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REVIEW

Update on the management of sigmoid diverticulitis

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Abstract

Diverticular disease and diverticulitis are the most common non-cancerous pathology of the colon. It has traditionally been considered a disease of the elderly and associated with cultural and dietary habits. There has been a growing evolution in our understanding and the treatment guidelines for this disease. To provide an updated review of the epidemiology, pathogenesis, classification and highlight changes in the medical and surgical management of diverticulitis. Diverticulitis is increasingly being seen in young patients (< 50 years). Genetic contributions to diverticulitis may be larger than previously thought. Potential similarities and overlap with inflammatory bowel disease and irritable bowel syndrome exist. Computed tomography imaging represents the standard to classify the severity of diverticulitis. Modifications to the traditional Hinchey classification might serve to better delineate mild and intermediate forms as well as better classify chronic presentations of diverticulitis. Non-operative management is primarily based on antibiotics and supportive measures, but antibiotics may be omitted in mild cases. Interval colonoscopy remains advisable after an acute attack, particularly after a complicated form. Acute surgery is needed for the most severe as well as refractory cases, whereas elective resections are individualized and should be considered for chronic, smoldering, or recurrent forms and respective complications (stricture, fistula, etc.) and for patients with factors highly predictive of recurrent attacks. Diverticulitis is no longer a disease of the elderly. Our evolving understanding of diverticulitis as a clinical entity has led into a more nuanced approach in both the medical and surgical management of this common disease. Non-surgical management remains the appropriate treatment for greater than 70% of patients. In individuals with non-relenting, persistent, or recurrent symptoms and those with complicated disease and sequelae, a segmental colectomy remains the most effective surgical treatment in the acute, chronic, or elective-prophylactic setting.

Key Words: Diverticulitis; Epidemiology; Antibiotics; Surgical resection; Laparoscopic lavage; Classification

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Core Tip: Diverticular disease and diverticulitis–specifically sigmoid diverticulitis-represent the most common non-cancerous pathology of the colon. It has traditionally been considered a disease of the elderly and associated with cultural and dietary habits. There is a constant evolution in our understanding and the treatment guidelines. This review provides an update of the epidemiology, pathogenesis, and classification, and it highlights changes in the medical and surgical management of diverticulitis.

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INTRODUCTION

Sigmoid diverticulitis is one of the leading gastrointestinal pathologies responsible for inpatient hospitalization in Western society[1,2]. With over 300000 hospital admissions resulting in 1.5 million days of inpatient care, diverticulitis places significant economic burden on the healthcare system at 2.4 billion dollars annually[3-5]. These numbers are expected to increase substantially with the increasing prevalence of this disease as the population ages and with a higher incidence in younger individuals. Concepts and management recommendations are in constant evolution. The aim of this article is to provide an update on epidemiology, pathogenesis, medical and surgical management of sigmoid diverticulitis.

EPIDEMIOLOGY AND RISK FACTORS

It has been historically reported that 10%-25% of patients with diverticulosis will eventually develop diverticulitis. This claim largely stems from a benchmark review of the natural history of diverticular disease by Parks[6]. In a 1947 review of 47000 radiological images of the colon, the prevalence of diverticulosis was 8.5% amongst the entire cohort, 15% of which were diagnosed with diverticulitis[7]. In 1958, a double-contrast barium enema study in 300 patients with diverticulosis estimated the risk of developing diverticulitis at 10% over a 1-5 year period and up to 25% after a 6-10 years follow up period[8].

More recent data suggest that the traditionally quoted lifetime risk of developing diverticulitis from diverticulosis is exaggerated[9-11]. In a study on veterans with diverticulosis with an 11 year follow up, there was a 1% risk of diverticulitis as confirmed by computed tomography (CT) or at time of surgery[9]. In a cohort of 2100 patients, there was a 4.3% risk of diverticulitis over a median follow up period of 7 years[12].

On the other hand, the overall incidence of diverticulitis has been trending upwards in recent years[13,14]. Furthermore, a substantial increase was observed in the prevalence of diverticulitis in younger patients[15]. When confining analysis to patients in their 40s, there was an 11% risk of diverticulitis–a 2.5 times higher risk than previously reported for the entire cohort of patients[12]. This is in stark contrast to older reports of 1%-2% incidence of diverticulitis in young patients[16,17]. In an analysis of the Nationwide Inpatient Sample of 267000 admissions for diverticulitis, the incidence of diverticulitis in patients between 18-44 years old nearly doubled over a 7 year period; of note, over the same time period, there was no change in patients older than 75 years of age and only a moderate increase in the age group in between[13]. Similarly, smaller single institution cohorts have also reported higher incidence rates of diverticulitis in young patients ranging from 20% to 26% in some studies[18,19].

Recent reports also suggest potential gender differences among patients with diverticulitis. Nationwide and single institution reviews have noted a male predominance in young patients as opposed to a female predominance in older patients with diverticulitis[14,18,20,21]. Lahat *et al*[21] demonstrated a male:female ratio of 3:1

in patients with diverticulitis under the age of 45, compared to a 1:2 ratio in patients over 45[21]. Similarly in patients admitted for diverticulitis, Schauer *et al*[18] noted a 2:1 male:female ratio in patients under 40 and a 1:1.5 ratio in older patients[18]. In reviewing hospital discharge data for diverticulitis in England from 1989 to 2000, the likelihood to be hospitalized under 50 years of age was higher for males, and over the age of 50 higher for females[20]. It should be noted that none of the authors offered a plausible explanation for these gender-related observations.

A diet low in fiber has long been regarded as the predominant exogenous or environmental factor leading to the development of diverticular disease. This hypothesis was initially put forth by Painter and Burkitt based on their observation of the extremely low rates of diverticular disease in rural Africa compared to developed Western countries[22,23]. Colonic transit studies by Burkitt demonstrated increased colonic transit times (80 h *vs* 34 h) and lower stool weights (110 g/d *vs* 450 g/d) in English individuals consuming a low fiber diet compared to individuals living in rural Uganda respectively[24]. It was speculated that the increased transit time would lead to elevated intraluminal pressure particularly on the left side of the colon, thereby promoting the development of diverticular disease. As countries become more developed and Westernized, an increase in the incidence of diverticulitis was commonly observed[25,26]. Analyzing a prospective cohort of 47888 United States men over a 4 year period, an inverse association between fiber intake and the development of diverticular disease (relative risks risk ratio 0.58, CI: 0.41-0.83, P = 0.01) was found[27]. This finding was corroborated in a study looking at 47033 men and women in the United Kingdom, 15459 (33%) of whom self-declared as vegetarians[28]. This subgroup as a whole was noted to have a 30% risk reduction of developing diverticular disease (risk ratio 0.7, CI: 0.56-0.87, P = 0.001), while the highest quintile of fiber intake was associated with a 41% risk reduction (risk ratio 0.59, CI: 0.46-0.78, P < 0.001) compared to non-vegetarians and individuals in the lowest quintile of fiber intake, respectively. Most recently, a study on 50019 healthy women in the Nurses' Health Study showed that a higher intake of various sources of dietary fiber, except from vegetables, was associated with a lower risk of diverticulitis[29].

Additional environmental factors implicated in diverticulitis include smoking[30,31], use of steroids and non-steroidal anti-inflammatory medications[31-33], and obesity[34]. A traditionally held belief was that patients with diverticulosis should avoid particulate foods such as nuts, popcorn, corn, or sunflower seeds for fear of particulate material obstructing diverticula and triggering diverticulitis[35]. This myth has since been solidly debunked *via* the benchmark study that analyzed 47288 men over a follow-up period of 18 years: The authors did not observe a negative effect, but surprisingly there was even a potential protective impact of nut, corn or popcorn consumption with regards to the development of diverticulitis[36].

In contrast to associating extrinsic and environmental factors as the leading cause for the development of diverticular disease, the contribution of potential endogenous, *i.e.* genetic factors, has largely been underreported - until recently. A Swedish national database analysis of over 104000 twins reported nearly 2300 hospitalizations for diverticular disease. An odds ratio of 7.15 (CI: 4.82-10.61) was noted for monozygotic twins, compared to 3.2 (CI: 2.21-4.63) for dizygotic twins; the overall contribution of genetics to diverticular disease was calculated to be 40%[37]. A review of the Danish national registry data from 1977-2011 found 10400 index siblings and 920 twins with diverticular disease. The authors calculated the risk ratio for siblings to be 2.92 (CI: 2.5-3.39) compared to the general population. Similar to the Swedish study, the risk ratio for diverticular disease was substantially higher in twins with 14.5 (CI: 8.9-23) and 5.5 (CI: 3.3-8.6) for monozygotic and dizygotic twins, respectively. The calculated heritable contribution in this study was 53% (CI: 0.45-0.61)[38]. Last but not least, yet unpublished data from our own institution showed an incidental prevalence of diverticula in young patients undergoing abdominal CT scans for unrelated suspected appendicitis; 14% of patients less than 20 years of age and 40% of patients between 20-39 years of age had evidence of diverticulosis, suggesting that there could not yet have been sufficient lifetime to allow for extrinsic factors alone, such as fiber-deficiency related constipation, to take effect.

In summary, the development of diverticula may have both constitutional/genetic as well as extrinsic environmental and nutritional factors[10]. The risk of developing diverticulitis in individuals with diverticulosis is lower than previously estimated. However, the overall incidence of diverticulitis is on the rise with an increasing proportion of cases seen in younger patients. Lifestyle factors may not be solely responsible for diverticulosis but play a more crucial role in initiating an inflammatory cascade once diverticula are present.

PATHOGENESIS AND PRESENTATION

The traditional concept of the pathogenesis of diverticulosis has been that the development of diverticula is associated with chronic constipation leading to increased intraluminal pressure, colonic muscular hypertrophy, and mucosal and submucosal herniation at the entry points of the penetrating blood vessels (vasa recta)[39,40]. While acquired diverticula may develop throughout the entire colon, the characteristic distribution predominates in the sigmoid and left-sided colon where the stool is most formed[23]. In contrast, isolated right-sided diverticula are comparably rare and likely represent a different entity (true diverticula, possibly congenital) that regardless of migration and dietary adjustments are more frequently seen in Asian patients[26,41,42].

The pathogenesis of diverticular disease is still not completely understood and appears to have multiple facets. Diverticulosis, *i.e.* the presence of diverticula alone without any inflammatory signs, is typically not thought to cause any symptoms. However, there may be a substantial overlap/coexistence with constipation-predominant irritable bowel syndrome (IBS). Hence, for diverticulosis with non-specific symptoms of pain and constipation, the term symptomatic uncomplicated diverticular disease (SUDD) was coined. Such patients may show visceral hypersensitivity whereas the bowel compliance is normal[43,44]. Colonic dysmotility has also been described in SUDD patients, possibly related to a reduction in the interstitial cells of Cajal (GI pacemaker cells) and increased duration of low contractile activity[45,46].

The two most common true complications of diverticulosis are (1) Bleeding (usually from non-inflamed diverticula); and (2) Diverticulitis. Bleeding or chronic anemia are not usually associated with diverticulitis, and the presence of either entity should raise the concern for an underlying malignancy.

Diverticular bleeding can be abrupt and range from severe to massive lower GI bleeding. Yet, it poses a common diagnostic and treatment challenge as the bleeding may be cyclic and difficult to localize, even if one might speculate the origin to likely be in the left-sided colon. However, the presence of diverticula alone may not be sufficient evidence for targeted treatment, particularly as the differential diagnosis of a bleeding arterio-venous malformation is more commonly found on the right side[47,48].

The pathway to diverticulitis includes stasis or obstruction within a diverticulum, alteration in the gut microenvironment/microbiome, local tissue ischemia, and a microperforation[49,50]. Depending on the location and evolution of the inflammatory process *vs* the promptness and effectiveness of the host response, varying degrees of local mucosal and pericolonic inflammation, abscess formation, or even macroperforation result. Clinically, acute diverticulitis usually presents with a basic triad of increased abdominal pain, fever and elevated inflammatory parameters (white blood cells, C-reactive protein) with varying degrees of aggravated symptoms[51,52]. More chronic forms of diverticulitis manifest as smoldering diverticulitis with or without formation of a fibrostenotic stricture (possibly causing a large bowel obstruction)[51], or of fistulae to other organs (bladder, vagina, occasionally small bowel and skin). Even a classical constellation of symptoms is far from specific for diverticulitis, and a wide differential diagnosis should be considered (Table 1). In particular, it should be highlighted that in 1%-5% of patients, the suggestive clinical and even radiological features are in fact caused by a locally advanced malignancy on the verge of micro/macro-perforation (see later discussion on colonoscopy)[53,54].

More recently, it has been recognized that a small subset of patients with chronic diverticulitis show signs of segmental colitis associated with diverticulosis (SCAD)[55]. The macroscopic colitis occurs as an epiphenomenon around an area of diverticulitis, but generally does not involve the diverticula themselves[56]. Histopathological analysis of SCAD demonstrates similarities to inflammatory bowel disease (IBD) in the form of crypt abscesses, granulomas and significant architectural disruption[57,58]. Non-corroborated case series have even suggested a progression to either Crohn's or ulcerative colitis in small subset of SCAD patients[56,59].

EVALUATION AND CLASSIFICATION OF DIVERTICULITIS

Initial evaluation of diverticulitis aims at defining the acuteness and severity of symptoms and patterns of complications or chronicity (Table 2). Amongst symptom analysis, it includes obtaining information about previous surgeries, colon evaluations, as well as comorbidities. The focus of the initial physical examination is to distinguish a localized disease process from diffuse peritonitis with systemic manifestations

Table 1 Differential diagnosis for acute and chronic presentations of diverticulitis

Acute presentation	Chronic presentation
Appendicitis	Malignancy
Anastomotic leak (post-surgical)	IBS/SUDD
Perforating malignancy	IBD
Constipation	Constipation
IBS	Post-surgical
IBD	
Ischemic colitis	
Post-radiation enteritis	
Urogynecological pathology (tubo-ovarian abscess, endometriosis, pyelonephritis, cystitis etc.)	
Non-malignant viscus perforation (peptic ulcer)	

IBD: Inflammatory bowel disease; IBS: Irritable bowel disease; SUDD: Symptomatic uncomplicated diverticular disease.

Table 2 Workup for acute and chronic presentations of diverticulitis

	Acute	Chronic
History	Onset, progression, severity, location. Previous colon evaluation (< 2 yr). Previous episodes. Bowel habits	Recurrent attacks. Previous hospitalizations.Previous imaging. Previous colon evaluation (< 2 yr). Change in bowel habits
Physical examination	Localized *vs* diffuse peritonitis?	Abdominal distension. Fistula
Lab tests	WBC, CRP	Anemia? UTI?
Imaging	CT with oral/rectal and intravenous contrast: (1) Phlegmon; (2) Abscess/contrast extravasation; (3) Free air; and (4) Findings suggestive of other diagnosis	CT with oral/rectal and intravenous contrast: (1) Wall thickening; (2) Extraluminal contrast/air; (3) Fistulization; (4) Proximal colon distention; and (5) Rule out cancer features
Endoscopy	Avoid in acute phase, plan after 6 wk à rule out malignancy/IBD and/or synchronous pathology	Always à assess for mucosal pathology at target site and for synchronous pathology in the rest of the colon
Additional	(1) Possible CT-guided abscess drainage; and (2) Possible water-soluble contrast enema	(1) If colon evaluation incomplete à CT colonography or barium double contrast enema; and (2) Potentially cystoscopy, colposcopy

WBC: White blood cell; CRP: C-reactive protein; UTI: Urinary tract infection; CT: Computed tomography; IBD: Inflammatory bowel disease.

(sepsis) which typically warrants an urgent surgical exploration. More than 90% of patients, however, have a less advanced disease and undergo further evaluation[60]. Apart from the clinical parameters and blood work, cross-sectional imaging with CT scans has universally become the radiological tool of choice with extremely high sensitivity (93%-97%) and specificity (near 100%) at evaluating the severity of the disease[33,60-62]. Analysis of 16 of the 528 patients included in the DIABOLO trial[63], who unexpectedly progressed from mild to complicated disease suggested that fluid collections and to a lesser extent the length of the inflamed colon segment might represent predictive factors[64]. In situations where there is a concern for an underlying malignancy, a limited water-soluble contrast enema may have a role in discerning diverticular from malignant morphologies (apple core lesion), but modern crossectional imaging has largely eliminated that need. Endoscopic evaluation of the colon is increasingly debated but overall still considered a crucial safety measure to assess the target area and rule out synchronous pathology. However, it should be avoided in the acute setting and deferred to about 6 wk later (see below)[13,65].

Optimizing treatment strategies and the ability to compare different cohorts of patients depends on defining the disease severity for each individual patient. Traditionally, the Hinchey classification (1978) was used to define the severity of acute diverticulitis based on clinical and operative findings[66]. However, this system lacks details on intermediate severities which are recognized by modern imaging[60]. A number of different classification systems have since been suggested[67-71]. The CT-based classification by Ambrosetti *et al*[72] distinguishes between mild and severe

(complicated) acute diverticulitis[72], but the system insufficiently distinguishes the different grades of complicated diverticulitis, thus limiting its usefulness in decision-making and tracking disease progression for outcome analysis. The modified (1999) and further adapted (2005) Hinchey classification system (Table 3) added subgroups for milder and complicated forms of acute diverticulitis as well as categories for chronic complications (obstruction, fistula) with the goal to define criteria for management decisions[69,71]. In an era of the near ubiquitous use of CT for diagnosis and evaluation of diverticulitis, the 2005 modification of the Hinchey classification appears well suited to correlate clinical and radiographic findings to serve as the basis for treatment strategies[71].

Work-up for chronic presentations of diverticulitis tends to focus more on defining the anatomy and disease configuration than its severity (Table 2). Endoscopic evaluation is relevant to rule out underlying or coexisting neoplastic conditions, but imaging modalities (CT, CT colonography, contrast enemas) are crucial to obtain "anatomical road maps" and information about fistulizing and stricturing segments. Characteristic features seen on imaging include thickening of bowel/bladder wall, adjacency of the inflamed colon to other structures as well as air in the bladder[73]. In addition, obtaining a CT colonography or contrast is superior in identifying the overall distribution of diverticula and presence of fistulas or a strictured segment.

DIVERTICULITIS, COLORECTAL CANCER, AND THE ROLE OF ENDOSCOPY

From epidemiological data, there is no indication to believe that diverticulitis could cause colorectal cancer. In that sense, cancer and diverticulitis are two completely independent entities that happen to have a parallel age-dependent increase in incidence. Occasionally, the two entities may present a similar symptomatology which typically points to a locally advanced cancer that penetrates the colon wall. It is therefore important that any patient with "characteristic" symptoms could potentially have an underlying cancer that mimics the diverticulitis or coexists in a different part of the colon. That concern is reflected in the call for colonoscopy after cooling off the acute episode. The probability to detect a cancerous nature responsible for the symptoms is certainly higher for complicated presentations than it is for mild forms which has led some authors question whether a colonoscopy is needed in all.

Current practice guidelines, including those from the from the American Society of Colon and Rectal Surgeons (ASCRS), continue to recommend an interval colonoscopy after an episode of diverticulitis[52,65,74]. The goal of endoscopic evaluation is to rule out underlying or coexisting conditions (neoplasm, IBD) that could mimic the index presentation or be independent entities. To avoid disease aggravation with avoidable associated healthcare cost, *i.e.* in converting the inflamed segment's micro- into a macro-perforation, the instrumentation and insufflation are typically postponed for 4-6 wk from the immediate time of an acute attack[75]. In recent years, the utility of routine colonoscopy after an attack of uncomplicated diverticulitis has come under scrutiny, predominantly in the European literature[76,77].

The incidence for cancer and adenomas was reported in the range of 1.6%-5.5% and 8%-30.9%, respectively[78]. A large population-based cohort study from Denmark with a total of nearly 41000 patients admitted with acute diverticulitis reported a two-fold higher incidence of colon cancer compared to individuals without diverticulitis (4.3% *vs* 2.3% respectively)[79]. A systemic review analyzing 9 studies with 2490 diverticulitis patients found that of the 1468 patients (59%) that underwent colonoscopy after a bout of uncomplicated diverticulitis, the prevalence of an underlying malignancy, advanced adenoma, or low grade adenoma was 1.2%, 2.2% and 6.1%, respectively[53]. Another metanalysis pooled 1970 patients from 11 studies in 7 countries and revealed a proportional estimate of malignancy of 1.6% (95%CI: 0.9–2.8); but in the subgroup of complicated diverticulitis, the pooled proportional estimate of malignancy increased to 10.8% (95%CI: 5.2–21.0)[80]. Lastly, a systematic review of 30 studies with a total of 29348 subjects with CT-diagnosed diverticulitis reported the overall rate of malignancy as 1.67% (95%CI: 1.24-2.14); compared to 1.22% (95%CI: 0.63-1.97) in uncomplicated diverticulitis, the rate was 6.14% (95%CI: 3.20-9.82) for complicated diverticulitis, representing a relative risk of 5.033 (95%CI: 3.194-7.930; $P < 0.001$)[81]. The rates of malignancy and advanced adenoma were across the literature significantly higher (5%-11% and 19%, respectively) in patients with complicated diverticulitis[54,80].

One might argue that the percentages of colon cancer in uncomplicated diverticulitis are relatively low but are higher than those seen in a general screening

Table 3 Hinchey classification and modified Hinchey classification of acute diverticulitis

Hinchey classification[66]		Modified Hinchey classification[60,69,91] (imaging- or surgery-defined)		Comment
I	Pericolic abscess or phlegmon	0	Mild clinical diverticulitis	LLQ pain, elevated WBC, fever, no confirmation by imaging or surgery
		I	Confined pericolic inflammation: Phlegmon	
		I	Confined pericolic abscess	In immediate adjacency to inflamed bowel segment
II	Pelvic, intra-abdominal or retroperitoneal abscess	II	Pelvic, distant intra-abdominal, or retroperitoneal abscess	
III	Generalized purulent peritonitis	III	Generalized purulent peritonitis	No open communication with bowel lumen (ruptured abscess)
IV	Generalized fecal peritonitis	IV	Fecal peritonitis	Free perforation, open communication with bowel lumen
		SMOL[1]	Smoldering diverticulitis/peridiverticulitis	Recurrent/intermittent or chronic
		FIST[2]	Colovesical/vaginal/enteric/cutaneous fistula	Chronic or acute
		OBST[3]	Large and/or small bowel obstruction	Chronic or acute

[1]Smoldering.
[2]Fistula formation.
[3]Obstruction. LLQ: Left lower quadrant; WBC: White blood cell.

population[82,83]. Furthermore, the yield is higher than the one obtained for evaluation of occult rectal bleeding.

Reconciling the current evidence suggests that interval endoscopy remains essential after complicated diverticulitis due to the higher risk of underlying malignancy even if the value and cost effectiveness remain to be determined.

NON-OPERATIVE MANAGEMENT OF DIVERTICULITIS

Antibiotics have been the cornerstone of treatment for acute diverticulitis with the goal to resolve the underlying bacterial inflammation and alleviate related symptoms. Whether the treatment is delivered in an outpatient or an inpatient setting depends on the healthcare setup, the presentation, and the severity of symptoms. With increasing severity, cases may require hospitalization with intravenous measures, potential temporary bowel rest and interventions. In many instances, the opposite strategy, *i.e.* gentle bowel stimulation and cleansing with small aliquots of magnesium citrate, might help reduce the stool load to promote resolution of symptoms and–if surgery should be needed–increase the chances for a primary anastomosis[84]. The duration of treatment is not universally defined but will primarily be related by the time to normalization of clinical and inflammatory parameters.

Due to the increased antibiotic stewardship in clinical practice, there has been a recent push against the standard antibiotic treatment of acute diverticulitis. A 2012 Cochrane Review of 3 studies showed insufficient evidence to confirm the safety of avoiding antibiotic therapy in diverticulitis[85]. Subsequently, a pivotal randomized controlled trial (RCT), the "Antibiotika Vid Okomplicerad Divertikulit" (AVOD, Antibiotics For Uncomplicated Diverticulitis) study, randomized 623 patients with CT-evidence of phlegmonous diverticulitis to at least 10 d of antibiotic therapy *vs* supportive IV fluids only[86]. There was no significant difference in the length of stay (3 d in both groups) and no difference in the incidence of abscess formation, recurrent diverticulitis or readmission at 1 year of follow-up (16% in both groups, $P > 0.05$)[86].

Furthermore, there was no differences in long-term recurrences (both 31.3%; P = 0.986), complications (4.4% *vs* 5.0%; P = 0.737), surgery for diverticulitis (6.2% *vs* 7.1%; P = 0.719) or colorectal cancer (0.4% *vs* 2.1%; P = 0.061). Quality of life was also not significantly different between the two groups[87].

The previously mentioned DIABOLO trial was another RCT with 528 patients with CT-proven uncomplicated acute diverticulitis randomized to a 10 d course of antibiotics *vs* observation as outpatient if patients met criteria[63]. The primary outcome was time to recovery during 6 mo of follow-up. Median time to recovery was 14 d for the observational and 12 d for the antibiotic treatment strategy (P = 0.151, hazard ratio for recovery of 0.91). Hospital stay was significantly shorter in the observation group (2 *vs* 3 d; P = 0.006), but there was no significant differences in secondary endpoints, such as complicated diverticulitis, ongoing diverticulitis, recurrent diverticulitis, readmission, adverse events and mortality (Table 4)[63,88]. However, more than one third of patients experienced persistent symptoms even though the long-term quality of life was comparable after initial antibiotic or observational treatment[89].

There is a growing body of high-level evidence to suggest that a more nuanced selective patient-centric approach is warranted as opposed to routine use of antibiotics in uncomplicated acute diverticulitis, but there remain significant obstacles to more widespread acceptance of the omission of antibiotics in the management of acute diverticulitis[90].

SECONDARY PROPHYLAXIS

With regards to secondary prevention of recurrent diverticulitis[91], it is hard to identify one specific effective strategy since the pathogenesis of diverticulitis is–as previously mentioned-multifactorial and encompasses both lifestyle and genetic factors[90]. Smoking cessation, decreased intake of red meat, increased physical activity and loss of excess body weight seem to be intuitively smart health improvement choices and may potentially reduce the risk of diverticulitis. High fiber diet has been associated with a risk reduction of the first episode of acute diverticulitis but has an unclear efficacy in secondary prevention of recurrent attacks[90].

Rifaximin has recently been advocated as candidate for secondary prevention of recurrent acute diverticulitis. A retrospective study of 142 patients with symptomatic disease who were treated with rifaximin noted a significant decrease in their symptoms including abdominal pain and tenderness[92]. A prospective randomized study of 165 patients, assigned to high fiber diet either with and without rifaximin, observed a 9% lower rate of recurrent diverticulitis on follow-up with addition of rifaximin[93]. Finally, a metanalysis of a total of 1660 patients found the combination of rifaximin and high fiber diet to be effective in secondary prevention with patients continuing to be symptom free at 1 year[50].

Probiotics have also been investigated with the goal to decrease the incidence of diverticulitis by adjusting the microbiome of the colon. A RCT of 210 patients found that patients who were randomized to mesalamine and Lactobacillus for 10 d *per* month had decreased recurrence of symptoms with a follow up of 12 mo[94].

Unfortunately, most studies investigating secondary prophylaxis were heterogeneous in nature and lacked a gold standard for comparison. As the evidence remains weak, most guidelines still refrain from recommending routine use of these medications to reduce the risk of diverticulitis recurrence. However, there is an increasing enthusiasm to use such measures for their benefit in reducing symptoms in patients with chronic ongoing disease[90].

PRACTICAL STAGE-BASED MANAGEMENT APPROACH TO AN ACUTE DIVERTICULITIS EPISODE (INDEX EPISODE)

When it comes to defining the role of medical *vs* surgical management for diverticulitis, it is important to distinguish between the single acute episode (index hospitalization) as opposed to subsequent disease, be it in form of recurrent events, smoldering disease, formation of complications (stricture, fistula). In this section, the focus is on getting control of the acute episode, regardless of the future risks.

Uncomplicated diverticulitis, modified Hinchey 0 and IA (phlegmon, SUDD)

The vast majority (> 70%) of acute presentations of diverticulitis are uncomplicated

Table 4 Randomized controlled trials of antibiotics *vs* omission of antibiotics in the treatment of uncomplicated diverticulitis

Ref.	Number of patients	Antibiotics group	Control group	Primary outcome	Conclusions
AVOD[86]	623	At least 10 d of Abx, IV initially, then PO after admission or upon discharge	IV fluids	Complications, length of stay, need for surgery	Complications: 1% in Abx arm, 1.9% in control group (P = 0.302). LOS: 3 d in both groups. Recurrent diverticulitis/readmission in 1 year: 16% in both groups (P = 0.881)
DIABOLO[63]	528	10 d course of Abx, IV for 2 d, then PO	Observation as outpatient if clinical criteria satisfied	Time to recovery	Median time (d) to recovery: Observation 14 (IQR 6-35); antibiotic 12 (7-30; HR 0.91; P = 0.151)

Abx: Antibiotics; LOS: Length of stay; IQR: Interquartile range; HR: Hazard ratio; AVOD: Antibiotika Vid Okomplicerad Divertikulit.

(modified Hinchey 0 and IA), for which the success rates of conservative management are high (> 90%)[38]. Antibiotics remain the mainstay treatment for the initial non-operative management, be it as outpatient or in hospitalized patients[36,95]. It is important and advisable to set a time frame for benchmarks expected to be reached. For example, within 72 h after initiation of appropriate treatment, symptoms, and objective parameters [pain, fever, leukocytosis, systemic inflammatory response syndrome (SIRS), *etc.*] must improve without exception or completely resolve/normalize. Failure to achieve this goal should prompt either (1) repeat imaging to discern whether a drainable abscess has formed; or (2) a surgical intervention. It remains poorly understood at the present time why a small 5%-10% fraction of patients with seemingly mild diverticulitis on initial imaging do not respond to standard conservative management and ultimately require a surgical intervention[60].

With the potential for a superimposed IBS component in SUDD, the use of probiotics in treatment of diverticular disease has been suggested and studied by various groups. A review included 4 studies with a total of 134 patients (104 SUDD, 30 uncomplicated diverticulitis) who were treated with probiotics over short term average follow-up of 12 mo[96]. For SUDD patients, probiotic administration was successful in rendering 70% of patients' symptom free in one study and maintained remission for 14.1 mo in another. Overall, the authors concluded that use of probiotics in SUDD is safe and has demonstrable efficacy in the short term. The fact however that most studies lacked a control arm and any randomized comparison significantly limits the strength of the recommendation; at best it paves the way for future studies of the role of probiotics for SUDD.

Use of rifaximin has been tested in recent years with the goal to restore the altered colon microflora that was attributed by some to the pathogenesis of diverticular disease. A meta-analysis of 4 randomized studies with a total of 1660 patients (970 rifaximin, 690 control) found significant symptom relief (rate difference 29%, CI: 24.5-33.6) in the rifaximin-treated group compared to controls[97]. The number needed to treat in order to attain symptom relief was 3 in SUDD patients; however, when patients with diverticulitis were included, the number needed to treat substantially increased to 50.

Utilization of anti-inflammatory medication (mesalamine) has been studied in the treatment of SUDD, SCAD, and prevention of recurrent diverticulitis. In a randomized study of 40 SUDD patients, daily doses of mesalamine compared to a cyclical regimen (10 d/mo dosing) resulted in 20% more patients remaining symptom-free at 24 mo[98]. Compared to rifaximin, mesalamine demonstrated improved efficacy at 3 mo in the treatment of 170 SUDD patients[99]. However, data on treatment of SCAD with mesalamine is limited to small case series. Of 24 patients with SCAD, 21 were treated with mesalamine, of which 17 (81%) demonstrated resolution within 6 mo; the remaining 4 patients took 2-7 years of therapy before symptom resolution[100]. Recurrence rates were high (30%) with a large variability in the time to recurrence. In the phase 3 controlled PREVENT trial on the use of mesalamine for the prevention of recurrence of diverticulitis, daily mesalamine compared to placebo achieved no significant difference in disease recurrence (53%-63% *vs* 65%, respectively)[101].

In summary, these limited data sets on various supportive measures currently provide only weak evidence for their use in the management of symptomatic diverticular disease or mild diverticulitis. It cannot be stressed enough that proper diagnostic elements should be used to distinguish true diverticulitis from SUDD or IBS. Finally, surgery is typically discouraged for IBS and SUDD, but a recent

retrospective study with 52 patients suggested an improvement in quality of life at 12 mo after laparoscopic sigmoid resection[102].

Complicated diverticulitis with abscess formation, modified Hinchey IB and II

Acute diverticulitis associated with an abscess (modified Hinchey IB, II) represents complicated diverticulitis and has been reported in up to 15%-25% of acute presentations[60,103,104]. Increased resolution and universal use of CT scanners unquestionably improved our ability to diagnose these abscesses and if amenable percutaneously drain them. Management invariably starts with broad-spectrum antibiotics[65,74,84]. Small abscesses (< 4 cm) may have a chance to resolve with antibiotic therapy alone. Larger abscesses, particularly if > 5 cm, warrant an attempt at an image-guided percutaneous drainage placement. Immediate success rates-defined as the ability to safely access and drain the abscess and achieve resolution of associated signs of sepsis–show a wide range between 49%-94%[60,104]. Treatment goals remain similar to conservative management: resolution of symptoms and normalization of objective clinical and inflammatory parameters within a defined time frame (*e.g.*, 72 h). Short-term failure during the index hospitalization of medical management including percutaneous abscess drainage occurs in 12%-30% and should prompt a surgical intervention[84,104]. Unfortunately, even after clinical resolution, there is a substantially higher risk of recurrent disease. In our systematic review, only 28% of patients with an abscess neither had surgery nor a recurrent attack[105]. Analysis of our own institutional data, one of the largest series, the recurrence rate after an episode of diverticulitis with an abscess was 61% overall and 71% in the subset of patients treated with CT-guided drainage. 42% experienced more than one recurrent episodes, 46% of the recurrences were more severe than during the index episode, and 63% of recurrences were complicated by abscess, fistula, stricture, or peritonitis[104]. This observation should trigger a much broader discussion about whether or not an elective surgery should be recommended (see below).

Complicated diverticulitis with free perforation and diffuse peritonitis, modified Hinchey III (purulent, non-feculent) and IV (feculent)

Historically, there was little debate about the immediate indication for surgery in patients with evidence of free perforation as evidenced by intraperitoneal gas distant from the originating bowel segment. More recently, however, the approach has become a bit more tailored and the optimal treatment for perforated diverticulitis with peritonitis continues to evolve, both in regards to the indication for surgery as well as the type of surgery required[74].

Perhaps the most crucial first step is to distinguish patients with clinical evidence of diffuse peritonitis from the subset of patients who on imaging show distant free air but display no clinical signs of sepsis or diffuse peritonitis. A combination of clinical judgment and analysis of the CT findings is quintessential for sound decision-making. A grading system was proposed that characterized CT findings of perforation based on the amount and location of air and peritoneal fluid and allowed the authors to defer acute interventions in 91% of grade III diverticulitis cases[106]. Other authors, however, found less utility in the CT scan and stressed the importance of clinical judgement when avoiding emergency surgery in 64% of patients[107].

Regarding the surgical strategy, the best source control is unquestionably achieved by resecting the inflamed and perforated bowel segment. Diversion alone and drainage alone have been associated with prolonged recovery and higher morbidity. In the past, a discontinuous resection (Hartmann resection) was most commonly performed and still to the present date represents an overall safe approach, particularly if the patient is unstable, frail, or if the tissue quality is poor. However, the chance of never having the stoma reversed is remarkably high, and an immediate reconstructive effort (primary anastomosis with or without proximal diversion) has been shown to be a safe approach in appropriately selected patients. Several mostly retrospective studies and systematic reviews attempted to define the outcome and concluded that the mortality was comparable at 14% for both, Hartmann resection *vs* primary anastomosis[108,109]. A modeling *via* decision-analysis suggested that a primary anastomosis with proximal diversion has the highest probability of a favorable short- and long-term outcome[110]. The utility of a non-resective surgery (lavage) has been extensively scrutinized for even localized abscesses and appears to be an inferior tool for diffuse peritonitis (see below).

RECURRENCES AFTER INITIAL CONSERVATIVE MANAGEMENT

Recurrence rates after a bout of acute diverticulitis depend on several intrinsic and extrinsic factors, but a key parameter is the severity of the initial index episode[91]. One Italian study on over 1000 patients with a mean follow-up period of 10.7 years reported a 17% recurrence rate with only a 6.9% need for eventual surgery[111]. In an even larger study of 3165 patients, a subset of 2551 patients (80.6%) who were treated conservatively over a mean of 8.9 years revealed that 2366 (92.7%) did not require a colectomy[71]. Recurrence of diverticulitis was reported only in 314 patients (13.3%), 71% of which had only a single recurrence[71]. Based on these data, both authors concluded that elective colectomy after acute diverticulitis is not warranted. While this conclusion may be true for a single uncomplicated episode of acute diverticulitis, the decision is a lot more nuanced when complicated or recurrent diverticulitis is considered.

For diverticulitis associated with an abscess (modified Hinchey IB, II), the literature has reported significantly higher recurrence rates (up to 40%-61%), particularly in patients with a pelvic abscess (modified Hinchey II)[60,104,105,112]. While some retrospective studies suggested even long-term success with expectant management after percutaneous abscess drainage[71,103], our systematic review including 1051 patients revealed a substantial bias in the data sets and reported that of all patients with abscesses, only 28% had neither any surgery nor a recurrence[105]. 30% underwent urgent surgery during the same hospitalization, 36% were scheduled for elective surgery (whereby two fifth of these had to undergo an urgent intervention before their scheduled elective surgery), and 6% were not planned for surgery but urgently needed one[105]. However, given the substantially high rates of recurrences, regardless of whether a CT guided drainage was successful or not, the ASCRS practice guidelines recommend consideration of an elective colectomy in these patients[65,74].

SURGICAL INDICATION FOR DIVERTICULITIS

The indication for surgery needs to distinguish between the acute, *i.e.* urgent or emergency setting, as opposed to the elective prophylactic setting that occurs after the acute symptoms have resolved.

With up to 20% of patients with acute diverticulitis failing non-operative management[71,109,113], it is important to understand the criteria that warrant surgical intervention. In general, patients who require emergency or urgent surgery for diverticulitis in the acute setting fall into 3 main categories: (1) Signs of diffuse peritonitis and/or free perforation; (2) Suspicion of underlying malignancy; and (3) inadequate response to non-operative measures (antibiotics, bowel rest, percutaneous abscess drainage) after 72 h as demonstrated by persistent symptoms and lack of normalization of objective findings (fever, tachycardia, leukocytosis). The presence of complicated diverticulitis (abscess, fistulae, stricture) by itself generally does not mandate emergency/urgent surgical intervention (70%-92%) as demonstrated in numerous prospective and retrospective studies[60,61,103-106,114-116].

A major paradigm shift in the elective surgical management of uncomplicated diverticulitis occurring in the 2000s was that strict criteria such as age and number of attacks were no longer considered absolute indications for prophylactic elective colectomy[74]. Large cohort studies demonstrated that the long-term recurrence rates for uncomplicated diverticulitis (13%-20%) and subsequent need for emergent surgery (6%-7%) was low[117-120]. Younger patients (< 50 years) were historically thought to have a more virulent form of the disease and hence elective surgery was meant of stave off worsening and increased attacks. A whole body of evidence, however, showed that this is not the case with younger patients having similar rates of recurrence and subsequent need for colectomy as older patients[119,121,122].

Instead of applying rigid criteria, the decision process for elective colectomy in uncomplicated diverticulitis should be individualized to each patient and take into account the severity of attacks, comorbidities, and the effect on quality of life. The prospective randomized DIRECT trial in patients with ongoing symptoms or at least 2 attacks suggested that offering surgery was associated with improved cost and quality of life in patients undergoing surgery[123,124]. Immunocompromised patients tend to have more masked symptoms, more complications and worse outcomes with recurrent attacks[125-127]. An analysis on 166 immunocompromised patients found that in severe diverticulitis, the risk of recurrence was significantly increased compared to controls (46% *vs* 20%), whereas it was not elevated in mild diverticulitis[128].

There is general consensus for elective surgery as the definitive treatment of complicated diverticulitis with fistulae, symptomatic colonic stricture and persistent inflammatory mass or chronic smoldering disease given that these conditions are unlikely to resolve without surgical intervention[129,130]. As mentioned earlier, there is observational data demonstrating successful long-term non-operative management of diverticular abscess. However, given the high recurrence rates of up to 61% with often more complicated than the index presentation[104], ASCRS guidelines adopted recommendations for elective colectomy in this scenario[74].

OPERATIVE APPROACH AND SURGICAL OUTCOMES

Resection

Resection of the inflamed colonic segment remains the goal of surgical intervention. While it is not necessary to resect all diverticula-bearing bowel segments, the appropriate extent of the resection begins in the healthy bowel wall proximal to the target area and distally must include the high-pressure zone of the rectosigmoid junction as identified by the coalescence of the teniae. In general, oncological principals should be employed unless a malignant cause of the inflammation has definitively been ruled out. Placement of ureteral stents has not been shown to lower ureteral injury rate in elective colectomy, but should be considered in the setting of significant retroperitoneal inflammation or evidence of a preexisting hydro-ureter on imaging[131,132].

Reconstruction vs diversion

After resection of the inflamed colon segment, there are essentially three reconstructive/divertive options to consider: (1) A primary anastomosis; (2) A primary anastomosis with upstream diversion; and (3) Creation of an end colostomy (Hartmann procedure).

Factors associated with the creation of an end colostomy include non-elective surgery, particularly for modified Hinchey class 3 or 4, poor performance index, immunosuppression, and obesity (Body mass index > 30)[110,133]. 30%-45% of patients with an end colostomy never have their stoma reversed. In addition, reversal of an end colostomy has been associated with some morbidity[134-136]. A small randomized study comparing rates of ostomy closure in patients with an end colostomy *vs* a diverting ileostomy demonstrated significantly higher reversal rates the latter group (58% *vs* 90%, respectively), and the post-operative morbidity was higher in the former (20% *vs* 0%)[136].

A decision analysis model suggested that the preferred strategy in the non-elective setting is to perform a primary colorectal anastomosis and an upstream diverting stoma[110]. A reality check, however, confirms that in the United States, more than 90% of surgeons performed a Hartmann resection in the acute setting, and only 7.6% of patients had a primary anastomosis with diverting loop ileostomy performed[137]. A systematic review revealed a 62% rate of restored intestinal continuity during damage-control surgery[138]. From a practical standpoint and as a rule of thumb, (1) 2 healthy bowel ends in healthy and stable patients merit a primary anastomosis without diversion; (2) 1 healthy and one suboptimal bowel end may allow for a primary anastomosis with an upstream diversion; and (3) 2 unhealthy bowel ends or an unstable/unfit patient warrant an end colostomy[84]. Under elective conditions, a primary anastomosis should always be the goal, even if the surgery is carried out for disease complications such as colo-vaginal/-vesical fistula formation[84].

