals. Eight series of mice were used in this work. Each set was comprised of different groups of mice, and each group was formed of 5-8 mice. The first series was used to develop the cross-organ sensitization model. Once the model was validated, a second series was used to analyze inflammatory parameters in the colon and the bladder and to assess colonic and bladder permeabilities. We then focused on the central nervous system, especially at lamina I and II of the dorsal horn at the L6-S1 segment, where pelvic extrinsic primary afferent neurons form synapses with spinal neurons. Our third series assessed the neuronal activity in this model using c-Fos expression, and inflammatory parameters were measured in the spinal cord of a fourth series. The model was then transposed on transgenic mice to assess changes in microglia cells. Finally, three different inhibitors/antagonists were used in the sixth, seventh and eighth series to gain a better understanding of which pathway could be involved in the cross-sensitization process.

Acetic acid sensitization

An injection of acetic acid (0.75%, 200 µL) or saline (NaCl 0.9%, 200 µL; Baxter, Deerfield, IL, United States) was made into the urinary bladder using ultrasound monitoring in mice under brief anesthesia (isoflurane: 3% in 1.5 L/min of air, Iso-Vet®; Piramal Critical Care, Voorschoten, The Netherlands).

Colorectal distension and visceral sensitivity measurement

We measured visceral pain to colorectal distension (CRD) using a non-invasive method, as previously

reported in mice[16]. Changes in intracolonic pressure, reflecting viscero-motor responses induced by CRD (nociceptive stimulus), were used as a surrogate marker of colorectal sensitivity[16].

An infinitely compliant distension balloon (diameter 0.7 cm) was made using a polyethylene bag attached to a PE-50 catheter (Intramedics, France) drilled in its end and taped 2 cm below the pressure sensor of a miniaturized pressure transducer catheter (SPR-524 Mikro-Tip catheter; Millar Instruments, Houston, TX, United States). Polypropylene 4-0 Ligatures (Prolène®; Ethicon Inc., Somerville, NJ, United States) were covered with parafilm to prevent any air leak.

On the experimental day, mice were briefly anesthetized with isoflurane (3% in air), and the lubricated "balloon-pressure sensor" was introduced into the colorectum, so that the balloon was inserted 2 cm upstream of the anal margin into the colon. Each mouse was placed in an adjustable mouse restrainer (30 mm diameter × 90 mm length, LE5016; *In Vivo* Research Instruments, Perkin Elmer, Waltham, MA, United States) and left to rest for 30 min before the CRD procedure. The balloon was then secured to the tail with tape and connected to an electronic barostat (Distender Series II; G & J Electronics Inc., Toronto, ON, Canada) to perform isobaric CRD. Our distension protocol consisted of a set of graded phasic distensions of 15, 30, 45 and 60 mmHg (two times each, 20 s duration, 4 min inter-stimulus interval) (Protocol Plus Deluxe; G & J Electronics, Toronto, ON, Canada). Voltage output was converted digitally using CED digital-analogic converter (Micro 1401; Cambridge Electronic Design, Cambridge, United Kingdom) and Spike 2 software (CED, Ltd., Cambridge, United Kingdom). The pressure sensor allowed the assessment of visceral pain *via* a custom-made script that allowed signals to be specifically extracted from abdominal muscle contractions (excluding those from colonic contractions).

Colonic sensitivity was measured in awake mice at 60 min, 3 d and 7 d following acetic acid urinary bladder injections.

Measurement of bladder and colonic paracellular permeabilities

After euthanasia, bladder and distal colon samples were removed on day 7. Samples were cut along the mesenteric border. Bladder and colonic permeabilities were assessed by measuring fluorescein isothiocyanate (FITC)-dextran (4 kDa) fluxes in Ussing chambers with an exchange surface of 0.07 cm^2 (Harvard Apparatus, Holliston, MA, United States) as previously described[17]. FITC-dextran (5 mg/mL) was loaded on the mucosal side. After 3 h at 37 °C, the medium from the contralateral side (serosa) was removed and stored at -80 °C. The fluorescence level of FITC-dextran (excitation at 485 nm, emission at 535 nm) was measured in a 96-well black plate using spectrometer Chameleon V (Hidex Co, Turku, Finland). The results were converted to concentrations of FITC-dextran (mg/mL) for analysis.

Myeloperoxidase measurement

After euthanasia, bladder and distal colon samples were removed on day 7. Colonic and vesical tissues (around 50 mg) were washed in phosphate-buffered saline and then homogenized (50 mg/mL) in 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, Steinheim, Germany) with 50 mmol/L of phosphate-buffered saline (pH 6.0). They were frozen at -80 °C and thawed at 37 °C three times, then sonicated (Vibra Cell ultrasonic processor 75115; Bioblock Scientific, Illkirch, France) and finally centrifuged (14000 rpm at 4 °C for 15 min). Myeloperoxidase (MPO) was assayed in the supernatant by adding 1 mg/mL of dianisidine dihydrochloride (Sigma-Aldrich) and 5 × 10^{-5}% of hydrogen peroxide (Sigma-Aldrich). The change in optical density was measured at 450 nm. One unit of MPO activity was defined as the amount that degraded 1.0 μmol of hydrogen peroxide per minute at 25 °C, and human neutrophil MPO (Sigma-Aldrich) was used as standard, as previously described[18,19].

c-Fos immunofluorescence

Seven days after the intrabladder injection of acetic acid 0.75%, c-Fos immunohistochemistry was performed after 120 min of CRD at 45 mmHg (20 s of distension every 4 min for 120 min). Under ketamine (100 mg/kg i.p.)/xylazine (10 mg/kg) anesthesia and upon thoracotomy, mice were perfused through a cardiac-aorta cannula with saline followed by 150 mL/mouse of ice-cold 4% paraformaldehyde and 14% saturated picric acid in a 0.1 M phosphate buffer solution (pH 7.2). After decapitation, the lumbo-sacral spinal cord (L6-S1) was post-fixed in the same fixative solution overnight at 4 °C, cryoprotected by immersion in 10% sucrose overnight and transferred to 30% sucrose overnight. The spinal cords were then embedded in Tissue-Tek® optimal cutting temperature compound (Sakura Finetek United States, Inc., Torrance, CA, United States), snap-frozen and cut with a cryostat. Fluorescent microscopy was used to identify activated neurons. The expression of c-Fos was assessed by immunofluorescence. We applied anti-c-Fos antibody (1:2000; Calbiochem, Darmstadt, Germany) overnight at 4 °C and then incubated Cy3-conjugated goat anti-rabbit IgG (1:400; Fisher, Invitrogen, Carlsbad, CA, United States) for 2 h at room temperature. Pictures were taken using a fluorescence microscope (DM5500 B; Leica Microsystem Ltd., Wetzlar, Germany) at magnification × 10, and the number of c-Fos immunoreactive cells in lamina I and II of the dorsal horn at the L6-S1 segment of the spinal cord was counted for each mouse. The average number of stained nuclei in three 20 μm thick slices for each mouse was used for analysis.

Semiquantitative PCR for the detection of TNF-α, IL-1β and IL-10 mRNA in the spinal cord

Total RNA from L6-S1 spinal cord segments was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). RNA was purified according to the manufacturer's instructions. Total RNA was treated with DNase I (Invitrogen) to remove any contaminating DNA. DNase I was stopped with DNase inactivation reagent (Invitrogen) according to the manufacturer's instructions. The quality and quantity of total RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, United States). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA. A ratio of ≥ 2.0 was accepted for analysis.

After reverse transcription of 1.5 µg total RNA into cDNA by using 200 units of SuperScript II Reverse Transcriptase (Invitrogen), quantitative PCR was performed using SYBR Green technology on a Bio-Rad CFX96 real time PCR system (Bio-Rad Laboratories, Marnes-la-Coquette, France). The *GAPDH* gene was chosen as the reference gene. All samples were performed in duplicate in a single 96-well reaction plate. Serially diluted cDNA samples were used as external standards. The absolute quantification of mRNA was performed by converting the sample Ct values to concentration (copies per µL) based on standard curves. The identity and purity of the amplified products were assessed using melting curve analysis at the end of amplification. The technique was used to assay *TNF-α*, *IL-1β* and *IL-10* mRNA in the spinal cord. The primer sequences for the targeted mouse genes are presented in Supplementary Table 1.

Analysis of microglia cells

Green fluorescent protein on the fractalkine receptor-positive transgenic mice were perfused as described for c-Fos immunofluorescence, and the dorsal horn of L6-S1 spinal cord was embedded in Tissue-Tek® optimal cutting temperature compound, snap-frozen and cut with a cryostat. Pictures were then taken with the Leica photonic microscope used for c-Fos experiments at magnification × 10, and the number of microglia cells expressing microglia green fluorescent protein in lamina I and II of L6-S1 dorsal spinal cord was counted using NIH ImageJ software (version 2.0.0-rc-43/1.51u)[20]. The average number of stained nuclei per field in three 20 µm thick slices for each mouse was used for statistics.

Pharmacologic studies

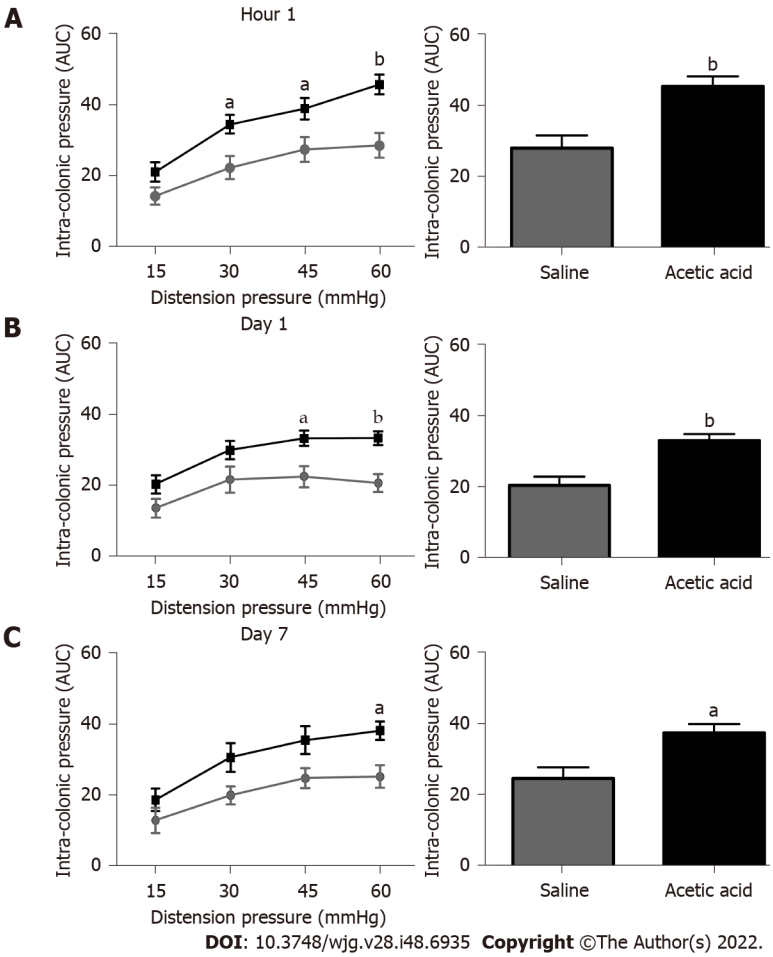
Minocycline (2.5 mg/mL; 1.25 mg/kg; Sigma-Aldrich), a microglia blocker, CP 99994 (15 mg/mL; 7.5 mg/kg; Sigma-Aldrich), a NK1R antagonist, SB203580 (5 mg/mL; 2.5 mg/kg; Calbiochem, Merck, EMD Millipore Corp., Billerica, MA, United States), a MAPK-p38 blocker, or saline were injected in the intracerebroventricular (ICV) region using a Hamilton syringe (Neuros™, Gastight, 1705, 33 gauge; Dutscher, Bernolsheim, France) 1 h before injecting acetic acid (0.75%) into the bladder. Intrathecal injections of minocycline at the L6-S1 Level of the spinal cord were also performed to demonstrate that microglia activation occurred at that level.

Western blot analysis

After euthanasia, the L6-S1 segment of the mice spinal cord was removed on ice and quickly frozen in liquid nitrogen. After thawing on ice, the spinal cord samples were homogenized at 4 °C in a lysis buffer (100 µL of buffer A × 2, 2 µL of 100 mmol/L dithiothreitol, 50 µL of 1% NP40, 1 µL of 1 × P8340 protease inhibitors, 2 µL of 1 × P2850 phosphatase inhibitors and 200 mL of H_2O). Samples were displayed on ice for 15 min, then centrifuged at 12000 × g for 15 min at 4 °C. The protein-containing supernatant was collected and stored at -80 °C until analysis. The proteins (25 µg) were loaded on a 4%-20% gradient polyacrylamide gel (Bio-Rad) and transferred onto a nitro-cellulose membrane (GE Healthcare, Orsay, France). Membranes were then blocked for 1 h at room temperature with 5% (w/v) nonfat dry milk in Tris buffered saline containing 0.05% Tween® 20 (Sigma-Aldrich). An overnight incubation at 4 °C was then performed with primary antibodies: anti-P-p38 (1:500; Cell Signaling Technology®, Leiden, The Netherlands; P/N 4511) or anti-NK1R (1:200; Atlas Antibodies, Bromma, Sweden; P/N HPA074573) from rabbit or anti-GAPDH (1:5000; Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France) from goat. All antibodies were diluted in a blocking solution. After three washes, a 1-h incubation was performed with a peroxidase-conjugated IgG secondary antibody from goat anti-rabbit or from rabbit anti-goat (1:5000; Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France). After three additional washes, immunocomplexes were revealed using the ECL detection system (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). Proteins were quantified by densitometry using ImageScanner III and ImageQuant TL software (GE Healthcare Life Sciences), and standardized against the intensity of GAPDH.

Statistical analyses

The data were expressed as mean ± standard error of the mean. Quantitative data was compared between groups using an unpaired *t*-test, with Welch's correction in case of unequal variances. Comparisons of multiple groups were performed using one-way analysis of variance (Kruskal-Wallis test) with post-hoc analysis (Dunn's multiple comparison post-test to compare all pairs of columns to each other or Dunnett's post-test to compare all pairs of columns to controls) to assess the difference among the groups, and two-way analysis of variance with post-hoc analysis (Bonferroni's correction)



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Figure 1 Assessment of visceromotor response to colorectal distensions. A-C: Changes in intracolonic pressure in response to isobaric colorectal distensions (15, 30, 45 and 60 mmHg) after intravesical injections of acetic acid (0.75%) *vs* saline (NaCl 0.9%) in mice at 1 h (A), 1 d (B) and 7 d (C) after injections, with details of the comparison at 60 mmHg of distension on the right. n = 8 mice per group. $^{a}P < 0.05$ and $^{b}P < 0.001$. Variability in the results is represented by the standard error of the mean (area under the curve ± standard error of the mean). AUC: Area under the curve.

was used to assess groups with repeated measures. Individual data for visceral sensitivity in mice, especially the kinetics of visceromotor responses to increasing CRD, was visually assessed for each experimental group separately; tracings of animals with aberrant or outlier responses to distensions were excluded from the analysis. A P value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 9.3.1 for Windows (GraphPad Software Inc., San Diego, CA, United States, www.graphpad.com). The statistical methods used in this study were reviewed by Fabien Wuestenberghs from CHU UCL Namur.

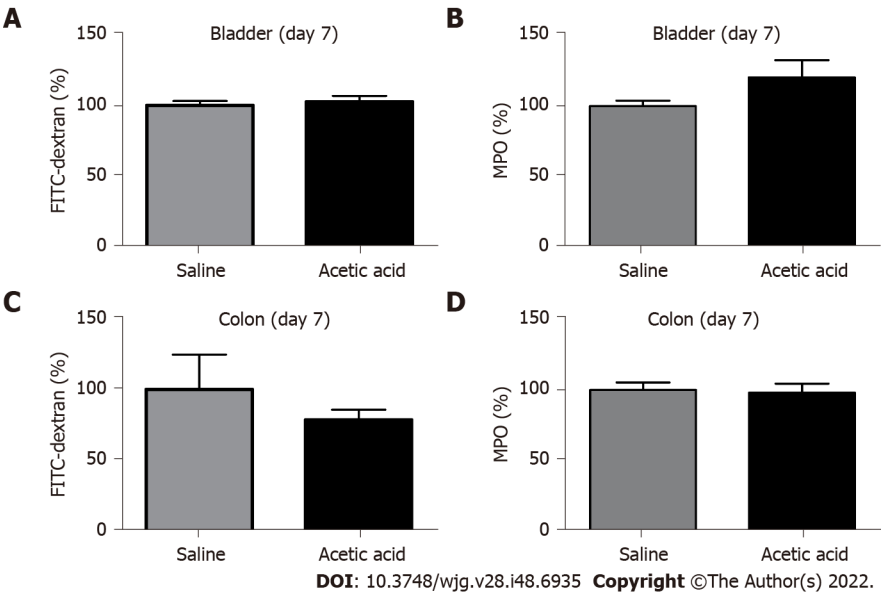
RESULTS

Development and validation of a mouse model of chronic bladder-colon cross-sensitization

Based on our previous rat study[9], we tested the intravesical administration of a 0.75% acetic acid solution in mice. A single intravesical injection of 0.75% acetic acid under ultrasound monitoring induced an increase of the colonic nociceptive response during CRD at 30 mmHg ($P < 0.05$), 45 mmHg ($P < 0.05$) and 60 mmHg ($P < 0.001$) 1 h after injection (Figure 1). An increased colonic nociceptive response during CRD was still observed at 60 mmHg ($P < 0.05$) on 7thd in the acetic acid group compared to the control group (Figure 1), confirming that colonic hypersensitivity persists up to 7 d after the intravesical injection in our model. Further experiments were therefore designed to understand the mechanisms of the chronicization of cross-sensitization in this model. No serious adverse events occurred during the experiments.

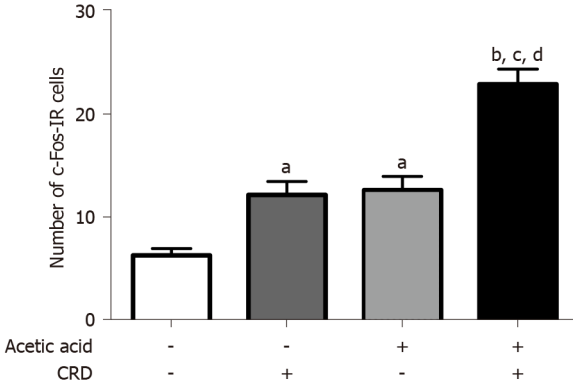
Permeability and inflammatory parameters of bladder and colon

No differences were found at day 7 in either bladder or colon permeabilities or MPO activities between animals treated with acetic acid or saline intravesically (Figure 2).



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Figure 2 Bladder and colorectal permeabilities and inflammation. A-D: Urinary bladder (A) and colorectal (B) permeabilities assessed by fluorescein isothiocyanate (FITC)-dextran, and urinary bladder (C) and colon (D) inflammatory levels assessed by myeloperoxidase (MPO) activities. They were not different between mice with intravesical injections of saline (NaCl 0.9%) or acetic acid (0.75%) at day 7. *n* = 4-5 mice per group. Results of mice treated by intravesical injections of acetic acid were normalized to those of mice treated with saline. Variability in the results is represented by the standard error of the mean.