Morbidity and mortality

Overall, outcomes data after damage-control surgery for diverticulitis demonstrate a 9.2% risk of mortality and high morbidity of close to 50% including a 7.4% leak rate[137,138]. Wound infection reported at 10%-20% was the most common morbidity with mortality rates being < 5%. Patient comorbidities and need for emergent surgery were the two main contributing factors to patient mortality[139-142]. Long term follow up data demonstrate low recurrence rates (6%-8%) and a long median time to recurrence at 29 mo[111,143,144].

Open surgery vs minimally invasive surgery (laparoscopic/robotic)

Of 3 randomized trials have demonstrated that minimally invasive surgery (laparoscopic, robotic) for diverticular disease is associated with lower short term

morbidity in terms of lower blood loss, decreased post-operative pain and shorter hospital stay compared to open surgery[145-147]. It would be important to draw a clear distinction between emergency damage-control surgery *vs* elective interventions for persistent or recurrent disease. Unfortunately, many studies in the literature either refer to the latter only or do not provide the necessary transparency of data. Long-term outcomes are comparable with an overall improvement in satisfaction scores with the minimally invasive approach[148,149].

Even though diverticular disease represents a benign process, it can at times pose significant technical challenges in surgery secondary to the chronic inflammatory process leading to distortion of anatomy. In such a setting, the application of hand-assisted laparoscopic surgery (HALS) with tactile sensation might have some benefit. Two small, randomized trials comparing laparoscopic to HALS for diverticular disease reported lower overall operative times but slightly increased length of stay (1 d longer in HALS). No significant difference in short term post-operative morbidity was noted[150,151].

Role of resection vs lavage

When patients require an emergent intervention for non-relenting diverticulitis with abscess formation or with diffuse peritonitis, laparoscopic lavage and drainage has emerged as a potential alternative to resection and for a while gained some popularity, primarily in European centers[152,153]. Early studies were all small retrospective series subject to inherent selection biases. A systematic review of 11 studies with 871 patients noted that some series included up to 25% Hinchey I patients that by all means should have been managed with standard non-operative measures rather than an operation in the first place; the reported 30 d postoperative mortality rate was 4.8%, the conversion rate in Hinchey IV patients was 45%; but most importantly the overall success rate was only 24.3% defined as the patients being alive without surgical treatment for recurrent symptoms, and notably 6.9% of rehospitalized patients were found to have a cancer[42].

Three prospective randomized controlled multicenter trials on laparoscopic peritoneal lavage were launched and resulted in high-quality evidence (Table 5).

The LADIES trial was conducted from 2010 to 2013 in Belgium, Italy and the Netherlands and consisted of the two sub-arms difficult intravenous access (comparing Hartmann *vs* primary anastomosis) and L-Ornithine L-Aspartate (lap lavage *vs* sigmoidectomy)[154]. The laparoscopic lavage arm of the trial, however, was terminated early upon governmental oversight pressure due to a significantly increased rate of perioperative morbidity, mortality, and need for surgical reintervention compared to the resection groups. Furthermore, an alarming and unexpected finding in the laparoscopic lavage group of 45 patients were 4 with cancer that were missed and thus required resection later.

The Scandinavian diverticulitis (SCANDIV) trial encompassed 101 laparoscopic lavage *vs* 98 resection patients in 21 centers in Norway and Sweden from 2010 to 2014[155]. In contrast to the LADIES trial, SCANDIV was continued to completion. The 90 d morbidity and mortality were not significantly different between the two groups. However, when compared to resection, the use of laparoscopic lavage did not reduce severe postoperative complications and rather led to worse outcomes in secondary end points[155]. The laparoscopic lavage group had a 14.6% significantly higher rate of reoperation within 90 d (20.3% *vs* 5.7%, *P* value 0.01). As with other trials, there were cases of missed cancer (4%) in the laparoscopic lavage group that required delayed resection[155].

Finally, the Scandinavian DILALA trial offered the most favorable spin of the three. From 2010 to 2014, patients with intraoperatively confirmed Hinchey III generalized purulent peritonitis were randomized to either lavage (39 patients) *vs* open Hartmann's procedure (36 patients)[156]. Patients in the lavage group had a 45% reduced risk of undergoing subsequent operations within 24 mo (relative risk 0.55, 95%CI: 0.36-0.84; *P* = 0.012) and had fewer operations (ratio 0.51, 95%CI: 0.31-0.87; *P* = 0.024) compared with those in the Hartmann's group. There was no difference in the mean number of readmissions (1.37 *vs* 1.50%; *P* = 0.221) or mortality between the two groups. Three patients in the lavage group and nine in the Hartmann's group had a colostomy at 24 mo[157].

Reconciling the currently available higher quality evidence, a systematic review concluded that the preservation of diseased bowel by laparoscopic lavage was associated with an approximately 3 times greater risk of persistent peritonitis, intraabdominal abscesses and the need for emergency surgery compared to a resection[158].

Table 5 Randomized controlled trials comparing laparoscopic peritoneal lavage for perforated diverticulitis

Ref.	Number of patients	Morbidity (%)	Mortality (%)	Nonresolution (%)	Recurrence (%)	Elective resection (%)	No resection needed (%)
LADIES/LOLA[154]							
-Laparoscopic lavage	47	44	2	20	13	2	52
-Sigmoidectomy	43	29	1	N/A	N/A	71	N/A
SCANDIV[155]							
-Laparoscopic lavage	101	26	8	Not recorded	Not recorded	Not recorded	Not recorded
-Sigmoidectomy	98	14	7	N/A	N/A	Not recorded	N/A
DILALA[156]							
-Laparoscopic lavage	43	21	8	Not recorded	0	0	Not recorded
-Open Hartmann's	40	17	11	N/A	N/A	Not recorded	N/A

N/A: Not applicable; LOLA: L-ornithine L-aspartate; SCANDIV: Scandinavian diverticulitis.

CONCLUSION

The prevalence of diverticulitis is expected to increase substantially in the future as the population ages and the disease becomes more prevalent in younger patient populations. The development of diverticula/diverticulosis may have both intrinsic and extrinsic factors. The pathogenesis from diverticulosis to diverticulitis has several components, many of which are extrinsic and related to lifestyle. Different sub-entities of diverticular disease have been recognized but are yet poorly understood; they may cause blurring and overlap of symptoms. CT imaging is essential in the diagnosis of diverticulitis and classification of diverticular disease severity as it carries prognostic and potentially therapeutic significance. Interval colonoscopy remains advisable after an acute attack, particularly after complicated diverticulitis to rule out malignancy or advanced adenomata at the target site and the rest of the colon. Non-operative management of diverticulitis remains primarily based on antibiotics, but those may be omitted in mild cases. Supportive measures may include probiotics or anti-inflammatories, as dietary changes. Secondary prophylaxis focuses broad health improvement goals whereas the evidence for targeted interventions remains weak. Failure of non-operative management is typically defined as persistent or worsening symptoms and objective findings (SIRS, leukocytosis) after 72 h of best treatment. This should prompt further intervention, either in the form of imaging-guided drainage of an abscess or surgery. Resection offers the best source control in the acute, chronic, or elective setting. Laparoscopic lavage is currently not considered non-inferior to a surgical resection and should not be used outside of specific circumstances or protocols. A primary colorectal anastomosis should be considered at time of a resection unless the patient is unstable or there is poor tissue quality on each bowel end. A protective diverting ostomy can allow for an anastomosis when not all parameters are optimal. The indication for an elective surgical resection should be individualized and not be based on rigid criteria such as young age or a specific number of attacks. Instead, high-risk constellations such as complicated diverticulitis or immunosuppression should lead the considerations for elective colectomy when there is no obvious objective disease pathology (stricture, fistula, inflammation) after resolution of acute symptoms. Minimally invasive surgery (robotic, laparoscopic) is the primary approach for all elective cases and may be used in select emergency cases.

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MINIREVIEWS

Interaction between hepatitis B virus and SARS-CoV-2 infections

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Abstract

Coronavirus disease 2019 (COVID-19) has become a global pandemic and garnered international attention. The causative pathogen of COVID-19 is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel, highly contagious coronavirus. Numerous studies have reported that liver injury is quite common in patients with COVID-19. Hepatitis B has a worldwide distribution as well as in China. At present, hepatitis B virus (HBV) remains a leading cause of cirrhosis, liver failure, and hepatocellular carcinoma. Because both viruses challenge liver physiology, it raises questions as to how coinfection with HBV and SARS-CoV-2 affect disease progression and mortality. Is there an increased risk of COVID-19 in patients with HBV infection? In this review, we summarize the current reports of SARS-CoV-2 and HBV coinfection and elaborate the interaction of the two diseases. The emphasis was placed on evaluating the impact of HBV infection on disease severity and clinical outcomes in patients with COVID-19 and discussing the potential mechanism behind this effect.

Key Words: COVID-19; Hepatitis B virus; Liver injury; SARS-CoV-2; Coinfection; Immune exhaustion

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Core Tip: Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has become a global public health crisis. Liver impairment is frequent in COVID-19 regardless of whether it is combined with hepatitis B virus (HBV) infection. Currently, there is no evidence to suggest that HBV increases susceptibility to SARS-CoV-2. HBV and SARS-CoV-2 coinfection does not increase the risk of severity and outcome of COVID-19. Nucleoside analogs are recommended due to the risk of HBV reactivation in COVID-19.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It has become a global pandemic and a major public health threat[1-3]. The disease mainly involves the respiratory system, causing flu-like symptoms such as fever, dry cough, and dyspnea, and severe cases may deteriorate to acute respiratory distress syndrome[4]. Apart from respiratory disorder, SARS-CoV-2 can also contribute to multiorgan dysfunction such as acute cardiac injury, acute renal insufficiency, and liver damage[5-7]. According to the previous reports, the incidence of abnormal liver function ranges from 14.8% to 53% in patients with COVID-19[8-10].

Hepatitis B virus (HBV), a prototypical member of the Hepadnaviridae family, has a worldwide distribution, especially in China[11]. Currently, 3.5% of the global population is chronically infected with HBV and 5%-6% (70 million) of the Chinese population are carriers of hepatitis B surface antigen (HBsAg)[12-14]. Although new infection with HBV is decreasing due to vaccination, HBV is still the primary cause of liver cirrhosis and hepatocellular carcinoma (HCC), resulting in many deaths each year[15,16].

Because SARS-CoV-2 and HBV can both cause abnormal liver function, one of the major concerns is whether people with pre-existing HBV infection have increased susceptibility to and severity of COVID-19, thus leading to a worse prognosis. Another concern is whether SARS-CoV-2 infection accelerates the course of hepatitis B progression and leads to active viral replication. It is important to figure out the interaction between the two diseases. To elucidate this complexity, we summarize the limited clinical research to compare the severity of organ injury and clinical outcome between coinfected patients and those with COVID-19 alone, to provide insights into early risk stratification, and follow-up disease management.

IMPACT OF HBV ON COVID-19

Liver injury

According to the data from two large cohorts, which enrolled 5700 and 1099 COVID-19 patients, respectively, 0.1%-2.1% of patients had HBV coinfection[6,17]. The first issue that attracts widespread attention is whether there is more serious liver damage in chronic hepatitis B patients after coinfection with SARS-CoV-2.

Based on the currently available research, the pattern and degree of liver injury in patients with HBV and SARS-CoV-2 coinfection are like those with SARS-CoV-2 alone. We know from previous studies that liver injury has a prevalence of 14.8%-53.0% in COVID-19. The characteristics of liver injury mainly manifest as different degrees of elevation in alanine aminotransferase (ALT, 2.5%-50.0%), aspartate aminotransferase (AST, 2.5%-61.1%), γ-glutamyl transferase, and total bilirubin (0%-35.3%), and patients with severe disease may also show reduced albumin[8,10,18-20]. In a retrospective study by Zou *et al*[21], 105 patients with COVID-19 and HBV coinfection were studied. Liver injury was observed in a small proportion of patients (14, 13.33%), which is within the range of incidence in patients with COVID-19 alone, and four (28.57%) patients with liver dysfunction progressed to acute-on-chronic liver failure. Moreover, other retrospective studies have shown that liver injury in COVID-19 patients with HBV coinfection also presents with varying degrees of elevated transaminases (such as ALT, AST, γ-glutamyl transferase, and total bilirubin), but most studies have found no significant difference in the degree of liver damage compared to that in patients with COVID-19 alone[22-26]. The characteristics of liver injury in patients with SARS-CoV-2 and HBV coinfection are listed in Table 1.

Although liver injury is common in COVID-19, severe liver damage is rare. With recovery of the disease, liver function in most patients gradually recover back to normal. Guo *et al*[27] recently showed that males, COVID-19 severity, low liver computed tomography density, and medication are risk factors closely related to liver injury. The possible mechanism of liver injury for individuals infected with SARS-

Table 1 Characteristics of liver injury in patients with coronavirus disease 2019 and hepatitis B virus coinfection

Reference	Number of analyzed cases	HBV cases, *n* (%)	HBV status	Anti-HBV therapy, *n* (%)	ALT (U/L)[1]	AST (U/L)[1]	TBil (μmol/L)[1]	GGT (U/L)[1]	Note
Zou *et al*[21]	105	105 (100)	HBsAg(+), 94% HBeAg (–)	13 (12.38); entecavir (9, 8.75); tenofovir (3, 2.86); lamivudine/defovir (1, 0.95)	23 (15-33)	28 (19-43)	8.3 (6.6-12.8)	24 (16-36)	Four patients developed ACLF and liver injury was associated with disease severity and worse prognosis
Chen *et al*[22]	326	20 (6.1)	HBsAg(+); HBeAg(–); HBV DNA < 100 IU/mL	NA	28.00 (16.25-42.25)	27.50 (22.00-42.25)	10.55 (6.83-15.73)	23.50 (15.50-35.25)	No differences in the level of liver function (HBV *vs* non-HBV)
Liu *et al*[23]	347	21 (6.4)	HBsAg(+); 95% HBeAg(–)	1 (4.8) tenofovir	30.40 (22.00-36.85)	34.15 (27.00-39.58	12.60 (10.50-16.43)	28.50 (17.25-43.42)	Three patients had HBV reactivation
Li *et al*[24]	342	7 (2)	HBsAg(+); 14% HBeAg(+)	2 (28.6)	31 (29-38)	31 (29-38)	12.7 (11.1-16.6)	NA	Liver injury was common but mild with no severe liver-related complications
[2]Chen *et al*[39]	123	15 (12.2)	HBsAg(+) 6.7% HBeAg(+); 67% HBV DNA; > 20 IU/mL	3 (20) entecavir	25 (16-44)	28 (19-58)	13.2 (10.0-17.4)	20 (14-28)	The level of TBil was higher in patients with HBV infection ($P < 0.05$)
Wu *et al*[40]	620	70 (11.3)	NA	NA	50 (28-69)	40 (25-54)	NA	NA	33% of patients had abnormal ALT and AST; ALT/AST levels were higher in patients with HBV ($P < 0.05$)
He *et al*[25]	571	15 (2.63)	NA	3 (20) entecavir	NA	NA	NA	NA	HBV infection was observed to have a lower risk of severe events ($P < 0.05$)
Zhang *et al*[26]	23	23 (100)	65.2% HBV carriers; 30.4% CHB; 4.3% cirrhosis	NA	38.6 (17.0-42.0)	31.6 (15.0-36.8)	24.9 (7.2-13.9)	32.3 (13.5-41.0)	26% of patients had abnormal liver function test results at admission

[1]Data are expressed as median and interval interquartile.
[2]Another person whose family name is Chen. ACLF: Acute-on-chronic liver failure; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CHB: Chronic hepatitis B; GGT: Gamma-glutamyl transferase; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; NA: Not available; TBil: Total bilirubin.

CoV-2 includes the following: (1) Direct damage caused by SARS-CoV-2 particles; (2) Immune-mediated organ damage; (3) Hypoxic-ischemic liver injury; (4) Drug-induced liver injury; and (5) Reactivation of pre-existing liver disease[28-31]. These factors may participate together to cause abnormal physiological function of the liver.

Disease severity and clinical outcome

A meta-analysis reported that 3% of patients with COVID-19 have underlying chronic liver disease (CLD)[32]. In addition, disease progression is higher in COVID-19 patients with CLD[33]. Metabolic-associated fatty liver disease, one of the etiologies of CLD, has been reported to increase the severity of COVID-19[34-37]. Although most studies have shown that HBV coinfection does not aggravate the liver injury, whether it affects disease severity and outcomes remains controversial.

Some studies have suggested that HBV coinfection does not aggravate the disease in patients with COVID-19. In an analysis of several large studies[4-6,38], the major complications in COVID-19 included acute respiratory distress syndrome (3.4%-29.0%), acute cardiac injury (4%-12%), acute kidney injury (0.5%-7.0%), and shock (1.1%-8.7%). Zou *et al*[21] and Zhang *et al*[26] showed that 8.7%-44.8% of patients had acute respiratory distress syndrome, 13.3% of patients had an acute cardiac injury, 3.81%-4.30% of patients had acute kidney injury, 2.81% patients had a shock, and 4.3% had deep venous thrombosis and upper gastrointestinal hemorrhage in patients with SARS-CoV-2 and HBV coinfection. According to these data, the percentage of organ

injury in patients with HBV coinfection was roughly parallel to that in patients with COVID-19 alone. It seems that SARS-CoV-2 and HBV coinfection does not exacerbate organ impairment in COVID-19.

Additionally, in a cohort of 326 confirmed COVID-19 patients, of which 20 (6.1%) had HBV coinfection, Chen *et al*[22] reported that there were no differences in discharge rate and length of stay between the two groups. HBV coinfection did not affect the course and prognosis of COVID-19. In another study by Liu *et al*[23], 21 (6.4%) patients with COVID-19 and HBV coinfection were included, and 51 matched COVID-19 patients without HBV were used for comparison. They explored the independent impact of chronic HBV infection on the progression to severe COVID-19 and found that HBV did not delay SARS-CoV-2 shedding and did not increase the risk of progression and poor outcomes related to SARS-CoV-2. Similarly, Li *et al*[24] and He *et al*[25] enrolled seven (2%) and fifteen (2.6%) patients with HBV infection out of 342 and 571 COVID-19 patients, respectively. They found that chronic HBV coinfection was not associated with disease severity or poor prognosis.

However, a few studies have reported conflicting results. A study of 15 (12.2%) patients with chronic hepatitis B and COVID-19 found that they had a more severe disease course and higher mortality rate (13.3% *vs* 2.8%) compared with those without HBV infection, suggesting that HBV coinfection may facilitate the development of liver injury, which is associated with adverse outcomes[39]. Another recent study involving 70 cases of coinfection by Wu *et al*[40] indicated that ALT, AST, and activated partial thromboplastin time were significantly higher in patients with COVID-19 and HBV coinfection. The proportion of severe/critically ill patients was also higher than that in the non-HBV infection group (32.86% *vs* 15.27%). Despite this, all patients with HBV coinfection in the study of Wu *et al*[40] were discharged, and the length of hospital stay and negative nucleic acid tests were both consistent with those without HBV coinfection, indicating no differences in clinical outcomes between the two groups. Comparison of disease severity and clinical outcome (discharge rate and mortality rate) in the above studies are shown in Figure 1. These studies appeared to suggest that in most cases, chronic HBV infection did not increase the risk of disease severity or lead to a worse prognosis in COVID-19.

Cirrhosis and HCC

Clinicians may be concerned about whether HBV-related cirrhosis and carcinoma are associated with poor outcomes in COVID-19. Data on this issue are currently scarce. Zhang *et al*[26] compared the impact of different hepatitis B status (HBV carrier group, hepatitis B/cirrhosis group) on COVID-19. Most HBV carriers do not develop severe or critical illness, and no significant differences were found in the length of hospital stay, disease severity, and prognosis between the two groups. It is worth mentioning that only one patient was cirrhotic in this study, although there may have been biasing in the results.

A large cohort[41] enrolled 745 CLD patients from 29 countries, of whom 386 had cirrhosis and 359 did not, and mortality was significantly higher in the cirrhotic patients (32% *vs* 8%). Mortality increased with Child-Turcotte-Pugh class, which showed for the first time that the stage of liver disease is strongly associated with COVID-19 mortality. The data from some other multicenter retrospective studies also supported the conclusion that patients with liver cirrhosis in COVID-19 had higher mortality and worse prognosis compared with patients without cirrhosis[42-45].

HBV-related cirrhosis only accounted for a small proportion of patients, and most cases of cirrhosis were attributed to nonalcoholic fatty liver disease (24%-32.5%), alcohol-related liver disease (4.6%-24%), and chronic hepatitis C virus infection (24%)[44,46]. More importantly, HBV accounted for the lowest proportion of severe cases and deaths compared with other etiologies. Alcohol-related liver disease rather than HBV was an independent risk factor associated with the outcome of COVID-19. Although the severity of cirrhosis is closely related to mortality and prognosis in COVID-19, the limited data about HBV-related cirrhosis are insufficient to confirm that HBV worsens the clinical outcome.

As for patients with HCC, they usually have a higher risk of infection and poor outcome due to their immunocompromised condition. Much of the research has revealed that individuals with cancer are more vulnerable to SARS-CoV-2 and have an increased risk of mortality[47,48]. However, there is no available data about HBV-related HCC in patients with COVID-19. Therefore, reducing exposure and preventing SARS-CoV-2 infection is important for these patients.

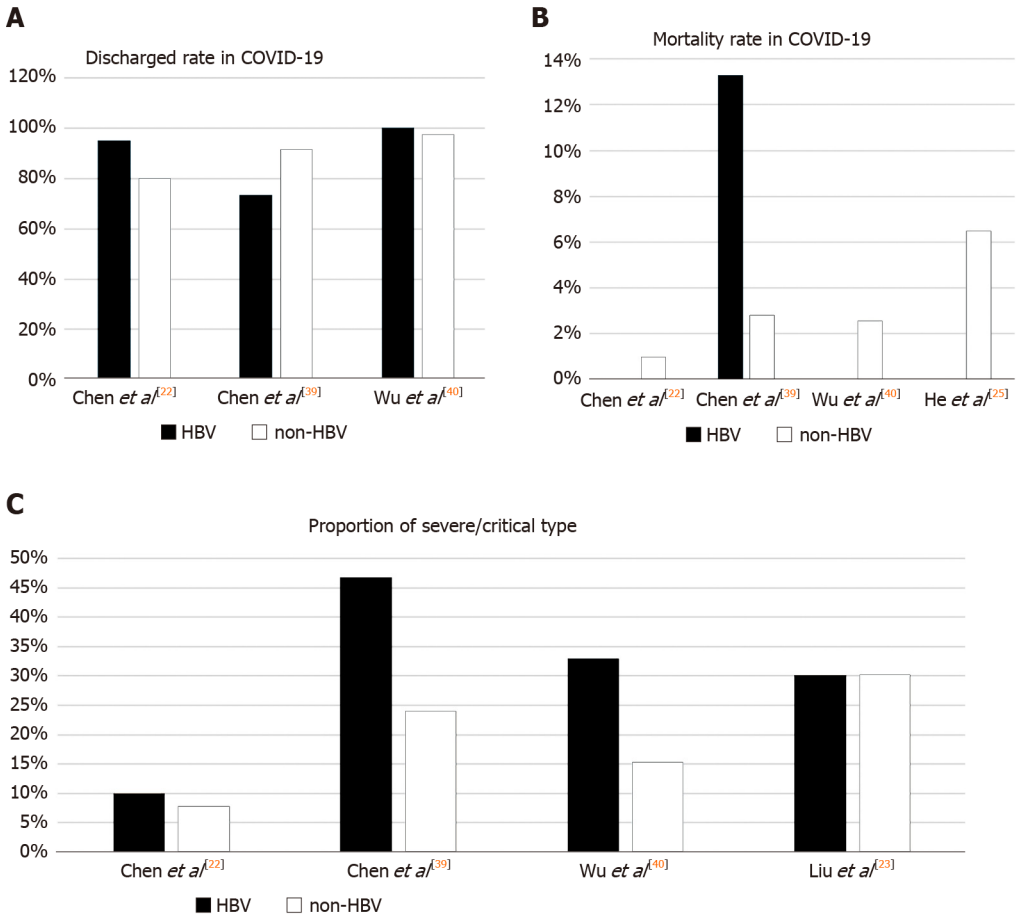


Figure 1 Comparison of clinical outcomes of coronavirus disease 2019 in hepatitis B virus and non-hepatitis B virus groups. A: Discharge rate; B: Mortality rate; C: Proportion of severe/critically ill patients. The data were collected from different clinical studies. COVID-19: Coronavirus disease 2019; HBV: Hepatitis B virus.

Interpretation of results

According to the limited research, HBV infection is not associated with the clinical outcome of COVID-19, although some patients may have a higher level of liver enzymes. We analyzed the underlying reasons behind this phenomenon.

(1) Some of the hepatitis B patients included in the study received nucleoside analogs as anti-HBV therapy (entecavir, tenofovir, *etc.*) in the long term, which may play a role in combating SARS-CoV-2 to some extent. Tenofovir tightly binds to SARS-CoV RNA-dependent RNA polymerase (RdRp) and terminates RNA synthesis catalyzed by SARS-CoV-2 RNA-dependent RNA polymerase[49,50]. These results provide a molecular basis for these nucleotide analogs to be viewed as a potential therapy for COVID-19. Additionally, a large cohort study in Spain found that the incidence of SARS-CoV-2 infection was low (0.4%, 8/1764) in patients with chronic hepatitis B who took tenofovir as anti-HBV therapy, which indirectly reflected that nucleoside analogs have a positive effect on resisting the novel coronavirus[51].

(2) Immune dysfunction caused by chronic HBV infection may play a crucial role in disease progression in COVID-19. Studies have proved that chronic HBV infection is associated with exhaustion of virus specific $CD4^+$ and $CD8^+$ T cells due to persisting viral antigens[52,53]. HBV-specific exhausted T cells lead to impaired secretion of cytokines, especially interleukin (IL) 2 and tumor necrosis factor-alpha, which is accompanied by progressive reduced antiviral function[54]. To our knowledge, the excessive immune response to SARS-CoV-2 infection (cytokine storm) results in overproduction of proinflammatory cytokines (such as IL-2, IL-6, and tumor necrosis factor-alpha), which is a critical factor associated with disease severity and mortality[55]. Under this circumstance, it is plausible that the exhaustion of HBV-specific T lymphocytes and the status of immunosuppression may avoid an overactive immune response to the novel coronavirus and reduce the cytokine storm, resulting in milder disease.

(3) Viral interference, which is defined as one virus in the host competitively

suppressing the replication of a second coinfecting virus, probably participates in the disease outcome in SARS-CoV-2 and HBV coinfection. Several studies have found that impaired type I interferon activity is a major feature in severe COVID-19 patients, which is associated with autoantibodies and genetic defects[56-58]. Viral interference can suppress coinfected viruses by enhancing type I interferon signaling[59]. Prior studies have proved that viral interference can occur in influenza virus, hepatitis virus, and human immunodeficiency virus[59-61]. For example, hepatitis C virus infection can limit the replication of HBV, and GB virus C and human immunodeficiency virus coinfection can reduce viral loads and prolong survival compared with patients with human immunodeficiency virus-1 infection. These observations support the hypothesis that HBV coinfection can affect replication and proliferation of SARS-CoV-2 by interferon-mediated viral interference.

(4) The number of patients with HBV coinfection in the published retrospective studies was small, which may have influenced the results. Almost all available studies about SARS-CoV-2 and HBV coinfection did not describe the baseline characteristics of HBV infection well, so the clinical stage of the patients could not be determined clearly. Hence, these results should be interpreted with caution and further conclusive research is needed.

IMPACT OF SARS-COV-2 ON HBV

For severe COVID-19 patients with HBV coinfection, there is a risk of HBV reactivation. There has been little consensus about the standardized definition of HBV reactivation. Primarily reactivation is defined as a sudden and rapid increase in HBV DNA levels in individuals with previously detectable HBV DNA or reappearance of HBV DNA viremia in individuals without detectable viral DNA[62]. HBV reactivation is usually associated with immunosuppressive therapy such as IL-6 receptor antagonists (tocilizumab and siltuximab), IL-1 receptor antagonists (anakinra), and high-dose corticosteroids[63-65]. In severe COVID-19 patients, these therapies may be used to control the cytokine storm, thus reducing the immune-mediated multiorgan injury.

In a retrospective study[23] of 21 patients with SARS-CoV-2 and HBV coinfection, 19 patients were tested for HBV DNA viral load at least twice during hospitalization. Of the 19 patients, three patients developed HBV reactivation and manifested as a rapid increase in HBV DNA viral load from undetectable to a high level. These three patients were negative for hepatitis B e antigen and did not receive any anti-HBV treatment before admission. During the hospitalization, two of the three patients received methylprednisolone, which may account for the reactivation, and one did not receive any corticosteroids. Another case report[66] showed that one patient with COVID-19 had acute HBV infection, and laboratory results showed AST (4933 U/L), ALT (4758 U/L), total bilirubin (183.9 mmol/L), HBsAg (+), hepatitis B core antibody immunoglobulin M (+), hepatitis B e antigen (–), hepatitis B e antibody (+), and HBV DNA viral load was 2490 IU/mL. The patient did not receive any immunosuppressive therapy. Regardless whether corticosteroids were used, the patient could have a risk of HBV reactivation.

The mechanisms of HBV reactivation following infection with SARS-CoV-2 are primarily due to a broken balance between the host's immune state and viral replication. In addition to the host baseline virological indicators, the intensity of glucocorticoids or immunosuppression therapies is a primary risk factor for reactivation of HBV during treatment of COVID-19[11,62].

Although infection with SARS-CoV-2 has a risk of HBV reactivation, the overall risk is low. One prospective study[67] evaluated the risk of HBV reactivation in 61 patients with severe COVID-19 and resolved HBV infection (HBsAg-negative, anti-hepatitis B core antibody-positive) undergoing immunosuppressive therapy. After at least 1 mo of follow-up, they found no cases develop HBsAg seroconversion and only two (3%) patients had detectable serum HBV DNA (< 15 IU/mL). Therefore, for patients with severe COVID-19 and coexistent HBV infection, corticosteroids and immunosuppressants can be selected clinically.

Given the risk of reactivation, the American Association for the Study of Liver Diseases guidelines strongly recommend that anti-HBV treatment should be initiated or continued once COVID-19 was diagnosed[68]. At the same time, routine HBV virologic indicators and liver-injury related indicators should be closely monitored during the disease.

CONCLUSION

In this review, we summarized reports about SARS-CoV-2 and HBV coinfection and explored the interaction between chronic hepatitis B and COVID-19. The limited clinical evidence reflects that chronic HBV infection does not increase the severity and outcome of COVID-19 in most cases (Figure 2). Given that the stages in patients with chronic hepatitis B are ambiguous (immune tolerance or low viral replication), these findings need to be confirmed in further studies. HBV reactivation is possible in the course of the disease. Therefore, liver function and hepatitis-B-related indicators should be monitored regularly.

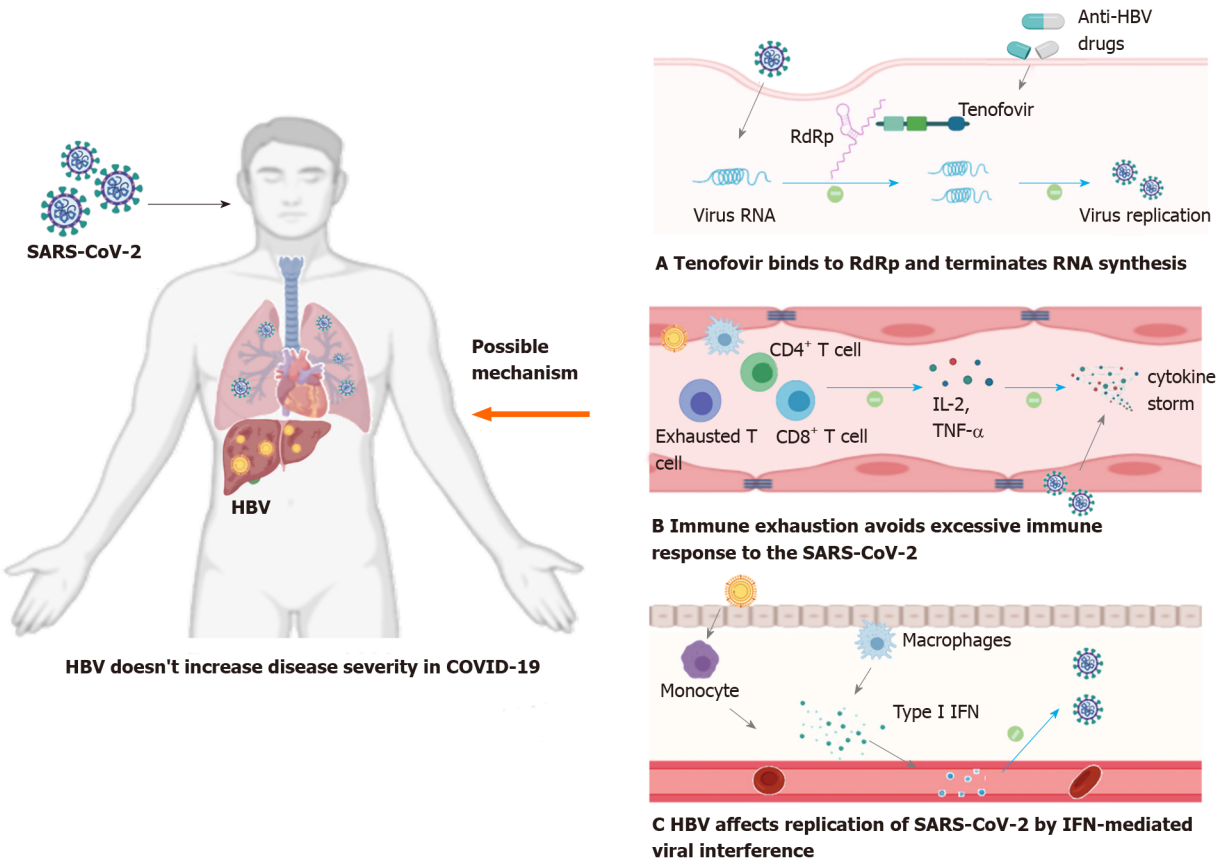


Figure 2 Graphical abstract. COVID-19: Coronavirus disease 2019; HBV: Hepatitis B virus; IFN: Interferon; IL: Interleukin; RdRp: RNA-dependent RNA polymerase; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; TNF-α: Tumor necrosis factor-alpha.

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ORIGINAL ARTICLE

Basic Study

Effect of acetyl-L-carnitine on hypersensitivity in acute recurrent caerulein-induced pancreatitis and microglial activation along the brain's pain circuitry

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Author contributions: McIlwrath SL performed the animal experiments, data analysis, produced the figures, wrote the manuscript; Starr ME assisted with animal caerulein dosing and read the manuscript; High AE analyzed the microglial morphology in the brain sections; Saito H designed the study, read and edited this manuscript; Westlund KN designed the study, read and edited this manuscript; all authors discussed the results, commented on the manuscript, and approved the final version of the article.

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Abstract

BACKGROUND

Acute pancreatitis (AP) and recurring AP are serious health care problems causing excruciating pain and potentially lethal outcomes due to sepsis. The validated caerulein- (CAE) induced mouse model of acute/recurring AP produces secondary persistent hypersensitivity and anxiety-like behavioral changes for study.

AIM

To determine efficacy of acetyl-L-carnitine (ALC) to reduce pain-related behaviors and brain microglial activation along the pain circuitry in CAE-pancreatitis.

METHODS

Pancreatitis was induced with 6 hly intraperitoneal (i.p.) injections of CAE (50 μg/kg), 3 d a week for 6 wk in male C57BL/6J mice. Starting in week 4, mice received either vehicle or ALC until experiment's end. Mechanical hypersensitivity was assessed with von Frey filaments. Heat hypersensitivity was determined with the hotplate test. Anxiety-like behavior was tested in week 6 using elevated plus maze and open field tests. Microglial activation in brain was quantified histologically by immunostaining for ionized calcium-binding adaptor molecule 1 (Iba1).

RESULTS

Mice with CAE-induced pancreatitis had significantly reduced mechanical withdrawal thresholds and heat response latencies, indicating ongoing pain. Treatment with ALC attenuated inflammation-induced hypersensitivity, but hypersensitivity due to abdominal wall injury caused by repeated intraperitoneal injections persisted. Animals with pancreatitis displayed spontaneous anxiety-like behavior in the elevated plus maze compared to controls. Treatment with ALC resulted in increased numbers of rearing activity events, but time spent in "safety" was not changed. After all the abdominal injections, pancreata were translucent if excised at experiment's end and opaque if excised on the subsequent day, indicative of spontaneous healing. Post mortem histopathological analysis performed on pancreas sections stained with Sirius Red and Fast Green identified wide-spread fibrosis and acinar cell atrophy in sections from mice with CAE-induced pancreatitis that was not rescued by treatment with ALC. Microglial Iba1 immunostaining was significantly increased in hippocampus, thalamus (intralaminar nuclei), hypothalamus, and amygdala of mice with CAE-induced pancreatitis compared to naïve controls but unchanged in the primary somatosensory cortex compared to naïves.

CONCLUSION

CAE-induced pancreatitis caused increased pain-related behaviors, pancreatic fibrosis, and brain microglial changes. ALC alleviated CAE-induced mechanical and heat hypersensitivity but not abdominal wall injury-induced hypersensitivity caused by the repeated injections.

Key Words: Acute recurrent pancreatitis; Neuropathic pain; Mechanical hypersensitivity; Heat hypersensitivity; Anxiety-like behavior; Ionized calcium-binding adaptor molecule 1

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Core Tip: The caerulein- (CAE) induced pancreatitis model requiring 6 wk of repeated injections is a model of recurrent acute bouts of pancreatitis that causes pancreatic tissue damage and fibrosis. Control repeated i.p. saline injections alone caused abdominal wall injury and hindpaw secondary mechanical hypersensitivity. Treatment with acetyl-L-carnitine significantly attenuated CAE-induced hypersensitivity without alleviating pancreatic histological disruption. Mice with CAE-induced pancreatitis with secondary mechanical and heat hypersensitivity had elevated plus maze anxiety-like behavior. Post-mortem analysis revealed microglial morphology changes indicative of activation in amygdala, hippocampus, hypothalamus, and thalamus, but not in primary somatosensory cortex. These data suggest that activated microglia in these brain regions contribute to chronic hypersensitivity and anxiety-like behaviors in mice with CAE-induced pancreatitis.

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INTRODUCTION

Pancreatitis is a progressive disease that when developing from acute to chronic pancreatitis increases the risk for pancreatic cancer[1-5]. A recent meta-analysis reported 10% of patients with acute and 36% with recurrent acute pancreatitis (AP) develop chronic pancreatitis[6,7]. This multifactorial disorder has a complex etiology including gene mutations/heritable factors, gallstones, poor and/or high fat diet, smoking, and regular alcohol consumption[8-10]. The recurrent inflammation of the pancreas results in progressive pancreatic insufficiency and replacement of exocrine parenchyma with

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fibrotic tissue and adipocytes. Chief complaint of patients with pancreatitis is intractable abdominal pain, present in approximately 90% of patients[11,12]. At present there is no cure for pancreatitis and pain management remains inadequate, yet pain which is the main cause of reduced quality of life is often ignored and neglected by specialists since it is poorly understood[13-15].

In the past, pain due to pancreatitis has been attributed to inflammation and distension pressure within the pancreas. Relieving pancreatic tissue pressure provides post-surgical pain relief, but no long-term pain relief[16]. More recently, studies have shown that severity of pancreatitis induced pain is independent of abdominal findings in clinical patients[14,17]. Pancreatitis pain is initially driven by peripheral inflammation, mitochondrial dysfunction in acinar cells, and release of reactive oxygen species[18-20]. The result is pathological activation of innervating sensory neurons and a vicious cycle of neurogenic inflammation[21]. Pre-treatment with acetyl-L-carnitine (ALC), a nutraceutical antioxidant and mitochondrial enhancer, has been shown to prevent biochemical and histological evidence of AP[22]. ALC is a free radical scavenger that can potentially repair oxidized polyunsaturated fatty acids esterified in membrane phospholipids[23,24]. With progression to recurrent acute and chronic pancreatitis, associated nerve pain becomes neuropathic and clinical study has demonstrated attenuation by pregabalin, the conventional pharmacological intervention for neuropathic pain[25]. Concurrent neuroplastic changes in the spinal cord and along the brain pain neuraxis cause a transition to central sensitization[26,27].

Microglia are resident immune cells in the central nervous system. After activation through neurochemical communication among neurons and glial cells, microglia can phagocytose cell debris and/or invading foreign cells, as well as activate circulating immune cells[28-31]. Microglial communication with neurons in the spinal cord has been identified as central in establishing and maintaining neuropathic pain[27,32]. Release of brain-derived neurotrophic factor from activated microglia in the spinal cord causes a neuronal anion shift that contributes to central sensitization[33]. In neuropathic pain models, ligating either spinal nerves or parts of the sciatic nerve induces selective microglial activation in the spinal cord but reportedly not in supraspinal regions 2 wk after nerve injury[34,35]. Four weeks after partial sciatic nerve ligation, however, migrated M2 bone-marrow derived microglia were localized in the amygdala concurrent with anxiety-like behavior[36]. Several studies using a chemically induced rat model of pancreatitis have demonstrated that activated spinal microglia are involved in the establishment and maintenance of hypersensitivity[27,31]. In clinical patients with chronic pancreatitis cortical reorganization within the pain neuraxis has been reported[37,38]. However, the role of microglia in this process is not yet understood.

In the present study we utilized a modified caerulein- (CAE) induced recurrent AP model[39-42]. This model causes pancreatic dysregulation of digestive enzymes, oxidative stress, fibrosis, acinar cell death, infiltration of inflammatory cells, leading to edema and fibrosis in mice[19,20,40,42]. In male C57BL/6J mice the CAE-induced pancreatitis model produces secondary hypersensitivity and anxiety-like behavioral changes. Morphological changes indicative of microglial activation in pain and anxiety-related brain regions were quantified after 6 wk of CAE-induced recurrent AP. Efficacy of ALC treatment to alleviate these symptoms in the last 3 wk of CAE-induced pancreatitis was determined.

MATERIALS AND METHODS

Animals

All animal procedures were conducted in accordance with the guidelines for the ethical treatment of experimental animals published by the International Association for the Study of Pain and approved by the University of Kentucky Institutional Animal Care and Use Committee. A total of 27 male 16- to 26-week-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, MA, United States) were used. All animals were maintained on a 12/12 h light/dark cycle with up to 5 animals in each pressurized intraventilated cage in a temperature (21-23 °C) and humidity (30%-70%) controlled room. Animals were given water and food *ad libitum*.

Body weight was measured daily to ascertain well-being. After 5 wk of repeated daily intraperitoneal injections, uninjected naïve control group animals weighed significantly more than the 3 other experimental groups whose body weight had plateaued (naïve control = 31.2 ± 0.5 g, VEH (vehicle) + ALC = 28.4 ± 0.7 g, CAE + VEH = 28.9 ± 0.8 g, CAE + ALC = 28.7 ± 0.5 g, $P < 0.05$ two-way ANOVA with Newman-Keuls *post hoc* test) (Supplementary Figure 1).

CAE-induced persistent pancreatitis model

Animals were randomly divided into 4 groups: CAE + VEH had CAE-induced pancreatitis and saline treatment, CAE + ALC had CAE-induced pancreatitis and ALC treatment, VEH + ALC control animals received saline injections and ALC treatment, and naïve controls that received no injections at all. Recurrent AP was induced using CAE, similar to previous description[39,41-43]. In brief, animals received 6 hly intraperitoneal (i.p.) injections of 100 μL of 0.9% sterile saline containing 50 μg/kg CAE (American Peptide, Sunnyvale, CA, United States) 3 times a week (Monday, Wednesday, Friday) for a total duration of 6 wk using a 1 mL syringe with a 1/2 inch long 27 gauge needle. Control vehicle animals were injected with the same volume of 0.9% saline vehicle[41]. Naïve control animals were not injected.

ALC treatment

In the present study we tested the efficacy of ALC post-treatment to reduce pain-related behaviors. Three weeks after the start of the CAE injections, animals received i.p. injections twice daily of either 50 μL 0.9% saline (VEH) or ALC (100 mg/kg per injection for a total daily dose of 200 mg/kg; Sigma-Aldrich, St. Louis, MO, United States). Injections continued in weeks 4-6 until experiment's end. A previous study demonstrated the efficacy of ALC pre-treatment given daily (200 mg/kg body weight, i.p.) for 7 d to prevent acute CAE-induced pancreatitis[22]. It has been shown that 100 mg/kg body weight results in a peak plasma carnitine concentration 3 h post and is completely removed within 24 h after administration[44].

Behavioral assessments

Animals were acclimated in their home cage to the behavior testing room for 1 h prior to starting experiments. Reflexive mechanical and heat assays were conducted weekly. Mechanical and heat sensitivity was tested several consecutive days in the week prior to the first CAE injection and baseline measurements were recorded when sensitivity measures were stable. The elevated plus maze and open field test were performed only once at the end of the experiment.

Mechanical withdrawal threshold measurement using von Frey filaments on hindpaws

The up-down method was used to determine mechanical sensitivity of the hindpaws[45]. Briefly, animals were placed on an elevated Teflon screen mesh (3 mm^2 holes) in individual clear lucite boxes and the hind paw glabrous skin was probed using a graded series of calibrated von Frey filaments (0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, 15.0 g or 3.9, 5.9, 9.8, 19.6, 39.2, 58.8, 98.0 mN). Withdrawal of the foot was considered a positive response. An algorithm was used to determine the mechanical sensitivity threshold for experimental group comparisons.

Hotplate test

Heat sensitivity was determined using a hot plate analgesiometer apparatus set at 50 ^oC (Columbus Instruments, Columbus, OH, United States). Animals were placed individually on the hotplate (25.4 cm × 25.4 cm heated surface) surrounded by a 30 cm high Plexiglas barrier. The latency until a nocifensive response (shaking or licking the paw, jumping, or running) occurred was recorded. Animals were immediately removed after the initial response. A mandatory cut-off time of 25 s prevented burn injury but was never employed. The heat sensitivity test was performed 3 times at 20 min intervals and latencies averaged.

Elevated plus maze test

The elevated plus maze consists of 2 closed (safe) and 2 open (threatening environment) arms and is widely used to measure anxiety-like behavior. The mouse is placed in the center of the elevated 4-arm maze and video recorded in isolation for 5 min (300 s) for post hoc analysis. Fear/anxiety-like behavior is determined by the number of open and closed arm entries, total open and closed arm occupancy, and by the number of exploratory rearing events. Anxiety-like behavior is related to open arm avoidance and reduced rearing events[46]. This test was consistently performed in all mice before the open field assay.

Open field exploratory behavior test

Exploratory behaviors were measured using the Flexfield Animal Activity System (San Diego Instruments, San Diego, CA, United States) that consisted of a Plexiglas

chamber (40 cm × 40 cm × 36 cm) equipped with a Photobeam Activity System (PAS) coupled to a Compaq 486 computer (Hewlett Packard, Palo Alto, CA, United States). An array of 16 evenly spaced infrared photobeam sensors in the X- and Y-axis (total of 32 sensors) was arranged at a distance of 1.25 cm above the chamber's floor. Obstruction of these light beams allowed the PAS software to localize the animal within the two-dimensional space to follow the animal's movement, distance traveled, and resting time within the chamber. A second array of 16 beams per axes was located 8 cm above the chamber's floor. Obstruction of these light beams indicated that the animal had moved in the z-axis, *i.e.*, rearing on its hindlegs[47]. Animals were tested after 6 wk of CAE injections with and without ALC treatment. Each session lasted for 45 min and data were collected in 5 min intervals to determine: (1) Number and duration of rearing events; (2) Active time *vs* rest time; (3) Overall distance traveled; and (4) Time spent in the central *vs* peripheral areas of the chamber.

Histology

At experiment's end, animals were deeply anesthetized with inhalant isoflurane (4%-5% in 1 L O_2) and euthanized by exsanguination through transcardial perfusion with heparinized 0.9% saline. The pancreas was excised and the animal perfused with 4% buffered paraformaldehyde (PFA). Whole mount pancreas tissue was photographed floating in 0.9% saline in a petri dish with charcoal-stained Sylgard covered bottom (Sigma, St. Louis, MO, United States). Tissue from cohort 2 was then immersed in 4% PFA overnight, embedded in paraffin, 5 μm sections cut and mounted on glass slides for microscopy. Sections were deparaffinized and reacted with Sirius Red and Fast Green[48]. Brightfield images were taken at 100 × magnification using a Zeiss AxioCam ICc 1 camera mounted on an Axio Observer Z1 microscope (Carl Zeiss Microscopy, White Plains, NY, United States). Semiquantitative histopathological scores on a scale of 1 (no damage) to 5 (severe damage) were made by three observers blinded to experimental groups. Scores were then averaged and graphed.

Brains were cryoprotected with 30% sucrose, stored at -80 °C, and sectioned (40 μm). Free floating sections were incubated for 30 min in 0.5% fresh sodium borohydride to break aldehyde bonds for improved antigen retrieval and to reduce background staining. Tissue was reacted with rabbit anti-Iba1 (ionized calcium-binding adaptor molecule 1) antibody (1:60000; Wako Chemicals United States, Richmond, VA, United States; Cat. #019-19741) overnight and visualized using the Pierce DAB substrate kit according to manufacturer instructions (ThermoFisher Scientific, Waltham, MA, United States). Images were taken of a brain slice at bregma -2.18 mm (interaural 1.62 mm) at 200 × magnification using a Nikon E1000 microscope (Nikon Instruments, Melville, NY, United States) and ACT1 software. Photomicrographs were analyzed with NIH ImageJ.

Microglial histological analysis

Histological analysis of the histological section taken at bregma -2.18 mm (interaural 1.62 mm) was conducted by a scientist blinded to experimental groups. Brain microglia were identified based on Iba1 immunoreactivity in 4 naïve control and 5 CAE + VEH animals. Microglial morphology was quantified from 10-20 cells per brain from each animal by measuring somal and convex hull area, the smallest convex polygon needed to surround the whole cell shape, soma and processes[49]. The somal area was outlined using the wand tool in NIH ImageJ. The microglial convex hull area, the smallest convex polygon needed to surround the entire cell (soma and processes) was drawn by hand. Area and intensity of staining were measured using NIH ImageJ.

Statistical analysis

All data are presented as the mean ± SE of the mean. Behavioral data and histopathological scores were compared using two-way ANOVA and Newman-Keuls Multiple Comparison *post-hoc* testing, one-way ANOVA or Student's *t*-test where appropriate. Microglial histological data were compared using Student's *t*-test. A P value of $P < 0.05$ was considered significant.

RESULTS

Behavioral responses to mechanical and heat stimuli

CAE-induced pancreatitis caused secondary mechanical and heat hypersensitivity on the hindpaws (Figure 1). At baseline, mechanical withdrawal thresholds were not

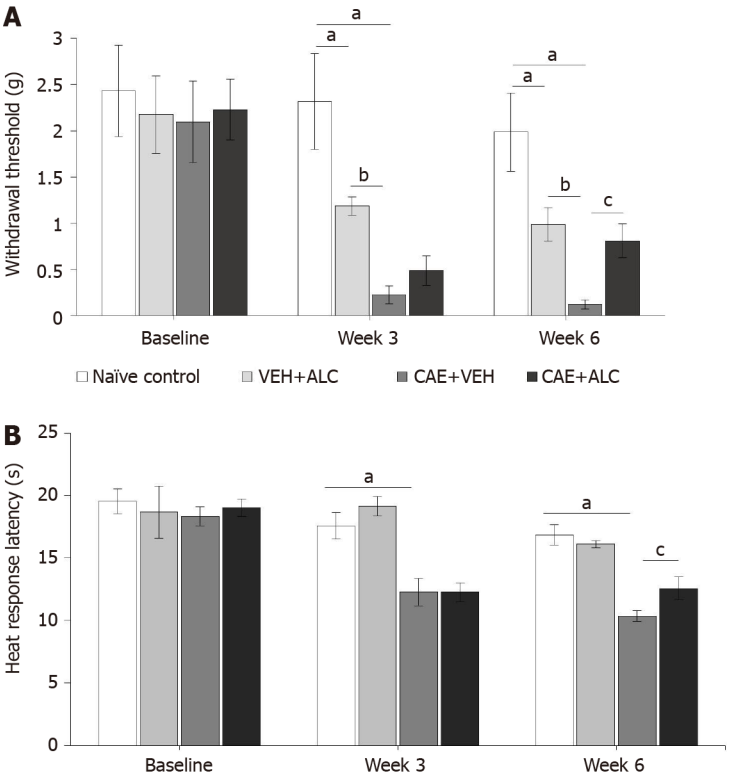


Figure 1 Caerulein-induced pancreatitis produced secondary hypersensitivity of the hindpaws that was attenuated by treatment with acetyl-L-carnitine. A: Mechanical withdrawal thresholds were significantly reduced after 3 wk of caerulein (CAE) injections indicating hypersensitivity and remained reduced after 6 wk of CAE injections; B: Response latencies to heat stimulation in the hotplate test were significantly reduced after 3 and 6 wk of CAE injections. Concurrent treatment with acetyl-L-carnitine (ALC) during the last 3 wk attenuated mechanical and heat hypersensitivity. n = 6/group; $^{a}P < 0.05$ compared to naïve control; $^{b}P < 0.05$ compared to VEH + ALC; $^{c}P < 0.05$ compared to CAE + ALC; two-way ANOVA with Newman-Keuls *post hoc* test. ALC: Acetyl-L-carnitine; CAE: Caerulein; VEH: Vehicle.

different between groups (Figure 1A). After 3 wk of a 3 × a week treatment regimen of 6 hly CAE injections, the force needed to elicit a hindpaw withdrawal response was significantly reduced in CAE + VEH (0.2 ± 0.1 g) and CAE + ALC (0.2 ± 0.1 g) groups, in comparisons to VEH + ALC (1.3 ± 0.1 g, $P < 0.05$) and naïve controls (2.3 ± 0.5 g, $P < 0.0001$; two-way ANOVA with Newman-Keuls *post hoc* test). However, using saline alone in this injection scheme was also able to cause significant mechanical hypersensitivity in VEH+ALC mice compared to naïve controls ($P < 0.05$). At the 6-wk timepoint, CAE + ALC and VEH + ALC mice had received twice daily ALC injections (i.p.) for 3 wk while CAE + VEH animals were given twice daily saline injections. ALC attenuated mechanical hypersensitivity in animals with CAE-induced pancreatitis while it did not alter mechanical withdrawal thresholds of VEH + ALC mice. Mechanical withdrawal thresholds of CAE + VEH (0.1 ± 0.1 g, $P < 0.001$), CAE + ALC (0.9 ± 0.2 g, $P < 0.05$), and VEH + ALC (1.1 ± 0.2 g, $P < 0.05$) were still significantly reduced compared to naïve controls (2.0 ± 0.4 g, two-way ANOVA with Newman-Keuls *post hoc* test). However, CAE + VEH responded to significantly lower mechanical stimuli compared to VEH + ALC ($P < 0.05$). Similarly, heat response latencies using the hotplate test were not different at baseline (Figure 1B). At the 3 wk timepoint, response latencies of both CAE + VEH (12.2 ± 1.1 s) and CAE + ALC (12.2 ± 0.8 s) were significantly reduced compared to VEH + ALC (17.2 ± 0.1 s, $P < 0.0001$) and naïve control (17.6 ± 1.1 s, $P < 0.0001$; two-way ANOVA with Newman-Keuls *post hoc* test). At the 6-week timepoint, response latencies of CAE + VEH (10.4 ± 0.4 s) were significantly lower compared to all other experimental groups. Treatment with ALC significantly attenuated heat response latencies in CAE + ALC animals (13.4 ± 0.9 s) compared to the untreated CAE + VEH group ($P < 0.05$, two-way ANOVA with Newman-Keuls *post hoc* test), however response was still significantly reduced compared to the two control groups (VEH + ALC = 15.5 ± 1.0 s; naïve control = 16.8 ± 0.8 s; $P < 0.01$, two-way ANOVA with Newman-Keuls *post hoc* test).

Anxiety-like responses

In week 6, anxiety-like behavior was determined using the elevated plus maze

(Figure 2). The CAE + VEH mice spent significantly more time in the closed arm (252.2 ± 3.8 s; $P < 0.05$) and significantly less in the center and open arm areas (center = 26.6 ± 3.5 s; open arm = 21.3 ± 4.3 s; $P < 0.05$) compared to uninjected naïve control animals (Figure 2A). This behavior was not different from the CAE + ALC group (closed arm = 240.7 ± 11.6 s; center = 32.4 ± 5.9 s; open arm = 26.8 ± 6.9 s) when compared to both control groups (VEH + ALC: closed arm = 209.5 ± 18.9 s; center = 47.0 ± 9.7 s; open arm = 43.5 ± 10.8 s; naïve control: Closed arm = 199.1 ± 14.3 s; center = 45.6 ± 4.9 s; open arm = 55.4 ± 13.0 s). No group differences were determined in the number of times animals entered the different zones of the elevated plus maze (Figure 2C). However, mice in the CAE + VEH group reared significantly less in the closed arm (Figure 2B) when compared to both control groups (CAE + VEH = 8.2 ± 1.6; VEH + ALC = 13.5 ± 3.7; naïve control = 13.6 ± 1.6, $P < 0.01$, one-way ANOVA) which was alleviated in the treatment group with ALC (14.3 ± 1.9).

In the open field test, all repeatedly injected animal groups, including those receiving saline, displayed altered behavior compared to uninjected naïve control animals. Compared to the other groups, naïve control mice traveled a significantly greater ambulatory distance (naïve control = 9466 ± 208, VEH + ALC = 7019 ± 720; CAE + VEH = 6592 ± 340; CAE + ALC = 6517 ± 817; $P < 0.05$, one-way ANOVA), reared significantly more often (naïve control = 176 ± 11, VEH + ALC = 97 ± 24; CAE + VEH = 102 ± 8; CAE + ALC = 116 ± 27; $P < 0.05$, one-way ANOVA), and spent significantly less time resting (naïve control = 282 ± 20 s, VEH + ALC = 579 ± 78 s; CAE + VEH = 606 ± 37 s; CAE + ALC = 588 ± 98 s; $P < 0.05$, one-way ANOVA). Thigmotaxis, a tendency to stay in the periphery close to the walls typically interpreted as an anxiety measure, was not detected in experimental groups.

Pancreas morphology

In both cohorts, pancreata were excised after perfusion with heparinized 0.9% saline and photographed floating in 0.9% saline (Figure 3A). Pancreas dissection occurred either one day (Figure 3A, top row) or two days (Figure 3A, bottom row) after the final CAE injection day. On day 1 after the final CAE injections, pancreata excised from animals with CAE-induced pancreatitis were significantly more translucent compared to both control groups (Figure 3B). The charcoal stained Sylgard filling in the dissection dish was more readily visible through the translucent tissue, and tissue brightness was reduced in pancreata from mice with CAE-induced pancreatitis (CAE + VEH = 146 ± 8 arbitrary units (a.u.); CAE + ALC = 151 ± 7 a.u.) compared to the two control groups (VEH + ALC = 171 ± 3 a.u.; naïve control = 194 ± 4 a.u., $P < 0.01$, one-way ANOVA, Tukey post hoc test). Treatment with ALC thus did not prevent the effect of CAE-induced pancreatitis when observed immediately post-treatment. However, when animals were dissected on day 2 after the final CAE injection, this translucence was significantly improved in pancreata from mice with CAE-induced pancreatitis (CAE + VEH = 192 ± 6 a.u.; CAE + ALC = 202 ± 3 a.u.). No difference of tissue opacity was determined in pancreata dissected on day 2 during group comparison (Figure 3C).

Histological staining of pancreatic tissue sections using Sirius Red and Fast Green (Figure 3D) identified extensive fibrosis and acinar cell atrophy in pancreata from mice of both groups with CAE-induced pancreatitis (Figure 3D). Histopathological scores of pancreatic tissue sections (Figure 3E) were significantly higher in CAE + VEH (CAE + VEH = 4.7 ± 0.4 a.u.) compared to both control groups (naïve control = 1.8 ± 0.2, VEH + ALC = 1.9 ± 0.7). While treatment with ALC reduced tissue damage (CAE + ALC = 3.8 ± 0.6 a.u.), the histopathological score remained significantly increased compared to both control groups and was not significantly different from the CAE + VEH group.

Histologically determined microglial responses

Post-mortem immunohistochemical detection of Iba1 staining, a biomarker for microglia and macrophages, was used to study several different higher brain regions involved in pain processing and anxiety. Due to the limited efficacy of ALC to reduce pain- and anxiety-related behaviors, the histological study was conducted solely on brain sections from animals with CAE-induced pancreatitis and naïve controls. Microglial morphology was quantified by measuring somal and convex hull area, the smallest convex polygon needed to surround the whole cell shape, soma and processes[49], and intensity of Iba1 immunostaining. In the following histological figures (Figure 4-8), the mouse brain atlas section at bregma -2.18 mm, interaural 1.62 mm[50,51] is shown as a reference with the analyzed area outlined in yellow.

Immunohistochemical localization of the microglial biomarker Iba1 in the basolateral (BLA) amygdala (Figure 4A) is shown in sample brain sections from naïve controls (Figure 4B) and CAE + VEH (Figure 4C). The microglial convex [Figure 4D;

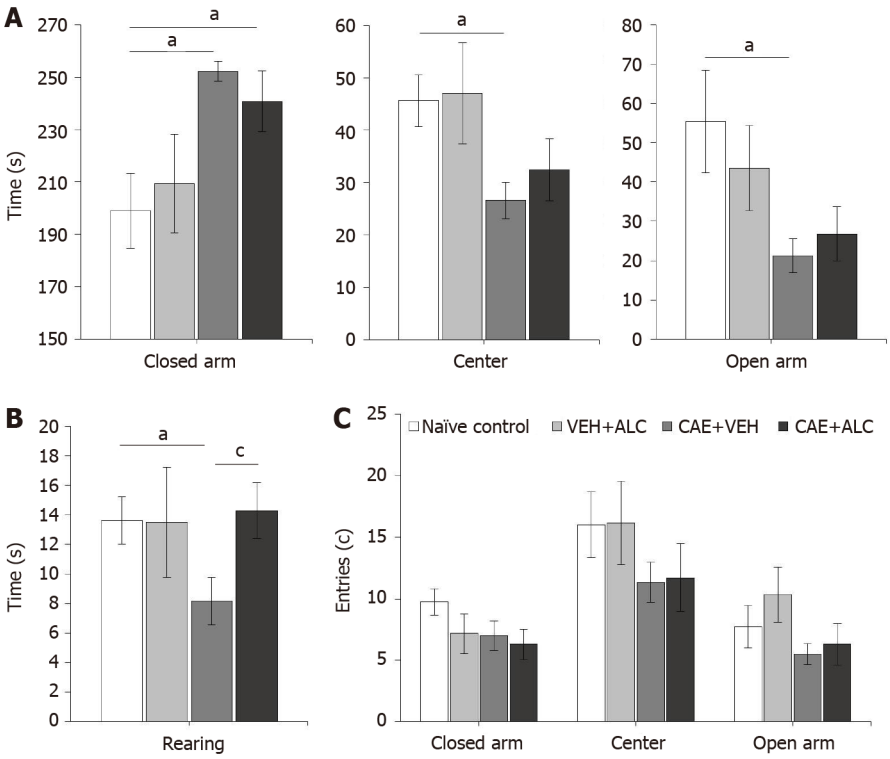


Figure 2 Anxiety-like behavior was measured in week 6 of caerulein-induced pancreatitis but acetyl-L-carnitine treatment only increased the number of rearing events. A: Mice with caerulein- (CAE) induced pancreatitis spent significantly more time in the closed arm, and less in the center and open arm of the plus maze compared to naïve control animals. Treatment with acetyl-L-carnitine (ALC) had no effect; B: Mice with CAE-induced pancreatitis reared significantly less compared to naïve control animals. This was alleviated after 3 wk of treatment with ALC; C: No differences were detected between groups in the number of entries to the different zones of the elevated plus maze. n = 6/group; $^{a}P < 0.05$ compared to naïve control; $^{c}P < 0.05$ compared to CAE + ALC; two-way ANOVA with Newman-Keuls *post hoc* test. ALC: Acetyl-L-carnitine; CAE: Caerulein; VEH: Vehicle.

CAE + VEH (n = 59) = 2157 ± 89 μm², naïve control (n = 52) = 1493 ± 89 μm², $P < 0.001$, Student's t-test] and somal areas (Figure 4F; CAE + VEH = 38.0 ± 1.4 μm², naïve control = 32.2 ± 1.7 μm², $P < 0.01$, Student's t-test) in CAE + VEH animals were significantly increased compared to controls. However, while microglial staining intensity for Iba1 was significantly increased when comparing the convex area (Figure 4E; CAE + VEH = 206 ± 1, naïve control = 181 ± 1, $P < 0.001$, Student's t-test), the somas alone were not different (Figure 4G and Supplementary Table 1).

The analyzed area in the dentate gyrus of the hippocampus (Figure 5A) and sample histological images are shown in the top of Figure 5. Microglial convex [CAE + VEH (n = 57) = 2355 ± 117 μm², naïve control, (n = 54) = 1833 ± 86 μm², $P < 0.001$] and somal areas (CAE + VEH = 38.5 ± 1.2 μm², naïve control = 33.1 ± 2.2 μm², $P < 0.05$) were significantly increased in the dentate gyrus of CAE + VEH animals compared to naïve controls (Figure 5). However, staining intensity for Iba1 was not different between groups (Figure 5D and F and Supplementary Table 1).

In the central lateral (CL)/paraventricular thalamus, the convex and somal areas were both significantly increased after CAE-induced pancreatitis (Figure 6C and E; convex area: CAE + VEH = 2674 ± 143 μm², naïve control = 2201 ± 108 μm², $P < 0.01$; somal area: CAE + VEH = 36.9 ± 1.3 μm², naïve control = 24.9 ± 1.2 μm², $P < 0.001$). The Iba1 staining intensity in the convex area was significantly increased while the soma in CAE-induced pancreatitis animals had decreased staining compared to naïve controls (Figure 6D and F; convex staining: CAE + VEH = 211 ± 1, naïve control = 192 ± 1, $P <$ 0.001; somal staining: CAE + VEH = 36.9 ± 1.3, naïve control = 24.9 ± 1.2, $P < 0.001$).

In the arcuate hypothalamic (Arc)/dorsomedial nuclei, both convex hull and somal area were significantly increased after CAE-induced pancreatitis (Figure 7C and E: convex area: CAE + VEH = 2512 ± 122 μm², naïve control = 1941 ± 101 μm², $P < 0.01$; somal area: CAE + VEH = 35.7 ± 1.7 μm², naïve control = 28.6 ± 1.3 μm², $P < 0.001$), while Iba1 staining intensity was not different (Figure 7D and F and Supplementary Table 1).

Surprisingly, microglia in the primary sensory cortex (S1) after CAE-induced pancreatitis had significantly smaller somal areas compared to naïve controls (Figure 8E; CAE + VEH = 37.2 ± 1.0 μm², naïve control = 42.4 ± 1.7 μm², $P < 0.01$) and

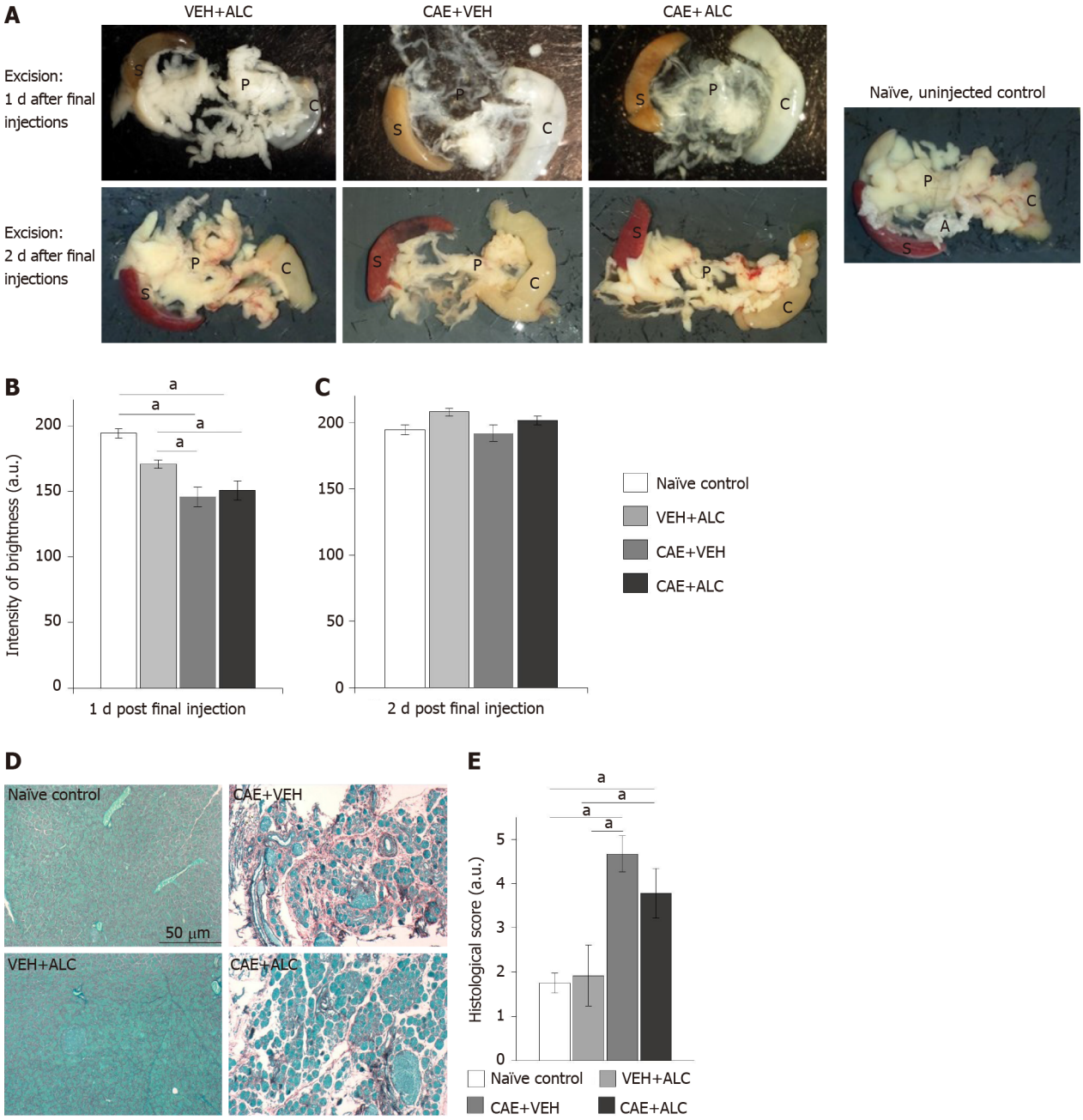


Figure 3 Caerulein-induced pancreatitis caused tissue translucence of the pancreas that was reversed within 2 d in the absence of pharmacological intervention as well fibrosis and acinar cell atrophy. A: Top row shows pancreata excised at experiments end in week 6, one day after the final injections. Bottom row shows pancreata excised at experiments end two days after final injections. On the far right is an opaque pancreas from a naïve control animal that was never injected; B: Tissue from caerulein (CAE) + vehicle (VEH) and CAE + acetyl-L-carnitine (ALC) were significantly darker, revealing the charcoal stained Sylgard in the dissection dish below, shining through the translucent pancreas compared to naïve controls when excised 1 d after the last injection days in week 6. Naïve control n = 6; VEH + ALC n = 3/timepoint; CAE + VEH n = 3/timepoint; CAE + ALC n = 3/timepoint; C: No group differences were noted on day 2 post final injection day in week 6; D: Pancreatic tissue sections stained with Sirius Red and Fast Green identified wide-spread fibrosis (red) and atrophy of the acinar parenchymal tissue only in CAE + VEH and CAE + ALC samples. Scale bar 50 μm; E: Average scores (1 = no damage to 5 = severe damage) of Sirius Red and Fast Green-stained pancreatic tissue sections were significantly higher in CAE + VEH and CAE + ALC samples compared to both control groups. Naïve control n = 4; VEH + ALC n = 4; CAE + VEH n = 3; CAE + ALC n = 3; $^{a}P < 0.05$; two-way ANOVA with Newman-Keuls *post hoc* test. A: Adiposita; C: Colon; P: Pancreas; S: Spleen; ALC: Acetyl-L-carnitine; CAE: Caerulein; VEH: Vehicle.

were otherwise not different from naïve controls (Supplementary Table 1).

The number of microglia was not different between the two groups in any of the sampled brain regions (amygdala, hippocampus, thalamus, hypothalamus, primary sensory cortex).

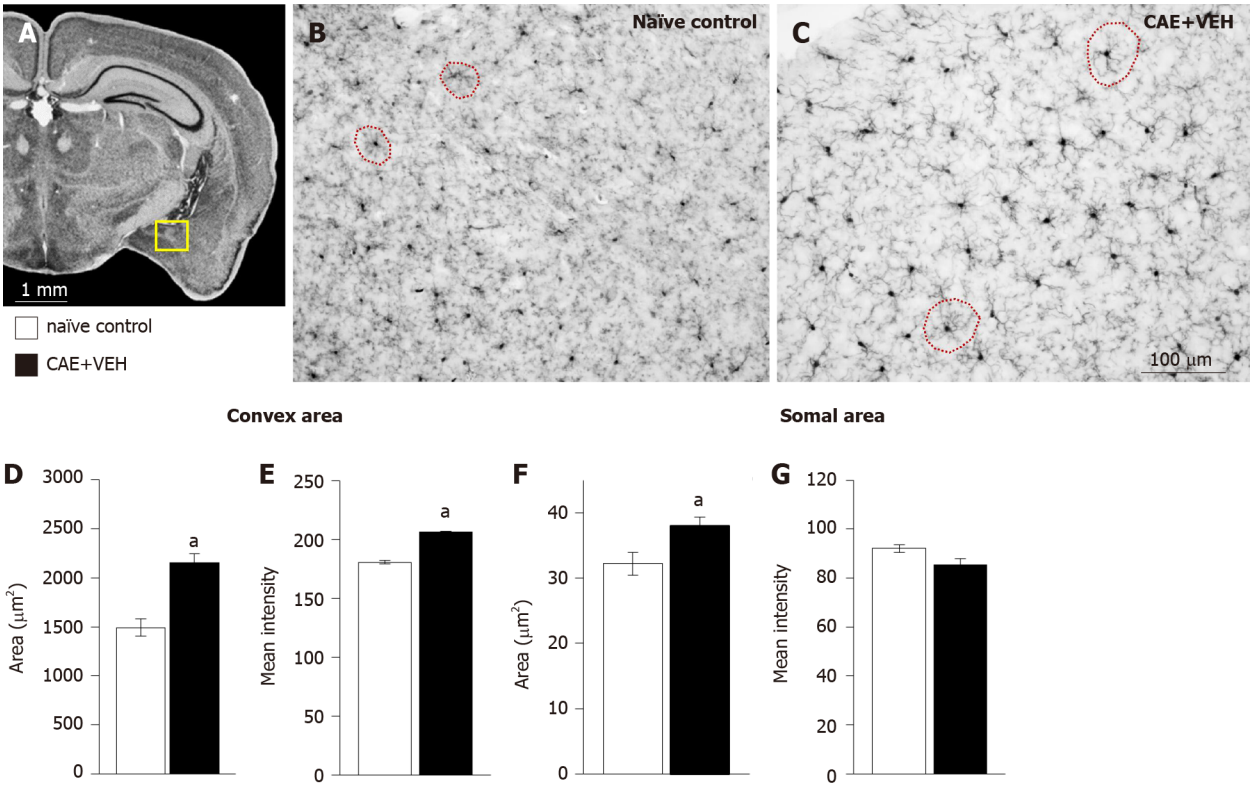


Figure 4 Microglia in the basolateral amygdala were activated only in mice with caerulein-induced pancreatitis. A: Overview of the mouse brain shown at bregma -2.18 mm, interaural 1.62 mm[50,51]. The quantified area, the basolateral amygdala (BLA), is outlined in yellow; B and C: Examples of BLA from naïve control and vehicle treated caerulein- (CAE) induced pancreatitis mice were stained for ionized calcium-binding adaptor molecule 1; D and E: The convex area, the hull region of the microglia (samples outlined in red in B and C), was significantly enlarged and more intensely stained in mice with CAE-induced pancreatitis; F and G: Similarly, the somal area was significantly enlarged in tissue from mice with CAE-induced pancreatitis, though, the intensity of staining was not different. Naïve control $n = 4$, CAE + VEH $n = 5$, $^{a}P < 0.05$ Student's *t*-test. CAE: Caerulein; VEH: Vehicle.

DISCUSSION

The present study identified microglial activation in supraspinal regions of the central nervous system in mice with CAE-induced recurrent AP pain and anxiety-like behaviors which were attenuated by ALC. Recurrent bouts of AP were modeled by repeated CAE injections into the abdomen[39-42] which caused extensive fibrosis and acinar cell atrophy in the pancreas. The number of weekly injection days in the present study was increased from the commonly used two to three days as the observed effect was too unstable using only two weekly injection days based on a previous report[41]. However, 3 wk of repeated control saline vehicle injections were also able to induce secondary mechanical and heat hypersensitivity measured on the hindpaws when compared to naïve control animals, despite conscious attempts to avoid repeated injections at the same site. This suggests that while the CAE-induced pancreatitis model induces inflammation in the pancreas[52,53], repeated abdominal injections alone can cause a painful, structural injury in the abdominal wall[54]. This chronic abdominal wall pain caused weight loss (Supplementary Figure 1) and induced pain-related behaviors that were similar to animals injected with CAE. Clinical reports estimate that approximately 2% of emergency room patients have abdominal wall pain caused by incisional or abdominal wall hernias which usually have a small trigger point[54,55]. These patients are typically treated with lidocaine and analgesics to block the development of chronic pain suggesting a potentially similar pain mechanism may contribute to hypersensitivity caused by repeated abdominal injection in the CAE-induced pancreatitis model[53,56].

The CAE-induced pancreatitis model produced secondary mechanical and heat hypersensitivity measured on the hindpaws that was attenuated by prolonged post treatment with the nutraceutical ALC in weeks 4-6. A previous study demonstrated that daily 7-d pre-treatment with ALC prior to induction of AP with CAE alleviated biochemical and histological symptoms[22]. ALC acts not only as an antioxidant but also increases/restores mitochondrial function in models of AP[23,24]. Other studies using CAE to induce pancreatitis have shown mitochondrial damage in parenchymal acinar

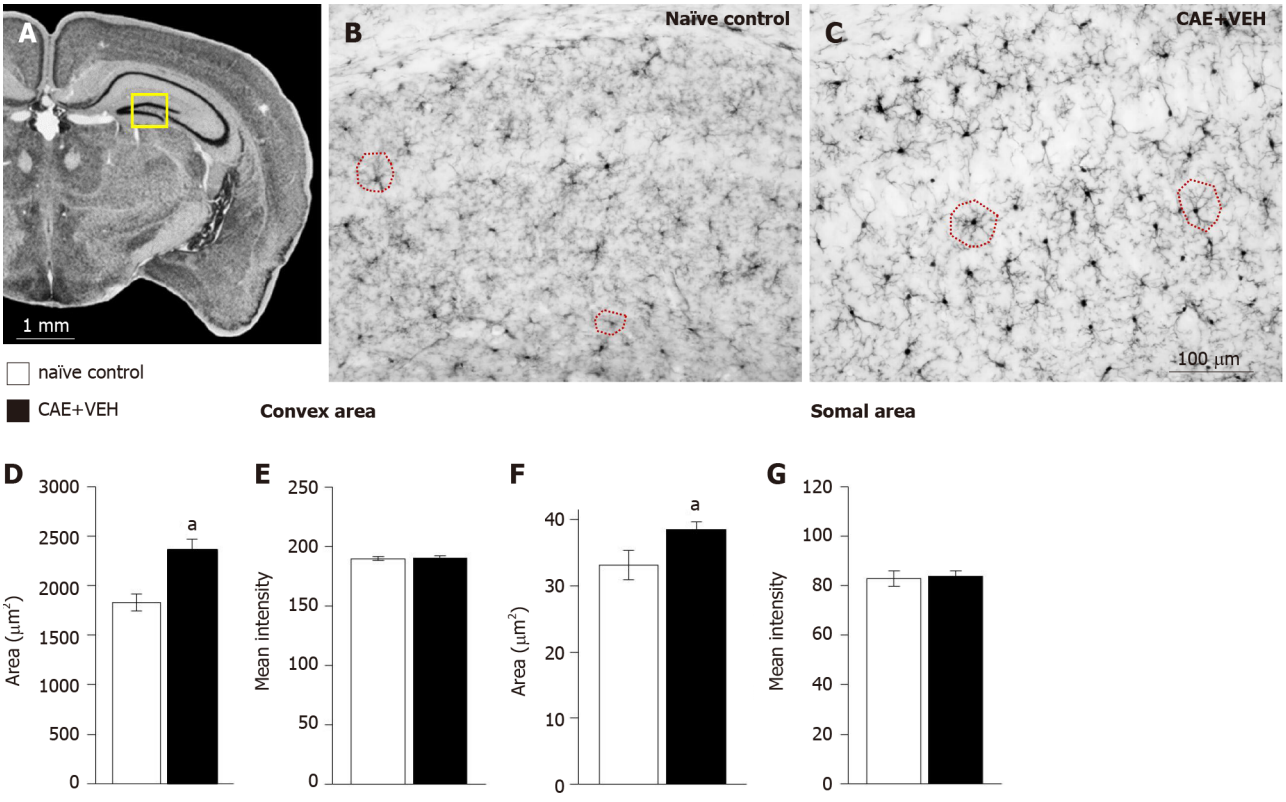


Figure 5 Enlarged microglial cells were measured in the dentate gyrus of mice with caerulein-induced pancreatitis compared to control samples. A: The analyzed area is outlined in yellow in an overview of the mouse brain at bregma -2.18 mm, interaural 1.62 mm[50,51]; B and C: Microglia in the dentate gyrus of naïve control and in the caerulein- (CAE) induced pancreatitis mice were measured; D-G: Microglial convex (examples circled in red in B and C) and (F) somal area were significantly enlarged after 6 wk of CAE-induced pancreatitis while (E and G) intensity of ionized calcium-binding adaptor molecule 1 (Iba1) immunoreactivity was unchanged. Naïve control $n = 4$, CAE + VEH $n = 5$, $^{a}P < 0.05$ Student's *t*-test. CAE: Caerulein; VEH: Vehicle.

cells and the release of reactive oxygen species[18-20]. Part of the pathogenesis of pancreatitis includes mitochondrial dysfunction and endoplasmatic reticulum stress which can progress to pancreatic cell death, fat vacuolization/necrosis, and fibrosis also observed here, symptoms of chronic pancreatitis[57,58]. Treatment with ALC attenuated pain-related behaviors, reducing them to the level equivalent to control animals injected with vehicle. The remaining hypersensitivity may reflect the contribution of the abdominal wall injury induced by the repeated i.p. injections needed for the CAE-induced pancreatitis model. However, since pancreatic tissue damage was not significantly improved in CAE-ALC mice compared to CAE-VEH animals, this may be an indicator of the low efficacy of ALC in this long-term model.

In the present study we used two different tests of anxiety-like behaviors in the CAE-induced recurrent AP model and received differing results. Anxiety-like behaviors were measured in both ALC and vehicle treated mice with CAE-induced pancreatitis with the elevated plus maze. However, only the number of rearing events was restored in the CAE + ALC group, likely due to the reduced hypersensitivity. The decrease in rearing events in the CAE-induced pancreatitis group is likely relevant to gravity pressure increase in abdominal hypersensitivity that is relieved by the ALC, rather than anxiety level. No differences were detected between the two control groups, VEH + ALC and naïve controls. However, in the open field test and all animals that received repeated injections had altered general ambulatory behavior compared to naïve control mice, while none displayed thigmotaxis, *i.e.*, avoidance of the arena center, an indicator of anxiety-like behavior. Differences in ambulatory behavior measured in the open field test is interpreted to be associated with abdominal wall injury pain caused by the repeated injections.