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Figure 3 Quantification of c-Fos immunoreactive cells. Quantification of c-Fos immunoreactive (IR) cells in lamina I and II of the dorsal horn per slice of the L6-S1 spinal cord on day 7 after intravesical injection of saline (NaCl 0.9%) or acetic acid 0.75%, with or without prior colorectal distensions (CRDs) at 45 mmHg for 120 min (*n* = 6-7 mice per group). [a]*P* < 0.05 and [b]*P* < 0.0001 (compared to mice treated with saline and no prior CRD); [c]*P* < 0.001 (compared to mice treated with saline and prior CRD); [d]*P* < 0.01 (compared to mice treated with acetic acid and no prior CRD). Results are expressed as the mean ± standard error of the mean.

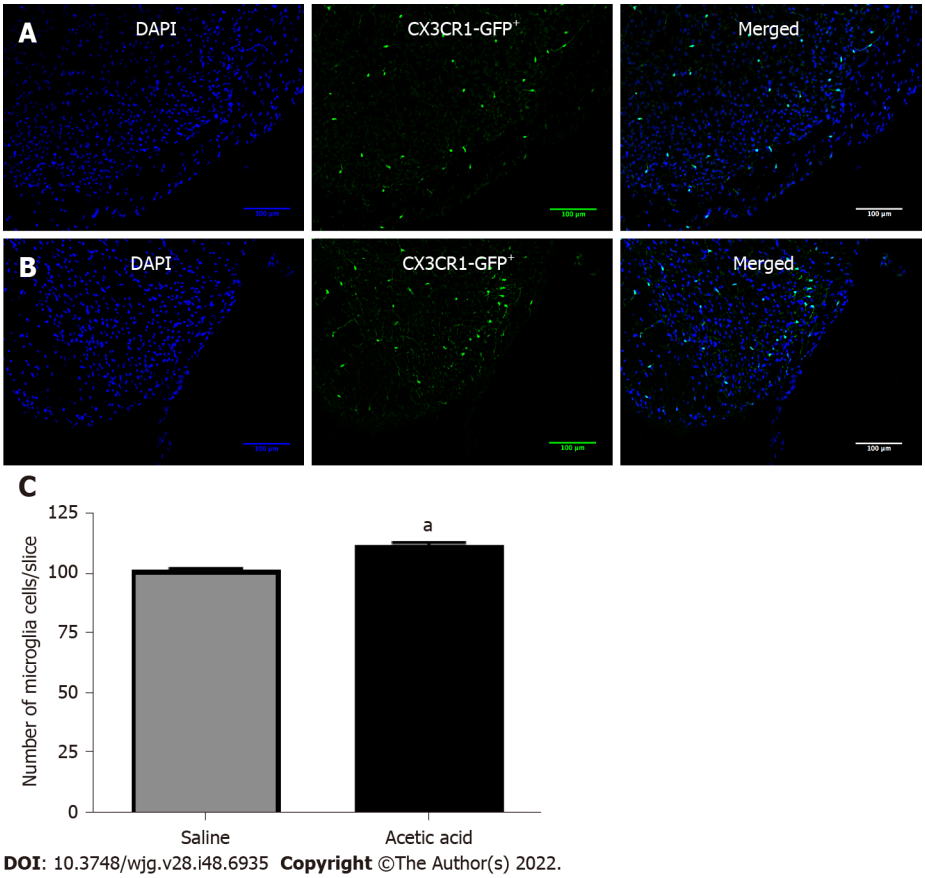
Neural activation in the dorsal horn of the spinal cord L6-S1

In the absence of CRD, the number of c-Fos immunoreactive cells in lamina I and II of the L6-S1 level of the dorsal horn was 6.5 ± 0.7 per slice in mice treated with saline. The CRD performed on the 7th d in these mice treated with saline increased the number of c-Fos immunoreactive cells to 12.4 ± 1.3 (P < 0.05). In mice treated with 0.75% acetic acid, the number of c-Fos immunoreactive cells was 12.9 ± 1.3 in the absence of CRD and increased to 23.2 ± 1.5 (P < 0.05 *vs* all other groups) after CRD (Figure 3).

Implication of central microglia from the dorsal horn of the spinal cord L6-S1 in bladder-colon cross-sensitization

The number of microglial cells has been observed in a transgenic mouse model that specifically expresses green fluorescent protein associated with the fractalkine receptor (Figure 4A and B). Seven days after injection of a 0.75% acetic acid solution into the bladder, the number of microglial cells increased compared to mice injected with saline (113.4 ± 13.0 *vs* 102.9 ± 11.2 per field, respectively, P < 0.05) (Figure 4C).

We administered minocycline, a microglial inhibitor, at the central level 1 h before the intravesical injection of acetic acid or saline to demonstrate the involvement of microglia cells. Both intrathecal and



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Figure 4 Number of microglia cells in the spinal cord. A and B: Microglial cells in the dorsal horn of the L6-S1 level of the spinal cord were stained green [green fluorescent protein on the fractalkine receptor-positive (CX3CR1^{gfp+}) mice], while nuclei appear in blue (DAPI) at day 7 after intravesical injections of saline (NaCl 0.9%) (A) or acetic acid 0.75% (B); C: The number of microglial cells per field was compared between the two groups using a Mann-Whitney bilateral test (n = 5 per group, average of 23 slices analyzed per mouse). $^{a}P < 0.05$. Results are expressed as the mean ± standard error of the mean.

ICV injections of minocycline prevented the development of cross-sensitization induced by intravesical administration of acetic acid (Figure 5A and B). ICV injections were favored for the following experiments because they are less traumatic and cause less stress to the animals.

Involvement of central NK1R in bladder-colon cross-sensitization

The ICV injection of CP 99994, a NK1R antagonist, 1 h before the intravesical injection of acetic acid, prevented the development of bladder-colon cross-sensitization compared to mice treated intracerebroventricularly by saline (Figure 6A). The intravesical administration of acetic acid increased the phosphorylation of MAPK-p38, a microglial protein involved in chronic pain generation[21], compared to mice injected intravesically with saline, but the ICV injection of CP 99994 prevented this phosphorylation, suggesting that microglial activation depends on the activation of NK1R (Figure 6B).

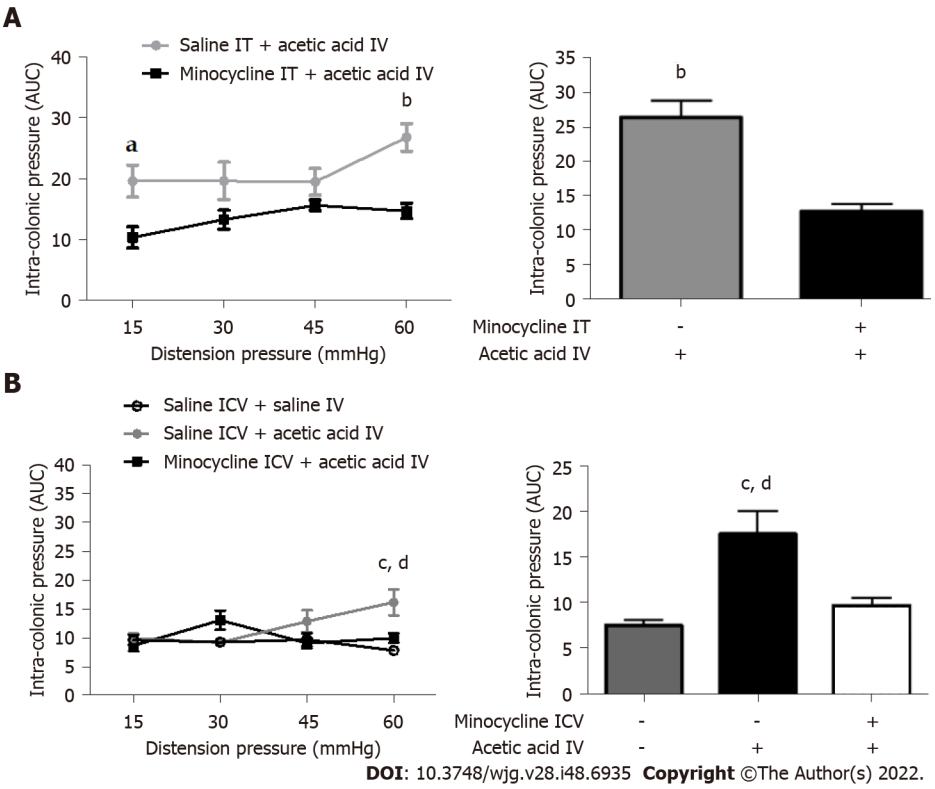
The ICV injection of SB203580, a MAPK-p38 inhibitor, 1 h before the intravesical injection of acetic acid prevented the occurrence of bladder-colon cross-sensitization (Figure 7A). The intravesical administration of acetic acid induced an increase in NK1R expression in the posterior horn of the L6-S1 level of the spinal cord compared to the administration of saline (Figure 7B). Acetic acid-induced spinal overexpression of NK1R was blocked by prior ICV administration of SB203580 (Figure 7B), suggesting that the MAPK-p38 pathway is involved in the development of bladder-colon cross-sensitization.

Assessment of the expression of spinal pro- and anti-inflammatory transcripts

Expression levels of *IL-1β*, *TNF-α* and *IL-10* mRNA did not differ between control and acetic acid groups (P = 0.22, 0.47 and 0.19, respectively) (Figure 8).

DISCUSSION

A mouse model of bladder to colon cross-sensitization with persistent visceral hypersensitivity in the colorectum has not been developed until now to our knowledge. Our study showed that pelvic visceral cross-sensitization involves central sensitization with microglia modulation following a peripheral



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Figure 5 Effect of minocycline injection at the central level on visceral sensitivity. A and B: Changes in intracolonic pressure in response to isobaric colorectal distensions (15, 30, 45 and 60 mmHg) intrathecal (IT) injections of saline (NaCl 0.9%) (A) or minocycline (1.25 mg/kg) prior to intravesical (IV) injections of acetic acid 0.75% and after intracerebroventricular (ICV) injections of saline or minocycline (1.25 mg/kg) (B) prior to IV injections of saline or acetic acid 0.75% in mice at day 7, both with details of the comparison at 60 mmHg of distension on the right. n = 6-8 mice per group. $^{a}P < 0.05$ and $^{b}P < 0.001$ (compared to the minocycline IT + acetic acid IV group); $^{c}P < 0.0001$ (compared to the saline ICV + saline IV group); $^{d}P < 0.05$ (compared to the minocycline ICV + acetic acid IV group). Variability in the results is represented by the standard error of the mean (area under the curve ± standard error of the mean). AUC: Area under the curve.