In the present study the elevated plus maze may have presented a more challenging situation to the animals. The plus maze is well established as a test well suited for the detection of anxiety-like behavior[59]. The use of this test in the recurrent AP model in week 6 may have been too early to detect anxiety-like behavior clearly. It is reported that anxiety- and depression-like behavior develop 6-8 wk after induction of animal models of chronic pain[60]. In many sciatic neuropathic pain models, pain behaviors resolve within 3-4 wk, so that anxiety- and depression-like behaviors have not yet

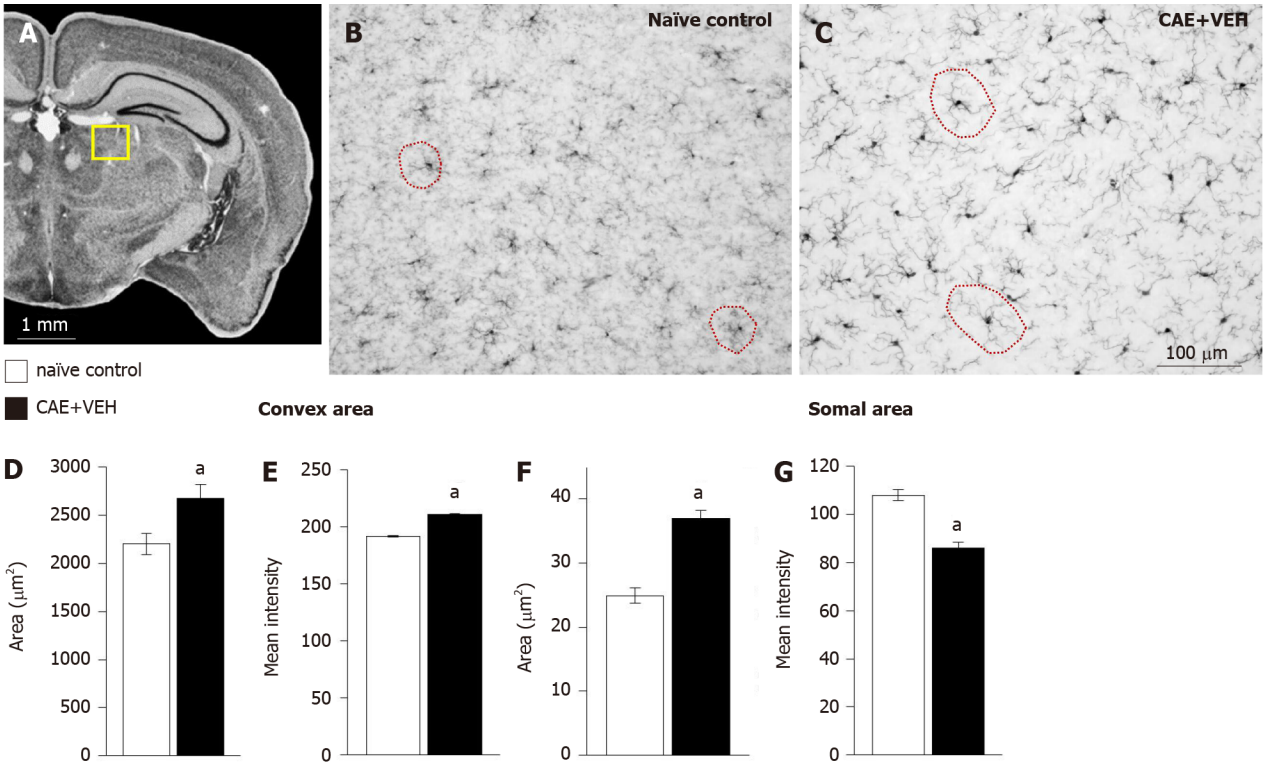


Figure 6 In the central lateral and paraventricular thalamic nuclei, microglial areas in mice with caerulein-induced pancreatitis were enlarged. A: An overview of the mouse brain at bregma -2.18 mm, interaural 1.62 mm[50,51] is shown with the analyzed central lateral and paraventricular thalamic nuclei thalamic area analyzed outlined in yellow. B and C: Examples of microglia of naïve control and caerulein- (CAE) induced pancreatitis mice with sample convex areas circled in red; D and E: Microglial convex area and ionized calcium-binding adaptor molecule 1 (Iba1) staining intensity were significantly enhanced in animals with CAE-induced pancreatitis; F and G: Similarly, somal areas were significantly enlarged, yet, their Iba1 staining intensity was decreased. Naïve control $n = 4$, CAE + VEH $n = 5$, $^{a}P < 0.05$ Student's *t*-test. CAE: Caerulein; VEH: Vehicle.

developed sufficiently and pain is not yet chronic[61,62]. Implication of developing anxiety is that the recurrent bouts in the conditions reported here are approaching a chronic pancreatitis state. Pancreatitis is a progressive disease and despite the unwanted abdominal wall pain induced by repeated CAE or saline injections, the CAE model of acute and recurrent AP adapted here is progressing to chronic pancreatitis[1-3,39-41,63].

In the present study of persistent pancreatitis, whole pancreas tissue translucence was used as an indirect measure of tissue healing since serum lipase levels are only elevated in patients with AP and normal in chronic pancreatitis[64]. Pancreata excised one day post final CAE injections at experiment's end were translucent, a sign we have previously reported for a model of chronic pancreatitis[58]. Two days after the final CAE injections, spontaneous healing even in the absence of pharmacological intervention was detectable as tissue opacity returned to normal. Pancreatic tissue regeneration after a single day of repeated CAE injections was demonstrated by reduced inflammatory cells within 2 d and healthy pancreatic morphology 7 d after injections[65]. In the present study, extensive fibrosis and acinar atrophy was detected in pancreata collected 2 d after the final CAE injections. The 3-wk treatment with ALC was not able to protect from CAE-induced pancreatic tissue damage, thus suggesting ongoing inflammation and tissue disruption in the pancreas was contributing to behavioral hypersensitivity.

Constant peripheral activation of nociceptors can lead to central sensitization, increase of ascending pain transmission and activation of numerous higher pain modulation center, as well as decreased descending pain inhibition and neurogenic inflammation. It is this combination that makes understanding the transition from acute to chronic pancreatitis pain so difficult to investigate and mitigate[66-68]. The present investigation focused on microglial activation as an indicator of regional central sensitization caused by persistent pancreatitis pain in the CAE-induced mouse model at 6 wk. Microglia had significantly increased Iba1 immunostaining with increased somal and convex areas, as well as a ramified appearance after 6 wk of untreated inflammation in the pancreas. These resident immune cells in the central nervous system have multiple functions including potential to increase neuronal

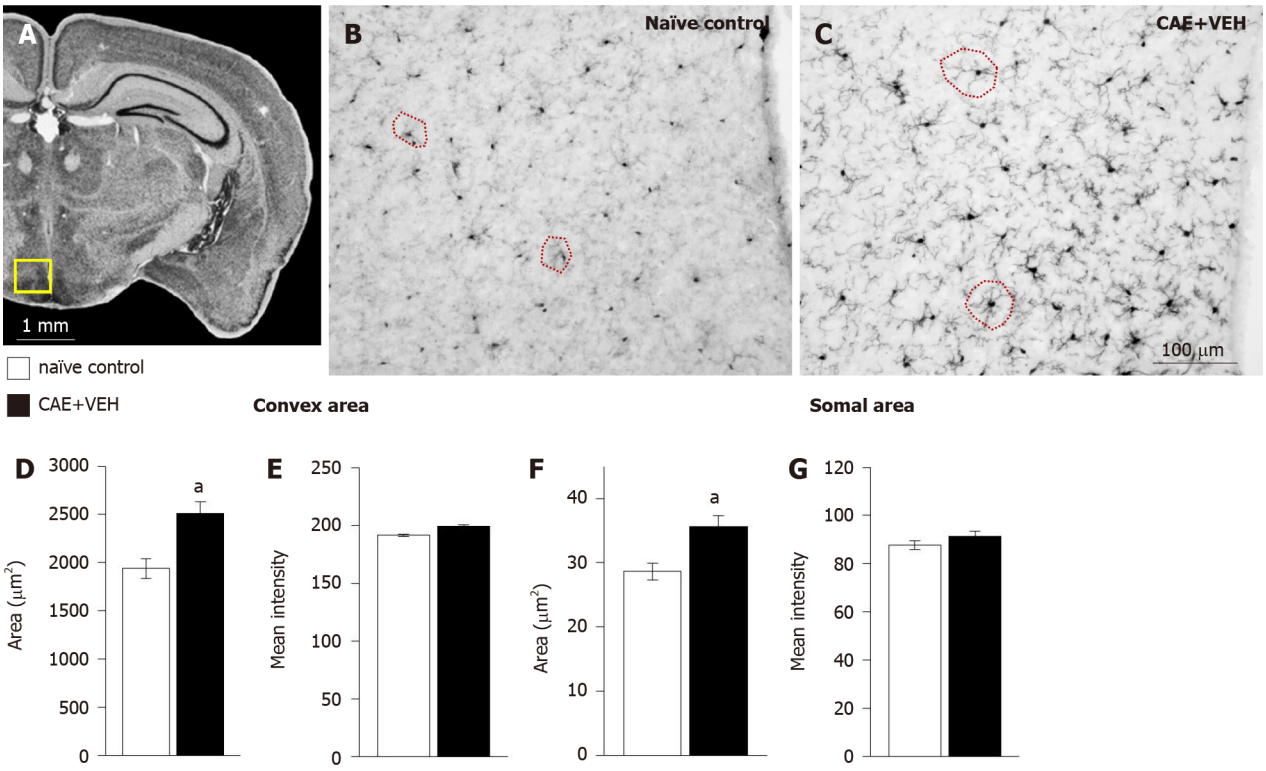


Figure 7 In the hypothalamic arcuate and dorsomedial nuclei, enlarged microglia were measured in mice with caerulein-induced pancreatitis. A: The analyzed area is outlined in yellow in an overview of the mouse brain at bregma -2.18 mm, interaural 1.62 mm[50,51]; B-D and F: Microglia in arcuate and dorsomedial nuclei of naïve control and (C) caerulein- (CAE) induced pancreatitis mice had (D) significantly different convex (samples circled in red in B and C) and (F) somal areas; E and G: Intensity of ionized calcium-binding adaptor molecule 1 (Iba1) immunoreactivity was not different. Naïve control $n = 4$, CAE + VEH $n = 5$, $^{a}P < 0.05$ Student's *t*-test. CAE: Caerulein; VEH: Vehicle.

activation. In their activated, pro-inflammatory M1 phenotype, they release cytokines and chemokines to attract other immune cells that alter neurogenesis and synaptic connectivity; while the invading macrophage M2 phenotype is anti-inflammatory[28-30,69]. Previous reports demonstrated activated microglia in the spinal cord after 3-4 wk of experimental pancreatitis[27,31]. Studies on somatic pain have identified activated microglia in the spinal cord of rats as early as 3-7 d after partial sciatic nerve ligation. However, reports on supraspinal regions are controversial with some finding no activated microglia[32,34,70], while others have found activated microglia[71,72]. This may be due to the post injury time point studied or indicative of differences between somatic and visceral pain related stress mechanisms[73,74].

Here we demonstrated that microglial activation in the CAE-induced pancreatitis model occurs at several sites within the brain associated with pain modulation and affective disorders. The spinothalamic tract relays noxious information to the thalamic ventral posterolateral (VPL) nucleus. However, cognitive awareness of deep tissue and organ interoception is "silent" until inflammation, chemical or mechanical distention initiates a visceral pain sensation as experienced by many patients with pancreatitis. Pain arising from inflamed viscera is relayed not only to the VPL but also to brainstem sites, the ventromedial, intralaminar CL, and parafascicular thalamic nuclei[75,76]. The CL has connectivity with the parietal/frontal cortex playing a role in arousal and excitability, responding to a variety of stimuli including intrapancreatic bradykinin, intraperitoneal dilute acetic acid, noxious colorectal distention, and greater splanchnic nerve electrical stimulation[76]. Electrophysiological studies have revealed that CL in particular receives information about pancreatic pain when it is stimulated by noxious input[76]. In fact, most CL neurons (93%) have increased responsivity to noxious bradykinin stimulation of the pancreas but do not respond to somatic stimuli. Responses of some CL and other intralaminar thalamic neurons are reported that have both visceral and somatosensory input when a cutaneous search stimuli is used as the initial search stimulus[77-79]. Activated microglia have been detected in the thalamus of patients with chronic lower back pain[80]. The activated microglia in the CL thalamus of CAE-induced pancreatitis are reflective of this noxious stimulation.

Neural circuitry remodeling during chronic pain is accompanied by comorbid anxiety and depression[67,71]. Brain remodeling during chronic pain recruits limbic and

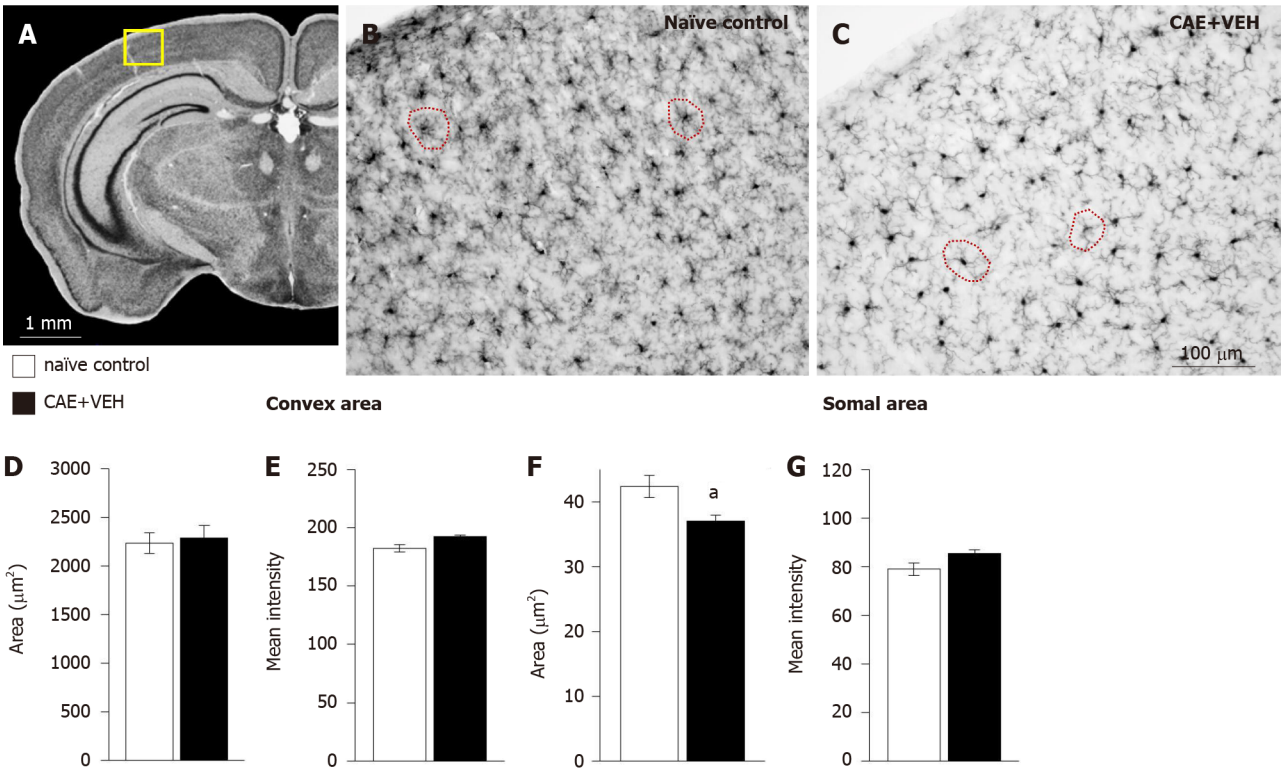


Figure 8 Microglia in the primary somatosensory cortex of mice with caerulein-induced pancreatitis were similar to those from controls.
A: The analyzed area is outlined in yellow in an overview of the mouse brain at bregma -2.18 mm, interaural 1.62 mm[50,51]. B-E: Microglia in (B) naïve control and (C) caerulein- (CAE) induced pancreatitis mice had (D) similar convex areas and (E) staining intensity for ionized calcium-binding adaptor molecule 1 (Iba1); F and G: In fact, their somal areas were significantly decreased in animals with CAE-induced pancreatitis compared to naïve mice, and their Iba1 staining intensity was not different. Naïve control $n = 4$, CAE + VEH $n = 5$, $^{a}P < 0.05$ Student's *t*-test. CAE: Caerulein; VEH: Vehicle.

reward/aversion circuitries, integral components of the chronic pain syndrome that provide an emotional context and suffering[81]. This includes the amygdala which is comprised of several nuclei of which the BLA, central (CeA) and lateral (LA) amygdala are centrally involved in modulating pain, emotional processing, the emotional aspect of pain, and neuropsychiatric disorders[82]. Activated microglia release pro-inflammatory cytokines and chemokines, contributing to physiological, as well as brain circuitry activation alterations during the chronification of pain[69]. Activated microglia provide indication that these limbic brain regions are activated by the ongoing pancreatic pain.

The spinoparabrachial pathway relays noxious information to the CeA, also called the nociceptive amygdala, where strengthening of this synaptic connection has been shown to contribute to neuropathic somatic pain[70,83,84]. However, the CeA forms a circuit with LA/BLA that has been shown to modulate pain related behaviors and affective behaviors. Activated microglia within the BLA in the CAE pancreatitis model may be an indirect indicator of dysfunctional neuronal activity within the BLA or the driving force for neuronal hyperexcitability through pro-inflammatory mediator release and thus contributing to anxiety-like behavior after 6 wk (Figure 4). Increased neuronal activity within the BLA has been recorded during chronic visceral hypersensitivity in rats[85]. During somatic neuropathic pain, the excitability of the synaptic connection from BLA to CeA has been shown to be increased and the reduction of this dysfunctional hyperexcitability alleviated pain-related behaviors[84]. In contrast, the inhibitory BLA-prefrontal cortex connection is increased during persistent pain which impairs emotion-based decision making, decreasing the ability to assess risk-benefit outcomes[82,86]. Most recently it was demonstrated that the BLA encodes the negative affective quality of chronic pain as opposed to only mediating the withdrawal from a noxious stimulus[87].

The BLA has direct, excitatory synaptic connections to the hippocampus, a brain structure involved in emotionality, learning, and memory[88]. In the spared-nerve injury rodent model pain behaviors were correlated with increased pro-inflammatory cytokines in the hippocampus[89]. Activated microglia as seen in our experiments in the dentate gyrus (Figure 5) could be contributing to this and result in aberrant behaviors. Chronic mild stress exposure causes hippocampal microglia activation and increased

pro-inflammatory mediators concurrent with anxiety- and depression-like behaviors[90]. Activated hippocampal microglia during sciatic nerve injury induced chronic stress have been proposed to suppress neurogenesis in the dentate gyrus[72]. Animals in the present study were unintentionally stressed by the repeated abdominal injections, possibly directly contributing to microglial activation in the hippocampus. Hippocampal abnormalities have been reported in an animal model of peripheral neuropathic pain[91]. In a previous study, persistent neuropathic pain influenced ability of animals to adapt to environmental demands suggesting impaired cognitive ability[92]. In patients with chronic back pain, the hippocampal volume was shown to be decreased using magnetic resonance imaging (MRI)[93]. These patients also had altered connectivity of amygdala, medial prefrontal cortex/anterior cingulate gyrus, and hippocampus evident with MRI have been demonstrated to be correlated with pain level and duration contributing not only to the chronification of pain but also to the development of comorbid anxiety[94-96].

Absence of microglial activation in S1 cortex in CAE-induced pancreatitis in the present study may be indicative of its normalized neural activity in persistent pain. This is in contrast to acute pain models which found increased neuronal activity at rest in the barrel field of S1 after chronic constriction of the infraorbital trigeminal nerve branch using the immunohistochemical neuronal activity biomarkers cFOS and pERK[97]. However, decreased regional cerebral blood flow, an indirect measure of neuronal activity, has been recorded in the cortex of clinical patients with longstanding trigeminal neuropathic pain reportedly associated with grey matter volume decrease in these regions[98,99]. Youssef *et al*[100] reported decreased regional cerebral blood flow in patients with chronic posttraumatic neuropathy compared to healthy subjects using the more accurate arterial spin labeling MRI technique, suggesting that dysfunctional central changes and loss of descending inhibition may be essential for maintenance of chronic pain[101].

Chronic pain can also be amplified through physiological stress caused by aberrant hypothalamic signaling[102]. Observed activated microglia in Arc and dorsomedial nuclei (DM) may be indicative of reduced descending pain control as has been reported in an arthritis model when the DM is activated[103]. Nociceptive spinal projection neurons synapse in the hypothalamus and thalamus[104]. Thalamus and hypothalamus are reciprocally connected. Excitatory connectivity from midline hypothalamic Arc and DM to the paraventricular nucleus in the thalamus also contributes to fear and anxiety-like behaviors. During chronic stress, the paraventricular nucleus regulates hypothalamic-pituitary-adrenal (HPA) axis and stress hormone release *via* CeA[105]. Activation of Arc neurons can suppress pain responses to acute noxious stimulation in rats[106]. However, enhanced synaptic connectivity was identified in hypersensitive rats with chemically induced persistent pancreatitis[107]. Patients with fibromyalgia suffering from chronic wide-spread pain were reported to have aberrant activation of the HPA axis. Their cortisol levels were significantly higher after stress tests than in healthy subjects, demonstrating dysfunction of the HPA axis in chronic pain[108].

CONCLUSION

The findings presented here identify multiple contributors to the CAE-induced recurrent AP model. These include continued activation of pro-nociceptive signaling caused by the repeated abdominal injections themselves, continued inflammation and resulting tissue damage in the pancreas, and activation of microglia in brain sites that signal persistent pain. Treatment with ALC significantly attenuated CAE-induced hypersensitivity but did not attenuate pancreatic histopathology. The activation of supraspinal microglia in pain and anxiety brain circuitry may be major contributors to the enhancement and perpetuation of a chronic pain state.

ARTICLE HIGHLIGHTS

Research background

Pancreatitis is a multifactorial, progressive disease developing from acute to recurring and chronic pancreatitis with increased risk of pancreatic cancer. Chief clinical complaint is intractable abdominal pain that does not respond to medication/narcotics.

Research motivation

Pancreatitis-induced pain is driven by peripheral tissue damage, inflammation, and oxidative stress. This abdominal pain is only poorly alleviated with present pharmacological interventions, including opioid analgesics, which pose a high risk of addiction and other serious adverse events such as bowel dysfunction, increasing of abdominal pain, and respiratory suppression.

Research objectives

The present investigation had two aims: (1) Can the antioxidant and mitochondrial enhancer ALC attenuate pain- and anxiety-like behavioral changes during 6 wk of recurrent acute pancreatitis; and (2) Does recurrent acute pancreatitis activate brain microglia along the pain neuraxis and contribute to central sensitization and the initiation/maintenance of chronic pain.

Research methods

The CAE-induced pancreatitis model with progression to chronic pancreatitis was employed in male C57BL/6J mice to investigate pain- and anxiety-like behaviors during a 6-week time course and the efficacy of ALC to alleviate them determined. Post-mortem analysis of microglial activation in pain- and anxiety-related brain regions from naïve animals was compared to vehicle-treated mice with CAE-induced pancreatitis.

Research results

The persistent recurring pancreatitis model induces mechanical and heat hypersensitivity, as well as pain related anxiety measures. Vehicle-treated animals with CAE-induced pancreatitis developed pain- and anxiety-like behaviors. Treatment with ALC attenuated hypersensitivity but had limited efficacy in reducing anxiety-like behaviors. Activated microglial were identified in hippocampus, thalamus, hypothalamus and amygdala of vehicle-treated mice with CAE-induced pancreatitis, but not in the somatosensory cortex.

Research conclusions

Acute recurrent pancreatitis is accompanied by brain microglia activation along the limbic pain- and anxiety-neuraxis in response to the persisting pancreatic pain.

Research perspectives

Pharmacological approaches to reduce microglial activation may identify novel non-opioid approaches for pain treatment during pancreatitis.

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ORIGINAL ARTICLE

Basic Study

Abdominal paracentesis drainage attenuates intestinal inflammation in rats with severe acute pancreatitis by inhibiting the HMGB1-mediated TLR4 signaling pathway

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Abstract

BACKGROUND

Our previous studies confirmed that abdominal paracentesis drainage (APD) attenuates intestinal mucosal injury in rats with severe acute pancreatitis (SAP), and improves administration of enteral nutrition in patients with acute pancreatitis (AP). However, the underlying mechanisms of the beneficial effects of APD remain poorly understood.

AIM

To evaluate the effect of APD on intestinal inflammation and accompanying apoptosis induced by SAP in rats, and its potential mechanisms.

METHODS

SAP was induced in male adult Sprague-Dawley rats by 5% sodium taurocholate. Mild AP was induced by intraperitoneal injections of cerulein (20 μg/kg body weight, six consecutive injections). Following SAP induction, a drainage tube connected to a vacuum ball was placed into the lower right abdomen of the rats to build APD. Morphological changes, serum inflammatory mediators, serum and

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ascites high mobility group box protein 1 (HMGB1), intestinal barrier function indices, apoptosis and associated proteins, and toll-like receptor 4 (TLR4) signaling molecules in intestinal tissue were assessed.

RESULTS

APD significantly alleviated intestinal mucosal injury induced by SAP, as demonstrated by decreased pathological scores, serum levels of D-lactate, diamine oxidase and endotoxin. APD reduced intestinal inflammation and accompanying apoptosis of mucosal cells, and normalized the expression of apoptosis-associated proteins in intestinal tissues. APD significantly suppressed activation of the intestinal TLR4 signaling pathway mediated by HMGB1, thus exerting protective effects against SAP-associated intestinal injury.

CONCLUSION

APD improved intestinal barrier function, intestinal inflammatory response and accompanying mucosal cell apoptosis in SAP rats. The beneficial effects are potentially due to inhibition of HMGB1-mediated TLR4 signaling.

Key Words: Abdominal paracentesis drainage; Severe acute pancreatitis; High mobility group box 1; Toll-like receptor 4; Nuclear factor-κB

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Core Tip: We provide the first evidence that abdominal paracentesis drainage (APD) plays a protective role in intestinal inflammation and accompanying apoptosis induced by severe acute pancreatitis (SAP). Our key findings were: (1) APD decreased serum levels of indices related to intestinal injury and improved intestinal barrier function; (2) APD alleviated intestinal inflammation and accompanying mucosal cell apoptosis; and (3) The beneficial effects of APD in ameliorating intestinal injury were due to suppressed activation of intestinal toll-like receptor 4/nuclear factor-κB signaling pathway mediated by high mobility group box protein 1. We elucidated the molecular mechanism underlying APD treatment of SAP and its complications.

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INTRODUCTION

Severe acute pancreatitis (SAP) is a rapidly progressive abdominal disease, often accompanied by multiple organ damage, with a mortality of up to 25%[1]. Intestine is the most vulnerable extrapancreatic organ and is closely related to the severity of SAP[2]. It is reported that the gut, especially the small intestine is not just a target organ for SAP but a trigger for the initiation of systemic inflammatory responses[3]. Available evidence suggests that intestinal injury, including inflammation and apoptosis, caused by SAP promotes changes in intestinal permeability and the release of inflammatory factors, resulting in bacterial translocation, secondary infection of pancreatic tissue and the amplification of systemic inflammation, eventually contributing to the high mortality rate of SAP in the early stage[4]. Therefore, preventing intestinal injury is an important part of SAP treatment. Our previous studies have proved that elimination of pancreatitis-associated ascitic fluid (PAAF) through early abdominal paracentesis drainage (APD) attenuate intestinal mucosal injury and preserves intestinal barrier function, but the potential molecular mechanisms responsible for the protective effect are still unclear[5,6].

Toll-like receptor 4 (TLR4) is a member of the mammalian pattern recognition receptor family, and it is believed to be a key transmembrane protein that transmits

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extracellular antigen recognition information to cells and triggers an inflammatory response[7]. Recent studies have shown that TLR4 plays a vital role in inflammatory initiation and organ injury during SAP[8]. Through the myeloid differentiation primary response gene 88 (*MyD88*)-dependent pathway, TLR4 can mediate the activation of nuclear factor (NF)-κB, which further induces the expression and release of inflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-6, resulting in a vicious cycle of positive feedback of inflammation[9]. The TLR4/NF-κB proinflammatory pathway has been reported to be involved in intestinal inflammation and apoptosis induced by SAP, but TLR4 can mediate downstream signal transduction only by binding to its specific ligand[10].

High mobility group box protein 1 (HMGB1) as a member of the damage-associated molecular patterns family has been confirmed to be the most important endogenous ligand for TLR4, particularly in SAP[11]. Extracellular HMGB1 as a late mediator of lethality in systemic inflammation participates in the development of multiple organ injury caused by SAP[12]. Active neutralization with anti-HMGB1 antibodies can prevent intestinal mucosal barrier dysfunction in mice with SAP[13]. Accordingly, our recent study reported that APD can significantly reduce the level of HMGB1 by draining PAAF rich in high concentrations of HMGB1[14]. Therefore, we speculate that the reduced effect of HMGB1 by early APD treatment may have a protective role in SAP-induced intestinal injury.

Here, we explored the effect of APD on intestinal inflammation and accompanying apoptosis in rats with SAP. Furthermore, experiments were conducted to determine whether HMGB1 in PAAF had a regulatory role in the intestinal TLR4 signaling pathway to confirm the protective effect *via* inhibiting HMGB1-mediated TLR4 signaling.

MATERIALS AND METHODS

Reagents

Sodium taurocholate was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The antibody specific for apoptosis regulator Bax (cat. no. ab32503) and MyD88 (cat. no. ab2064) were purchased from Abcam (Cambridge, United Kingdom). The antibodies for caspase-3 (cat. no. 9662S), transcription factor p65 (cat. no. 8242) and phosphorylated (p-)p65 (serine 536; cat. no. 3033) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, United States). The antibodies for apoptosis regulator Bcl-2 (cat. no. GTX100064) and TLR4 (cat. no. GTX13867) were purchased from GeneTex Inc. (Irvine, CA, United States). The antibody for HMGB1 (cat. no. BM3965) was purchased from Boster Biological Technology, Inc. (Wuhan, China). All other chemicals and kits employed in this study were for experimental study and were commercially available.

Animals and experimental design

Adult male Sprague-Dawley rats (weight, 190-210 g) were provided by Dashuo Animal Science and Technology Co., Ltd. (Chengdu, China). Three days prior to the experiments, all rats were housed in ventilated plastic cages, and rats were acclimated to a 12-h light/dark cycle under a standard room temperature of 23°C, and had free access to laboratory chow and water.

The animals were fasted overnight prior to the experiment but had free access to water. In the first part of the experiment, 45 rats were anesthetized with 5% isoflurane before surgery, and were randomly divided into three groups with 15 rats in each of the following groups: Sham operation, SAP, and SAP + APD. The SAP rat model was established under aseptic conditions through the retrograde infusion of 5% sodium taurocholate into the biliopancreatic duct using a pump (0.1 mL/100 g body weight, 12 mL/h) according to our previous study[15]. For the SAP + APD rat model, following SAP induction, we placed a drainage tube at the lower right abdomen which was connected to a negative-pressure suction ball. After the operation, rats received a subcutaneous injection of sterile saline every 6 h for fluid resuscitation (4 mL/100 g body weight). For the sham operation group, rats were treated in the same way without puncturing the biliopancreatic duct. All rats were killed under anesthesia at 24 h; blood samples and PAAF were obtained, and stored at -80 °C after centrifugation (1000 × *g* for 10 min). The pancreas and intestine were quickly harvested and washed with cold phosphate buffer saline (PBS) solution, and part of the pancreas and intestine were flash-frozen in liquid nitrogen or placed into 4% paraformaldehyde for subsequent experiments.

In the second part, experiments were conducted to evaluate the effect of HMGB1 in PAAF on the activity of the TLR4 signaling pathway in intestinal tissues. Fresh PAAF was aseptically collected from the SAP rats in the first part, and then centrifuged for 15 min at 4000 × g at 4 °C to obtain the sterile supernatant. Another 24 rats were injected intraperitoneally with cerulein (six consecutive injections, 20 μg/kg body weight, at 1-h intervals) to induce mild acute pancreatitis (MAP) as previously described[15], and were then randomly divided into the following four groups with 6 rats in each group: Controls (CN group), PAAF injection (PI group), PAAF + anti-HMGB1 neutralizing antibody (PIH group) and PAAF + control lgY (PIC group). CN group rats received an intraperitoneal injection of 8 mL saline solution; PI group rats received an intraperitoneal injection of 8 mL sterile supernatant of PAAF; PIH group rats received an intraperitoneal injection of 8 mL sterile supernatant of PAAF containing 200 μg anti-HMGB1 neutralizing antibody; PIC group rats received an intraperitoneal injection of 8 mL sterile supernatant of PAAF containing 200 μg control lgY. The dosage of anti-HMGB1 neutralizing antibody (rabbit anti-HMGB1 monoclonal antibody) was based on the results of our previous studies and has been proved to be effective in an *in vivo* experimental model[14]. Western blot analysis was adopted to confirm the neutralizing activity and specificity of this antibody. Before the intraperitoneal injection, PAAF and anti-HMGB1 neutralizing antibodies were incubated for 30 min at 36 °C. The rats were anesthetized 8 h after the injections, blood and intestinal tissue samples were obtained and stored at -80 °C for subsequent detection.

Histological assessment

The terminal pancreas and distal ileum tissue samples were treated with 4% paraformaldehyde, paraffin-embedded and then cut into sections 4 μm thick. Hematoxylin and eosin (HE) staining was used to examine the histology of pancreatic and intestinal samples under a Leica DM 3000 light microscope (Leica Microsystems GmbH, Wetzlar, Germany). Pathological scoring was performed by a consultant histopathologist blinded to the groups. Pathological damage of the pancreas, such as edema, hemorrhage, fat necrosis, acinar necrosis and inflammatory cell infiltrate were evaluated according to the literature[16]. With regard to the severity of intestinal injury, the grading system was utilized according to Chiu's standard[17].

Analysis of biochemical indices, D-lactate, diamine oxidase and endotoxin in serum

Biochemical indices included TNF-α, IL-6, IL-1β, HMGB1, amylase and lipase. Serum HMGB1, TNF-α, IL-6, D-lactate, diamine oxidase (DAO) and HMGB1 in PAAF were detected using an enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Institute of Biological Engineering, China). Serum endotoxin was measured using a chromogenic limulus amebocyte lysate kit (Xiamen Bioendo Technology Co., Ltd., China). The serum levels of amylase and lipase were measured using an automatic biochemistry analyzer (TC6010L; Tecom Science Corporation, Jiangxi, China).

Analysis of TNF-α, IL-6 and myeloperoxidase in distal ileum

To determine the degree of inflammatory response in the intestine, TNF-α and IL-6 protein levels and myeloperoxidase (MPO) activity were measured in distal ileal homogenates. Distal ileal tissue (0.1 g) was obtained and homogenized in ice-cold 0.9% sodium chloride solution (weight/volume, 1:9). The homogenates were centrifuged at 800 g and the supernatants were collected. The concentrations of supernatants were measured using an enhanced BCA protein assay kit. MPO activity and TNF-α and IL-6 protein concentrations in the samples were measured with ELISA kits (both Nanjing Jiancheng Institute of Biological Engineering).

Immunohistochemical staining

Immunohistochemistry was used to detect the expression of TLR4 in intestinal tissue of each group after 24 h of SAP induction. Briefly, the paraffin-embedded distal ileal tissue sections were dewaxed in xylene and rehydrated in gradient ethanol solutions, respectively, according to standard procedures. After peroxide-blocking enzyme reaction in 3% hydrogen peroxide for 10 min, the sections were boiled in sodium citrate solution (0.01 mol/L) for 5 min to repair the antibody. Subsequently, the antibody was blocked by 5% bovine serum (Boster Biological Technology Co., Ltd., Wuhan, China) for 30 min, and then incubated with rabbit anti-TLR4 at a 1:200 dilution overnight at 4 °C. After rinsing with PBS, sections were incubated with biotin-labeled goat anti-rabbit IgG (Boster Biological Technology) for 30 min at 37 °C, and

horseradish peroxidase (HRP)-labeled streptavidin (Boster Biological Technology) for 30 min at 37 ℃. Finally, the specimens were stained with diaminobenzidine tetrahydrochloride (Boster Biological Technology), nuclei were counterstained with hematoxylin, and images were captured under a Leica DM 3000 microscope which were analyzed by two pathologists in a blind manner.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assay

We used a terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay kit (Fluorescein *in situ* Cell Death Detection Kit; Boster Biological Technology) to detect intestinal mucosa cell apoptosis in frozen intestinal sections (10 μm). All cells exhibited blue nuclear 4′,6-diamidino-2-phenylindole staining, but the TUNEL-positive cells displayed green/yellow nuclear staining. The stained specimens were captured and analyzed by confocal laser scanning microscopy (Eclipse Ti2; Nikon Corporation, Tokyo, Japan).

Fluorescent real-time quantitative polymerase chain reaction

TRIzol reagent (Beyotime Institute of Biotechnology, China) was used to extract total RNA of tissues, and the Thermo NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, United States) was adopted to determine the concentration and purity of the extracted RNA. The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and their sequences were as follows: *TLR4* forward 5′-TTTATTCAGAGCCGTTGG-3′ and reverse 5′-AGGCGATACAATT CCACC-3′; *GAPDH* forward 5′-GCAGAATTCCTGGCC AAGGTCATCCATGACA-3′ and reverse 5′-GCAGGTACCGGGGCCATCCA CAGTCTTCTG-3′. The One Step SYBR® PrimeScript™ RT-PCR kit II (Takara Biotechnology Co., Ltd., Dalian, China) was used to perform real-time quantitative polymerase chain reaction (qPCR) on a C1000™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, United States) according to the manufacturer's instructions. Each reaction was performed 3 times for each sample. The expression of *TLR4* was analyzed using CFX Manager™ software version 1.6 (Bio-Rad Laboratories) and expressed as the ratio of *TLR4* over the levels of *GAPDH* in the samples.

Western blotting

Proteins from the distal ileal tissues were extracted using ice-cold lysis buffer, and subsequently we used an enhanced BCA protein detection kit (both Nanjing Jiancheng Bioengineering Institute) to determine the concentrations. Equal amounts of protein samples (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel in a MiniPROTEAN II minigel apparatus (Bio-Rad Laboratories) and were transferred to a 0.22- or 0.40-μm polyvinylidene difluoride membrane using wet transfer at 300-mA or 200-mA. Protein was blocked with 0.05% Tween-20 Tris-buffered saline containing 5% nonfat milk or bovine serum albumin for 60 min at room temperature, and then incubated with the primary antibodies such as anti-caspase-3 (1:1000 dilution), anti-Bax (1:2000 dilution), anti-Bcl-2 (1:1000 dilution), anti-TLR4 (1:500 dilution), anti-p65 (1:1000 dilution), anti-p-p65 (1:1000 dilution) and anti-GAPDH (1:5000 dilution; loading control) antibodies overnight at 4 ℃. The HRP-conjugated secondary antibody was then used, the bands were developed with an electrochemiluminescence solution (Millipore, Billerica, MA, United States), and exposed to the BioSpectrum4 apparatus (UVP, Upland, CA, United States).

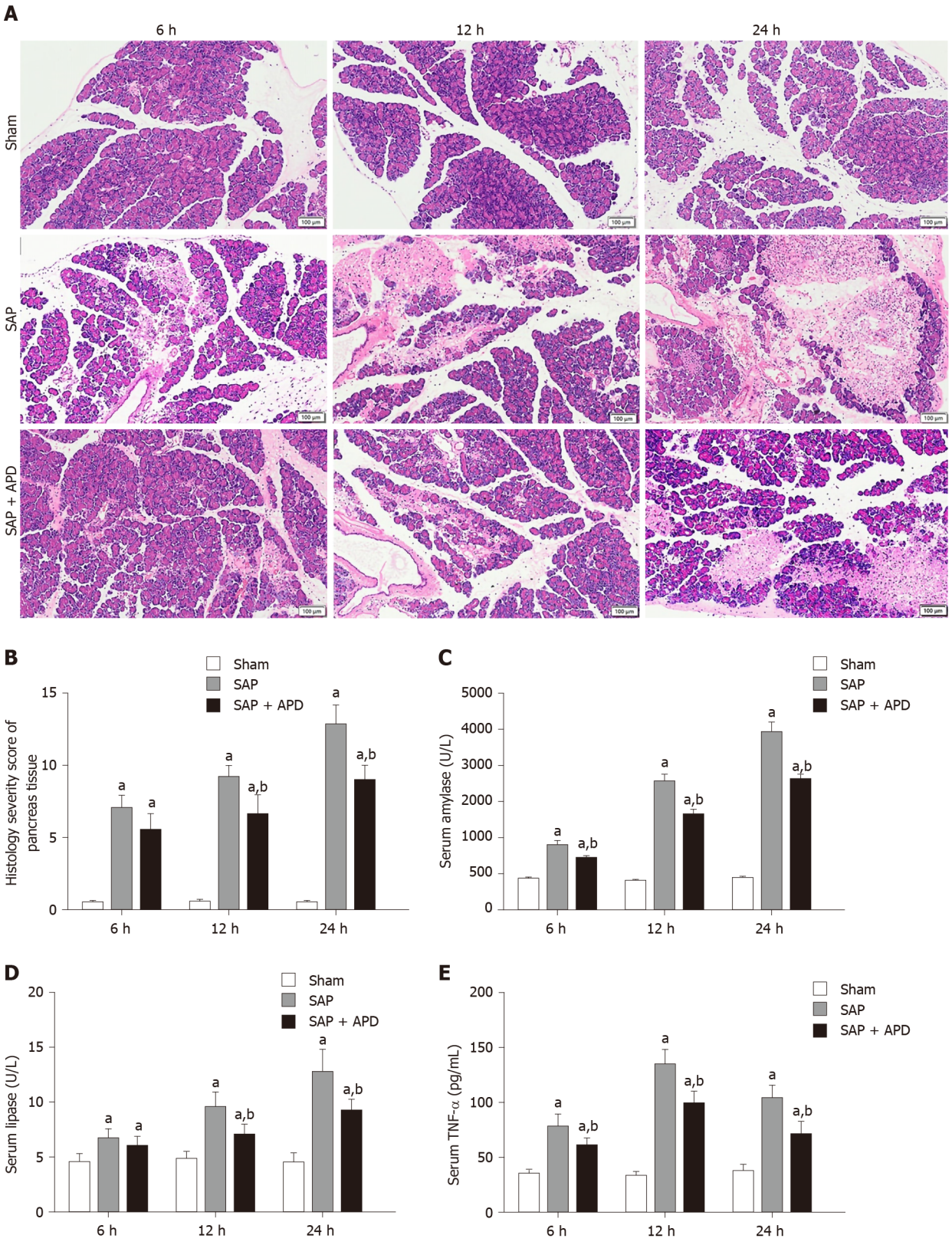
Statistical analysis

SPSS version 19 statistical software (SPSS Inc., Chicago, IL, United States) was used for statistical analysis. Measurement data are presented as mean ± SD, one-way analysis of variance (Tukey method) was used for comparison between multiple groups, and $P < 0.05$ was considered statistically significant.