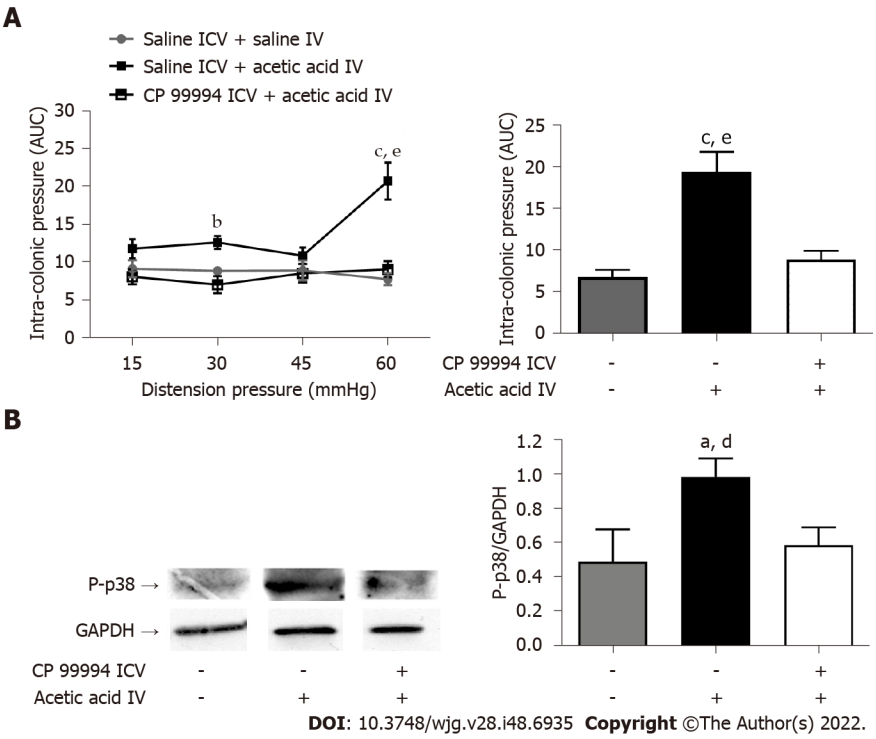
inflammatory event and is mediated by the NK1 and MAPK-p38 pathways.

The pathophysiology of colon-bladder cross-sensitization is complex and probably involves both peripheral and central mechanisms including: the sensitization of sensory nerve terminals in both organs; cross-sensitization of adjacent sensory primary afferent neurons within dorsal root ganglia (involving satellite glia cells and macrophages); axon reflexes *via* primary sensory afferent neurons with a dichotomizing axon explaining neurogenic inflammation if there is a convergence of sensory information from distinct organs to a single neuron and antidromic release of inflammatory mediators in the unaffected organ (pre-spinal convergence); the sensitization of second order spinal neurons in which there is a convergence of inputs from both colon and bladder, involving spinal interneurons (convergence-projection theory or dorsal root reflex); and supraspinal mechanisms (modified central processing of visceral stimuli in the amygdala, *etc.*)[10]. Our work adds to the current understanding that central sensitization in the spinal cord involves microglia modulation and is mediated by the NK1 and MAPK-p38 pathways.

It is already known that an increase in proinflammatory factors within the bladder tissue can sensitize the colon[22-24]. Several mechanisms have been proposed to explain this inter-organ sensitization. These mechanisms include the increased mechanical sensitivity of visceral muscle afferents, a higher proportion of chemosensitive visceral afferents[23], brainstem neurons integrating somatovisceral messages after bladder irritation[25], and central sensitization resulting from stressful life events[26].

In our study, we used a mouse model of colonic visceral hypersensitivity induced by cross-organ sensitization to demonstrate that microglia play a central role in the development of this hypersensitivity. Indeed, we showed that the ICV injection of minocycline, which is known to be an inhibitor of microglial cells, blocks the cross-sensitization process. It has already been shown that minocycline also induces a decrease in neuronal excitability by preventing phosphorylation of the ERK protein and MAPK, which are expressed in the spinal cord[27]. The analgesic effect of minocycline is mediated by its action in both microglial activation and neuronal activation. Microglial cells have already been shown to be recruited during colonic sensitization induced by chronic stress in rats[12], but our study is the first to identify the crucial role of microglia in bladder to colon cross-sensitization and in a mouse model.

We showed that the role of microglia in the development of cross-sensitization from the bladder to the colon is mediated by the tachykininergic pathway, particularly involving NK1R. This receptor activates, directly or indirectly, the MAPK-p38 protein *via* its phosphorylation. Several studies have



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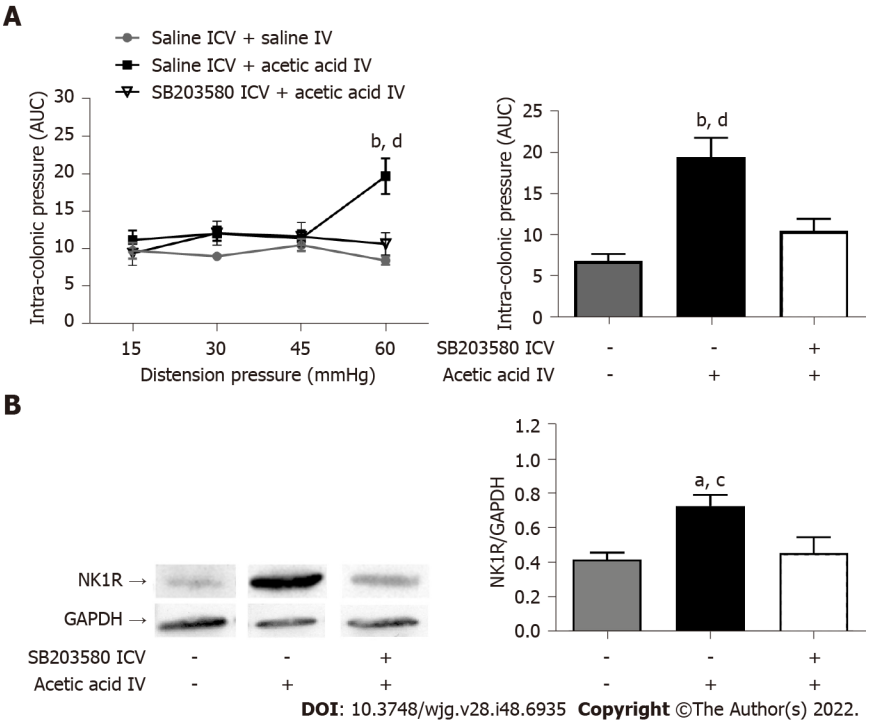
Figure 6 Effect of CP 99994 injection at the central level on visceral sensitivity and the expression of phosphorylated MAPK-p38. A: Changes in intracolonic pressure in response to isobaric colorectal distensions (15, 30, 45 and 60 mmHg) after intracerebroventricular (ICV) injections of saline (NaCl 0.9%) or CP 99994 (7.5 mg/kg) prior to intravesical (IV) injection of saline or acetic acid 0.75% in mice at day 7, with details of the comparison at 60 mmHg of distension on the right. *n* = 8 mice per group; B: Expression of phosphorylated MAPK-p38 (P-p38) at the L6-S1 level of the spinal cord, with quantitative analysis (ratio to the reference protein GAPDH) on the right. Samples were run on separate gels and compiled for the figure. *n* = 5-8 mice per group. [a]*P* < 0.05, [b]*P* < 0.01 and [c]*P* < 0.001 (compared to the saline ICV + saline IV group); [d]*P* < 0.05 and [e]*P* < 0.001 (compared to the CP 99994 ICV + acetic acid IV group). Variability in the results is represented by the standard error of the mean (area under the curve ± standard error of the mean). AUC: Area under the curve.

confirmed that MAPK-p38 is expressed only by microglial cells[21,28,29]. Its involvement in the course of chronic stress-induced colonic sensitization has already been demonstrated in rats[12]. Another study suggested that the activation of microglial MAPK-p38 protein at the ventromedial nucleus of the spinal cord (rostral ventromedial medulla) was responsible for uterocolonic cross-sensitization in an acute model[30]. In our work, we found that visceral hypersensitivity induced by chronic stress was associated with the phosphorylation of MAPK-p38 at the microglial cell level in mice and that this effect was inhibited by the ICV injection of a MAPK-p38 inhibitor (SB203580).

Bradesi *et al*[12] also showed that the fractalkine receptor potentiates the development of visceral hypersensitivity *via* a chemokine function on NK1R. The activation of MAPK-p38 is known to be associated with the increased synthesis and secretion of several neurotransmitters of inflammation, such as COX2, IL-1β, inducible nitric oxide synthase, PLA2 and PGE2[31]. The mediators of inflammation involved in our model are probably different from those involved in the trinitrobenzenesulfonic acid-induced colitis models, in which expression of IL-1β is upregulated in the spinal cord[32] since we did not demonstrate any changes at the *TNF-α*, *IL-1β* and *IL-10* mRNA level.

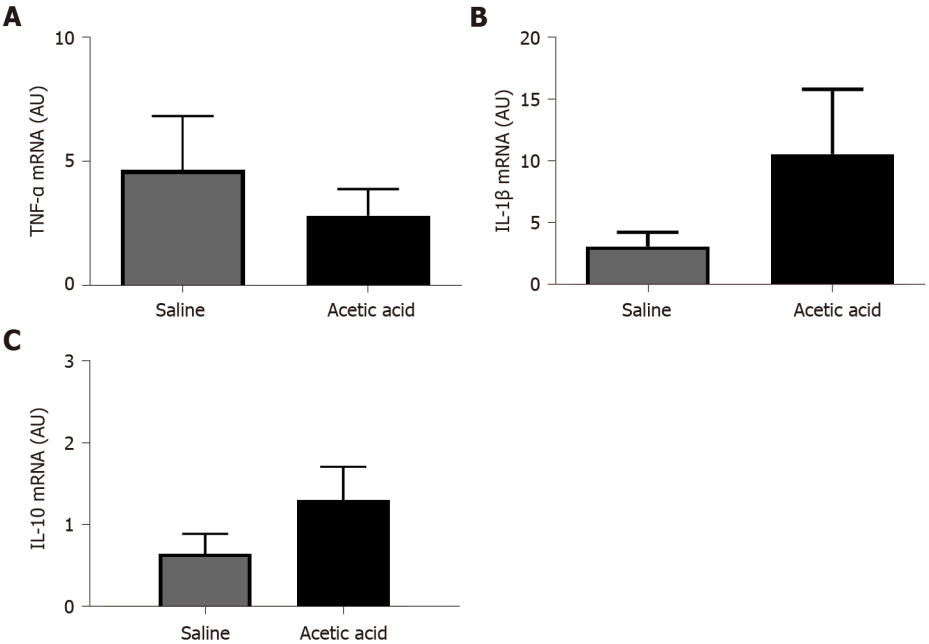
We therefore propose a mechanistic view of the molecular and cellular mechanisms underlying the development of colonic hypersensitivity by bladder-to-colon cross-sensitization in which the inflammatory reaction following irritation of the urothelium induces the activation of extrinsic primary afferent neurons, some of them co-innervating the bladder and the colon and giving rise to axon reflexes, while others innervating the colon are activated by paracrine interactions. Convergent neurons in the dorsal root ganglia and the spinal cord, and those innervating the colon secondarily sensitize the colon. Some activated extrinsic primary afferent neurons relaying to other neurons in the dorsal horn of the spinal cord specifically secrete substance P, a member of the tachykinin family, which binds to NK1R activating the MAPK-p38 by phosphorylation. This in turn could either directly or indirectly induce the synthesis of mediators involved in neuroplasticity and neuroinflammation in the spinal cord.

A mini-invasive approach that specifically targets the bladder without inducing structural lesions in neighboring areas (especially the colon and peritoneum) is a prerequisite for cross-sensitization. Indeed, abdominal surgery is known to induce stress, involving a hormonal stress response at the peripheral and central levels, and implicating the corticotropin-releasing factor pathways[33]. It can induce visceral sensitization in the absence of signs of overt inflammation in mice[16]. Similarly, we decided to use measurements of the intracolonic pressure as a surrogate marker of visceromotor responses induced by CRD to assess visceral sensitivity in our study. This technique was an alternative to the measurement of



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Figure 7 Effect of SB203580 injection at the central level on visceral sensitivity and the expression of NK1 receptors. A: Changes in intracolonic pressure in response to isobaric colorectal distensions (15, 30, 45 and 60 mmHg) after intracerebroventricular (ICV) injections of saline (NaCl 0.9%) or SB203580 (2.5 mg/kg) prior to intravesical (IV) injection of saline or acetic acid 0.75% in mice at day 7, with details of the comparison at 60 mmHg of distension on the right. *n* = 8 mice per group; B: Expression of NK1R at the L6-S1 level of the spinal cord, with quantitative analysis (ratio to the reference protein GAPDH) on the right. Samples were run on separate gels and compiled for the figure. *n* = 5-8 mice per group. [a]*P* < 0.05 and [b]*P* < 0.001 (compared to the saline ICV + saline IV group); [c]*P* < 0.05 and [d]*P* < 0.01 (compared to the SB203580 ICV + acetic acid IV group). Variability in the results is represented by the standard error of the mean (area under the curve ± standard error of the mean). AUC: Area under the curve.



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Figure 8 Expression of inflammatory cytokines in the spinal cord. A-C: Expression levels of *TNF-α* (A), *IL-1β* (B) and *IL-10* (C) mRNA in the L6-S1 level of the spinal cord on day 7 were not different between mice with intravesical injections of acetic acid 0.75% or saline (NaCl 0.9%) on day 7. *n* = 7 per group. Results were expressed as the mean ± standard error of the mean. AU: Arbitrary unit.

the electromyographic activity of the abdominal muscles[34] and had the advantage of being minimally invasive.

When we planned our study, the available animal models for the study of bladder-bowel interactions included acute[11] and chronic[35,36] colon irritation with 2,4,6-trinitrobenzenesulfonic acid in mice[36] and in rats[11] and acute bladder irritation by cyclophosphamide, an antitumoral drug known to induce hemorrhagic cystitis in mice[35] and by infusing protamine sulphate and potassium chloride in rats[11]. In those studies, colonic sensitivity was assessed on day 5 after bladder irritation in mice and on the day of the irritation in rats.

The main limitation of our model is that the chronicity of the cross-organ sensitization was considered at day 7 following bladder irritation in mice. This period may seem short in view of clinical situations that evolve over several years. However, no other inflammatory model of cross-sensitization from the bladder to the colon published in the literature exceeds 48 h[35]. Furthermore, stress-induced chronic colonic sensitization models with 11 d of homotypic stress[12] were shown to be sufficient to induce changes in colonic tenderness and spinal microglia modulation. Our 7-d period in mice therefore seems to be long enough to attest to the chronicity of the process. Further studies with a longer longitudinal assessment of visceral sensitivity are necessary to confirm our results. Our results can only be extrapolated to males because sex-dependent differences in the responses to the sensitization process[37,38] and microglia[39] have been reported. Since pelvic neuroanatomy is similar in other rodents and in humans, we could expect similar results in those species, which could explain in part the common overlap between BPS and IBS in the clinic.

CONCLUSION

In conclusion, we have developed the first model of cross-organ chronic visceral sensitization between bladder and colon in mice. Pelvic cross-sensitization involves central sensitization with microglia modulation and is mediated by the NK1 receptor pathway and MAPK-p38 activation.

ARTICLE HIGHLIGHTS

Research background

Limited mechanistic data is available in the context of overlap between bladder pain syndrome and irritable bowel syndrome. Based on the fact that pelvic organs share common sensory pathways, a few studies have offered evidence that cross-sensitization between the bladder and the colon may explain sensitization of both organs. This may involve primary extrinsic afferents or central sensitization, both at the spinal and supraspinal levels.

Research motivation

The precise pathophysiology involved in cross-sensitization of the bladder and the colon remains poorly understood.

Research objectives

The objectives of this study were to develop a model of chronic bladder-colon cross-sensitization and to investigate the mechanisms involved.

Research methods

Chronic cross-organ visceral sensitization was obtained in C57BL/6 mice using ultrasound-guided intravesical injections of acetic acid under brief isoflurane anesthesia. Colorectal sensitivity was assessed in conscious mice by measuring intracolonic pressure during isobaric colorectal distension. Three different inhibitors/antagonists were assessed to gain a better understanding of which pathway could be involved in the cross-sensitization process.

Research results

Visceral hypersensitivity to colorectal distension was observed after the intravesical injection of acetic acid. This effect started 1 h post-injection and lasted up to 7 d post-injection. Colorectal sensitization was prevented by intrathecal or intracerebroventricular injections of minocycline, a microglia inhibitor, by intracerebroventricular injection of CP-99994 dihydrochloride, a NK1 antagonist, and by intracerebroventricular injection of SB203580, a MAPK-p38 inhibitor.

Research conclusions

We described a new model of cross-organ visceral sensitization between the bladder and the colon in mice lasting up to 7 d. Intravesical injections of acetic acid induced colorectal hypersensitivity to

distension, mediated by neuroglial interactions, MAPK-p38 phosphorylation and the NK1 receptor.

Research perspectives

A bladder-colon chronic cross-sensitization mouse model using intravesical injections of acetic acid can be used as a preclinical model of overlap between bladder pain syndrome and irritable bowel syndrome.

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FOOTNOTES

Author contributions: Gourcerol G and Leroi AM designed the research study; Atmani K, Meleine Mathieu, Baron M, Bouleté I, Guérin C, Vaudry D, do Rego JC and Bahlouli W performed the research; Atmani K, Wuestenberghs F, Meleine M and Gourcerol G analyzed the data; Atmani K, Wuestenberghs F and Gourcerol G drafted the manuscript; Atmani K, Wuestenberghs F, Baron M, Bouleté I, Guérin C, Bahlouli W, Vaudry D, do Rego JC, Cornu JN, Leroi AM, Coëffier M, Meleine M and Gourcerol G critically reviewed the manuscript for important intellectual content; All authors approved the final version of the article, including the authorship list; Gourcerol G accepts official responsibility for the overall integrity of the manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Clinical features and long-term outcomes of patients with colonic oligopolyposis of unknown etiology

Dan Feldman, Linda Rodgers-Fouche, Stephanie Hicks, Daniel C Chung

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Abstract

BACKGROUND

Colonic adenomatous polyposis of unknown etiology (CPUE) is an adenomatous polyposis phenotype that resembles Familial Adenomatous Polyposis (FAP) even though no germline pathogenic variant is identified.

AIM

We sought to better characterize the clinical features and outcomes in a cohort of CPUE patients.

METHODS

This is a retrospective case series of patients 18 years old or older with adenomatous oligopolyposis (between 10-100 adenomas) and negative genetic testing, identified through the Hereditary Gastrointestinal Cancer Database at Massachusetts General Hospital, a tertiary academic referral center. A retrospective chart review was performed with a focus on demographics, alcohol and tobacco use, medication use, familial malignancy and polyp burden, genetic testing information, endoscopic surveillance data including the corresponding histopathology, colonic and extracolonic malignancies, mortality events, and their etiology. Spearman correlation and Pearson Chi-square test (or Fisher's exact test) were used for continuous and categorical variables respectively.