RESULTS

APD ameliorated pancreatic injury and reduced inflammatory indices in SAP rats

To evaluate the effects of APD treatment in a rat model of SAP induced by the retrograde injection of 5% sodium taurocholate, the changes in pancreatic histopathology, amylase and lipase activity, and levels of inflammatory mediators were measured. For pancreatic histopathology, HE-stained sections of rat pancreatic tissue were analyzed (Figure 1A and B). The samples obtained from the sham group


A
6 h
12 h
24 h
Sham
SAP
SAP + APD
B
Sham
SAP
SAP + APD
Histology severity score of pancreas tissue
15
10
5
0
a
a
a
a,b
a
a,b
6 h
12 h
24 h
C
Sham
SAP
SAP + APD
Serum amylase (U/L)
5000
4000
3000
2000
1000
500
0
a
a,b
a
a,b
a
a,b
6 h
12 h
24 h
D
Sham
SAP
SAP + APD
Serum lipase (U/L)
20
15
10
5
0
a
a
a
a,b
a
a,b
6 h
12 h
24 h
E
Sham
SAP
SAP + APD
Serum TNF-α (pg/mL)
200
150
100
50
0
a
a,b
a
a,b
a
a,b
6 h
12 h
24 h

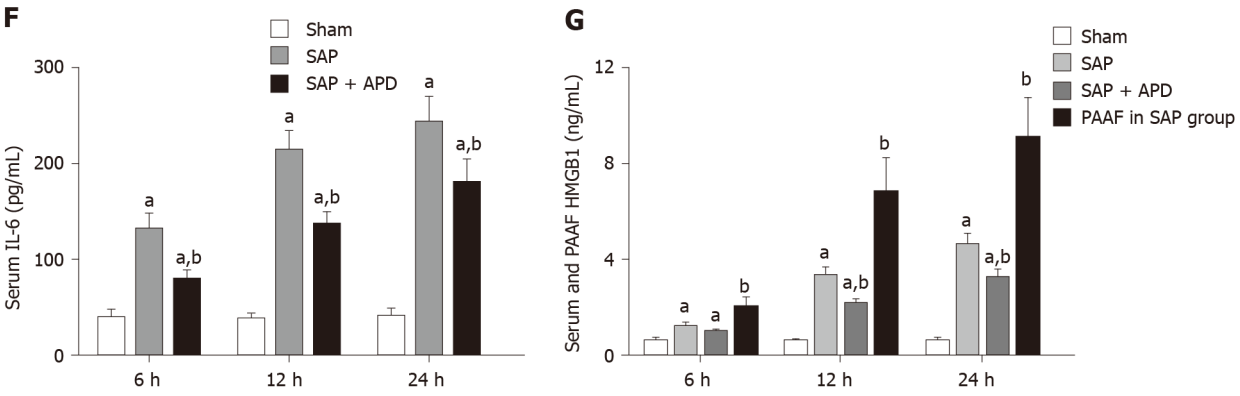


Figure 1 Effects of abdominal paracentesis drainage on pancreatic histopathology and proinflammatory cytokines. A: Representative images of rat pancreatic tissue from different groups following hematoxylin and eosin staining (bar = 100 μm); B: Histology severity score of pancreas tissue; C: Amylase; D: Lipase; E: Tumor necrosis factor-α; F: Interleukin-6; G: High mobility group box 1. All data are presented as mean ± SD (n = 6). $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* severe acute pancreatitis group. SAP: Severe acute pancreatitis; APD: Abdominal paracentesis drainage; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; PAAF: Pancreatitis-associated ascitic fluid; HMGB1: High mobility group box 1.

exhibited intact pancreatic characteristics with normal structures. However, the SAP group samples showed large areas of tissue necrosis and notable inflammatory infiltration and hemorrhage, supported by the raised histological severity scores. Compared with the histological features of the SAP group, samples from the SAP + APD group exhibited reduced pancreatic injury and had a lower severity score.

In addition, compared with the sham group, serum amylase and lipase activity and levels of IL-6, TNF-α and HMGB1 in the SAP group were significantly increased which were consistent with pathological changes. In particular, HMGB1 level in PAAF was higher than that in serum in the SAP group (Figure 1C-G) ($P < 0.05$). APD treatment following SAP induction resulted in a marked decrease in the levels of these indices ($P < 0.05$). These results indicate that APD treatment reduces tissue damage and alleviates the inflammatory process induced by SAP.

APD treatment alleviated intestinal mucosa injury induced by SAP in rats

To evaluate the therapeutic effects of APD on intestinal injury in rats with SAP, distal ileal histopathology and intestinal mucosal-injury-associated indices, such as serum D-lactate, DAO activity and endotoxin were investigated. Tissue from the sham group displayed regular intestinal architecture and villous shape, whereas the samples from the SAP rats showed notable changes in morphology and structure, including denuded villi, mucosal and submucosal swelling, congestion, inflammatory infiltration and increased granules in Paneth cells (Figure 2A and B). In contrast, the intestinal lesions in the SAP + APD group were mild with relatively normal structure, and their severity score was significantly lower compared with the SAP group ($P < 0.05$). Serum DAO activity, D-lactate and endotoxin levels proved to be important indicators of intestinal mucosal integrity and barrier function. Compared with the sham group, the serum levels of the above indicators in the SAP group were obviously elevated following SAP induction at different time points ($P < 0.05$) (Figure 2C-E), suggesting that the intestinal integrity was partially destroyed and the permeability was markedly increased. However, APD treatment significantly decreased serum DAO activity, D-lactate and endotoxin levels ($P < 0.05$). These results indicate that APD treatment improves histopathology and preserves the integrity of intestinal mucosa during SAP.

APD attenuated intestinal inflammation and intestinal mucosal cell apoptosis during SAP

Given that inflammation is the main initiator of intestinal injury caused by SAP, we sought to explore whether APD treatment influenced intestinal inflammation and apoptosis during SAP. Expression of TNF-α, IL-6 and MPO activity was examined to evaluate the level of inflammation in the distal ileum. After SAP induction, protein expression of TNF-α and IL-6 and MPO activity were significantly elevated compared with the sham group ($P < 0.05$) (Figure 3A-C). However, APD treatment significantly inhibited this elevation ($P < 0.05$). Intestinal epithelial apoptosis has a direct link with the inflammatory response, which can reflect the severity of intestinal inflammation. Thus, a TUNEL assay was used to measure apoptosis in the myocardium, and western blot analysis was conducted to determine the expression of apoptosis-associated

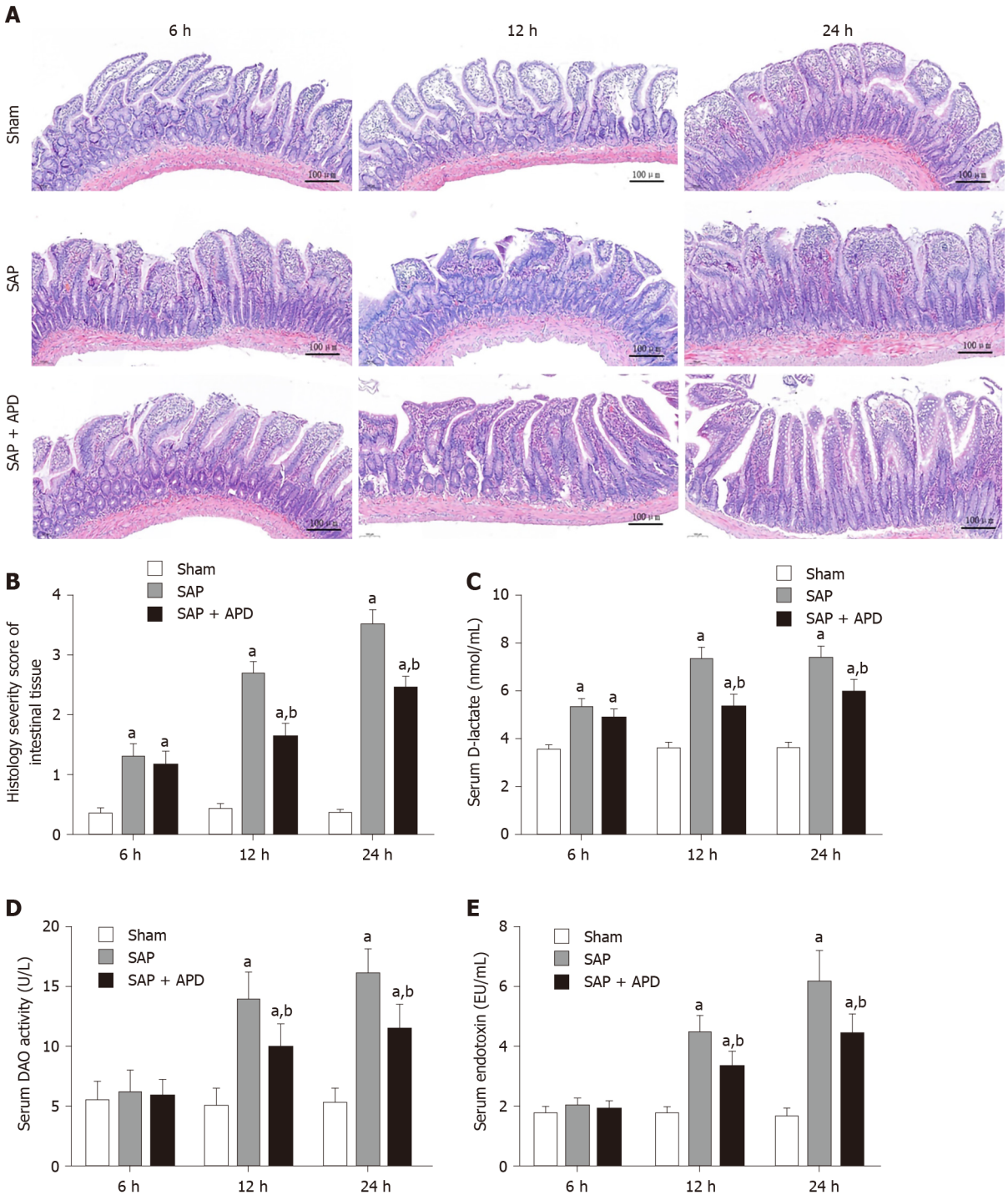
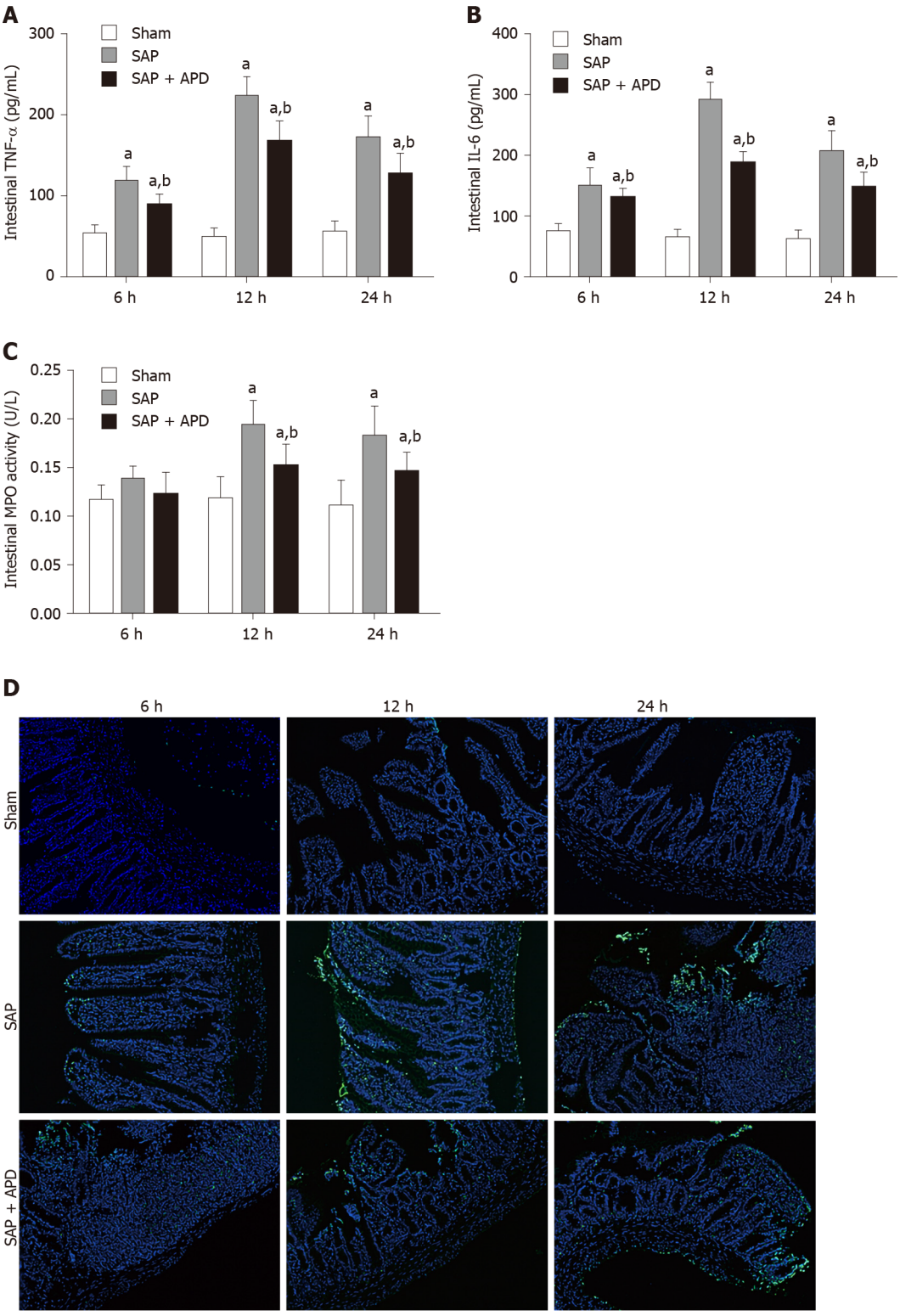


Figure 2 Effects of abdominal paracentesis drainage on intestinal mucosa injury induced by severe acute pancreatitis. A: Representative images of rat distal ileal tissue from different groups following hematoxylin and eosin staining (bar = 100 μm); B: Histological severity score of intestinal tissues; C: D-lactate; D: Diamine oxidase; E: Endotoxin. All data are presented as mean ± SD (n = 6). $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* severe acute pancreatitis group. SAP: Severe acute pancreatitis; APD: Abdominal paracentesis drainage; DAO; Diamine oxidase.

proteins. The apoptotic indices were significantly increased in the SAP group compared with the sham group at different time points ($P < 0.05$), and following the intervention of APD, the incidence of apoptosis was significantly decreased compared with the SAP group ($P < 0.01$) (Figure 3D and E). Western blotting revealed that APD treatment led to downregulation of the proapoptotic markers cleaved caspase-3 and Bax, and upregulation of the antiapoptotic protein Bcl-2 ($P < 0.05$) (Figure 3F-I). These data suggested that APD treatment lessened SAP-induced intestinal inflammation and accompanying apoptosis of intestinal epithelial cells.


A
Intestinal TNF-α (pg/mL)
300
200
100
0
Sham
SAP
SAP + APD
a
a,b
a
a,b
a
a,b
6 h
12 h
24 h
B
Intestinal IL-6 (pg/mL)
400
300
200
100
0
Sham
SAP
SAP + APD
a
a,b
a
a,b
a
a,b
6 h
12 h
24 h
C
Intestinal MPO activity (U/L)
0.25
0.20
0.15
0.10
0.05
0.00
Sham
SAP
SAP + APD
a
a,b
a
a,b
6 h
12 h
24 h
D
6 h
12 h
24 h
Sham
SAP
SAP + APD

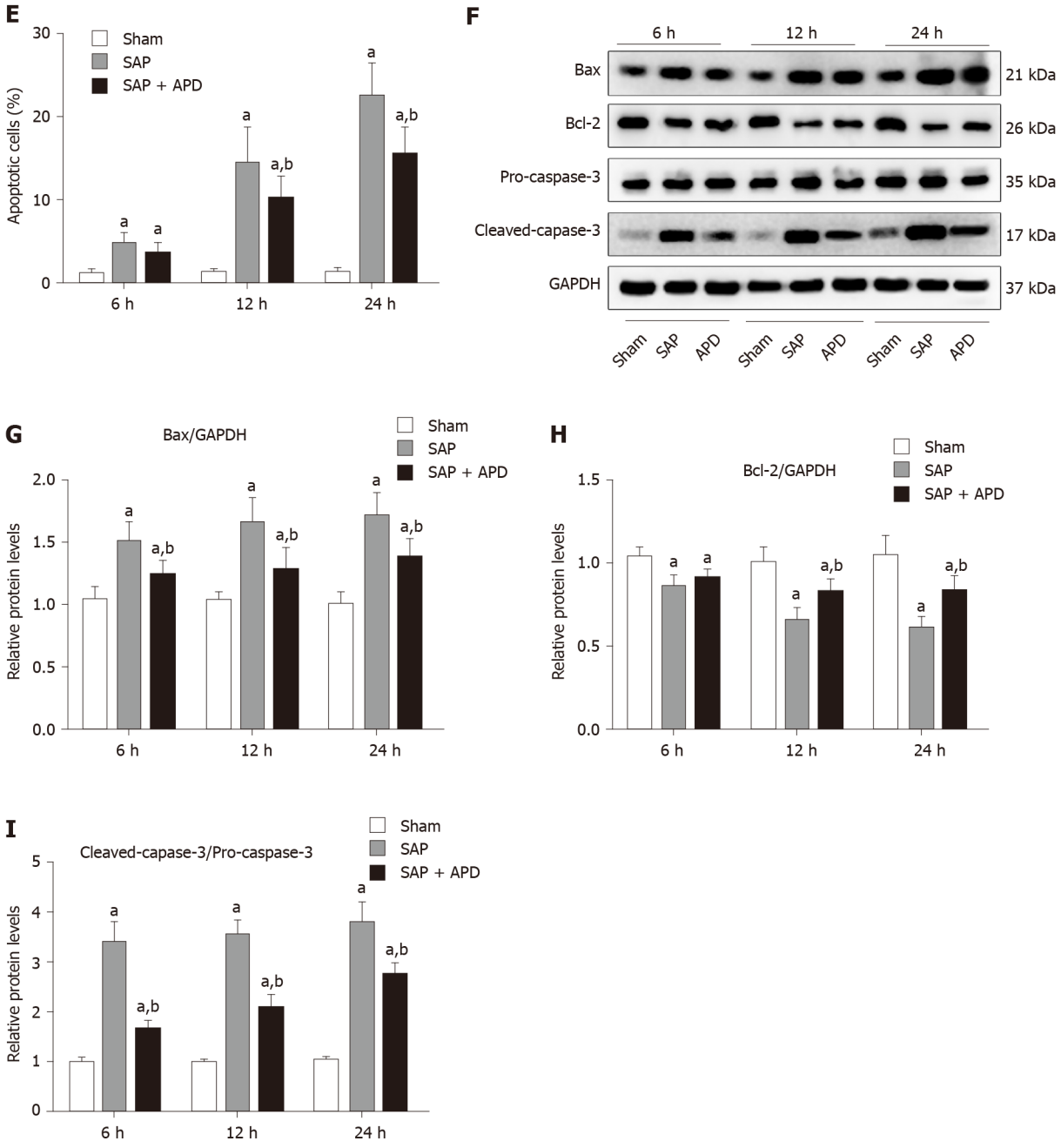
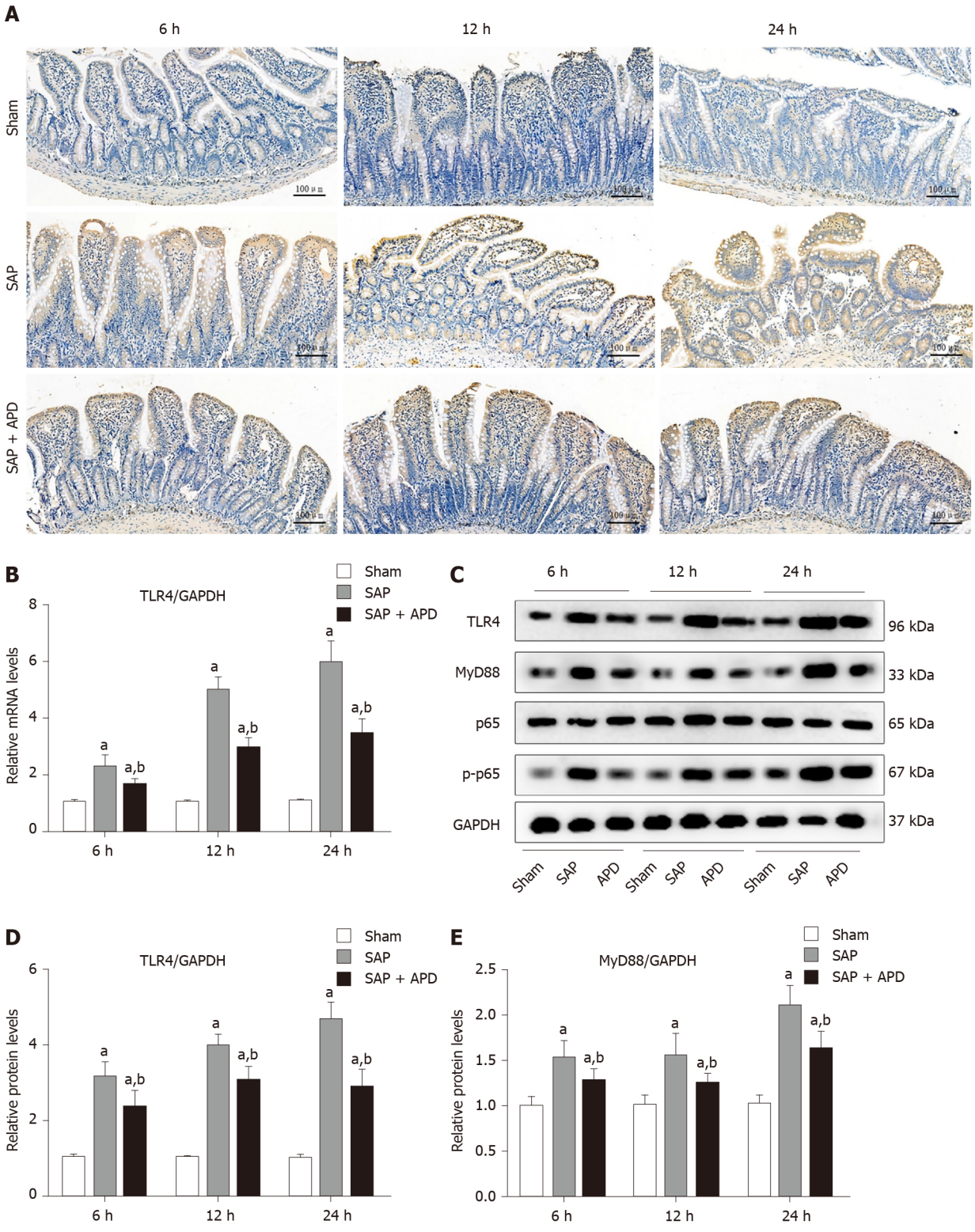


Figure 3 Effects of abdominal paracentesis drainage on intestinal inflammation and mucosal cell apoptosis induced by severe acute pancreatitis. A: Intestinal tumor necrosis factor-α; B: Intestinal interleukin-6; C: Intestinal myeloperoxidase activity; D: Representative images (400 × magnification) of terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay; E: Statistical results of the number of apoptotic intestinal mucosal cells in each group; F: Immunoblot of Bax, Bcl-2, pro-caspase-3 and cleaved-caspase-3 protein expression from intestinal samples; G-I: Densitometry analysis of the immunoblot data of apoptosis-related proteins in intestinal tissue. Data are expressed as means ± SD, n = 6 enzyme-linked immunosorbent assay and TUNEL assay results per group; means ± SD, n = 3 immunoblot results per group. $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* severe acute pancreatitis group. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; MPO: Myeloperoxidase; SAP: Severe acute pancreatitis; APD: Abdominal paracentesis drainage; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

APD-mediated protection involves suppressing TLR4/MyD88/NF-κB signaling

Activation of TLR4 and its downstream signaling pathway has been reported to exert an essential role in the regulation of intestinal inflammation and accompanying cell apoptosis induced by SAP. Therefore, we sought to investigate whether the signaling pathway was involved in the molecular mechanism of APD-mediated protection. Western blotting showed a significant decline in the expression of intestinal TLR4 in the APD group compared with the SAP group from 6 h to 24 h ($P < 0.05$) (Figure 4). This effect of APD treatment on TLR4 expression was further confirmed by immunohistochemical detection that showed the same trend. Compared with the SAP group, expression of MyD88 and phosphorylation of NF-κB p65, known as the


A
6 h
12 h
24 h
Sham
SAP
SAP + APD
B
TLR4/GAPDH
Sham
SAP
SAP + APD
Relative mRNA levels
C
6 h
12 h
24 h
TLR4
MyD88
p65
p-p65
GAPDH
96 kDa
33 kDa
65 kDa
67 kDa
37 kDa
Sham
SAP
APD
D
TLR4/GAPDH
Relative protein levels
E
MyD88/GAPDH
Relative protein levels

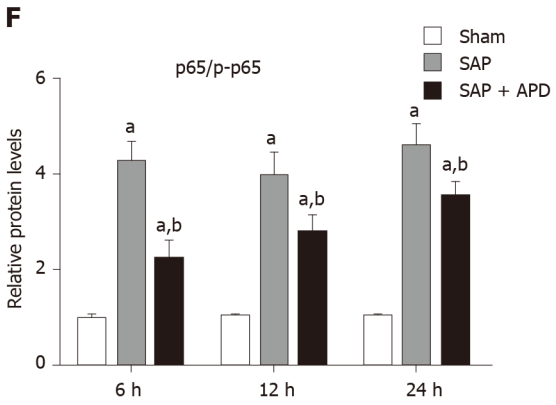


Figure 4 Effects of abdominal paracentesis drainage on intestinal toll-like receptor 4 signaling pathway in rats with severe acute pancreatitis. A: Representative immunohistochemistry images of toll-like receptor 4 (TLR4) in intestinal tissue (bar = 100 μm); B: TLR4 mRNA measurement by real-time polymerase chain reaction (PCR); C: Immunoblotting of TLR4, myeloid differentiation factor 88 (MyD88) and p65 protein expression and p65 phosphorylation level from intestinal samples; D-F: Densitometry analysis of TLR4, MyD88 and ratio of p65 to phosphorylated p65. Data are expressed as means ± SD, n = 6 real-time PCR results per group; means ± SD, n = 3 immunoblotting results per group. $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* severe acute pancreatitis group. SAP: Severe acute pancreatitis; APD: Abdominal paracentesis drainage; MyD88: Myeloid differentiation factor 88; TLR4: Toll-like receptor 4; p-p65: Phosphorylated p65; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

downstream signaling molecules, were markedly reduced in the APD group ($P < 0.05$). The above results showed that APD treatment suppressed the TLR4/MyD88/NF-κB signaling pathway in SAP rat intestine, indicating that the inhibition of TLR4-mediated signaling may be involved in the protection of APD in SAP-induced intestinal injury.

By draining PAAF, APD treatment inhibited intestinal TLR4 signaling by removing intra-abdominal HMGB1

HMGB1 is the most important natural ligand of TLR4 and acts as a key inflammatory mediator during SAP; therefore, we determined whether the PAAF which contains a high concentration of HMGB1 participated in regulation of the TLR4 signaling pathway. Therefore, rats were intraperitoneally injected with the supernatant of PAAF (PI group) or supernatant containing the anti-HMGB1 neutralizing antibody (PIH group) and TLR4 signaling was detected in intestinal samples from rats with MAP. We then assessed the HMGB1 level in rat circulation in each group. As shown in Figure 5A, compared with the control group, the serum level of HMGB1 in the PI group was obviously increased ($P < 0.05$), indicating that HMGB1 following intraperitoneal injection of PAAF could permeate into the circulation under a microinflammatory environment. With blockade of HMGB1 in the PAAF, the level of HMBG1 in serum was significantly decreased compared with that in the PI group ($P < 0.05$). Next, we evaluated the effect of HMGB1 in PAAF on the TLR4 signaling pathway (Figure 5B-G). Eight hours after injection, compared with the MAP control group, expression of TLR4, MyD88 and NF-κB p65 phosphorylation level were significantly increased in the PI group, suggesting that PAAF activated the TLR4 signaling pathway in intestinal tissues during MAP. When treated with the anti-HMGB1 neutralizing antibody, the TLR4 signaling pathway was significantly inhibited compared with that in the PI group. These data demonstrated that APD treatment attenuated intestinal injury *via* inhibition of HMGB1-mediated TLR4 signaling by draining PAAF.

By draining PAAF, APD attenuated intestinal inflammation and apoptosis by suppressing HMGB1-mediated TLR4 signaling

The present data showed that APD treatment reduced intestinal inflammation and accompanying cell apoptosis, and inhibited HMGB1-mediated TLR4 signaling in intestinal tissues during SAP. However, we are still unclear whether there is any potential relationship between these results. To clarify this, the effects of PAAF on intestinal inflammation and cell apoptosis in rats with MAP were investigated. The expression of TNF-α and IL-6 was significantly increased in the PI group compared with the CN group, along with upregulated apoptosis detected by TUNEL staining, which was confirmed by the changes in caspase-3, Bax and Bcl-2 expression measured by western blotting (Figure 6). However, the increased expression of proinflammatory

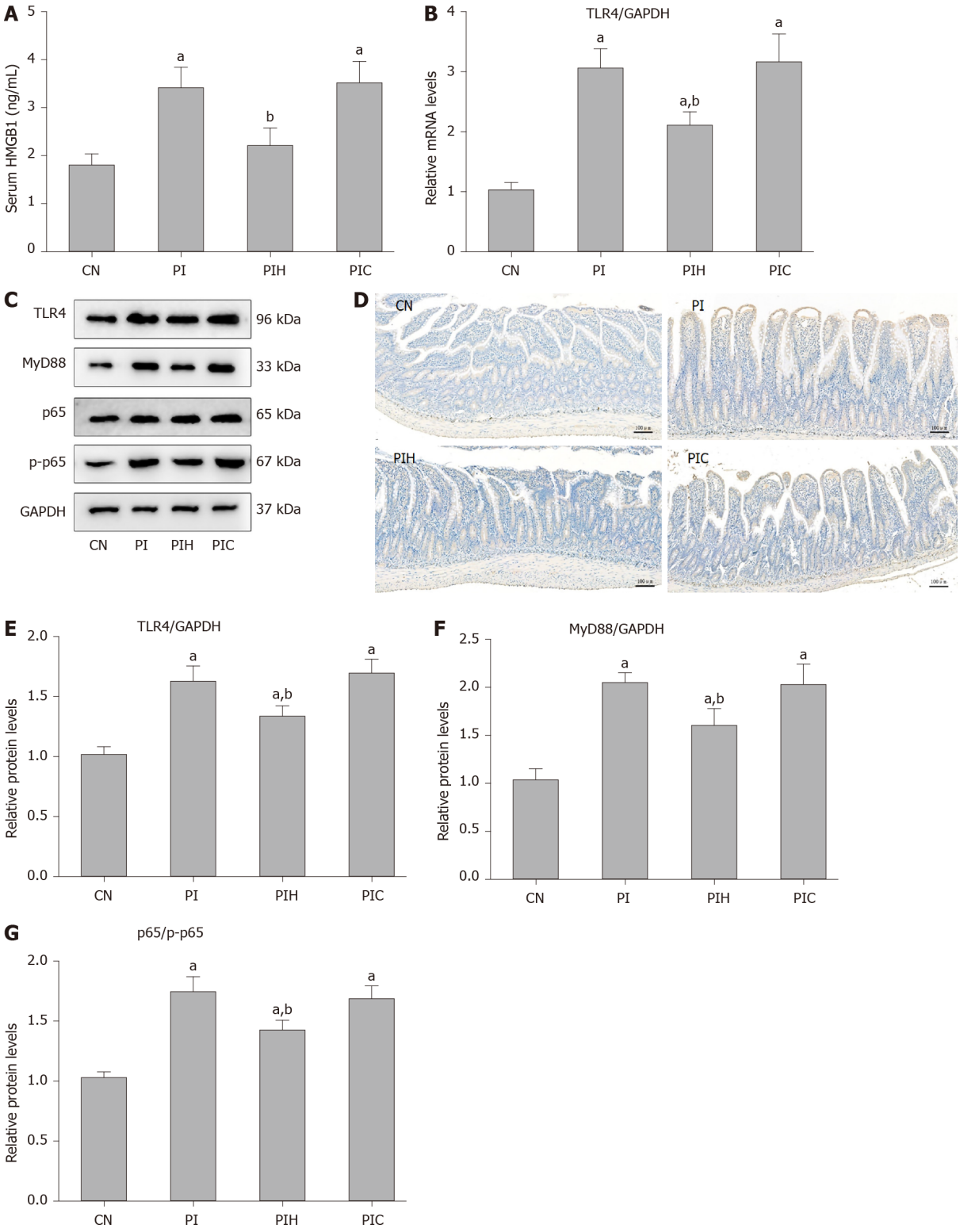


Figure 5 Effects of pancreatitis-associated ascitic fluid intraperitoneal injection with or without anti-high mobility group box protein 1 neutralizing antibody on intestinal toll-like receptor 4 signaling pathway in cerulein-treated rats. A: High mobility group box protein 1 in serum; B: Toll-like receptor 4 (TLR4) mRNA measurement by real-time polymerase chain reaction; C: Immunoblotting of TLR4, myeloid differentiation factor 88 (MyD88) and p65 protein expression and p65 phosphorylation level from intestine samples; D: Representative immunohistochemical images of TLR4 in intestinal tissue (bar = 100 μm); E-G: Densitometry analysis of TLR4, MyD88 and ratio of p65 to phosphorylated p65. Data are expressed as means ± SD, n = 6 enzyme-linked immunosorbent assay results per group; means ± SD, n = 3 immunoblotting results per group. [a]$P < 0.05$ *vs* control group; [b]$P < 0.05$ *vs* pancreatitis-associated ascitic fluid injection group. HMGB1: High mobility group box protein 1; CN: Control; PI: Pancreatitis-associated ascitic fluid (PAAF) injection; PIH: PAAF and 200 μg anti-HMGB1 neutralizing antibody; PIC: PAAF + control IgY; TLR4: Toll-like receptor 4; MyD88: Myeloid differentiation factor 88; p-p65: Phosphorylated p65; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

cytokines and the incidence of apoptosis in intestinal samples were significantly downregulated in the PIH group compared with the PI group. These results indicated that administration of PAAF aggravated the intestinal inflammatory response and promoted apoptosis in MAP. This suggested that APD treatment significantly attenuated inflammation and apoptosis of intestinal tissues by suppressing HMGB1-mediated TLR4 signaling by removing PAAF.

According to the above research results, we proposed a schematic diagram of the underlying mechanism of the influence of APD treatment on SAP-induced intestinal injury (Figure 7). Once SAP occurs, a large amount of HMGB1 enters the blood circulation through peritoneal reabsorption, which is involved in the development of remote organ injury. Following the implementation of APD treatment, HMGB1 concentrations in the circulation decrease markedly, inducing a downregulation of TLR4 expression in intestinal tissues, which can effectively alleviate intestinal inflammation and accompanying cell apoptosis *via* inhibiting activation of the NF-κB signaling, and thereby protecting against SAP-induced intestinal injury.

DISCUSSION

In the present study, the primary goal was to investigate the effect of APD treatment on intestinal inflammation and accompanying apoptosis in rats with SAP, and explore the potential mechanism of this condition. The important findings were that (1) APD treatment lessened the intestinal inflammatory response and accompanying mucosa cell apoptosis by removing PAAF; and (2) the protective effects of APD treatment in attenuating SAP-induced intestinal injury were due to inhibition of HMGB1-mediated TLR4 signaling. The above findings present new insights into the therapeutic mechanism underlying APD.

SAP is a dangerous acute abdominal disease that is usually complicated by multiple organ injury[18]. The small intestine is most frequently affected by SAP[19,20]. In the present study, we found that rats with SAP exhibited serious pancreatic injury and changes in the structure of the small intestine mucosa, which is consistent with previous research[21,22]. Simultaneously, following SAP induction, serum DAO activity, D-lactate and endotoxin levels in rats were significantly increased, and these are useful markers of intestinal mucosal integrity and barrier function. In particular, DAO is specific in the cytoplasm of mammalian intestinal mucosal epithelial cells and its basal level in peripheral blood is positively correlated with integrity of the intestinal mucosa[23]. Disruption of the integrity of the intestinal mucosa during SAP is accepted as the main reason for bacterial translocation and endotoxin into the circulation, which further stimulates activated monocytes and macrophages to release excessive cytokines and inflammatory mediators, promote the occurrence of systemic inflammatory reaction syndrome, and constitute a serious "second blow" to the pancreas and other organs[4]. Therefore, it is important to control the occurrence and development of intestinal mucosal injury in SAP to improve prognosis. We demonstrated that APD attenuated intestinal mucosal injury in SAP rats *via* elimination of PAAF, which has been shown to play an important role in the severity of SAP[24]. Clinically, APD improves the administration of enteral nutrition in acute pancreatitis (AP) patients[25]. Thus, we devoted our attention to further exploration of the potential therapeutic mechanisms of APD in SAP-evoked intestinal injury.

TLR4 is the best-characterized member of the pattern-recognition receptor family, and has been shown to play important roles in innate immunity and in inducing the inflammatory response by acting as a transmembrane signal transduction receptor during SAP[26]. It is reported that the downstream effects of TLR4 are mainly dependent on the interaction with the adaptor molecule MyD88, through a series of signal transductions leading to activation of NF-κB, ultimately resulting in massive synthesis and release of inflammatory cytokines, such as TNF-α and IL-6, which is widely accepted as the primary mode of action[27,28]. Under physiological conditions, to avoid an excessive inflammatory response caused by some stimuli, TLR4 expression in the intestinal epithelium is low. However, the expression of intestinal TLR4 can be upregulated significantly in the course of SAP[21,29]. In this study, we verified that TLR4 expression is markedly increased in distal ileal tissue in rats with SAP. Correspondingly, the activation of MyD88/NF-κB was demonstrated to aggravate the inflammatory response in the intestinal mucosa, along with the upregulated expression of TNF-α and IL-6. In contrast, previous studies have reported that inhibition of the TLR4 signaling pathway can significantly reduce the expression of inflammatory cytokines and ameliorate intestinal injury induced by SAP, indicating

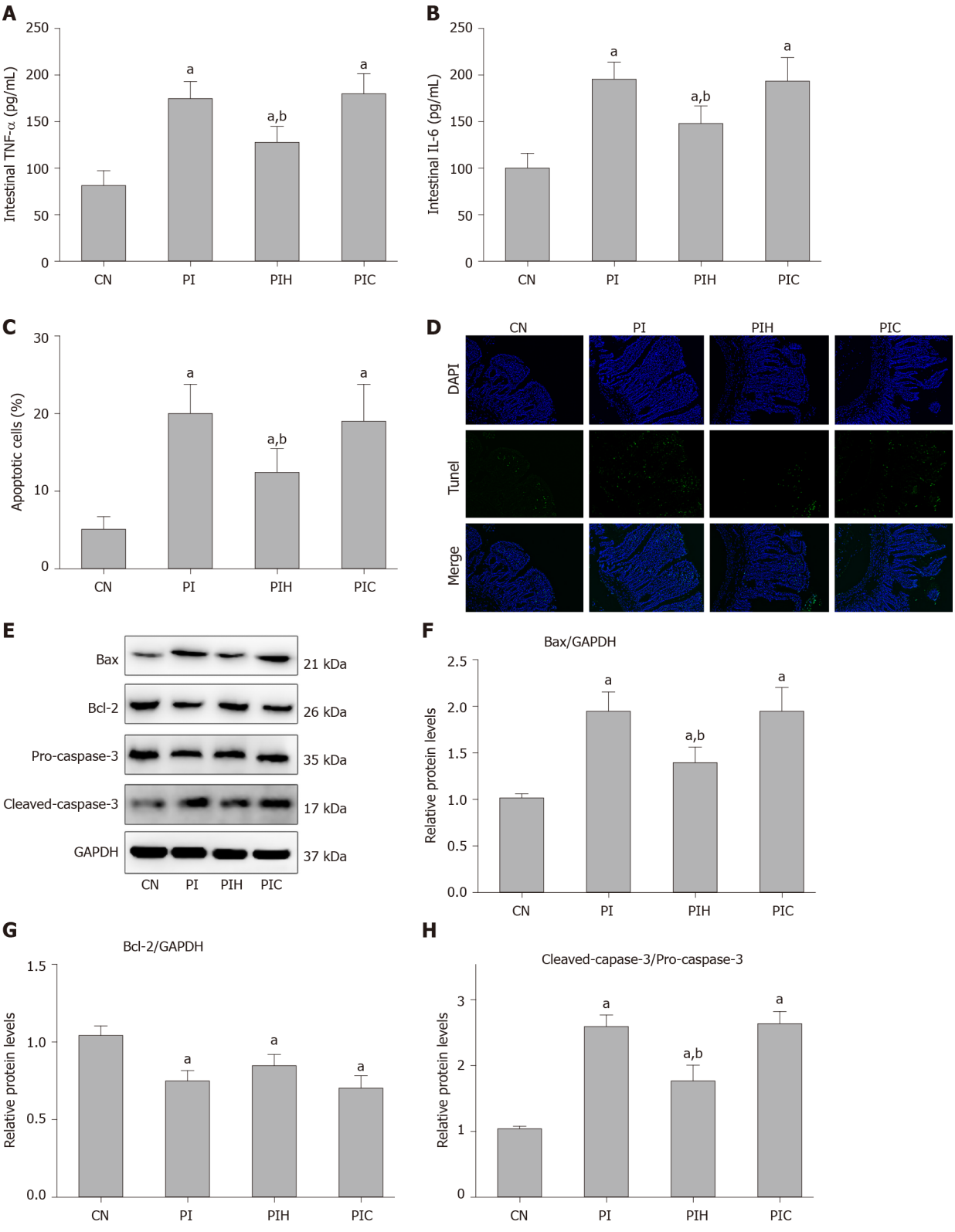


Figure 6 Effects of intraperitoneal pancreatitis-associated ascitic fluid injection with or without anti-high mobility group box protein 1 neutralizing antibody on intestinal inflammation and cell apoptosis in cerulein-treated rats. A: Intestinal tumor necrosis factor-α; B: Intestinal interleukin-6; C: Statistical results of the number of apoptotic intestinal mucosal cells in each group; D: Representative images (400 × magnification) of terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay; E: Immunoblotting of Bax, Bcl-2, pro-caspase-3 and cleaved-caspase-3 protein expression from intestinal samples; F-H: Densitometry analysis of the immunoblotting data of apoptosis-related proteins in intestinal tissue. Data are expressed as means ± SD, n = 6 enzyme-linked immunosorbent assay and TUNEL assay results per group; means ± SD, n = 3 immunoblotting results per group. $^{a}P < 0.05$ *vs* control group; $^{b}P < 0.05$ *vs* pancreatitis-associated ascitic fluid group. TNF-α: Tumor necrosis factor-α; CN: Control; PI: Pancreatitis-associated ascitic fluid (PAAF) injection; PIH: PAAF and 200 μg anti-high mobility group box protein 1 neutralizing antibody; PIC: PAAF + control IgY; IL-6: Interleukin-6; TUNEL: Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DAPI: 4',6-diamidino-2-phenylindole.

that activation of TLR4 plays a critical role in intestinal barrier disruption and inflammation[8,21]. When measurements were implemented at different timepoints after SAP induction, we found that APD treatment markedly decreased intestinal TLR4 and MyD88 expression detected by western blotting, thereby inhibiting involvement of downstream NF-κB in the nucleus, which was supported by the decreased expression of TNF-α and IL-6. Intestinal mucosal cell apoptosis secondary to the inflammatory response that accounts for destroyed integrity and reduced defense has been confirmed in the course of SAP[30,31]. Activation of the NF-κB signaling pathway can lead to an imbalance in the expression of apoptosis-related proteins[32]. In particular, the proapoptotic protein caspase-3 is a key protein that induces cell death, and its activated lysates further activate the caspase signaling pathway, ultimately leading to apoptosis[33]. In the present study, we observed through TUNEL staining that the number of apoptotic cells in intestinal mucosa was significantly increased along with aggravation of intestinal inflammation in rats with SAP, which was further demonstrated by the increased expression of proapoptotic cleaved caspase-3 and Bax, and decreased expression of antiapoptotic protein Bcl-2. After treatment with APD, intestinal mucosal cell apoptosis and the imbalance between proapoptotic and antiapoptotic proteins were markedly improved. These data demonstrated that downregulation of TLR4 expression *via* APD treatment alleviated inflammatory injury and intestinal mucosal cell apoptosis caused by SAP, which indicates a therapeutic mechanism of APD in preserving intestinal barrier function. However, it is still unclear how APD treatment suppresses intestinal TLR4 signaling by removing PAAF, and it may be related to the reduction in the levels of some lethal factors by APD that can act on intestinal TLR4.

Activation of TLR4 requires binding to its specific ligand. Extracellular HMGB1 has been confirmed as the most important endogenous ligand for TLR4 during SAP and to act as a key inflammatory mediator[11,34]. Accumulated evidence indicates that HMGB1 promotes the development of AP from local inflammation to a lethal systemic inflammatory response, and participates in multiple organ injury[11,35]. Clinical studies have shown that elevated serum level of HMGB1 is positively related to the outcome of patients with SAP as well as organ dysfunction[36]. It is reported that blockade of HMGB1 attenuates intestinal mucosal barrier dysfunction in experimental AP, suggesting a contribution by HMGB1 to intestinal mucosal biological dysfunction[13]. In the present study, the serum levels of HMGB1 in SAP rats were significantly increased with time after SAP induction, which was in line with earlier studies[37,38]. In particular, HMGB1 concentration in PAAF was higher than that in serum at the same time point, which may be because HMGB1 was mainly derived from activated intraperitoneal macrophages and damaged pancreatic tissue during the course of SAP[37]. PAAF is a common local complication of SAP, and our previous studies have shown that timely drainage of PAAF through APD is an important part of SAP therapy and plays a significant role in organ protection[7]. Therefore, we speculate that the high concentration of HMGB1 in PAAF may have a detrimental effect on the intestine in SAP, and the protective mechanism of APD treatment may involve HMGB1 mediation of the TLR4 signaling pathway.

To confirm that the effect of HMGB1 in PAAF drained by APD treatment can alleviate hyperactivity of the intestinal TLR4 signaling pathway, rats with MAP received an intraperitoneal injection of PAAF or PAAF with anti-HMGB1 neutralizing antibody. Eight hours after PAAF injection, compared with the MAP control group, we found that the serum level of HMGB1 in the PI group was significantly increased, suggesting that HMGB1 in PAAF enters the bloodstream under a microinflammatory state in the abdominal cavity induced by cerulein pancreatitis. In line with that, mRNA and protein expression of intestinal TLR4 was significantly increased, along with expression of MyD88 protein and NF-κB p65 phosphorylation level, indicating that administration of PAAF activates the TLR4 signaling pathway in intestinal tissues during MAP. When treated with the anti-HMGB1 neutralizing antibody, we observed that the level of active HMGB1 in serum was significantly reduced, and the TLR4 signaling pathway was also significantly inhibited in the PIH group compared with the PI group, in conjunction with the downregulated expression of TNF-α and IL-6, and reduced intestinal mucosal cell apoptosis. Overall, our results indicate that APD treatment can suppress overactivation of the intestinal TLR4 signaling pathway by draining HMGB1 from PAAF, thereby reducing the release of proinflammatory cytokines and the incidence of apoptosis, and ultimately reducing the severity of SAP-induced intestinal injury.

Our study had several limitations. Firstly, we focused on the effect of APD on intestinal inflammation and apoptosis, but whether APD also has an effect on intestinal motility remains to be studied. Secondly, we did not detect changes in the

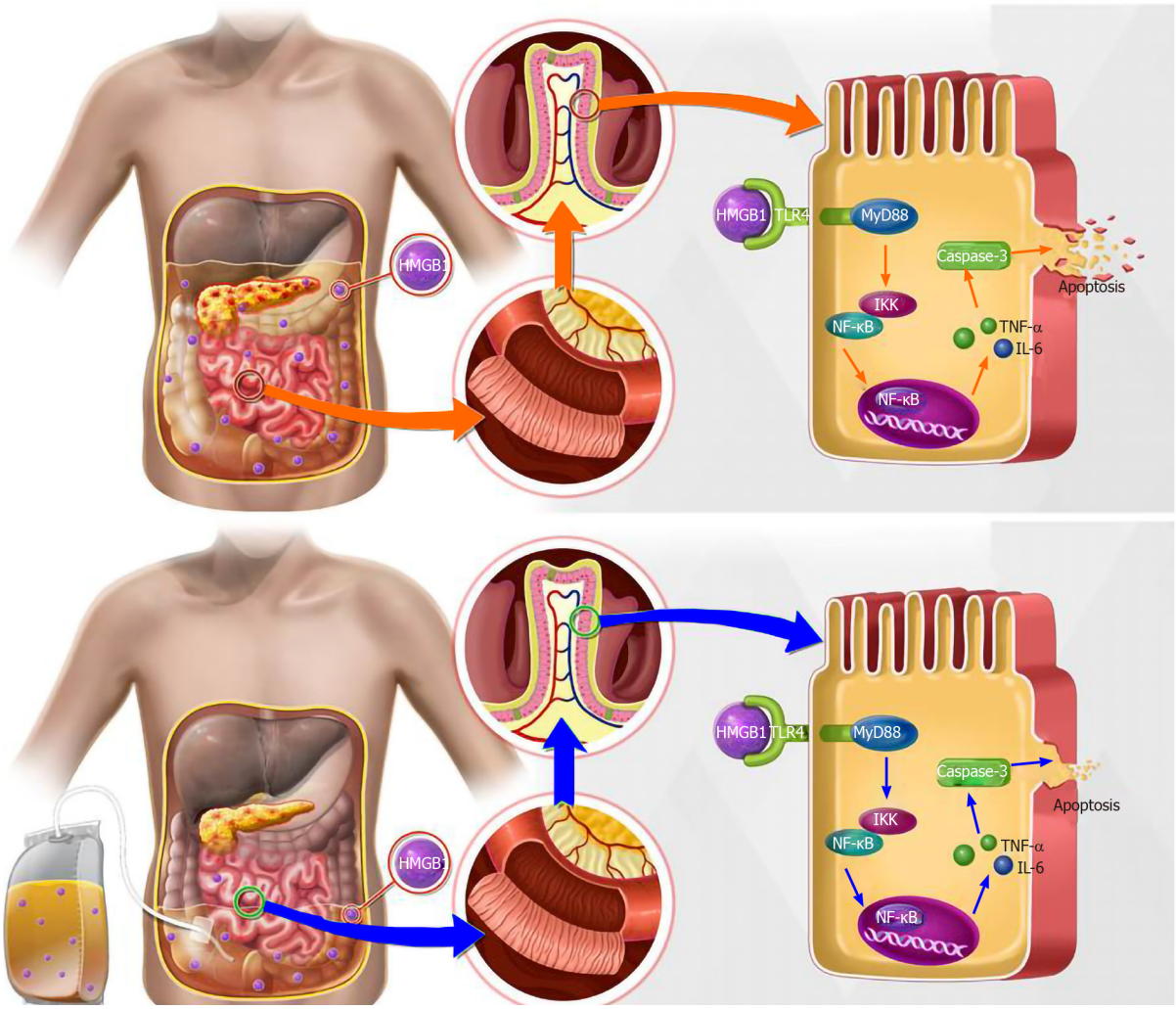


Figure 7 Possible mechanisms responsible for the protective effects of abdominal paracentesis drainage treatment on severe acute pancreatitis-induced intestinal inflammation and accompanying apoptosis. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; HMGB1: High mobility group box protein 1; IKK: IkappaB kinase; NF-kB: Nuclear factor kB; TLR4: Toll-like receptor 4.

expression of HMGB1 in intestinal tissue, and future experiments should be carried out to verify the relationship between intestinal HMGB1 and PAAF.

CONCLUSION

Our data revealed that APD treatment ameliorated intestinal injury and improved intestinal barrier function in an SAP rat model, and ultimately improved outcome. The protective effect was potentially achieved through reducing intestinal inflammatory response and accompanying apoptosis of mucosal cells by downregulation of HMGB1-mediated TLR4 signaling. This study provides a novel molecular mechanism associated with the effect of APD on SAP-induced intestinal injury. SAP is characterized by extensive inflammation of the pancreas with tissue necrosis.

ARTICLE HIGHLIGHTS

Research background

Severe acute pancreatitis (SAP) is an abdominal disease characterized by extensive inflammation and tissue necrosis of the pancreas and usually accompanied by multiple organ damage, resulting in high mortality. Intestinal injury is the most common extrapancreatic complication and is closely related to the severity of SAP. Our previous studies proved that elimination of pancreatitis associated ascitic fluids

(PAAF) through early abdominal paracentesis drainage (APD) attenuates intestinal mucosal injury and protects intestinal barrier effectively, but the potential molecular mechanisms responsible for the beneficial effect are yet to be clarified.

Research motivation

The inflammatory response plays an essential role in the pathological process of SAP-induced intestinal injury. High concentration of high mobility group box protein 1 (HMGB1) participates in remote organ damage and has been confirmed during SAP. Based on our previous research, we further investigate whether HMGB1 in ascites is related to intestinal inflammation and apoptosis under SAP conditions, which may reveal the underlying mechanism of APD in SAP.

Research objectives

The purpose of the present experiment was to explore the effect of APD treatment on intestinal inflammation and apoptosis induced by SAP in rats, and its potential mechanisms.

Research methods

SAP was induced in male adult Sprague-Dawley rats by 5% sodium taurocholate. Mild acute pancreatitis was induced by intraperitoneal injections of cerulein (20 μg/kg body weight, six consecutive injections). Following SAP induction, a drainage tube connected to a vacuum ball was placed into the lower right abdomen of the rats for APD. Morphological staining, serum amylase, lipase and inflammatory mediators, serum and ascites HMGB1, serum indices which reflect intestinal barrier function, apoptosis and associated proteins in intestinal tissue were assessed. The expression levels of key proteins in the toll-like receptor 4 (TLR4) signaling pathway were also examined.

Research results

The results demonstrated that APD notably alleviated the changes in pancrease and intestinal mucosa, attenuated the alterations in serum amylase, lipase and inflammatory mediators, improved intestinal barrier function, lessened intestinal inflammation and accompanying apoptosis of mucosal cells, and reversed the expression of apoptosis-associated proteins. APD significantly inhibited activation of the intestinal TLR4 signaling pathway mediated by HMGB1 in intestinal tissue, and thus plays a protective role in SAP-associated intestinal injury.

Research conclusions

APD treatment effectively improved intestinal barrier function, ameliorated the intestinal inflammatory response and mucosa cell apoptosis in rats with SAP. The protective effects are potentially due to the inhibition of the HMGB1-mediated TLR4 signaling pathway.

Research perspectives

The present study provided new evidence of the efficacy and safety of APD treatment on SAP, and provided a novel molecular mechanism associated with the effect of APD on SAP-induced intestinal injury. However, in this research we did not detect the expression change of HMGB1 in intestinal tissue. In our next experiments, we will attempt to verify the relationship between intestinal HMGB1 and PAAF to further reveal the precise mechanisms of APD treatment in SAP.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Progressive liver injury and increased mortality risk in COVID-19 patients: A retrospective cohort study in China

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Author contributions: All authors conceptualized and designed the study; Zhang SS, Dong L, Wang GM, Ye XF, Tian Y, Ge QG, Xiu DR, and Shen N contributed to the literature search; Zhang SS, Dong L, Wang GM, Tian Y, Ye XF, Zhao Y, Liu ZY, Zhai JY, Zhao ZL, Wang JH, Zhang HM, Li XL, Wu CX, Yang CT, Yang LJ, Du HX, and Wang H contributed to data collection; Zhang SS, Dong L, Wang GM, Ye XF, Tian Y, Ge QG, Xiu DR, and Shen N contributed to

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data analysis and interpretation; Zhao Y, Liu ZY, Zhai JY, Zhao ZL, Wang JH, Zhang HM, Li XL, Wu CX, Yang CT, Yang LJ, Du HX, and Wang H contributed to preparing the figures and tables; Zhang SS, Dong L, and Wang GM contributed to writing of the manuscript; all authors revised the final manuscript and approved the final version of the article, including the authorship list; Zhang SS, Dong L, Wang GM, Tian Y, and Ye XF should be considered joint first authors; Xiu DR, Ge QG, and Shen N should be considered co-corresponding authors.

Institutional review board statement: The study was approved by the Ethics Committee of Peking University Third Hospital (IRB00006761-M2020060).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment. Due to the particularity of the COVID-19 epidemic, all the data of patients could not be taken out and were sealed in place.

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

Data sharing statement: No additional data are available.

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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Abstract

BACKGROUND

Liver injury is common and also can be fatal, particularly in severe or critical patients with coronavirus disease 2019 (COVID-19).

AIM

To conduct an in-depth investigation into the risk factors for liver injury and into the effective measures to prevent subsequent mortality risk.

METHODS

A retrospective cohort study was performed on 440 consecutive patients with relatively severe COVID-19 between January 28 and March 9, 2020 at Tongji Hospital, Wuhan, China. Data on clinical features, laboratory parameters, medications, and prognosis were collected.

RESULTS

COVID-19-associated liver injury more frequently occurred in patients aged ≥ 65 years, female patients, or those with other comorbidities, decreased lymphocyte count, or elevated D-dimer or serum ferritin ($P < 0.05$). The disease severity of COVID-19 was an independent risk factor for liver injury (severe patients: Odds ratio [OR] = 2.86, 95% confidence interval [CI]: 1.78-4.59; critical patients: OR = 13.44, 95%CI: 7.21-25.97). The elevated levels of on-admission aspartate aminotransferase and total bilirubin indicated an increased mortality risk ($P < 0.001$). Using intravenous nutrition or antibiotics increased the risk of COVID-19-associated liver injury. Hepatoprotective drugs tended to be of assistance to treat the liver injury and improve the prognosis of patients with COVID-19-associated liver injury.

CONCLUSION

More intensive monitoring of aspartate aminotransferase or total bilirubin is recommended for COVID-19 patients, especially patients aged ≥ 65 years, female patients, or those with other comorbidities. Drug hepatotoxicity of antibiotics and intravenous nutrition should be alert for COVID-19 patients.

Key Words: COVID-19; Liver injury; Prognosis; Risk factors; Drugs; Alanine amino-transferase

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Core Tip: The prevalence of liver injury in hospitalized patients with coronavirus disease 2019 (COVID-19) is high and also can be fatal. Therefore, high-risk population, especially patients aged ≥ 65 years, female patients, or patients with other comorbidities should be intensively monitored. On-admission total bilirubin has the strongest correlation with the prognosis of COVID-19 patients, which can be used for monitoring of COVID-19 patients at risk of liver injury. Intravenous nutrition and antibiotics are associated with abnormal liver biochemistry; these drugs should be given with caution. Hepatoprotective drugs are favorable for patients with liver injury.

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INTRODUCTION

The World Health Organization declared the coronavirus disease 2019 (COVID-19) outbreak on March 11, 2020, and the numbers of cases and deaths of COVID-19 globally are soaring[1]. As of March 2, 2021, more than 114000000 confirmed cases of COVID-19 and more than 2500000 deaths had been reported worldwide. The global spread of COVID-19 is an enormous threat to humanity.

Liver injury has been reported as a common clinical manifestation in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection[2,3]. Between 14% and 53% of patients have elevated levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)[4-7], and a recent study reported that patients with abnormal liver tests were more likely to progress to severe disease[8]. It becomes extremely emergent to treat severe patients and reduce mortality. Some studies have focused on severe cases with liver injury and their survival outcome, but the proportion of severe and critical cases is low.

Comprehensive and detailed studies on the clinical features, risk factors, and prognosis of patients with liver injury are still lacking, and it is urgent to clarify predictive factors and therapeutic approaches for liver injury.

Here we conducted a retrospective cohort study to identify the risk factors related to liver injury in COVID-19 patients, to evaluate the impact of liver injury on prognosis and to determine whether it is a reliable and independent predictor of disease prognosis, and to investigate the efficacy of hepatoprotective drugs in patients with liver injury. Our results will provide relatively comprehensive and reliable references for clinical decisions, and are expected to improve the outcomes of COVID-19 patients.

MATERIALS AND METHODS

Study participants

This study was a retrospective observational study. All patients with COVID-19 consecutively admitted to six wards at the Sino-French New City Branch of Tongji Hospital, Huazhong University of Science and Technology from January 28 to March 9, 2020 were enrolled. Tongji Hospital was designated to treat severe and critical COVID-19 patients by the Wuhan government in January 2020. Follow-up of the clinical outcomes of all patients was censored on March 27, 2020.

Data collection

Sociodemographic characteristics, clinical symptoms, laboratory parameters, chest computed tomography (CT) features, outcomes, and medications were extracted from the electronic medical records. We followed laboratory-specific thresholds for all laboratory parameters and categorized each into two or three strata. To quantify the severity of pneumonia, we utilized two scores, *i.e.*, confusion, uremia, respiratory rate, blood pressure, and age ≥ 65 (CURB-65)[9], and the quick Sequential Organ Failure Assessment (qSOFA)[10].

Definition

COVID-19 patients and disease severity: We included all patients with confirmed COVID-19 according to "Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia" (trial seventh edition) by the Chinese National Health Commission[11]. Severity of COVID-19 was categorized into three stages: Severe cases were defined as (1) respiratory rate ≥ 30 breaths/min; (2) oxygen saturation ≤ 93%; or (3) PaO2/FiO2 ratio ≤ 300 mmHg. Critical cases were defined as including one or more of the following criteria: Shock, respiratory failure requiring mechanical ventilation, in combination with other organ failures, or admission to intensive care unit. Or else, other COVID patients were general cases. In-hospital disease severity was the severest grade during hospitalization.

Liver biochemistry abnormality or injury and acute liver injury: COVID-19-related liver injury was divided into three stages *as per* the Chinese Guideline for COVID-19-

related liver injury[12]. Stage 0, normal liver biochemistry (LB), was defined as markers indicating liver function, *i.e.*, ALT, AST, and total bilirubin (TBIL), within normal limits. Stage 1, LB abnormality (LBA), was defined as liver function markers outside the normal limits but not reaching the thresholds for stage 2. Stage 2, acute liver injury (ALI), was defined as an ALT or AST increase of ≥ 3 times the upper limit of normal, or TBIL increase of ≥ 2 times the upper limit of normal. In our study, LB abnormality or injury (LBAI) was defined as either stage 1 or stage 2. Worsening of LB was defined as any stage elevation during hospitalization. Cases were excluded from the study if no data on ALT, AST and TBIL were available.

Other terms: We discriminated liver injury associated with COVID-19 by temporal patterns, *e.g.*, admission to the hospital, in-hospital, and cumulative incidence that took both time points into account. We also linked the time of illness onset that refers to the onset of symptoms. Hepatoprotective drugs used in the study included magnesium isoglycyrrhizinate, diammonium glycyrrhizinate, and polyene phosphatidylcholine.

Ethics approval

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki and the Harmonized Tripartite Guideline for Good Clinical Practice from the International Conference on Harmonization. The study was approved by the ethics committee of Peking University Third Hospital (IRB00006761-M2020060).

Statistical analysis

Categorical variables are summarized as counts (percentages) and continuous variables are presented as the median with interquartile range (IQR). Univariate binary logistic regression was used to explore the factors correlating with cumulative LBAI. The cumulative incidence rates of mortality were assessed using the Kaplan-Meier method and significant differences between the subgroups were determined using the standard log-rank test. Univariable and multivariable Cox proportional hazard regression was used for the survival analysis associated with liver injury markers and other risk factors for mortality. All descriptive statistical analyses were conducted with SPSS version 23.0 (IBM Corp, United States) and analytical analyses were done using R version 4.0.0 (R Foundation for Statistical Computing, Austria). A two-sided *P* value less than 0.05 was considered statistically significant.

RESULTS

Demographic and clinical characteristics associated with LBAI

A total of 440 patients with COVID-19 were included in the final analysis. The median age was 63 years (IQR 18) and 233 (53%) were female. Median follow-up time was 17 d (IQR 21). The total incidence rate of cumulative LBAI was 57.7%, including 15.7% with ALI. The different incidence rates of cumulative LBAI stratified by sociodemographic and clinical characteristics are shown in Table 1. The likelihood of cumulative LBAI increased with the age of patients, with a 2-fold risk in patients aged ≥ 65 years compared with those aged 50 years or younger [odds ratio (OR) = 1.83; 95% confidence interval (CI): 1.03 to 3.26]. Female patients were at a higher risk of cumulative LBAI (OR = 2.09; 95%CI: 1.46 to 3.06) than males.

Additional significant associated risk factors with LBAI included comorbidities, especially diabetes, vital signs on admission (including temperature, respiratory rate, pulse oximeter O_2 saturation, and heart rate), and severity of illness (qSOFA and CURB-65 scores). Compared to general COVID-19 patients, LBAI occurred more often in severe and critical patients with a 2-fold (OR = 2.86; 95%CI: 1.78 to 4.59) and 13-fold (OR = 13.44; 95%CI: 7.21 to 25.07) higher likelihood, respectively.

Laboratory and imaging features associated with LBAI

The likelihood of cumulative LBAI among COVID-19 patients varied with the laboratory parameters and chest CT imaging features, as shown in Table 1. Patients with abnormal counts of blood cells at admission were more prone to later develop cumulative LBAI, especially decreased lymphocyte count (OR = 4.10; 95%CI: 2.74 to

Table 1 Demographics, clinical characteristics, laboratory parameters, and computed tomography imaging features of 440 patients with coronavirus disease 2019, stratified by severity of cumulative liver abnormality or injury

	All patients (*n* = 440)	Normal cumulative LB (*n* = 186)	Cumulative LBA (*n* = 185)	Cumulative ALI (*n* = 69)	Cumulative LBAI (*n* = 254)	OR (95%CI)[1]	*P* value[2]
Characteristics							
Age, yr, median (IQR)	63 (25)	61 (18)	63 (23)	65 (17)	64 (18)	2.536	0.012
< 50	65 (14.8)	33 (50.8)	23 (35.4)	9 (13.8)	32 (49.2)	Ref	Ref
50-64	203 (46.1)	91 (44.8)	85 (41.9)	27 (13.3)	112 (55.2)	1.27 (0.73 to 2.22)	0.404
≥ 65	172 (39.1)	62 (36.0)	77 (44.8)	33 (19.2)	110 (64.0)	1.83 (1.03 to 3.26)	0.040
Sex, female	233 (53.0)	79 (33.9)	109 (46.8)	45 (19.3)	154 (66.1)	2.09 (1.42 to 3.06)	< 0.0001
BMI, ≥ 24 kg/m^2	124 (49.8)	63 (50.8)	45 (36.3)	16 (12.9)	61 (49.2)	0.95 (0.58 to 1.57)	0.849
Epidemiological exposure[3]	268 (60.9)	123 (45.9)	104 (38.8)	41 (15.3)	145 (54.1)	0.68 (0.46 to 1.01)	0.055
Smoking history	62 (20.9)	21 (33.9)	31 (50.0)	10 (16.1)	41 (61.1)	1.48 (0.84 to 2.61)	0.176
Comorbidities[4]	285 (64.8)	109 (38.2)	123 (43.2)	53 (18.6)	176 (61.8)	1.59 (1.07 to 2.37)	0.021
Hypertension	181 (41.1)	69 (38.1)	83 (45.9)	29 (16.0)	112 (61.9)	1.34 (0.91 to 1.97)	0.141
Diabetes	136 (30.9)	48 (35.3)	53 (39.0)	35 (25.7)	88 (64.7)	1.52 (1.00 to 2.31)	0.048
Cardiovascular disease	56 (12.7)	22 (39.3)	19 (33.9)	15 (26.8)	34 (60.7)	1.15 (0.65 to 2.04)	0.628
Cerebrovascular disease	16 (3.6)	4 (25.0)	8 (50.0)	4 (25.0)	12 (75.0)	2.26 (0.72 to 7.11)	0.165
Chronic renal disease	21 (4.8)	11 (52.4)	9 (42.9)	1 (4.8)	10 (47.6)	0.65 (0.27 to 1.57)	0.340
Chronic liver disease	12 (2.7)	4 (33.3)	7 (58.3)	1 (8.3)	8 (66.7)	1.48 (0.44 to 4.99)	0.527
Chronic hepatitis B	8 (1.8)	2 (25.0)	5 (62.5)	1 (12.5)	6 (75.0)	2.23 (0.44 to 11.15)	0.331
Other chronic liver disease[5]	4 (0.9)	2 (50.0)	2 (50.0)	0	2 (50.0)	0.73 (0.10 to 5.23)	0.754
Chronic respiratory disease	31 (7.0)	16 (51.6)	9 (29.0)	6 (19.4)	15 (48.4)	0.67 (0.32 to 1.39)	0.278
Signs and symptoms at disease onset							
Fever (temperature ≥ 37.3 °C)	369 (85.8)	149 (40.4)	159 (43.1)	61 (16.5)	220 (59.6)	1.63 (0.95 to 2.81)	0.079
Sputum	236 (53.6)	93 (39.4)	104 (44.1)	39 (16.5)	143 (60.6)	1.29 (0.88 to 1.88)	0.191
Dyspnoea	243 (55.2)	82 (33.7)	116 (47.7)	45 (18.5)	161 (66.3)	2.2 (1.49 to 3.23)	< 0.0001
Haemoptysis	63 (14.3)	25 (39.7)	26 (41.3)	12 (19.0)	38 (60.3)	1.13 (0.66 to 1.95)	0.653
Chest pain/tightness	227 (51.6)	88 (38.8)	100 (44.1)	39 (17.2)	139 (61.2)	1.35 (0.92 to 1.97)	0.125
Pharyngalgia/nasal congestion/running	94 (21.4)	47 (50.0)	33 (35.1)	14 (14.9)	47 (50.0)	0.67 (0.42 to 1.06)	0.088

Headaches/dizziness	135 (30.7)	57 (42.2)	54 (40.0)	24 (17.8)	78 (57.8)	1.00 (0.67 to 1.51)	0.989
Myalgia/fatigue	289 (65.7)	122 (42.2)	120 (41.5)	47 (16.3)	167 (57.8)	1.01 (0.68 to 1.50)	0.973
Nausea/vomiting	133 (30.2)	53 (39.8)	59 (44.4)	21 (15.8)	80 (60.2)	1.15 (0.76 to 1.75)	0.498
Abdominal pain	70 (15.9)	29 (41.4)	26 (37.1)	15 (21.4)	41 (58.6)	1.04 (0.62 to 1.75)	0.876
Diarrhea	185 (42.0)	78 (42.2)	83 (44.9)	24 (13.0)	107 (57.8)	1.01 (0.69 to 1.48)	0.968
On-admission vital signs							
Temperature, ≥ 37.3 °C	103 (23.8)	26 (25.2)	50 (48.5)	27 (26.2)	77 (74.8)	2.74 (1.67 to 4.49)	< 0.0001
Respiratory rate, ≥ 30 breaths per min	53 (12.1)	13 (24.5)	21 (39.6)	19 (35.8)	40 (75.5)	2.50 (1.30 to 4.82)	0.006
Pulse oximeter O_2 saturation, ≤ 93%	146 (35.0)	25 (17.1)	82 (56.2)	39 (26.7)	121 (82.9)	6.37 (3.89 to 10.43)	< 0.0001
Systolic blood pressure, < 90 mmHg	4 (0.9)	2 (50.0)	2 (50.0)	0	2 (50.0)	0.74 (0.10 to 5.27)	0.760
Heart rate, > 125 beats per min	23 (5.2)	5 (21.7)	10 (43.5)	8 (34.8)	18 (78.3)	2.77 (1.01 to 7.61)	0.048
Severity of illness							
In-hospital disease severity status, general	128 (29.1)	88 (68.8)	37 (28.9)	3 (2.3)	40 (31.3)	Ref	Ref
Severe	184 (41.8)	80 (43.5)	84 (45.7)	20 (10.9)	104 (56.5)	2.86 (1.78 to 4.59)	< 0.0001
Critical	128 (29.1)	18 (14.1)	64 (50.0)	46 (35.9)	110 (85.9)	13.44 (7.21 to 25.07)	< 0.0001
qSOFA score, 0	231 (52.7)	114 (49.4)	95 (41.1)	22 (9.5)	117 (50.6)	Ref	Ref
1	179 (40.9)	59 (33.0)	80 (44.7)	40 (22.3)	120 (67.0)	1.98 (1.32 to 2.97)	< 0.0001
2-3	28 (6.4)	13 (46.4)	9 (32.1)	6 (21.4)	15 (53.6)	1.12 (0.51 to 2.47)	0.770
CURB-65 score, 0-1	330 (75.3)	159 (48.2)	132 (40.0)	39 (11.8)	171 (51.8)	Ref	
2	71 (16.2)	17 (23.9)	41 (57.7)	13 (18.3)	54 (76.1)	2.95 (1.64 to 5.31)	< 0.0001
3-5	37 (8.4)	10 (27.0)	11 (29.7)	16 (43.2)	27 (73.0)	2.51 (1.18 to 5.35)	0.017
Routine blood results							
White blood cell count, < 3.5 × 10^9/L	44 (10.0)	17 (38.6)	19 (43.2)	8 (18.2)	27 (61.4)	0.68 (0.35 to 1.29)	0.234
3.5-9.5	313 (71.3)	151 (48.2)	126 (40.3)	36 (11.5)	162 (51.8)	Ref	Ref
> 9.5	82 (18.7)	18 (22.0)	40 (48.8)	24 (29.3)	64 (78.0)	2.24 (1.00 to 4.99)	0.049
Lymphocyte count, < 1.1 × 10^9/L	232 (52.8)	62 (26.7)	115 (49.6)	55 (23.7)	170 (73.3)	4.1 (2.74 to 6.12)	< 0.0001
Neutrophil count, > 6.3 × 10^9/L	149 (33.9)	44 (29.5)	73 (49.0)	32 (21.5)	105 (70.5)	2.29 (1.50 to 3.49)	< 0.0001
Hemoglobin, < 130 g/L for male; < 115 g/L for female	194 (44.4)	85 (43.8)	76 (39.2)	33 (17.0)	109 (56.2)	0.90 (0.61 to 1.31)	0.576
Platelet count, < 125 × 10^9/L	59 (13.5)	17 (28.8)	31 (52.5)	11 (18.6)	42 (71.2)	1.98 (1.09 to 3.6)	0.026
Biochemical results-basic values							

Albumin, < 35 g/L	232 (52.7)	58 (25.0)	118 (50.9)	56 (24.1)	174 (75.0)	4.80 (3.19 to 7.22)	< 0.0001
ALT, > 41 U/L for male; > 33 U/L for female	113 (25.7)	0	77 (68.1)	36 (31.9)	113 (100.0%)	-	-
AST, > 40 U/L for males; > 32 U/L for females	144 (32.7)	0	91 (63.2)	53 (36.8)	144 (100.0)	-	-
Total bilirubin, > 26 mmol/L for males; > 21 mmol/L for females	25 (5.7)	0	12 (48.0)	13 (52.0)	25 (100.0)	-	-
Direct bilirubin, > 8.0 mmol/L	61 (13.9)	4 (6.6)	29 (47.5)	28 (45.9)	57 (93.4)	13.16 (4.68 to 37.01)	< 0.0001
Lactate dehydrogenase, > 225 U/L for males; > 214 U/L for females	332 (75.5)	105 (31.6)	162 (48.8)	65 (19.6)	227 (68.4)	6.49 (3.96 to 10.62)	< 0.0001
γ-Glutamyl transferase, > 71 U/L for males; > 42 U/L for females	115 (26.4)	20 (17.4)	62 (53.9)	33 (28.7)	95 (82.6)	5.12 (3.01 to 8.7)	< 0.0001
Creatinine, > 104 μmol/L for males; > 84 μmol/L for females	70 (16.0)	22 (31.4)	31 (44.3)	17 (24.3)	48 (68.6)	1.73 (1.01 to 2.99)	0.048
C-reactive protein, ≥ 1 mg/L	369 (87.6)	145 (39.3)	161 (43.6)	63 (17.1)	224 (60.7)	2.92 (1.59 to 5.36)	0.0010
Coagulation and inflammation index results							
Prothrombin time, ≤ 14.5 s	289 (66.3)	144 (49.8)	116 (40.1)	29 (10.0)	145 (50.2)	0.35 (0.23 to 0.54)	< 0.0001
Prothrombin activity, < 75%	82 (18.8)	14 (17.1)	43 (52.4)	25 (30.5)	68 (82.9)	4.39 (2.38 to 8.09)	< 0.0001
Activated partial thromboplastin time, > 42.0 s	166 (38.2)	46 (27.7)	89 (53.6)	31 (18.7)	120 (72.3)	2.69 (1.77 to 4.07)	< 0.0001
D-dimer, > 0.5 μg/mL	295 (68.4)	87 (29.5)	145 (49.2)	63 (21.4)	208 (70.5)	5.17 (3.33 to 8.03)	< 0.0001
Serum ferritin, > 400 μg/L for males; > 150 μg/L for females	319 (81.0)	111 (34.8)	153 (48.0)	55 (17.2)	208 (65.2)	6.91 (3.80 to 12.57)	< 0.0001
Interleukin-6, ≥ 7 pg/mL	255 (62.3)	73 (28.6)	123 (48.2)	59 (23.1)	182 (71.4)	4.62 (3.01 to 7.08)	< 0.0001
Interleukin-10, ≥ 5 pg/mL	177 (44.1)	41 (23.2)	90 (50.8)	46 (26.0)	136 (76.8)	4.67 (3.01 to 7.25)	< 0.0001
Procalcitonin, ≥ 0.5 ng/mL	57 (13.6)	10 (17.5)	29 (50.9)	18 (31.6)	47 (82.5)	3.76 (1.84 to 7.68)	< 0.0001
Imaging features							
Bilateral	381 (93.2)	155 (40.7)	162 (42.5)	64 (16.8)	226 (59.3)	2.62 (1.18 to 5.84)	0.018
Ground-glass opacity	306 (74.8)	119 (38.9)	131 (42.8)	56 (18.3)	187 (61.1)	1.73 (1.10 to 2.72)	0.017
Consolidation	89 (21.8)	29 (32.6)	36 (40.4)	24 (27.0)	60 (67.4)	1.69 (1.03 to 2.78)	0.037
Pleural effusion	47 (11.5)	6 (12.8)	25 (53.2)	16 (34.0)	41 (87.2)	5.85 (2.42 to 14.13)	< 0.0001

Data are presented as medians (interquartile range) or *n* (%) according to the corresponding conditions.

[1]ORs were calculated using univariate unconditional logistic regression.

[2]All *P* values were calculated using univariate unconditional logistic regression for the characteristics between the patients with normal cumulative liver biochemistry and those with cumulative liver biochemical abnormality or injury, but two independent samples (unpaired) Student's *t*-test was used to compare the median ages.

[3]Referring to exposure to confirmed coronavirus disease 2019 patients.

[4]Comorbidities were defined as history of at least one disease out of the following: Hypertension, diabetes, cardiovascular disease, cerebrovascular disease, chronic renal disease, chronic respiratory disease, and chronic liver disease.

[5]Four patients reported history of either fatty liver or cirrhosis. "-": It is not applicable due to the no individuals with abnormal alanine aminotransferase or aspartate aminotransferase or total bilirubin in normal cumulative liver biochemistry group. COVID-19: Coronavirus disease 2019; LB: Liver biochemistry; LBA: Liver biochemical abnormality; ALI: Acute liver injury; LBAI: Liver biochemical abnormality or injury; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CURB-65: Confusion, uremia, respiratory rate, blood pressure, and age ≥ 65; IQR: Interquartile range; OR: Odds ratio; CI: Confidence interval; qSOFA: Quick Sequential Organ Failure Assessment; BMI: Body mass index.

6.12) and elevated neutrophil count (OR = 2.29; 95%CI: 1.50 to 3.49). Most biochemical indices at admission indicated the subsequent incidence of cumulative LBAI, such as elevated γ-glutamyl transferase (GGT), decreased albumin, and elevated C-reactive protein. Abnormal coagulation parameters were strongly associated with cumulative LBAI, especially the increase of D-dimer levels, which suggested a 5-fold increase in the likelihood of cumulative LBAI. Serum ferritin, an index of inflammation, also showed a positive association with the cumulative incidence of LBAI (OR = 6.91; 95%CI: 3.8 to 12.57). The presence of bilateral lesions, ground-glass shadows, consolidation, or pleural effusion on CT images all suggested an increased risk of cumulative LBAI ($P < 0.05$).

On-admission LBAI is associated with cumulative ALI

Liver function on admission predicted the subsequent risk of cumulative occurrence of ALI among COVID-19 patients to a great extent, as shown in Figure 1. The cumulative incidence of ALI over 2 mo was 41.9% (95%CI: 31.4 to 51.9) in patients with on-admission LBAI, dramatically higher than the 9.6% (95%CI: 4.7 to 16.5) in patients with on-admission normal LB ($P < 0.0001$); the risk was 8-fold higher in the LBAI group than in the normal LB group [hazard ratio (HR) = 8.07; 95%CI: 4.23 to 5.37]. We also observed similar patterns albeit with a moderately reduced effect (HR = 4.83; 95%CI: 2.45 to 9.54) when on-admission LBA associated with the subsequent risk of in-hospital incidence of ALI was analyzed (Supplementary Figure 1).

Drugs and treatment associated with LBAI

To address the issue of whether liver dysfunction was caused by drugs commonly used to treat COVID-19, such as antibiotics, antivirus, traditional Chinese medicine/Chinese patent drug, and intravenous nutrition (IVN), we analyzed the association of drugs with subsequent incidence of ALI or LBAI or worsening LB (Figure 2 and Supplementary Figure 2). The results showed that patients who received IVN either on admission or in hospital were associated with cumulative LBAI or ALI; a similar association was found between in-hospital use of antibiotics and subsequent ALI or worsening LB.

Treatment with oxygen therapy by mask, high-flow nasal cannula, or mechanical ventilation were used approximately 6-fold more in patients with cumulative LBAI than those with normal cumulative LB, indicating that hypoxia was an important risk factor for LBAI (Supplementary Table 1).

On-admission and cumulative LBAI is associated with increased risk of in-hospital mortality

In this cohort predominated by severe or critical COVID patients, a mortality of 22.3% (98/440) was observed from admission to thereafter 2 mo with a substantial difference in mortality between patients with cumulative LBAI and normal cumulative LB (34.3% *vs* 5.9%, $P < 0.0001$) (Table 2). With the worsening status of liver function on-admission, the mortality risk significantly increased from double in on-admission LBA (adjusted HR = 1.78; 95%CI: 1.03 to 3.06) to up to four times the risk in on-admission ALI (adjusted HR = 4.00; 95%CI: 1.68 to 9.50; Figure 3A and Supplementary Table 2). An even stronger association was noticed when cumulative liver injury instead of on-admission liver injury was used (Figure 3B).

In-depth analysis into single markers indicating liver function revealed that the levels of AST and TBIL but not ALT at admission or at peak were strong risk indicators of mortality (Figure 3C-E,Supplementary Figure 3, and Supplementary Table 2). To clarify the potential impact of existing chronic liver disease on the prognosis, such as hepatitis, fatty liver, or cirrhosis, we conducted a sensitivity analysis by removing 12 cases with chronic liver diseases, and found similar results (Supplementary Table 2). Meanwhile, to clarify the effects of 38 censored patients on the survival analysis, we also conducted another sensitivity analysis and confirmed similar conclusions (Supplementary Table 2).

In addition, shorter intervals between illness onset and hospital admission (12 d *vs*

Table 2 Outcomes for 440 patients of coronavirus disease 2019, stratified by severity of cumulative liver abnormality or injury

	All patients (*n* = 440)	Normal cumulative LB (*n* = 186)	Cumulative LBA (*n* = 185)	Cumulative ALI (*n* = 69)	Cumulative LBAI (*n* = 254)	*P* value[1]
Outcomes						
Discharge	304 (69.1)	166 (54.6)	117 (38.5)	21 (6.9)	138 (45.4)	< 0.0001[2]
Death	98 (22.3)	11 (11.2)	45 (45.9)	42 (42.9)	87 (88.8)	
Still in hospital	38 (8.6)	9 (23.7)	23 (60.5)	6 (15.8)	29 (76.3)	
ICU admission	91 (20.7)	11 (12.1)	45 (49.5)	35 (38.5)	80 (88.0)	< 0.0001
Time from illness onset to hospital admission (median, IQR), d	438	19 (11 to 35)	13 (9 to 19)	11 (8 to 16)	12 (9 to 18)	< 0.0001
Hospital stay time (median, IQR), d	402	12 (6 to 22)	19 (11 to 29)	17 (8 to 28)	19 (10 to 29)	< 0.0001
Time from illness onset to death (median, IQR), d	93	19 (9 to 26)	23 (16 to 33)	28 (18 to 33)	25 (17 to 33)	0.037
Time from illness onset to discharge (median, IQR), d	303	40 (32 to 49)	40 (34 to 51)	42 (31 to 51)	40 (34 to 50)	0.088
Time from illness onset to last day of follow-up (median, IQR), d	37	61 (52 to 66)	59 (49 to 64)	58 (52 to 67)	59 (51 to 65)	0.689
Time from illness onset to ICU admission (median, IQR), d	90	15 (13 to 25)	18 (11 to 30)	19 (14 to 26)	18 (12 to 26)	0.478
Duration of viral shedding after illness onset (median, IQR), d	298	33 (24 to 42)	30 (25 to 38)	26 (21 to 36)	30 (24 to 38)	0.172

Data are presented as *n* (%).