RESULTS

CPUE patients were primarily male (69%) and presented for genetic counseling at

63.7 years. Only 2 patients (2.9%) reported a first-degree relative with polyposis. During an average surveillance period of 12.3 years, 0.5 colonoscopies *per* year were performed. Patients developed 2.3 new adenomas *per* year. 4 (5.7%) were diagnosed with colorectal cancer (CRC) at a mean age of 66 years, and 3 were diagnosed prior to the onset of oligopolyposis. 7 (10%) required colectomy due to advanced dysplasia or polyp burden. With respect to upper gastrointestinal manifestations, 1 patient had a gastric adenoma, but there were no cases of gastric or small bowel polyposis. During surveillance, 10 (14%) patients died at a mean age of 72, and none were due to CRC.

CONCLUSION

CPUE is distinct from familial adenomatous polyposis (FAP) syndrome and the use of FAP surveillance guidelines may result in unnecessarily frequent upper and lower endoscopies.

Key Words: Colonic polyposis of unknown etiology; Multigene cancer panel; Colorectal cancer; Colectomy; Surveillance; Mortality

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Core Tip: Colonic adenomatous polyposis of unknown etiology (CPUE) resembles familial adenomatous polyposis (FAP) syndrome, but no genetic alterations are identified. The optimal management of CPUE is uncertain. Patients with CPUE are typically older males that exhibit a low rate of new adenoma formation without upper gastrointestinal polyposis during long-term surveillance. 10% required colectomy for polyposis, and none died from colon cancer. The clinical behavior of CPUE is distinct from FAP, and the current application of FAP surveillance guidelines for CPUE may result in unnecessarily frequent upper and lower endoscopies.

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INTRODUCTION

Colorectal cancer (CRC) is the 4th most frequently diagnosed cancer and the second leading cause of cancer death in the United States[1,2]. Most CRCs are considered sporadic and the lifetime cumulative risk for CRC in the general risk population is estimated as 5%[3]. Approximately 5% of CRCs are attributable to a hereditary CRC syndrome. These are broadly classified into polyposis and nonpolyposis syndromes.

Current clinical guidelines suggest a minimal set of genes that should be tested in all patients suspected of hereditary CRC or polyposis, preferably by using a multigene panel because of overlapping clinical phenotypes, inconsistent definitions for oligopolyposis, challenges with accurately classifying polyp histology, and variable modes of inheritance[4,5]. Adenomatous polyposis syndromes are the most common polyposis syndromes and typically result from a germline mutation in *APC* or biallelic variants in *MUTYH*. Rarely, adenomatous polyposis that may be phenotypically indistinguishable from *APC/MUTYH*-related polyposis, can be observed secondary to germline mutations in *AXIN2, GREM1, NTHL1, POLE, POLD1*, or *MSH3*[5-7]. Germline mutations in one of these genes are not always identified, and the term "colonic adenomatous polyposis of unknown etiology (CPUE)" has been coined to describe cases of adenomatous polyposis in which no pathogenic variant is found in a polyposis gene. This occurs in as many as 30-50% of all polyposis cases[8,9]. *APC* promoter 1B mutations[10] and somatic mosaicism for *APC*[11] are possible mechanisms, but this is likely to account for only a small fraction of these cases. Recent data also suggested that missed germline mutations can be potentially revealed by retesting[12].

CPUE appears to be more common in those with lower polyp numbers. 48% of cases of polyposis with over 100 adenomas were explained by a germline mutation, most commonly in *APC* or *MUTYH*. However, only 13.6% of individuals with 20-99 adenomas and 6.4% of those with 10-19 adenomas exhibited a germline mutation in a polyposis gene. Thus, a diagnosis of CPUE is more common in individuals who exhibit oligopolyposis (10-99 adenomas)[13].

The clinical features of individuals with adenomatous oligopolyposis of unknown etiology are not well-defined, and management recommendations are largely extrapolated from guidelines for familial

adenomatous polyposis (FAP) syndrome[14]. Previous reports of CPUE are mostly small and heterogeneous. While some have described a benign course[6], others found duodenal adenomas and fundic gastric polyposis in addition to an approximately 30% risk of extracolonic malignancies including skin cancer, leukemia, breast, bladder, and prostate cancer[9,15,16]. We sought to better describe the clinical characteristics and outcomes in a large cohort of CPUE patients.

MATERIALS AND METHODS

Study population

The study population of CPUE patients was identified through the Hereditary Gastrointestinal Cancer Database at Massachusetts General Hospital. Patients 18 years old or older who were documented to have at least 10 cumulative adenomas and less than 100 adenomas and completed sequencing of at least *APC* and the 2 common mutations (*Y179C* and *G396D*) of *MUTYH* without evidence of a pathogenic germline mutation were included.

Data collection

Patient charts were reviewed utilizing the EPIC Electronic Health Record from the first available endoscopic surveillance documentation through November, 2021. Data were retrospectively collected including demographics (age, gender, race maternal and paternal ethnicities), self-reported tobacco and alcohol use, metabolic comorbidities (diabetes mellitus, obesity, metabolic syndrome), and medication usage (as documented at the time of genetic counseling) associated with possible chemoprevention effect (Statins, Aspirin, and Glucophage). Genetic data and relevant family history were collected and included the date and age at the time of genetic consultation, results of genetic testing, and family history of polyps and malignancy up to 3rd degree relatives.

Data from colonoscopies, sigmoidoscopies, esophagogastroduodenoscopies, and video capsule endoscopies (VCE) including indication, quality of bowel preparation, polyps, and other significant findings were recorded. Histopathology reports were reviewed. Colonic and extra-colonic malignancies, mortality, and causes of death were also documented.

A Research Electronic Data Capture platform, a secure, password-protected database, was utilized to store data, and these data were later exported as Excel sheath files (saved on encrypted drives) for analysis.

Data analysis

Categorical variables were described as frequencies and percentages. Pearson Chi-square test (or Fisher's exact test if > 20% of cells had expected count < 5) were used to test correlations of dichotomous and categorical variables. Continuous variables were described as a mean ± standard deviation (SD), median, and range. Student's *t*-test and non-parametric Mann-Whitney *U* test were used to compare means or mean ranks across scale variables of two independent samples. A univariate logistic regression analysis was performed to assess the impact of a set of predictors on extra colonic malignancies (dependent variable). Limited by the low number of cases observed we did not have the power to estimate their confounding effect using multiple regression. All statistical tests were two-sided and $P < 0.05$ was considered as statistically significant. SPSS software [IBM SPSS Statistics for Windows, ver. 28.0.1.0(142)] was utilized for statistical analysis. A statistical review of the study was performed by a biomedical statistician.

This study was approved by the institutional review board and was carried out in accordance with the ethical principles described in the Helsinki Declaration.

RESULTS

Patient demographics

70 patients met the inclusion criteria and comprised our cohort of CPUE patients with oligopolyposis. The last clinical surveillance was documented at a mean age of 69.3 (range 30.6 - 85.5. median 70.6). 48 (69%) patients were male and 62 (89%) were Caucasian, predominantly represented by Irish and English ancestry. 29 patients (41.4%) were diagnosed with any metabolic comorbidity. 34 patients (49%) reported any history of alcohol usage. 7 patients (21.2%) were documented to consume more than 1 alcoholic beverage *per* day. 14 of these patients (42.4%) consumed between 1-7 drinks *per* week. 41 patients (58.6%) reported any smoking history. Data concerning current use of tobacco could be retrieved in 39 patients, and 15 of these patients (38.4%) were reported as active smokers. 17/41 (41.4%) patients who smoked had reported a mean of 30.1 packs/year (range 0.5 - 100 packs/year, median 25) (Table 1).

Table 1 Colonic adenomatous polyposis of unknown etiology cohort characteristics, *n* (%)

Cohort characteristics	Number (% of cohort)
Male	48 (69%)
Ethnicity	
White non-Hispanic	62 (88.5%)
Hispanic or Latino	5 (7.1%)
Black or African American	2 (2.9%)
Asian	1 (1.4%)
Paternal lineage	
Irish	25 (24%)
English	14 (13%)
French	12 (11%)
Scottish	11 (10%)
Italian	9 (9%)
Maternal lineage	
Irish	20 (23%)
English	14 (16%)
Italian	14 (16%)
Canadian	6 (7%)
Scottish	6 (7%)
German	5 (6%)
Any metabolic comorbidity reported (Obesity/diabetes mellitus)	29 (41.4%)
Any alcohol usage	34 (49%)
1-7 drinks/week	14 (20%)
More than 1 drink/day	7 (10%).
Any smoking history	41 (58.5%)
Active smokers	15 (21.4)
Mean pack years	17 (30.1)

Data include demographic and clinical features for all 70 patients in the cohort. Leading five paternal and maternal lineages are presented (Full data in supplementary table). Presence of metabolic comorbidities were combined. Data concerning alcohol consumption and smoking were available for 69 patients. Detailed data concerning smoking burden (Pack/years) were available for 17 patients.

Genetic test results

All 70 CPUE patients had genetic counseling and testing at a mean age of 63.7 years (range 27 - 83, median 65.5). Each patient had documentation of at least 10 adenomas as an indication for counseling, and most presented with a cumulative polyp burden of 10-20 polyps (36 patients; 51.4%), followed by 21-30 polyps (18 patients; 25.7%), 31-50 polyps (12 patients; 17.1%) and 4 patients (5.7%) with 51-100 polyps.

All patients had sequencing of the *APC* (full) and *MUTYH* genes (24% had sequencing of the 2 common mutations *Y179C* and *G396D*, 76% had full sequencing). 26 patients (37%) had sequencing of only these 2 genes, 5 patients (7%) had 4-7 additional genes analyzed (including *BRCA1, BRCA2*, *MLH1, MSH2, MSH6, PMS2,* and *EPCAM*), and the majority (39 patients, 56%) had multi-gene panels with a mean of 35.2 (3.6) genes tested (range 12 - 91; Median 28). The CRC-related genes that were sequenced are described in Table 2 (full list in Supplementary Table 1).

None of the patients carried a pathogenic *APC* or *MUTYH* mutation, and no other polyposis-related gene mutations were identified when tested as part of a multi-gene panel. 2 patients were identified as carriers of a non-polyposis associated pathogenic variant [*APC* I1307K and *CFTR* (TG)11-5T]. 5 patients (7.1%) carried a *VUS* (in *RAD 50*, *ATM*, *BARD1* or *APC*).