[1]Comparison of the characteristics between normal cumulative liver biochemistry and cumulative liver biochemical abnormality or injury was calculated by Pearson's chi-square test for outcomes and a nonparametric Mann-Whitney *U* test was used for time.

[2]Comparison of the distribution of disease outcomes (discharge, death, and still in hospital) between normal cumulative liver biochemistry and cumulative liver biochemical abnormality or injury. COVID-19: Coronavirus disease 2019; LB: Liver biochemistry; LBA: Liver biochemical abnormality; LBAI: Liver biochemical abnormality or injury; ALI: Acute liver injury; ICU: Intensive care unit; IQR: Interquartile.

19 d, $P < 0.0001$), longer hospital stays (19 d *vs* 12 d, $P < 0.0010$), and prolonged time from illness onset to death (25 d *vs* 19 d, $P = 0.037$) were observed in the patients with cumulative LBAI compared to the patients with normal cumulative LB (Table 2).

Dynamic variations of liver function monitoring of the risk of in-hospital mortality

The temporal patterns of LB parameters over time from illness onset differed by different survival outcomes of COVID-19 patients (Figure 4). In general, the levels of ALT, AST, and TBIL varied in a wider range over time in the non-survivors than in the survivors. Although no pronounced elevation of ALT levels was observed especially at the time of admission, there was a smooth decrease over time in the survivors compared to a sharp elevation at the fourth week in the non-survivors. AST and TBIL levels at the time of admission tended to be higher in the non-survivors and showed a sharp elevation at the fourth and sixth weeks or continuous elevation until the fifth week, respectively, in contrast with almost no variation over time in survivors (Figure 4). The peak of ALT, AST, and TBIL happened at the fourth, sixth, and fifth weeks from illness onset, respectively. Additional LB markers such as GGT, direct bilirubin, and albumin also presented different time-varying tendency in the non-survivors from that of survivors (Supplementary Figure 4).

Hepatoprotective drugs associated with a reduced risk of in-hospital mortality

The use of hepatoprotective drugs in the patients with cumulative LBAI tended to attenuate the risk of in-hospital mortality by 38% in the univariate model (crude HR = 0.62; 95%CI: 0.41 to 0.96; Figure 3F and Supplementary Table 2) compared to their normal LB counterparts, although this association was not significant after adjustment for confounding risk factors such as age, sex, comorbidities, disease severity,

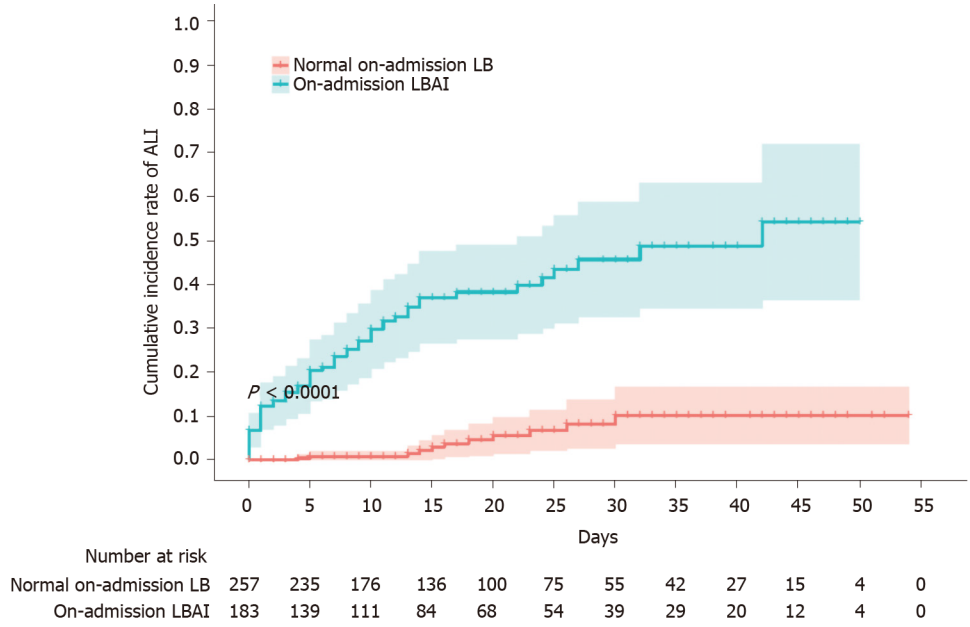


Figure 1 Cumulative incidence rate of acute liver injury stratified by on-admission liver biochemistry. Shadows indicate the 95% confidence intervals of the corresponding estimates cumulative incidence rate. ALI: Acute liver injury; LB: Liver biochemistry; LBAI: Liver biochemical abnormality or injury.

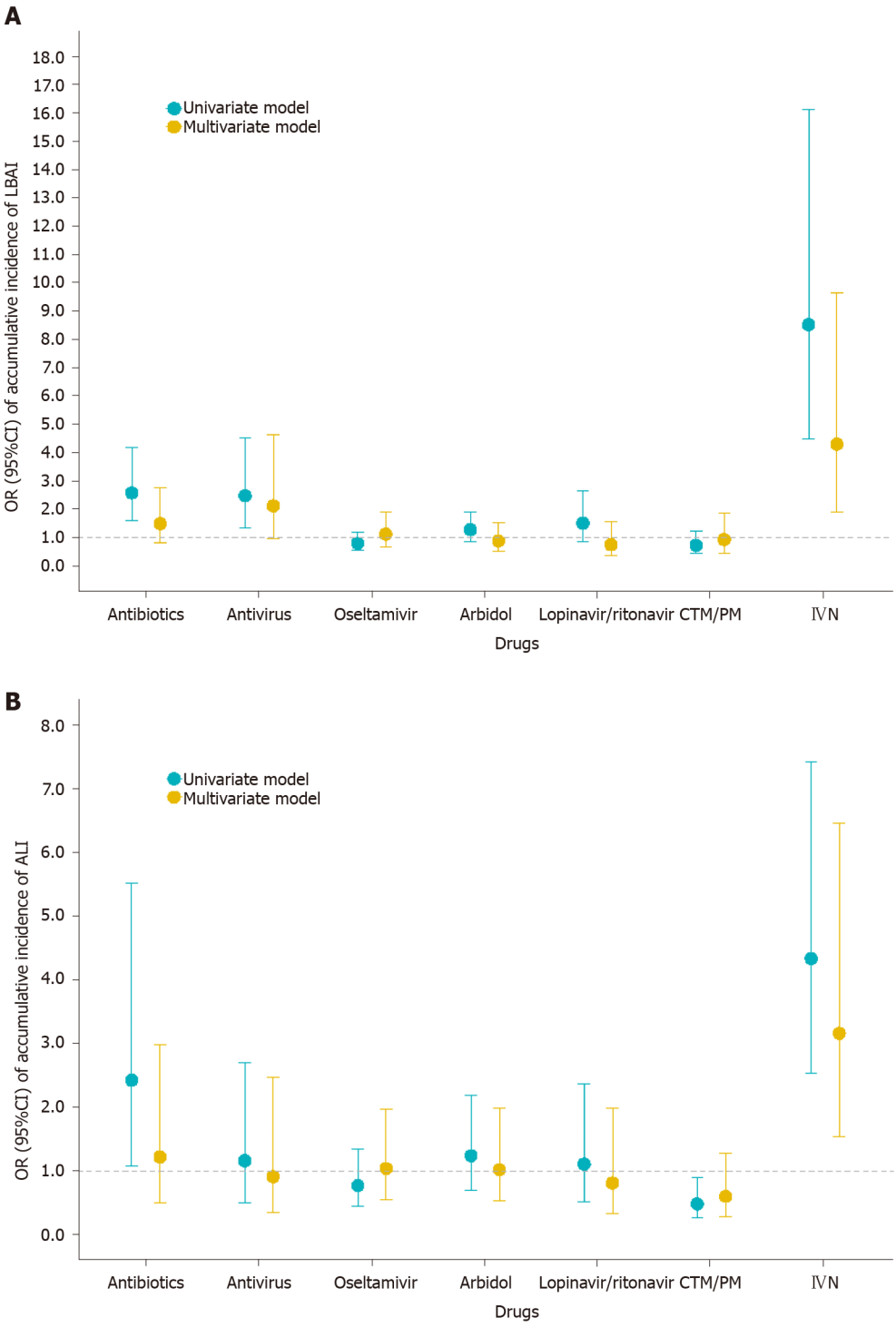
lymphocyte count, D-dimer, and serum ferritin (adjusted HR = 0.67; 95%CI: 0.4 to 1.08).

DISCUSSION

In the present retrospective cohort study, we observed a high LBAI prevalence of up to 57.7% and a high mortality of 22.3% in hospitalized patients with relatively severe COVID-19. Some key demographics, clinical features, and lab parameters, *e.g.*, age ≥ 65 years, female gender, comorbidities, the severity of disease, depleted lymphocyte count, abnormal D-dimer, and elevated serum ferritin levels, were found to be significantly associated with liver injury. On-admission liver function is a reliable predictor of subsequent liver injury. Patients with liver injury had up to four times the risk of mortality. AST and TBIL were identified to be factors that were most strongly related to prognosis and therefore could aid in prognosis monitoring. The use of antibiotics and IVN were associated with liver injury and therefore should be used with caution among COIVD-19 patients. Hepatoprotective drugs tended to favor survival, and their uses deserve recommendations for patients with abnormal liver function.

In some recent studies, elevated levels of ALT and AST were reported in patients with COVID-19, with rates ranging from 14% to 53%[4-7]. The prevalence of liver injury in the present study was higher, partly due to the high proportion of severe and critical COVID-19. Previous studies found that liver injury was common in critically ill patients with COVID-19[4,6]. Several indices of liver function, including ALT, AST, and TBIL, were significantly elevated in severe COVID-19 cases compared with mild cases[1]. These results were coincident with ours. Besides this, we found some key demographics, *e.g.*, age 65 years or above, female gender, and comorbidities, were significant indicators of liver injury. These demographic characteristics and comorbidities are wind vanes for liver injury and are cue signals which are helpful for intensive care and individualized tailored surveillance.

Multiple explanations for the mechanism of liver injury have been proposed to date[13]. One hypothesis is that COVID-19 itself is a likely cause of liver injury. Liver biopsy specimens of patients who died from COVID-19 show degeneration of hepatocytes and focal necrosis[8]. Abundant SARS-CoV-2 viral particles were observed in hepatocytes[14]. Angiotensin-converting enzyme 2 (ACE2) receptor is the cell entry receptor of SARS-CoV-2, and according to a recent study using single-cell RNA sequencing, ACE2 was highly expressed not only in type II alveolar epithelial cells, but also in bile duct cells[15]. Hence, it is hypothesized that SARS-CoV-2 may infect the liver and cause abnormal liver function in these patients[16]. Our findings that the elevated


A
Univariate model
Multivariate model
OR (95%CI) of accumulative incidence of LBAI
18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Antibiotics
Antivirus
Oseltamivir
Arbidol
Lopinavir/ritonavir
CTM/PM
IVN
Drugs
B
Univariate model
Multivariate model
OR (95%CI) of accumulative incidence of ALI
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Antibiotics
Antivirus
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Arbidol
Lopinavir/ritonavir
CTM/PM
IVN
Drugs

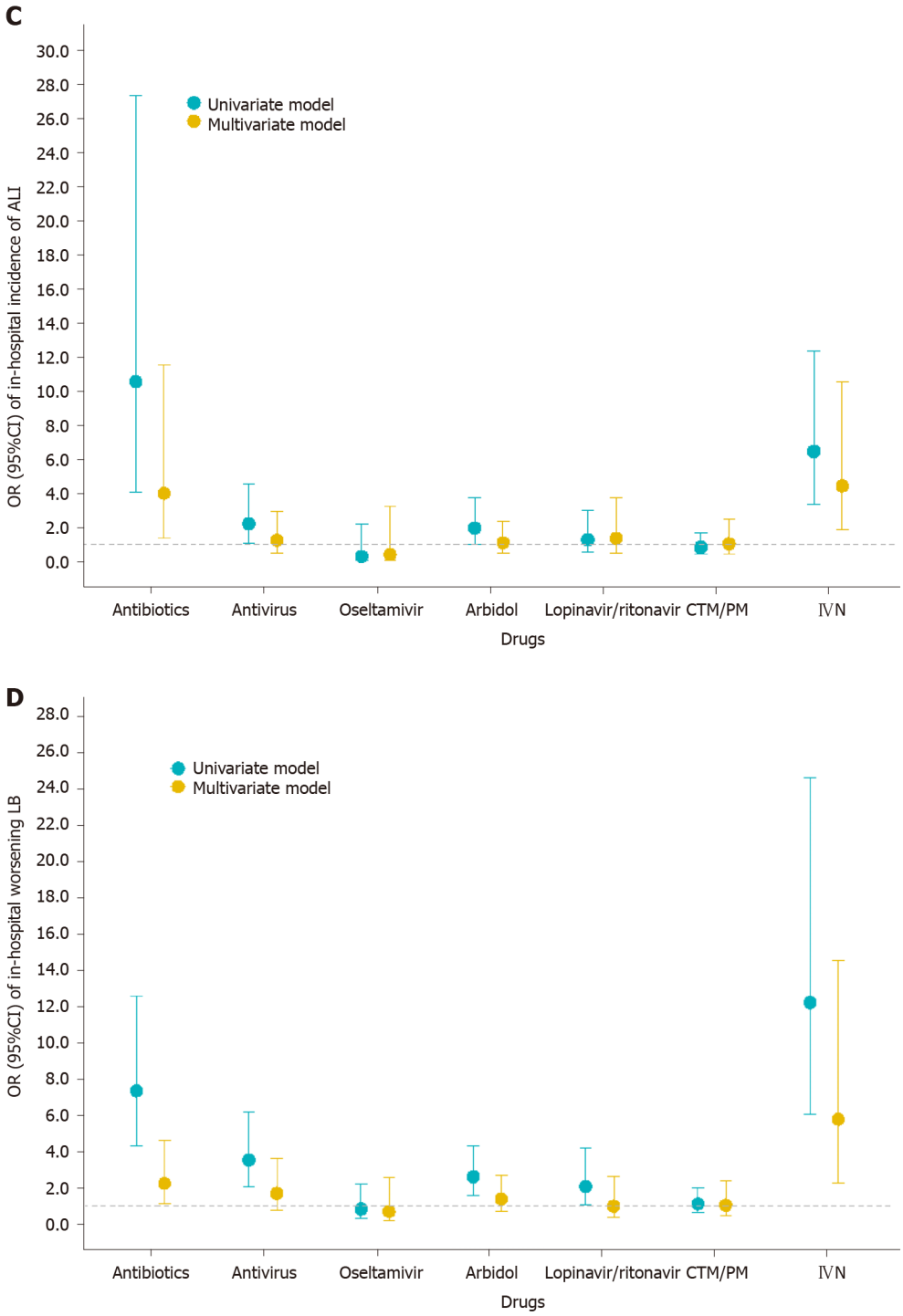
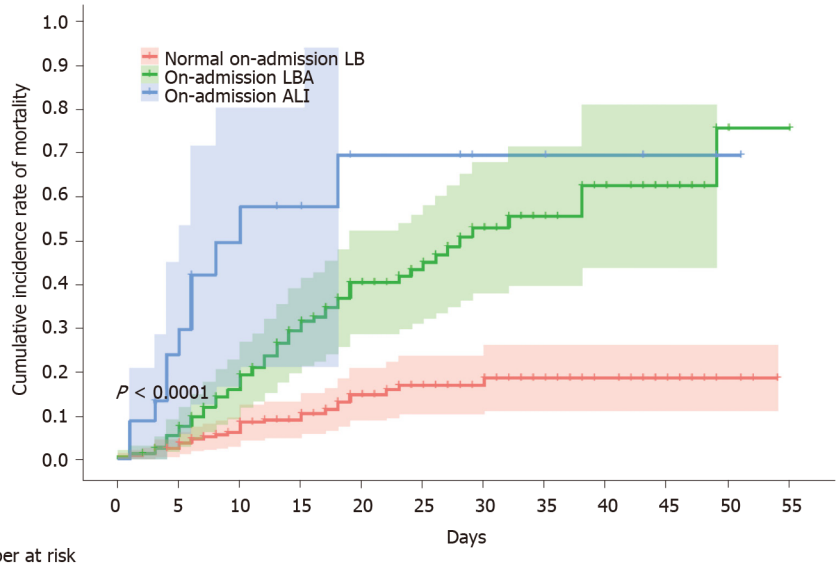


Figure 2 Association between drugs and liver injury. A: Cumulative incidence of liver biochemical abnormality or injury and drugs used during the whole course; B: Cumulative incidence of acute liver injury and drugs used during the whole course; C: In-hospital incidence of acute liver injury and drugs used after admission; D: In-hospital worsening liver biochemistry and drugs used after admission. Univariate model refers to univariate binary logistic regression model. Multivariate model refers to multivariate binary logistic regression model adjusted by age, sex, comorbidities (defined as history of at least one disease out of hypertension, diabetes, cardiovascular disease, cerebrovascular disease, chronic renal disease, chronic respiratory disease, and chronic liver disease), in-hospital disease severity status, lymphocyte count, D-dimer, and serum ferritin. Antivirals included oseltamivir, arbidol, lopinavir/ritonavir, and some other uncommonly used antiviral drugs. OR: Odds ratio; CI: Confidence interval; ALI: Acute liver injury; LB: Liver biochemistry; LBAI: Liver biochemical abnormality or injury.

GGT, a cholangiocyte-related enzyme, was strongly associated the increased risk of liver injury may provide supportive evidence for the hypothesis.

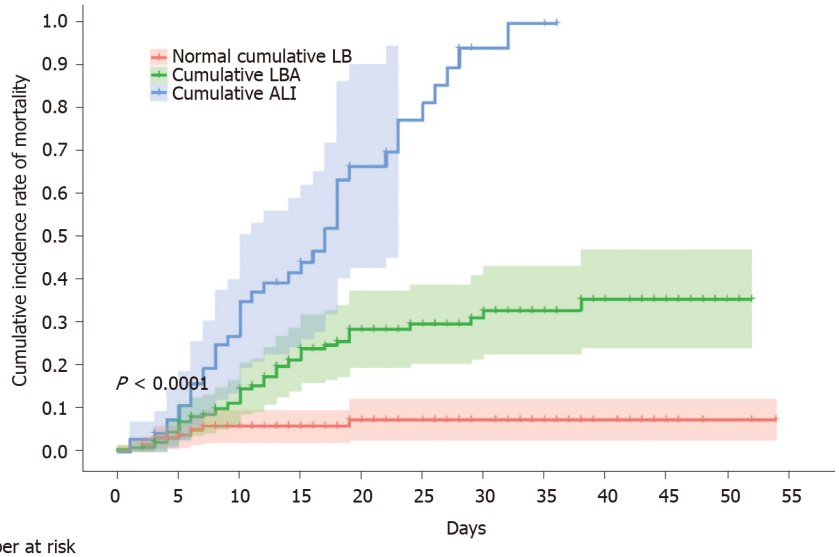
Other causes of ALI may be systemic inflammatory response, hypoxemia, and drug usage[17]. During the course of COVID-19, immune cells can release a group of inflammatory cytokines[18], which cause systemic inflammatory response syndrome (SIRS) and acute respiratory distress syndrome (ARDS). SIRS and ARDS can lead to more cell damage and necrosis. This vicious cycle can lead not only to lung damage

A



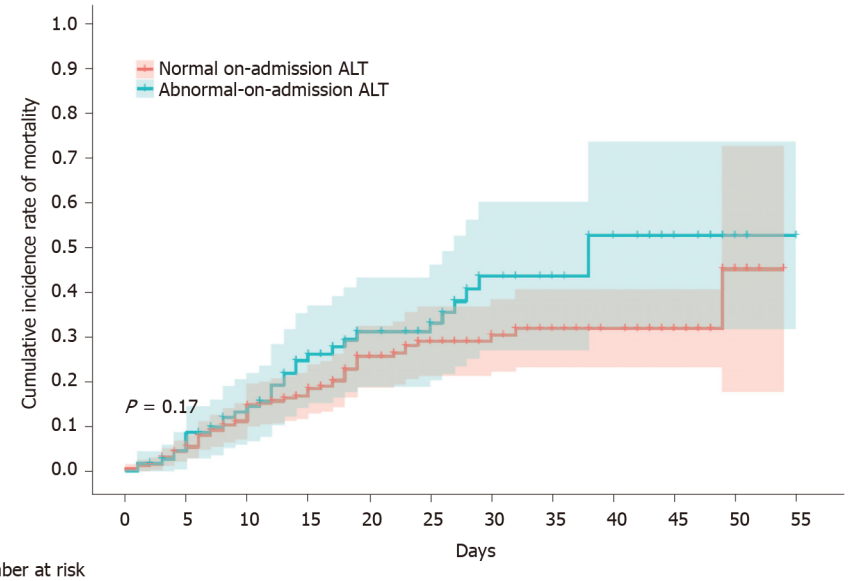
Number at risk												
Normal on-admission LB	257	235	177	139	104	79	60	48	32	17	4	0
On-admission LBA	159	143	122	99	77	63	45	35	24	14	5	1
On-admission ALI	24	18	13	10	7	7	4	4	3	2	1	0

B



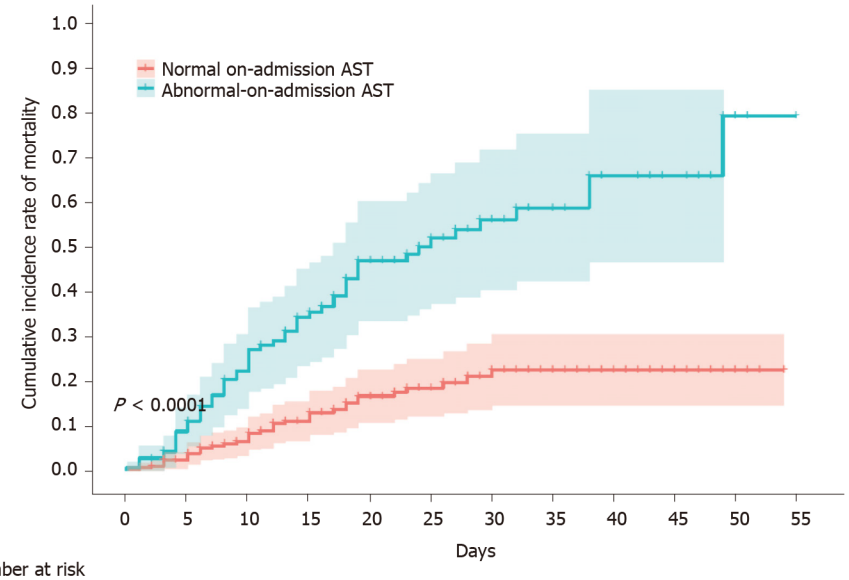
Number at risk												
Normal cumulative LB	186	165	111	84	60	43	31	24	15	8	2	0
Cumulative LBA	185	169	150	122	98	80	68	46	31	19	6	0
Cumulative ALI	69	63	51	42	30	26	18	17	13	6	2	1

C



Number at risk												
Normal on-admission ALT	327	297	226	180	135	101	78	61	39	22	6	0
Abnormal-on-admission ALT	113	100	86	68	53	48	31	26	20	11	4	1

D



Number at risk												
Normal on-admission AST	296	268	205	159	120	92	67	52	36	20	5	0
Abnormal-on-admission AST	144	129	107	89	68	57	42	35	23	13	5	1

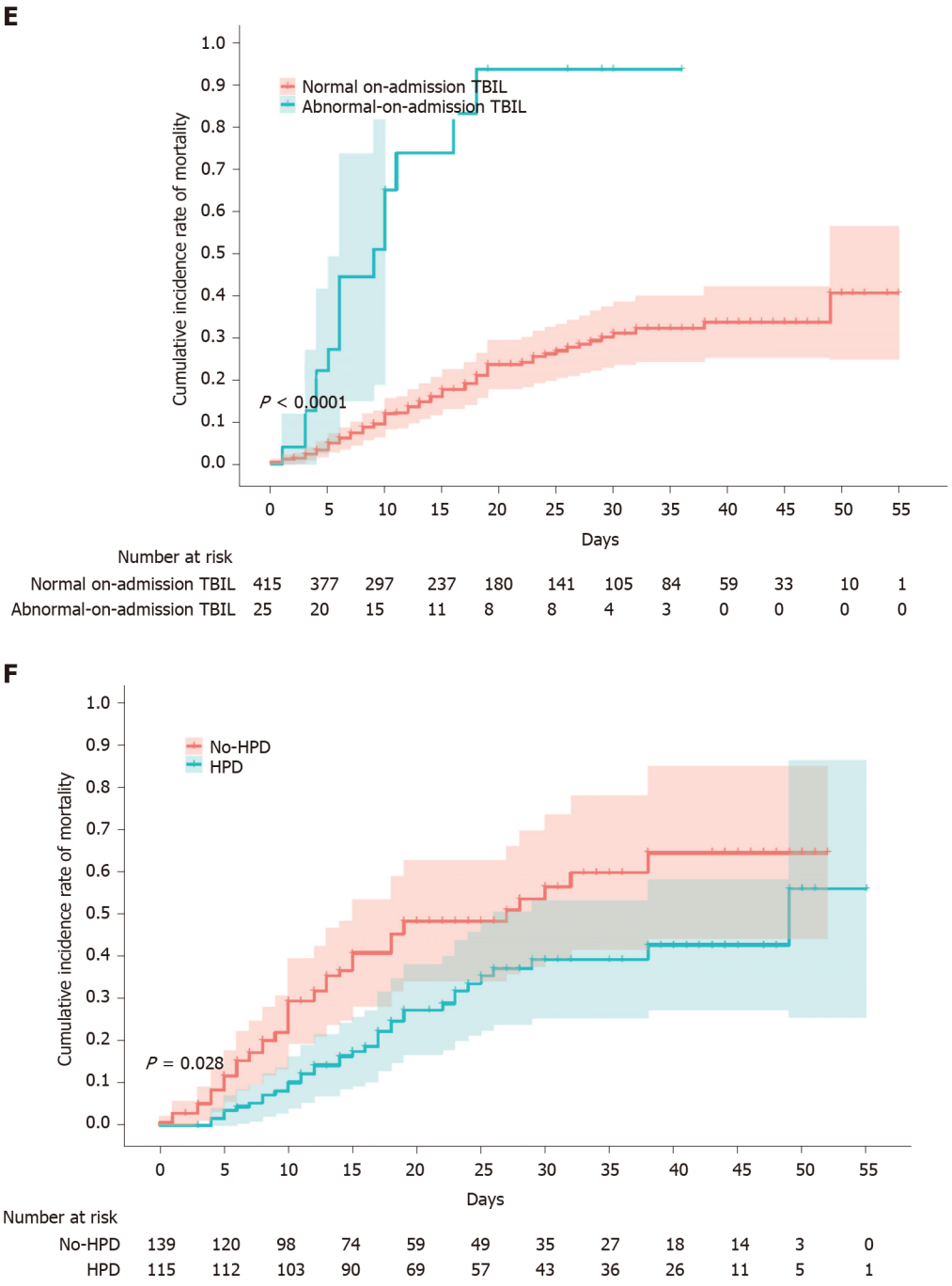


Figure 3 Cumulative incidence of in-hospital mortality of patients with coronavirus disease 2019, stratified by liver disease indicators or hepatoprotective drugs. A-F: Shadows indicate the 95% confidence intervals of the corresponding estimates: Stages of on-admission liver injury (A), stages of cumulative liver injury (B), on-admission alanine aminotransferase (C), on-admission aspartate aminotransferase (D), on-admission total bilirubin (E), and hepatoprotective drug uses in patients with abnormal liver function (F). LB: Liver biochemistry; LBA: Liver biochemical abnormality; ALI: Acute liver injury; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; HPD: Hepatoprotective drugs.

but also to liver damage[19]. Our study observed that depleted lymphocytes, elevated procalcitonin, elevated interleukin-6, and elevated serum ferritin levels are significant indicators of liver injury, and these markers should be monitored. Many COVID-19 patients who experience different degrees of hypoxemia need to receive oxygen therapy. In theory, hypoxemia in severe COVID-19 pneumonia may lead to hypoxia of liver tissue and abnormalities of liver function[5].

Many COVID-19 patients, especially those who are severe and critically severe, are treated with multiple drugs. Liver injury emerging during the course of disease could be the side effect of drugs or drug interactions. In the present study, we explored the effect of drugs on liver function, and found two commonly used drugs, IVN and antibiotics, related with increased risk of LBAI. This indicated that IVN and antibiotics

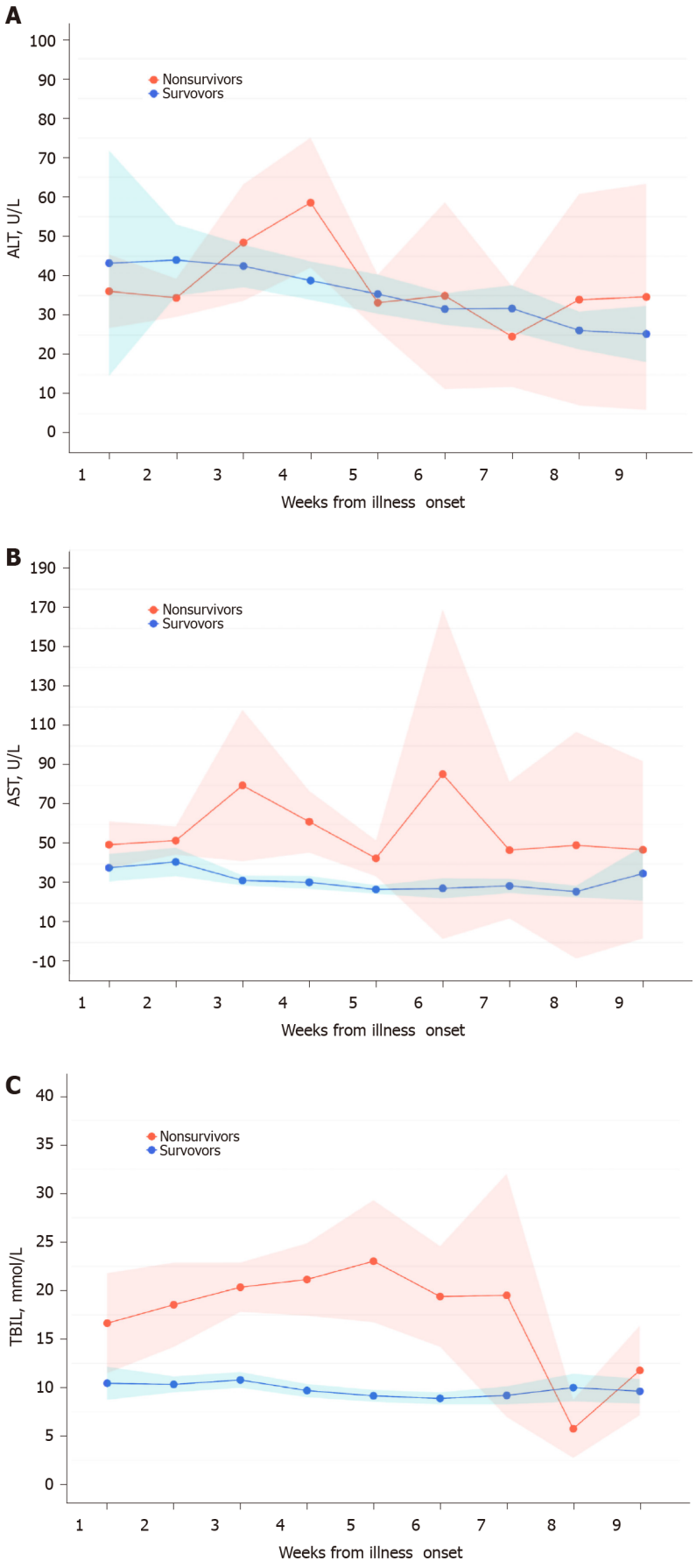


Figure 4 Dynamic variations of liver biochemistry from illness onset. A-C: Shadows indicate the 95% confidence intervals of the corresponding estimates: Alanine aminotransferase (A), aspartate aminotransferase (B), and total bilirubin (C). TBIL: Total bilirubin.

should be given with great caution in patients with COVID-19.

The patients included in the present study were severely ill and the overall mortality was high. The studied wards were rebuilt during the epidemic for severe or critical patients, although in the later part of the study period, we received some general patients. In addition, patients were sometimes transferred to the studied wards late in their illness, contributing to the high rate of poor clinical outcomes. Protecting high-risk individuals to reduce the mortality of COVID-19 patients is a fundamental challenge in managing the pandemic. In addition to old age and underlying comorbidities as the well-established predictors of high risk[20], we found that patients with liver abnormalities were at high risk. In particular, acute liver injury was an independent risk factor with a mortality four times higher than normal liver function. Moreover, progressive liver injury, indicated by liver function markers which were continuously abnormal over the course of disease, strongly predicted mortality in COVID-19. Our findings were supported by a recent study in which AST abnormality was found to be strongly associated with risk of mortality[21]. However, the design of our study, which included more complete baseline clinical characteristics to effectively adjust for bias, leads to more extensive findings than that of previous studies. For instance, TBIL abnormality, not a significant indicator in the earlier study at admission or peak, sensitively indicated subsequent mortality risk in our study; liver injury panel results rather than single indices such as AST were more informative for evaluating both the severity of liver injury and the mortality risk.

To manage the liver injury and thereafter reduce the mortality risk, several tips might be of great assistance. First, the underlying liver diseases should be treated properly. Second, the therapeutic regimen, *e.g.*, abovementioned antibiotics, with respect to the varieties, doses, and duration should be used cautiously and moderately for reducing the risk of drug-induced liver injury. Third, all COVID-19 patients, particularly at severe or critical stage, were advised to receive meticulous monitoring of liver associated biochemical indicators, such as AST and TBIL, so as to catch liver injury in time. Fourth, for COVID-19 patients, any prophylactic use of drugs to protect the liver is not mandatory. But for patients with LBAI, as the evidence of this present study, the commonly used hepatoprotective drugs could improve survival to some degree.

Our study, which is among the first studies of survival analysis associated with liver injury in COVID-19, provided valuable information on the effective prevention, intensive monitoring, and individualized treatment for COVID-19 patients. However, there are several limitations to be addressed. First, any retrospective single-center study could have selection biases in identifying and recruiting participants. Second, the population size was relatively small. Third, we found that comorbidities were related to liver injury, but a small fraction of patients had various degrees of consciousness disorders at admission which made it difficult to collect detailed medical history. Fourth, we did not have enough medical resource to assess clinical-relevant injury such as ascites and encephalopathy.

CONCLUSION

In conclusion, liver injury is highly prevalent among COVID-19 patients and also can be fatal, particularly in severe or critical patients. More intensive monitoring of AST or TBIL and individualized tailored therapeutics are highly recommended for COVID-19 patients with liver injury, especially in patients aged ≥ 65 years, female patients, or those with other comorbidities. Drug hepatotoxicity should be alert for the use of antibiotics and IVN for COVID-19 patients. When severe liver damage occurs, liver protective drugs are favorable for improved prognosis. Further research should focus on the mechanism of liver injury in COVID-19 and more effective measures to prevent progressive liver injury and mortality risks of COVID-19.

ARTICLE HIGHLIGHTS

Research background

The prevalence of liver injury in hospitalized patients with coronavirus disease 2019 (COVID-19) is high.

Research motivation

Comprehensive and detailed studies on the clinical features, risk factors, and prognosis of patients with liver injury are still lacking, and it is urgent to clarify predictive factors and therapeutic approaches for liver injury.

Research objectives

We aimed to conduct an in-depth investigation into the risk factors of liver injury and into the effective measures to prevent subsequent mortality risk.

Research methods

A retrospective cohort study was performed on 440 consecutive patients with relatively severe COVID-19 between January 28 and March 9, 2020 at Tongji Hospital, Wuhan, China. Data on clinical features, laboratory parameters, medications, and prognosis were collected.

Research results

High-risk population, especially patients aged ≥ 65 years, female patients, or those with other comorbidities should be intensively monitored. On-admission total bilirubin has the strongest correlation with the prognosis of COVID-19 patients, which can be used for monitoring of COVID-19 patients at risk of liver injury. Intravenous nutrition (IVN) and antibiotics are associated with abnormal liver biochemistry; these drugs should be given with caution. Hepatoprotective drugs are favorable for patients with liver injury.

Research conclusions

More intensive monitoring of aspartate aminotransferase or total bilirubin is recommended for COVID-19 patients, especially patients aged ≥ 65 years, female patients, or those with other comorbidities. Drug hepatotoxicity of antibiotics and IVN should be alert for COVID-19 patients.

Research perspectives

IVN and antibiotics are associated with abnormal liver biochemistry; these drugs should be given with caution. Hepatoprotective drugs are favorable for patients with liver injury.

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ORIGINAL ARTICLE

Retrospective Study

Surgical resection of esophagogastric junction stromal tumor: How to protect the cardiac function

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Abstract

BACKGROUND

Various surgical procedures have been described for gastrointestinal stromal tumors (GISTs) at the esophagogastric junction (EGJ) close to the Z-line. However, surgery for EGJ-GIST involving Z-line has been rarely reported.

AIM

To introduce a novel technique called conformal resection (CR) for open resection of EGJ-GIST involving Z-line.

METHODS

In this retrospective study, 43 patients having GISTs involving Z-line were included. The perioperative outcomes of patients receiving CR (n = 18) was compared with that of proximal gastrectomy (PG) (n = 25).

RESULTS

CR was successfully performed in all the patients with negative microscopic margins. The mean operative time, time to first passage of flatus, and postoperative hospital stay was significantly shorter in the CR group ($P < 0.05$), while the intraoperative blood loss was similar in the two groups. The postoperative gastroesophageal reflux as diagnosed by esophageal 24-h pH monitoring and quality of life at 3 mo were significantly in favor of CR compared to PG (both $P < 0.001$). The 5-year disease-free survival between the two groups was similar ($P = 0.163$). The cut- off value for the determination of CR or PG was 7.0 mm above the Z-line (83.33% sensitivity, 84.00% specificity, 83.72% accuracy).

CONCLUSION

CR is safe and feasible for EGJ-GIST located within 7.0 mm above the Z-line.

University (Liaoning Cancer Hospital and Institute) Institutional Review Board (approval No. 20190461).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Core Tip: We retrospectively enrolled 43 cases of esophagogastric junction-gastrointestinal stromal tumor (EGJ-GIST) involving Z-line, including 25 cases in the proximal gastrectomy (PG) group and 18 cases in the conformal resection (CR) group. The operation CR was introduced, and the following indicators were analyzed: Clinicopathological characteristics, perioperative outcomes, postoperative esophageal 24-h pH, postoperative quality of life, and 5-year disease-free survival. Finally, the cut-off value above the Z-line for the determination of CR or PG was determined. Our results confirm that CR is safe and feasible for EGJ-GIST located within 7.0 mm above the Z-line. CR was associated with lower incidence of postoperative gastroesophageal reflux and better quality of life with similar oncological outcomes compared to PG.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) contribute to 1%-3% of all gastrointestinal malignancies. They originate primarily from the interstitial cells of Cajal or from their stem cell precursors[1]. The most common sites of GIST are the stomach and small intestine. Rarely, they can arise in the esophagus, appendix, gallbladder, pancreas, and the retroperitoneum[2]. Surgery is the mainstay of treatment for localized and resectable GIST. The main principles of the surgical procedure are complete excision with tumor-free resection margins and preservation of the organ functions as much as possible[3].

The esophagogastric junction (EGJ) is an uncommon site of GIST. Surgery in such cases is often difficult due to the anatomical location of these tumors with the need to perform proximal gastrectomy (PG). Resection of the EGJ with cardia results in severe postoperative gastroesophageal reflux (GER) and poor quality of life. The main hurdle to understanding and treating these tumors is their relative rarity and the subsequent shortage of literature. Recently, many different surgical procedures have been described to resect EGJ-GIST near the Z-line called type B lesions (Figure 1)[4-10]. However, there are limited studies on resection of type A lesions (when the tumor is located at the EGJ and the upper edge of the tumor is invading the Z-line) (Figure 1). For type A GIST, surgical approach is technically challenging because of the complex anatomical factors and the difficulty of functional preservation of lower esophageal sphincter[11,12].

In this article we have described a novel technique for EGJ-GIST involving Z-line (type A) called conformal resection (CR). The main purpose of this surgical technique was to achieve R0 resection of GIST with preservation of the sphincter function. We have also compared the outcomes of CR with PG.

MATERIALS AND METHODS

Patients

This study is a retrospective analysis of the prospectively maintained database of sarcoma patients at Liaoning Cancer Hospital and Institute. In this study, we included patients undergoing resection of primary localized EGJ-GIST involving Z-line from January 2009 to December 2017. All cases were diagnosed as GIST after preoperative endoscopic ultrasound-guided fine needle aspiration or core needle biopsy. Patients

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were excluded if they underwent concomitant resection for other malignancies, *e.g.*, patients with incidentally discovered GISTs in specimens resected for gastric, esophageal, or pancreatic carcinomas. The data of the patients collected were as follows: Clinical and pathologic findings, intra-operative and postoperative outcomes, esophageal 24-h pH parameters, the 36-item short-form health survey (SF-36), and disease-free survival (DFS). This study was approved by the Ethics Committee of Cancer Hospital of China Medical University (Liaoning Cancer Hospital and Institute) (ethics number: 20190461). Written informed consent was obtained from each patient at our center. The decision to perform CR or PG was taken by the treating surgeon based on the preoperative endoscopy and intraoperative findings.

General surgical technique

The patient was placed in the supine position. The median upper abdominal incision of 15-20 cm was taken from the xiphoid process after induction of general anesthesia. The abdominal wall was retracted by a self-retaining retractor. To facilitate subsequent dissection, the triangular ligament of the left lobe of the liver was taken down with electrocautery to expose the esophageal hiatus and the gastric cardia. We made a precise and irregular excision according to the shape of the type A GIST and preserved the Z-line as much as possible and then performed manual suturing. We used the concept of conformal radiotherapy for reference and named the operation CR. This procedure is different from previous simple wedge resection or PG.

The surgical technique of CR of the type A GIST lesions located on the anterior wall and posterior wall of EGJ has been described below.