Table 2 Genes sequenced in the colonic adenomatous polyposis of unknown etiology cohort

Gene name	Number of patients tested	Percentage (%)
APC	70	100
MUTYH (full seq.)	53	75.7
MLH1	43	61.4
MSH2	43	61.4
MSH6	42	60.0
PMS2	42	60.0
EPCAM	41	58.6
CHEK2	39	55.7
TP53	39	55.7
BMPR1A	38	54.3
CDH1	38	54.3
PTEN	38	54.3
SMAD4	38	54.3
STK11	38	54.3
GREM1	36	51.4
POLD1	36	51.4
POLE	36	51.4
ATM	33	47.1
AXIN2	28	40.0
MUTYH (*Y179C* and *G396D* mutations only)	17	24.3
NTHL1	15	21.4
MSH3	13	18.6
BLM	7	10.0
GALNT12	7	10.0
RPS20	4	5.7
MLH3	3	4.3
RNF43	1	1.4

Colorectal cancer related genes sequenced in the colonic adenomatous polyposis of unknown etiology cohort. All had at least *APC* and *MUYTH* sequenced. Full list of genes tested in Supplementary Table 1.

Family history of cancer and polyps

At the time of initial genetic consultation, 54 patients (77.1%) reported a family history of any malignancy in a first-degree relative (FDR). There was a total of 113 cases of malignancy in FDRs in our cohort. Of these, the most prevalent were CRC (25 cases, 22.1%), breast (20 cases, 17.7%), and prostate (14 cases, 12.4%) cancer (Table 3). 14 patients (20%) had at least 1 FDR with CRC and 6 patients (8.6%) had at least 1 FDR and 1 second-degree relative (SDR) with CRC. The mean age of CRC diagnosis in a FDR was 66 (range 44 - 97, median 66). Most of these patients (30) had only 1 FDR (55.6%) with any cancer. With respect to SDRs, 94 cases of any malignancy were found in our cohort, mostly represented by 17 cases of CRC (18%) and 11 cases (11%) of breast cancer.

22 patients (31.4%) were reported to have a FDR with any colon polyp, and most of these patients were reported to have 1 or 2 FDR (36% and 41%, respectively) with any colonic polyp. Only 2 patients (2.9%) were reported to have a family history of multiple polyps in a FDR. In one patient, the father required a partial colectomy for multiple polyps.

Polyp and adenoma burden during colonoscopy surveillance

Among the 70 patients, 430 colonoscopy reports were reviewed, resulting in a calculated mean of 6.1 ±

Table 3 Malignancies in first-degree relatives of patients with colonic adenomatous polyposis of unknown etiology

Type of cancer in FDR	Number of cases (Total = 113)	Percentage (%)
CRC	25	22.1
Breast	20	17.6
Prostate	14	12.3
Non-melanoma skin cancer	10	8.8
Lung	8	7.0
Gastric	4	3.5
Bladder/Ureter	4	3.5
Brain	4	3.5
Renal	3	2.6
Melanoma	3	2.6
Cervical	3	2.6
Pancreas	2	1.7
Unknown type	2	1.7
Leukemia	2	1.7
Ovarian	2	1.7
Thyroid	2	1.7
Lymphoma	1	0.8
Liver	1	0.8
Malignant meningioma	1	0.8
Esophageal	1	0.8
Liposarcoma	1	0.8

54 patients reported a family history of cancer. Highest frequencies were noted for CRC (22.1%), Breast (17.6%) and Prostate (12.3%). 10 patients (18.5%) had 2 FDRs, 8 patients (14.8%) had 3 FDRs, 1 patient (1.9%) had 4 FDRs, 4 patients (7.4%) had 5 FDRs and 1 patient (1.9%) had 7 FDRs with any malignancy. FDR: First-degree relative; CRC: Colorectal cancer.

3.4 colonoscopies (range 1 - 15; median 6) performed per patient. The first documented colonoscopy was in March 1992 and the last one was documented in November 2021. Among the 63 patients who had more than one colonoscopy performed over more than one year of surveillance, 418 colonoscopies were documented, with a calculated mean of 6.6 ± 3.2 colonoscopies (range 2 - 16, median 6) *per* patient, during a mean surveillance period of 12.3 ± 6.2 years (range 1.3 - 24.8, median 11.8 years). The calculated average frequency of colonoscopy surveillance among this group was 0.5 colonoscopies *per* year.

For these 63 patients, there was a total of 2547 documented polyps in 408 colonoscopies and 1826 documented adenomas in 394 colonoscopies, with a mean total cumulative burden of 39 ± 24.7 polyps (range 10 - 111; median 29) and 29.0 ± 18.9 adenomas (range 10 - 102; median 24). Over the entire surveillance period, this translates to a mean of 3.2 polyps diagnosed per year and 2.3 adenomas *per* year.

With respect to the distribution of polyps and adenomas in the colon, the right colon was the most prevalent location (75% and 54%, respectively), followed next by the transverse colon (49.7% and 38.8%, respectively). For polyps in general, the next most prevalent locations were the sigmoid colon (41.6%), left colon (39.0%), and rectum (26.6%), while for adenomatous polyps the locations and prevalence were left colon (31.1%), sigmoid colon (22.4%) and the rectum (10.5%).

The most prevalent adenoma histology found was tubular adenoma with low-grade dysplasia (90%). High-grade dysplasia in a tubular adenoma or tubulovillous adenoma was seen in 11 exams (3.2%) (Supplementary Table 2). In 142 of the 428 colonoscopies (33%), at least one non-adenomatous polyp was reported. Except for one colonoscopy in which only an inflammatory polyp was described, serrated polyps were reported in 141 of these 142 colonoscopies (99%), and these included hyperplastic polyps (74%), sessile serrated polyps/adenomas (24%) and traditional serrated adenoma (1.3%). Most of the serrated polyps were located in the sigmoid colon (33.3%) followed by the right colon (22.5%), rectum (20%), transverse colon (13.3%), and left colon (10.7%). No hamartomas were reported.

Incidence of invasive CRC

Four patients were diagnosed with invasive CRC (5.7%; mean age 66). Three underwent colectomy and one had a malignant polyp that was resected endoscopically. Among these four cases, three patients were diagnosed with CRC prior to the development of oligopolyposis. The first was a male diagnosed with rectal carcinoma at the age of 63 along with 4 adenomas; this patient later developed 23 more adenomas over 17 years of surveillance. The second patient was a male diagnosed at the age of 70 with a malignant polyp (T1N0M0, well-differentiated adenocarcinoma) in the sigmoid colon that was completely resected at colonoscopy. He presented with a polyp burden of only 6 adenomas over a 15-year period prior to the diagnosis of CRC. The third patient was a female diagnosed with a sigmoid colon CRC at the age of 71 at her first colonoscopy with a polyp burden at that time of 5 adenomas. This patient later developed 33 adenomas over 4.5 years of surveillance. The fourth patient was a female diagnosed with transverse colon CRC at the age of 69, one year after her first colonoscopy with a cumulative burden of approximately 30 adenomas. Immunohistochemical stains for DNA mismatch repair proteins were available for two of these patients, and both demonstrated preserved expression of all proteins (hMLH1, hMSH2, hMSH6, and hPMS2).

Rates of colectomy for high polyp burden or advanced dysplasia

Four additional patients (5.7%, mean age 64) underwent colectomy due to a high polyp burden without cancer. Two had a subtotal colectomy for multiple tubular and tubulovillous adenomas, some of which were large and unresectable endoscopically. Another had a subtotal colectomy due to a cumulative burden of approximately 50 adenomas as well as recurrent diverticulitis. One had a total proctocolectomy due to a cumulative burden of more than 80 adenomas in addition to adenomatous polyps with high-grade dysplasia. These patient characteristics are summarized in Table 4. Three patients (4.3%, mean age 52) were diagnosed with intramucosal carcinoma during colonoscopy, and all underwent colectomy.

When comparing these 11 patients who had a significant clinical outcome (intramucosal cancer, invasive cancer, or risk-reducing colectomy for polyposis) to the rest of the cohort (59 patients), no difference was found in any clinical parameters including gender, tobacco use, metabolic comorbidities, familial malignancy burden, personal malignancy burden or duration of colonoscopy surveillance (Supplementary Table 3).

Extracolonic findings

With respect to upper gastrointestinal findings, 39 patients (55.7%) had at least 1 upper endoscopy performed (first exam at mean age 62.3, range 22 – 83, median 65). 10 patients (14%) were found to have any gastric polyp. In 11 gastroscopies, there were up to 5 polyps documented and none was above 1 cm. Among cases in which histologic sampling was performed, the most common histology was fundic gland polyp without dysplasia (72%) followed by hyperplastic polyp (22%), and there was one case of 1 gastric adenoma that also exhibited high-grade dysplasia (6%). This patient also had low-grade dysplasia that arose in a background of chronic gastritis and intestinal metaplasia secondary to H. pylori.

No duodenal adenomas were detected. 4 patients (5.7%) had a formal small bowel evaluation with VCE, and no small bowel polyps were identified.

A total of 49 extra colonic malignancies (ECM) were documented in 35 patients (50%), and 9 patients (20%) had more than 1 ECM. The mean age of first ECM diagnosis was 60 (range 23- 82, median 62) with non-melanoma skin cancer (51%) and prostate cancer (12%) as the most common. (Table 5). Gender, age, and cumulative adenoma burden were evaluated by univariate logistic regression analysis for their potential contribution to the development of an ECM. Age was found to have a correlation with breast cancer and melanoma occurrence with an odds ratio of 0.8 (P = 0.01) and 0.9 (P =0.01). Otherwise, cumulative adenoma burden was not found to be a predictor. (Supplementary Table 4). No correlation was found between the cumulative adenoma burden and the total number of extra colonic malignancies reported (P = 0.18).

Mortality

10 patients (14%) died during follow-up. The mean age of death was 72 (range 61 - 78, median 73.5). 5 patients (50%) died of malignancy, but none was from CRC. The mean age of death from cancer was 74.4 (range 71 - 77, median 75) and 5 patients (50%) died from non-malignancy causes at a mean age of 69.6 (range 61 - 78, median 69). None of these causes were directly related to the underlying polyposis (*i.e.*, complications from colonoscopy or colectomy) (Table 6).

DISCUSSION

CPUE is a colonic adenomatous polyposis syndrome in which no germline mutation is detected. Our relatively large CPUE cohort is comprised primarily of older white males without a family history of polyposis but a modest family history of colon cancer and personal history of tobacco use. The adenoma

Table 4 Clinical features of 11 patients with a significant clinical outcome (cancer, advanced dysplasia, or colectomy)

ID (Gender)	Colectomy (age) (yr)	Colectomy – indication	Surveillance (No. yr to colectomy; Total yr)	Total adenoma burden	No. FDR with CRC	No. SDR with CRC
26 (F)	RHC (62)	IMC	12; 16	Multiple (at least 34)	0	0
29(M)	SIG (52)	IMC	0; 12	Multiple at least 15	0	0
76(F)	IPAA (42)	IMC	0; 14	Multiple (at least 15)	0	0
32(M)	IRA (64)	Polyp burden	0; 1	Multiple (>30, many > 1 cm)	0	0
46(M)	IRA (65)	Polyp burden + recurrent diverticulitis	15; 24	Multiple (at least 47)	0	0
72(M)	IRA (69)	Polyp burden	12; No data post colectomy	Multiple (at least 31)	0	0
62(F)	IPAA (58)	Polyp burden	3; 8.5	Multiple (approx. 83)	0	1 (65)
37(M)	APR (63)	Rectal CRC a	0; 17	27	1 (66)	0
56(F)	SIG (71)	Sigmoid CRC[1]	0; 4.5	38	1 (68)	0
68(F)	Colectom[3](69)	Transverse CRC[2]	1; No data post colectomy	Multiple (at least 28)	1 (70)	0
82(M)	None(70)	SigmoidMP[4]	15; 16	16	0	0

[1]CRC diagnosed at 1st colonoscopy.
[2]CRC diagnosed at 3rd colonoscopy.
[3]No data about the type of colectomy.
[4]Resected endoscopically.
Adenoma burden, surgical data and familial burden of CRC (1st and 2nd degree relatives) of 11 patients who had a significant clinical outcome. CRC: Colorectal cancer; F: Female; M: Male; MP: Malignant polyp; IMC: Intramucosal carcinoma; RHC: Right hemicolectomy; IRA: Subtotal colectomy with ileorectal anastomosis; IPAA: total proctocolectomy with ileal pouch anal anastomosis; APR: abdominoperineal resection; SIG: sigmoidectomy; FDR: First-degree relative.

Table 5 Extra colonic malignancies in the colonic adenomatous polyposis of unknown etiology cohort

Type of malignancy	Number	%	Incidence rate (*Per* 1000 person-years)
Non-melanoma skin cancer	25	51	5.1
Prostate	6	12.2	1.2
Melanoma	5	10.2	1
Breast	4	8.2	0.8
Lung	2	4.1	0.4
Uterine	1	2	0.2
Non-Hodgkin's Lymphoma	1	2	0.2
Gallbladder	1	2	0.2
Ovary	1	2	0.2
Bladder	1	2	0.2
Pancreas	1	2	0.2
Merkel cell tumor	1	2	0.2

35 patients reported an extra-colonic malignancy. Highest frequencies were noted for Non-melanoma skin cancer (51%), Prostate (12.2%) and Melanoma (10.2%)

burden is modest and is characterized by a relatively low rate of adenoma growth (average of 2.3 adenomas *per* year). However, 15.7% were considered to have a significant outcome, which included colectomy due to polyp burden, advanced polyp histology of intramucosal carcinoma, or a diagnosis of CRC. There were no deaths related to CRC or polyposis.

Table 6 Causes of death in the colonic adenomatous polyposis of unknown etiology cohort

Malignancy causes (n = 5)	Age of death (yr)
Lung	71
Unknown (suspected) malignancy	72
Metastatic Merkle cell carcinoma	75
Gallbladder	77
Pancreas	77
Non- malignancy causes (n = 5)	
Pulmonary failure.	61
Ruptured aortic aneurysm	78
Unknown	65
Shock and multi-system organ failure.	69
Cardiac arrest	75

There were 10 deaths among CPUE patients. 5 (50%) were secondary to malignancy. CRC was not reported as a cause of death. CPUE: Colonic adenomatous polyposis of unknown etiology cohort; CRC: Colorectal cancer.

Interestingly, three patients had a CRC diagnosis before developing at least ten cumulative adenomas, However, all did exhibit colonic adenomas either prior to or at the time of CRC diagnosis, demonstrating that a predisposition to polyp formation was present at the same time. These findings reveal the heterogeneity of disease presentation associated with CPUE.

Although CPUE is often considered an attenuated variant of FAP, our findings in a large CPUE cohort over an extended period of surveillance (12.3 +/-6.2 years) suggest that CPUE is quite dissimilar from FAP given the gender distribution, age of onset, absence of family history, low rate of colon adenoma growth, and absence of upper gastrointestinal (GI) and other extra-colonic manifestations. Others have described CPUE cohorts to have higher rates of colon polyp formation, family history of polyposis and CRC, and upper GI polyposis. For example, CRC was observed in 19.3% of a different CPUE cohort. However, more comprehensive multi-gene panel testing was not performed. A low rate of upper GI findings in CPUE was also observed[17], consistent with our findings. The relatively high rates of metabolic co-morbidities as well as alcohol and tobacco use in our cohort suggest that there may be significant environmental and lifestyle contributors in patients with CPUE.

Because data are limited with respect to clinical features and outcomes in CPUE, it has been difficult to formulate definitive management recommendations. The National Comprehensive Cancer Network describes CPUE as a potential attenuated subgroup of familial adenomatous polyposis with possible FAP-related extra colonic manifestations. In addition to recommending short colonoscopy intervals (every 1-2 years), consideration is given to the evaluation of the upper GI tract, with specific attention to the duodenum and the ampullary area[5]. Our findings suggest that most with CPUE do not exhibit features suggestive of a FAP-related syndrome. Annual colonoscopy and routine upper GI surveillance may therefore not be required.

Our study has some limitations. Due to its retrospective nature, complete endoscopic and pathology data in some patients could not be retrieved, and not all endoscopy reports reliably quantified polyp burden. Thus, our results might reflect an underestimation of the cumulative polyp burden. In addition, approximately 40% had only *APC* and *MUTYH* genes sequenced, so alternative genetic etiologies for polyposis may not have been recognized. However, the frequency with which mutations in these other novel intermediate-risk genes are identified is very low[13,18], and it is unlikely that a significant number of these cases would be explained by one of these mutations. Finally, our cohort was comprised mostly of Caucasian men of Irish and English descent and may not be representative of the broader CPUE population. This may result in a selection bias that could be attributable to lower rates of referrals for genetic counseling and testing in non-white populations[19-20].

CONCLUSION

Most individuals with CPUE in our cohort exhibited a relatively benign course, characterized by a generally modest colonic adenoma burden, dominance of non-advanced histology, low rates of CRC during surveillance, negligible upper GI involvement, and low rates of mortality due to polyposis or CRC. We suggest that colonoscopy surveillance intervals could be extended, and that routine upper GI

screening may not be required.

ARTICLE HIGHLIGHTS

Research background

Colonic polyposis syndromes typically result from germline mutations in the *APC* or *MUTYH* genes and less commonly from other low/intermediate-risk genes. When no pathogenic variant is identified, a diagnosis of colonic polyposis of unknown etiology (CPUE) is made.

Research motivation

The existing literature on CPUE is limited, and the precise clinical features and long-term outcomes are not well-defined.

Research objectives

To characterize the natural history of CPUE by defining the malignancy risk, long-term colonic adenoma burden, and risk of extra-colonic tumors over an extended period of surveillance.

Research methods

We performed a retrospective detailed chart review of demographic, lifestyle habits, endoscopic, genetic, and clinical data of patients aged 18 years old or older meeting the criteria for CPUE in the Hereditary Gastrointestinal Cancer Database at Massachusetts General Hospital.

Research results

70 patients met the inclusion criteria and were predominantly Caucasian males. During an extended surveillance period, a very low cumulative colonic adenoma burden was observed, with no evidence for duodenal adenomas. 4 patients were diagnosed with colorectal cancer (CRC), but none had extra-colonic malignancies that are typically seen in familial adenomatous polyposis (FAP) syndrome (*i.e.*, gastric, duodenal, or thyroid cancer). There was no mortality attributable to CRC.

Research conclusions

Individuals with CPUE exhibited a relatively mild course with respect to polyp burden and cancer risk, which differs significantly from the FAP syndrome. The modest colonic burden implies colonoscopy surveillance intervals could be extended, and regular gastroscopic exams may not be necessary.

Research perspectives

CPUE is an underdiagnosed and heterogeneous clinical entity. The current findings should be validated in large-scale multi-center prospective studies, with greater representation of non-Caucasian populations in order to better define this unique condition in an evidence-based approach.

FOOTNOTES

Author contributions: Feldman D contributed to conceptualization and design, formal analysis and interpretation, investigation, resources and acquisition of data, methodology, visualization, writing, revising, and editing the draft critically for important intellectual content; Rodgers-Fouche L contributed to conceptualization, resources and acquisition of data, writing, revising, and editing the draft critically for important intellectual content; Hicks S contributed to conceptualization, resources and acquisition of data, writing, revising, and editing the draft critically for important intellectual content; Chung DC contributed to conceptualization and design, formal analysis and interpretation, investigation, methodology, resources and acquisition of data, supervision, visualization, writing - original draft, writing, revising, and editing the draft critically for important intellectual content; All authors have read and approve the final manuscript.

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