Surgical technique for CR of type A GIST located in the anterior wall of EGJ

The tumor location was confirmed by visualization and palpation (Figure 2A and B). The lesser omentum at the EGJ was separated, and the cardia was clearly exposed on the right side. According to the size and shape of the tumor, wide local excision was performed in a caudo-cranial fashion from the gastric side of the tumor along the edge of the tumor to the esophageal side of the tumor. The resected specimen included part of the anterior wall of the stomach and part of the anterior wall of the esophagus (Figure 2C). The esophageal resection margin was kept as small as possible so as to preserve the cardiac function. Esophageal margin was examined by intraoperative frozen section to ensure a negative margin. After the complete removal of the tumor, the defect was sutured with 3-0 absorbable interrupted full thickness sutures. The esophageal wall was sutured to the gastric wall beginning from the gastric greater curvature and progressed towards the lesser curvature so as to prevent the risk of cardiac stenosis (Figure 2D and E).

Surgical technique for CR of type A GIST located in the posterior wall of EGJ

The tumor location on the posterior wall of EGJ was confirmed by palpation (Figure 3A). The anterior gastrotomy was made close to the EGJ of about 10 cm (Figure 3B). After entering the gastric cavity, the tumor was visualized and excised along the edge of the tumor, including the part of the posterior wall of the stomach and the left lateral wall of the esophagus (Figure 3C). The defect was sutured accordingly to the above-mentioned principles for the anterior wall tumors (Figure 3D). Finally, the anterior gastrotomy was sutured.

Follow-up care of the patients

Follow-up of the patients was conducted by telephone or in the outpatient department. Gastroduodenal endoscopic examination was done to look for deformity or stenosis of the EGJ at 2 mo postoperatively (Figure 4). All patients received esophageal 24-h pH monitoring and SF-36 at the third month after surgery, and regular abdominal enhanced computed tomography every 3-4 mo within 2 years, and every 6 mo in 2-5 years. Medium-risk and high-risk GIST patients received imatinib targeted therapy after surgery for 1 year and 3-5 years, respectively.

Definitions

Esophageal 24-h pH monitoring: Esophageal 24-h pH monitoring was measured with an antimony electrode (Medtronic Functional Diagnostics A/S, Copenhagen, Denmark). Data were digitized with a Macintosh computer (Apple Computer Inc, Cupertino, CA, United States) and the digitized signals were displayed and analyzed using AcqKnowledge software (Biopac Systems, Goleta, CA, United States). The patients were advised not to consume any food or drink below pH 4 during the week before monitoring. All patients were studied after an overnight fast. The pH electrode

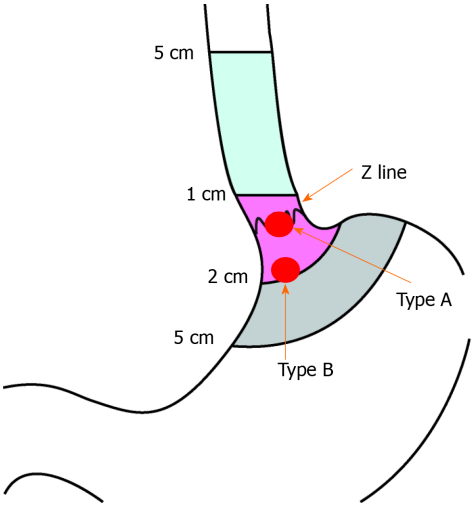


Figure 1 Schematic diagram of esophagogastric junction-gastrointestinal stromal tumor. Z-line: Squamocolumnar junction; Type A: Defined as esophagogastric junction-gastrointestinal stromal tumor involving Z-line; Type B: Defined as esophagogastric junction-gastrointestinal stromal tumor near Z-line.

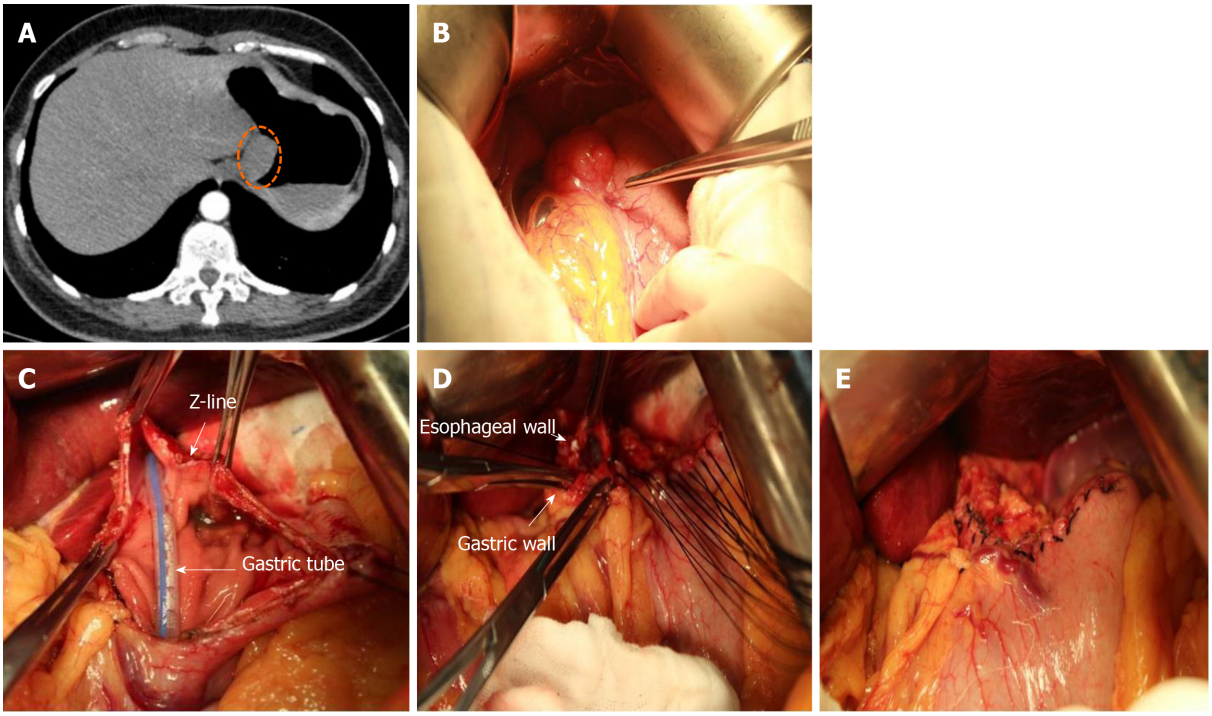


Figure 2 Conformal resection of type A esophagogastric junction-gastrointestinal stromal tumor located in the anterior wall of esophagogastric junction. A: The abdominal computed tomography showing the lesion located in the anterior wall of the esophagogastric junction; B: Exploratory laparotomy; C: Removal of the tumor with preservation of esophagogastric junction and esophageal wall as much as possible; D: Suturing of the esophageal wall to the gastric wall to prevent stenosis; E: The final appearance after suturing.

was positioned 5 cm above the cardia. Ambulatory pH recordings were then undertaken for 24 h. Monitoring indicators included mean of 24-h pH, total time for pH < 4, number of acid reflux events in 24 h (standing, lying), and number of regurgitations (> 5 min)[13].

SF-36: The SF-36 is an extensively used generic questionnaire for the assessment of health-related quality of life (QoL), which contained eight dimensions [physical function (PF), role physical (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role-emotional (RE), and mental health (MH)]. The score ranges from 0 to 100; the higher score indicating a better health related QoL[14].

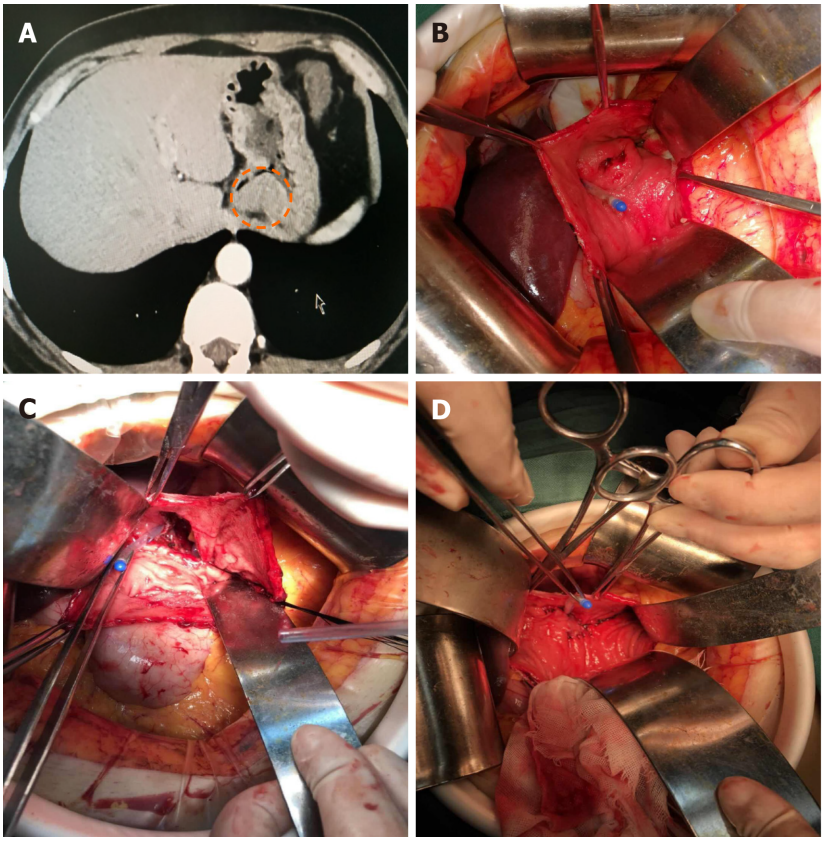


Figure 3 Conformal resection of type A esophagogastric junction-gastrointestinal stromal tumor located in the posterior wall of esophagogastric junction. A: The abdominal computed tomography showing the lesion located in posterior wall of the esophagogastric junction; B: Anterior gastrotomy to visualize the lesion; C: Removal of the tumor; D: Suturing of the posterior wall of the esophagus to the gastric wall.

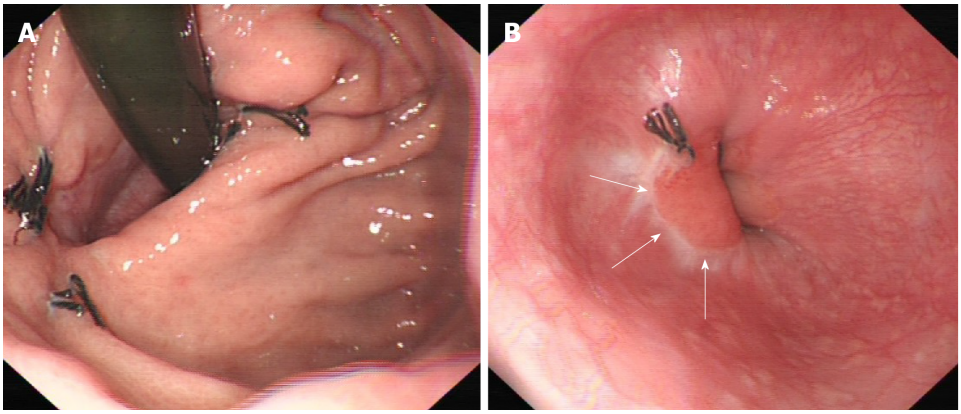


Figure 4 Gastroduodenal endoscopy 2 mo after the operation. A: Gastric view; B: Esophageal view.

Statistical analyses

All statistical analyses were performed using SPSS software version 19.0 (SPSS Inc., Armonk, NY, United States). In this study, continuous variables are expressed as the mean ± standard deviation. The χ^2-test and Fisher exact test were applied to compare categorical data of CR and PG groups. The statistical analysis of intra-operative and postoperative correlation coefficients, the esophageal 24-h pH parameters, and the SF-36 parameters were performed by *t*-test. The cut-off value to discriminate surgical method was analyzed by the Youden index from the receiver operating characteristic (ROC) analysis. Estimations for DFS were obtained using the Kaplan-Meier method and differences between Kaplan-Meier curves were investigated by log-rank test. Statistical significance was set at P value < 0.05.

RESULTS

A total of 43 patients were included in this study. Eighteen patients underwent CR, and 25 patients underwent PG including gastric cardia (PG).

Clinicopathological characteristics

The clinical and pathologic variables of patients are summarized in Table 1. The median age of all patients was 59.4 years, and 24 were males. There was no significant difference in the mean age and gender between the CR and PG groups. The median tumor size in the CR and PG groups was 6.4 cm (1.8-12.5 cm) and 8.3 cm (2.5-14 cm), respectively. Most tumors were of spindle histologic subtype in both groups (13/18 in the CR and 18/25 in the PG groups), had Ki67 index ≤ 5% (15/18 in the CR and 16/25 in the PG groups), and had mitotic rate < 5/50 HPF (high-power field, 30/43 in both the CR and PG groups). Immunohistochemistry showed positivity for CD117, CD34, and DOG-1 in 38 (88.37%), 39 (90.70%), and 36 (83.72%) cases, respectively. The two groups were comparable with respect to the modified National Institutes of Health risk classification[15].

Perioperative outcomes of the CR group and PG group

Table 2 compares the intra-operative and postoperative outcomes of the two surgical procedures for type A GISTs. The results showed that except for intraoperative blood loss (59.44 ± 23.08 *vs* 90.60 ± 35.62, $P = 0.069$), the other indicators were significantly different between the two groups ($P < 0.05$), suggesting that CR group was significantly superior to the PG group. The negative rate of surgical margin was 100% in both groups.

Esophageal 24-h pH monitoring between the CR group and PG group

Table 3 summarizes the postoperative esophageal 24-h pH parameters such as the mean 24-h pH, total time for pH < 4, acid reflux number of 24 h, and regurgitation times (> 5 min) after both surgical procedures for type A GISTs. The results of the two groups were compared, and it was found that the postoperative reflux of CR group was significantly less than that of PG group (all $P < 0.001$).

SF-36 between the CR group and PG group

On comparison of the postoperative health-related quality of life, it was found that CR group had higher PF, RP, BP, GH, VT, SF, RE, and MH scores compared with PG group (all $P < 0.01$) (Table 4).

Cut-off point of distance of involving Z-line for discrimination of the surgical method

We generated ROC curves to find best the Youden index to detect the cut-off value for predicting the surgical method. The area under the curve (AUC) was 0.907, indicating that the cut-off value of the length of the upper edge of the GIST crossing the Z-line (or invading the esophagus) was 7.0 mm. It can also be understood that when the invasion of the esophagus is greater than 0.7 cm, it is not suitable for CR. The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy rates were 83.33%, 84.00%, 78.90%, 87.51%, and 83.72%, respectively (Figure 5).

Comparison of DFS between the two groups

A total of 37 patients were included for the analysis of DFS (CR group: 17, PG group: 20) after excluding one case of preoperative liver metastasis from the CR group and 3, 1, and 1 case of liver metastasis, peritoneal metastasis, and diaphragm invasion from the PG group, respectively. Median follow up was 46.2 mo (range, 11-102 mo). The 5-year DFS was 100% in the CR group and 85% in the PG group, and the difference was not statistically significant ($P = 0.163$, Figure 6).

DISCUSSION

In this study, we described a novel surgical technique of CR for type A EGJ-GIST in order to retain the gastric cardia function as much as possible and prevent postoperative GER. We found that the perioperative outcomes of CR were superior to PG including 24-h pH monitoring and SF-36. The results showed that CR was safe and feasible.

PG has been the only recommended operation in the National Comprehensive

Table 1 Comparison of clinical and pathologic variables of study patients

Variables	CR group	PG group	*P* value
Age, median (range), in yr	58 (32-73)	61 (28-75)	0.532
Sex			0.168
Male	8	16	
Female	10	9	
Size in cm			0.549
≤ 2	1	0	
2.1-5.0	6	6	
5.1-10	7	13	
> 10	4	6	
Histologic type			0.71
Spindle	13	18	
Epitheloid	1	3	
Mixed	4	4	
Mitotic index, per 50 HPF			0.766
≤ 5	13	17	
> 5	5	8	
Ki-67			0.163
≤ 5%	15	16	
> 5%	3	9	
CD117			0.929
Positive	16	22	
Negative	2	3	
CD34			0.159
Positive	15	24	
Negative	3	1	
DOG-1			0.083
Positive	13	23	
Negative	5	2	
NIH risk category			0.476
Very low risk	1	0	
Low risk	5	4	
Intermediate risk	7	12	
High risk	5	9	
Length of the upper edge of the GIST crossing the Z-line, mean ± SD (cm)	0.639 (0.29)	1.024 (0.395)	0.193
Circumference of the EGJ involvement			0.716
T ≤ 1/4	10	13	
1/4 < T ≤ 2/4	7	8	
2/4 < T ≤ 3/4	1	3	
T > 3/4	0	1	
Heartburn, postoperative			
Yes	3	16	0.004

No	15	9	
Oral antacids, postoperative			
Yes	4	18	0.002
No	14	7	

CR: Conformal resection; EGJ: Esophagogastric junction; GIST: Gastrointestinal stromal tumor; HPF: High-power field; PG: Proximal gastrectomy; T: Tumor.

Table 2 Comparison of intra-operative and postoperative variables between two surgical procedures for type A esophagogastric junction-gastrointestinal stromal tumors

	CR group, *n* = 18	PG group, *n* = 25	*P* value	95%CI	
				Lower	Upper
Operating time in min	108.61 ± 30.13	137.80 ± 51.04	0.029	-56.41	-0.97
Intraoperative blood loss in mL	59.44 ± 23.08	90.60 ± 35.62	0.069	-53.53	-11.78
First passage of flatus time in h	29.11 ± 15.35	74.24 ± 40.39	0.013	-65.389	-24.87
Days to oral intake	3.56 ± 1.65	8.68 ± 3.88	0.006	-7.09	-3.16
Postoperative hospital stay in d	6.00 ± 2.40	10.96 ± 4.15	0.049	-7.17	-2.75
Negative margin, %	100	100			

CI: Confidence interval; CR: Conformal resection; PG: Proximal gastrectomy.

Table 3 Postoperative esophageal 24-h pH parameters of patients who underwent surgery for type A esophagogastric junction-gastrointestinal stromal tumors

	Mean 24-h pH	Total time for pH < 4 (min)	Acid reflux number of 24 h		Regurgitation events, > 5 min
			Standing	Lying	
CR group, *n* = 18	4.97 ± 1.44	31.83 ± 22.65	17.83 ± 11.25	35.62 ± 15.71	1.44 ± 1.68
PG group, *n* = 25	3.41 ± 1.15	184.48 ± 118.74	32.68 ± 15.89	58.81 ± 19.13	6.04 ± 1.92
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

CR: Conformal resection; PG: Proximal gastrectomy.

Table 4 Postoperative health-related quality of life of study patients

	PF	RP	BP	GH	VT	SF	RE	MH
CR group, *n* = 18	53.72 ± 2.13	54.61 ± 1.61	60.94 ± 1.86	61.56 ± 1.75	61.72 ± 3.49	55.11 ± 2.24	50.61 ± 2.93	55.00 ± 2.27
PG group, *n* = 25	48.00 ± 3.64	48.36 ± 2.89	49.80 ± 4.12	50.72 ± 5.15	52.20 ± 4.06	44.60 ± 2.63	37.16 ± 4.08	46.12 ± 3.95
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

BP: Bodily pain; GH: General health; MH: Mental health; PF: Physical function; RE: Role-emotional; SF: Social functioning; RP: Role physical; VT: Vitality.

Cancer Network guidelines[3] and related studies on EGJ-GIST[8,16]. PG is associated, however, with high risk of postoperative GER. The incidence of reflux esophagitis after PG is high and usually accompanied by symptoms such as acid reflux and retrosternal burning[17]. Besides, GER seriously affects the quality of life of patients and requires them to take drugs such as omeprazole for a long time. In the present study, the symptoms of heartburn (3/18 *vs* 16/25, $P < 0.05$) and need for acid-suppressive drugs (4 /18 *vs* 18/25, $P < 0.05$) were significantly less in the CR group compared to the PG group suggesting that CR was significantly superior to the traditional PG in

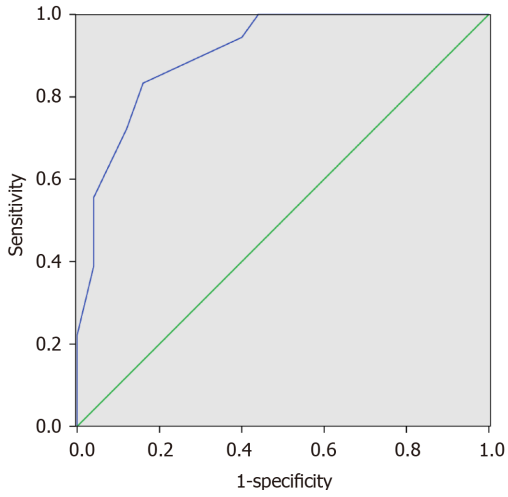


Figure 5 The receiver operating characteristic curve analyses showed that the best cut-off value of distance of involving Z-line was 7.0 mm.

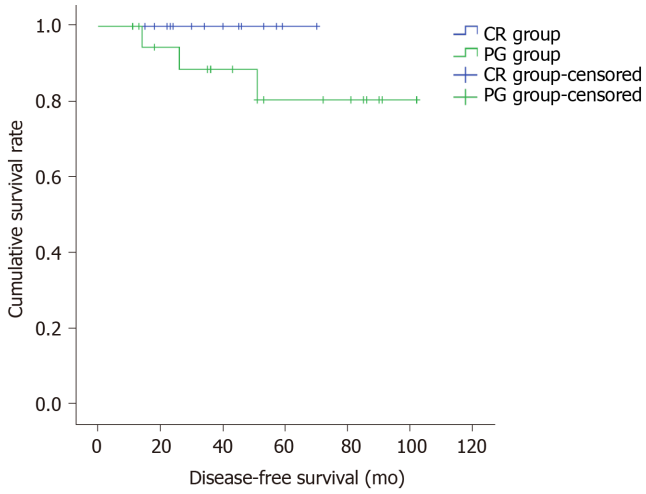


Figure 6 The disease-free survival between conformal resection group and proximal gastrectomy group (*P* = 0.163). CR: Conformal resection; PG: Proximal gastrectomy.

controlling GER.

The gold standard for evaluating GER is 24-h pH monitoring[18]. It objectively measures the change of pH value in the esophagus under physiological state. pH < 4 total time percentage and the number of consecutive > 5 min are the main parameters used to diagnose and assess the severity of acid reflux[13]. In the current study, the CR group was significantly superior to the PG group based on the 24-h pH study findings including the mean 24-h pH, total time for pH < 4, acid reflux number of 24 h (standing), acid reflux number of 24 h (lying), and regurgitation times (> 5 min). These findings provide objective evidence for the reduction in GER by CR.

Impairments of health-related QoL in patients with heartburn have been reported previously[14,19]. We adopted the SF-36 questionnaire, which is an extensively used generic questionnaire for assessing physical and mental related QoL[14]. We found that the patients in PG group displayed a much poorer QoL (both mental and physical) in comparison with those in CR group. This difference might be partly because of the high incidence of heartburn in PG group, which contributed to the high levels of psychological burden[20].

CR was performed according to the shape of the tumor and the extent of invasion of the cardiac muscle, so as to minimize the damage to the anatomical structure of the gastric cardia. In our experience, we found that the distance of the tumor across the Z-line was the key determinant of whether CR can be performed. The cut-off value calculated by ROC curve was 7.0 mm, which was the maximum adaptive distance of CR. If it exceeds 7.0 mm, then it will inevitably result in excessive resection of

esophageal tissue, which is not suitable for this operation. Also, if the tumor is involving the EGJ circumferentially, then CR is not possible. However, none of the patients in our study had circumferentially involvement of the EGJ by the tumor. At the same time, it is important to limit the resection of esophagus and cardiac muscles as much as possible by using intraoperative frozen section and preserve the cardiac function to the maximum extent. With this technique we achieved negative margins in all the cases operated in this study. Another important aspect of CR is the suturing of the defect after resection. In order to prevent stenosis, we sutured the esophageal wall to the gastric wall and not to the esophageal wall. All these goals of CR can be best achieved by open surgery. Laparoscopic resection and suturing for CR can be technically very challenging. Previous studies have shown that the ratio of conversion from laparoscopy to laparotomy was high for EGJ-GIST that did not involve Z-line[21-23]. Laparoscopic surgery has not been recommended by the National Comprehensive Cancer Network guidelines[3]. Future studies by experienced laparoscopic surgeons will help to determine whether CR can be safely performed by laparoscopy.

In this study, the operative time, time for first passage of flatus, and postoperative hospital stay were significantly less in the CR group suggesting that the CR group induced less surgical damage and faster recovery of patients. Moreover, the long-term outcomes such as DFS was similar between CR and PG, indicating that CR helped in preserving gastric cardiac function without compromising the oncological outcomes.

There are some limitations of this study. First, it was a retrospective study with potential for selection bias. Second, the sample size of this study was small. Third, this study was a single center study. Future larger prospective multicenter studies are needed to validate the findings of this study.

CONCLUSION

CR is a safe and feasible alternative to PG for non-circumferential localized EGJ-GIST within 7.0 mm above the Z-line. CR is associated with lower risk of postoperative GER and better quality of life compared to PG.

ARTICLE HIGHLIGHTS

Research background

For gastrointestinal stromal tumors (GISTs) at the esophagogastric junction (EGJ) involving the Z-line, previous proximal gastrectomy (PG) results in serious postoperative reflux and poor quality of life.

Research motivation

To explore and improve the surgical methods of GIST in this part, so as to preserve the cardiac sphincter as much as possible and reduce the incidence of postoperative reflux esophagitis.

Research objectives

We aimed to describe a novel technique to achieve R0 resection of EGJ-GIST involving Z-line with preservation of the sphincter function. We have also compared the outcomes of conformal resection with PG.

Research methods

In this study, 43 patients having GISTs involving Z-line were included. The perioperative outcomes of patients receiving conformal resection (CR) (n = 18) was compared with that of PG (n = 25). The data of the patients collected were as follows: Clinical and pathologic findings, intra-operative and postoperative outcomes, esophageal 24-h pH parameters, the 36-item short-form health survey, and disease-free survival.

Research results

CR was successfully performed in all the patients with negative microscopic margins. The mean operative time, time to first passage of flatus, and postoperative hospital stay was significantly shorter in CR group (P < 0.05), while the intraoperative blood

loss was similar in the two groups. The postoperative gastroesophageal reflux as diagnosed by esophageal 24-h pH monitoring and quality of life at 3 mo were significantly in favor of CR compared to PG (both $P < 0.001$). The 5-year disease-free survival between the two groups were similar ($P = 0.163$). The cut- off value for the determination of CR or PG was 7.0 mm above the Z-line (83.33% sensitivity, 84.00% specificity, 83.72% accuracy).

Research conclusions

CR is safe and feasible for EGJ-GIST located within 7.0 mm above the Z-line. CR was associated with lower incidence of postoperative GER and better quality of life with similar oncological outcomes compared to PG.

Research perspectives

Our surgical team will continue to conduct survival follow-up for enrolled patients and cooperate with multiple centers to explore further the advantages and disadvantages of this surgical approach.

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ORIGINAL ARTICLE

Observational Study

Serum 1,3-beta-D-glucan as a noninvasive test to predict histologic activity in patients with inflammatory bowel disease

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Abstract

BACKGROUND

1,3-beta-D-glucan (BG) is a ubiquitous cell wall component of gut micro-organisms. We hypothesized that the serum levels of BG could reflect active intestinal inflammation in patients with inflammatory bowel disease.

AIM

To determine whether the serum BG concentrations correlate with intestinal inflammation.

METHODS

A prospective observational study was performed in a tertiary referral center, from 2016 to 2019, in which serum BG was determined in 115 patients with Crohn's disease (CD), 51 with ulcerative colitis (UC), and 82 controls using a photometric detection kit. Inflammatory activity was determined by ileocolonoscopy, histopathology, magnetic resonance enterography, and biomarkers, including fecal calprotectin (FC), C-reactive protein, and a panel of cytokines. The ability of BG to detect active *vs* inactive disease was assessed using the area under the receiver operating characteristic curve. In subgroup analysis, serial BG was used to assess the response to therapeutic interventions.

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Conflict-of-interest statement: The authors declare that they have no competing interests to report related to the work submitted for consideration of publication.

Data sharing statement: Technical appendix, statistical code, and dataset supporting the conclusions of this study will be made available from the corresponding author at [hsouza@hucff.ufrj.br], without undue reservation, to any qualified researcher.

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RESULTS

The serum BG levels were higher in CD patients than in controls (P = 0.0001). The BG levels paralleled the endoscopic activity in CD patients and histologic activity and combined endoscopic and histologic activity in both CD and UC patients. The area under the curve (AUC) in receiver operating characteristic analysis to predict endoscopic activity was 0.694 [95% confidence interval (CI): 0.60-0.79; P = 0.001] in CD, and 0.662 (95%CI: 0.51-0.81; P = 0.066) in UC patients. The AUC in receiver operating characteristic analysis to predict histologic activity was 0.860 (95%CI: 0.77-0.95; P < 0.001) in CD, and 0.786 (95%CI: 0.57-0.99; P = 0.015) in UC patients. The cut-off values of BG for both endoscopic and histologic activity were 60 μg/mL in CD, and 40 μg/mL in UC patients. Performance analysis showed that the results based on BG of 40 and 60 μg/mL were more specific for predicting endoscopic activity (71.8% and 87.2% for CD; and 87.5% and 87.5% for UC, respectively) than FC (53.3% and 66.7% for CD; and 20% and 80% for UC, respectively); and also histologic activity (60.5% and 76.3% for CD; and 90.0% and 95.0% for UC, respectively) than FC (41.7% and 50.0% for CD; and 25% and 50% for UC, respectively). Regarding the clinical, endoscopic, and histologic activities, the BG levels were reduced following therapeutic intervention in patients with CD (P < 0.0001) and UC (P = 0.003). Compared with endoscopic (AUC: 0.693; P = 0.002) and histologic (AUC: 0.868; P < 0.001) activity, no significant correlation was found between serum BG and transmural healing based on magnetic resonance enterography (AUC: 0.576; P = 0.192). Positive correlations were detected between BG and IL-17 in the CD (r: 0.737; P = 0.001) and the UC group (r: 0.574; P = 0.005), and between BG and interferon-gamma in the CD group (r: 0.597; P = 0.015).

CONCLUSION

Serum BG may represent an important novel noninvasive approach for detecting mucosal inflammation and therapeutically monitoring inflammatory bowel diseases, particularly in CD.

Key Words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Beta-glucan; Histologic activity; Noninvasive test

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Core Tip: This study investigated whether serum concentrations of beta-glucan (BG), which originate from the gut microbiota, could reflect active intestinal inflammation in inflammatory bowel diseases patients. BG levels paralleled the endoscopic activity in Crohn's disease (CD) patients and histologic activity and combined endoscopic and histologic activity in both CD and ulcerative colitis patients. The BG results were better for predicting histologic inflammation than fecal calprotectin. Regarding the endoscopic and histologic activities, the BG levels were reduced following therapeutic intervention in both CD and ulcerative colitis patients. Serum BG levels may represent a novel noninvasive approach to detect mucosal inflammation and therapeutically monitor inflammatory bowel diseases.

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INTRODUCTION

In the last decade, therapeutic targets in inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), have evolved from simple clinical

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remission to more objective parameters to confirm the resolution of inflammation. Mucosal healing defined by endoscopic evaluation has been consistently associated with better disease outcomes[1,2]. Hence, the recommended goals of treatment endpoints have changed toward deep remission and include a combination of clinical remission and endoscopic healing[3-8]. However, because microscopic inflammation may persist even in patients with endoscopically normal mucosa, histologic assessment has been proposed recently as a more precise predictor of disease outcomes[9-11].

The approach for monitoring mucosal or histologic remission in IBD demands frequent endoscopic evaluations, which are costly and invasive procedures. Thus, alternative methods have been investigated, including cross-sectional imaging, such as magnetic resonance enterography (MRE)[12]. However, in addition to the high cost, MRE relies on semiquantitative analysis and experience with recommended indexes as an endpoint in clinical trials is still lacking[10]. To circumvent these issues, several biomarkers in blood and stools have been investigated. C-reactive protein (CRP), for example, has long been utilized as a systemic marker of inflammation with increased levels associated with disease activity[13]. However, it is generally accepted that CRP has both low sensitivity[14] and specificity[15]. Nevertheless, in the last decade, fecal calprotectin (FC) has become the most used biomarker in the follow-up of IBD due to practical features and the correlation with disease activity[16,17]. However, the predictive value of FC in mucosal healing is not well established, and no clear cut-off level has been defined to assess mucosal inflammation or remission[18]. Moreover, the relationship between FC and histologic inflammation, particularly in colonic CD, remains to be determined[19,20]. In this context, a reliable noninvasive biomarker that accurately identifies mucosal inflammation continues to be necessary in the follow-up of patients with IBD.

Among several factors that participate in the pathogenesis of IBD, the gut microbiota has gained increased attention in the last decade[21,22]. Although whether dysbiosis is a primary or secondary phenomenon in IBD remains to be fully elucidated, gut microbial composition and function appear to be altered in IBD[23], and a growing body of evidence supports the idea that IBD might result from a complex interaction among host, environmental, and microbial factors[24,25]. In this regard, immune reactivity to microbial antigens, such as circulating antibodies against *Saccharomyces cerevisiae*[26], *Escherichia coli* outer membrane porin C[27], bacterial flagellin[28], and antibodies against glycans, commonly present in microbial cells surface[29], has long been reported in IBD. Bacterial cell wall components, such as the endotoxin lipopolysaccharide (LPS) of Gram-negative strains, are potent inflammatory agents[30] whose levels were shown to be increased in IBD[31]. LPS may also play an important role in altering gut homeostasis, including the control of cell death processes that permit translocation among the gut, blood, and other tissues[32]. In line with this, Guo *et al*[33] detected increased levels of LPS and 1,3-beta-D-glucan (BG) in the serum of patients with CD that were positively correlated with the clinical intensity of disease[33]. BG is a ubiquitous cell wall component present in several microorganisms, including *Candida* and *Aspergillus* spp, within the gut microbiota[34]. Fungal dysbiosis was recently reported to be associated with CD patients compared with healthy controls[35]. Additionally, lamina propria mononuclear cells derived from the intestinal specimens of patients with CD showed increased release of pro-inflammatory cytokines in response to stimulation with BG *in vitro*[36], suggesting a potential contribution of BG to the pathogenesis of intestinal inflammation. Therefore, we hypothesized that high BG concentrations in the serum of patients with IBD could reflect active intestinal inflammation. Thus, this prospective study aimed to determine whether the serum BG concentrations correlate with intestinal inflammation based on other inflammatory biomarkers and endoscopic, histological, and imaging criteria and to establish an optimal cut-off level of BG to predict mucosal healing.

MATERIALS AND METHODS

Ethical considerations

The Ethical Committee of the Hospital Copa D'Or, with the co-participation of the University Hospital of the Federal University of Rio de Janeiro, approved the study protocol (CAAE: 53351116.1.0000.5249), which was implemented in agreement with the ethical standards described in the 1964 Declaration of Helsinki. All the enrolled patients provided written informed consent before participating in the study.

Study population

This prospective observational study involved patients followed up regularly at the outpatient unit for Intestinal Diseases of the Division of Gastroenterology at the University Hospital of the Federal University of Rio de Janeiro, a tertiary referral center, from March 2016 to June 2019. Eligible patients were adults (18-80 years of age) with a diagnosis of IBD (CD and UC), supported by routine clinical, imaging, endoscopic, and histologic parameters. A total of 115 patients with CD, 51 with UC, and 82 controls were included in the study. The sample size, considering a two-tailed variance analysis (fixed effects, special, main effects and interactions), including three groups, with an effect size f of 0.25, an alpha of 0.05, and power of 0.95, was estimated as 210. Patients were consecutively selected to participate, at least one week before a scheduled ileocolonoscopy. The demographics and clinical information based on the Montreal classification (including disease duration, age at diagnosis, disease extension and localization, and the predominant disease behavior[37]), presence of perianal involvement, extraintestinal manifestations, primary sclerosing cholangitis, history of surgeries due to IBD, and medical therapy at the time of enrollment, were all registered during consultation. The exclusion criteria were patients with CD with exclusive upper gastrointestinal disease (not accessible to ileocolonoscopy and/or histopathological analysis), patients with unclassified IBD, patients in the postoperative period of less than 6 mo or with total colectomy, pregnant patients, patients with evidence of abdominal abscess or colonic mucosal dysplasia, patients with cancer or acute or chronic enteric infection (*e.g.*, *Clostridioides difficile*), and individuals who had received concomitant antibiotics, nonsteroidal anti-inflammatory agents, proton-pump inhibitors, probiotics, prebiotics, and/or synbiotics in the previous 3 mo.

The control group comprised 25 men (30.5%) and 57 women (69.5%), with a median age of 51.5 years (range: 36.5-60.0 years), including 64 non-IBD patients (24 with irritable bowel syndrome, 20 with noncomplicated colonic diverticular disease, and 20 with benign polyps), and 18 healthy controls. All non-IBD patients followed up in the outpatient unit for intestinal diseases who were scheduled for ileocolonoscopy also had undergone blood and fecal sampling. In the control group, all endoscopic examinations and histological analyses were normal. None of the control individuals were taking any medication at the time of the study, and none had taken antibiotics and/or nonsteroidal anti-inflammatory agents in the previous 3 mo.

Assessment of disease activity

Clinical activity of disease was recorded at the time of ileocolonoscopy using the Harvey-Bradshaw index (HBI)[38] for CD (remission defined as HBI < 5), and the Mayo clinical score[39] for UC (remission defined as Mayo score < 2). The assessment of endoscopic activity was performed using the Simple Endoscopic Score-CD (SES-CD)[40] for CD (remission defined as SES-CD < 3) and the Mayo endoscopic subscore (MES)[41] for UC (remission defined as MES < 2). For patients who had undergone ileocolonic surgery due to CD, ileocolonoscopies were classified according to the Rutgeerts score into remission (i0-i1) and endoscopic activity (i2-i4)[42]. Endoscopic biopsies obtained during ileocolonoscopy were evaluated for histologic disease activity according to the global histologic disease activity score for CD (histologic healing defined as global histologic disease activity score < 2)[43] and the Geboes score for UC (histological healing defined as a Geboes score < 3.1)[44]. In subgroup analysis involving CD, MRE was used to obtain information on transmural inflammation whenever patients had undergone ileocolonoscopy within an interval of 30 d. MRE analysis was based on descriptive parameters, distinguishing among the predominant disease patterns (inflammatory, fibrostenosing or penetrating). The criteria for the presence of inflammatory activity included mucosal enhancement, parietal thickening, mesenteric fat infiltration, mesenteric vein engorgement, and lymphadenopathy, as previously published[45,46]. Deep healing was defined as a combination of histologic and transmural healing.

To further investigate the potential association of serum BG with disease activity in patients with IBD, subgroup analysis was performed to analyze serial alterations. Patients with IBD were selected consecutively depending on the presence of active disease based on clinical, endoscopic, and histologic evaluation. The assessment of serum BG was performed at week 0, and between weeks 12 and 16 after the medical intervention. The interval chosen was based on the practical feasibility of performing a second round of examinations and the minimum time necessary for therapeutic changes to be effective. The new therapeutic regimens included the initiation of new medications, such as biological agents, immunosuppressants (azathioprine), and

salicylates, with or without the association of a short course of steroids or the optimization of previous therapy.

Routine laboratory tests and potential new biomarkers

Peripheral blood and fecal samples were collected within 2 to 10 d before the preparation for ileocolonoscopy from all patients with IBD and controls after an overnight fast. To measure BG, serum was separated by centrifugation and processed within 10 min. The BG serum concentrations were determined at the Mycology Laboratory of the hospital using a photometric detection commercial kit (Fungitell® assay; Associates of Cape Cod, Inc., East Falmouth, MA, United States). The assay is a highly sensitive, microplate-based test that detects (1,3)-beta-D-glucan in serum at 405 nm (range: 31-500 pg/mL).

In addition to routine erythrocyte sedimentation rate and ultrasensitive C-reactive protein measurements, FC was measured using a quantitative, commercially available enzyme-linked immunosorbent assay (ELISA) kit (range: 31.2-2000 pg/mL) (Biomatik, Wilmington, DE, United States). In another subgroup analysis, blood samples were also used to assess potential circulating biomarkers. For such purpose, we analyzed a panel of cytokines, LPS-binding protein (LBP) and zonulin. The BD™ cytometric bead array human Th1/Th2/ T helper 17 (Th17) cytokine kit (BD Biosciences, San Jose, CA, United States) was used to assess interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17A, tumor necrosis factor-alpha (TNF-alpha), and interferon-gamma (IFN-gamma) protein levels, whose performance has been optimized to detect physiologically relevant concentrations (pg/mL). The measurements were performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, United States), and the results were analyzed with BD cytometric bead array analysis software. To measure serum LBP, we used a commercial ELISA kit and the absorbance was detected at 450 nm (range: 4.4-50 ng/mL) (Hycult Biotech Inc., Wayne, PA, United States). To assess serum zonulin, we used a commercial human zonulin ELISA kit with the absorbance read at 450 nm (range: 0.625-40 ng/mL).

Statistical analysis

Statistical analysis was performed using Statistic Package for Social Science statistical software for Windows (Version 20, Statistic Package for Social Science, Inc., Chicago, IL, United States) and Prism 8 for OS X (Version 8.4.3, GraphPad Software, LLC, San Diego, CA, United States). The distribution of individual features was determined using simple descriptive statistics. Sample size was estimated using G*Power free software for statistical analyses (University of Düsseldorf, Germany). Receiver operating characteristic analysis was used to evaluate the sensitivity and specificity of BG in reference to clinical, endoscopic, histologic and combined endoscopic and histologic remission and to determine optimal cutoff values for generating dichotomous variables. Optimal cut-off values were determined by the maximum sum of sensitivity and specificity of BG values for screening intestinal inflammatory activity. Differences among the experimental groups were assessed using the Kruskal-Wallis on ranks tests, in which multiple comparisons were performed using Dunn's test, as appropriate. Differences between the distributions of the selected variables were evaluated using chi-squared test or Fisher's exact test for categorical variables. Spearman's rank correlation was used to assess relationships between continuous variables. A pairwise Wilcoxon rank-sum test was used to compare the effect of medical intervention on the BG levels between two different time points. Preference was given to exact and nonparametric tests to avoid assumptions of normal distribution in the collected data. All the tests were two-tailed, and statistical significance was set at a P value of less than 0.05.

RESULTS

Patients' characteristics and results of serum BG

One hundred fifteen patients with CD (sixteen with ileal, thirty-nine with colonic, and sixty with ileocolonic CD), and fifty-one patients with UC (fourteen with proctitis, seventeen with left colitis, and twenty with pancolitis) were enrolled. Age; sex; disease duration; erythrocyte sedimentation rate (ESR); CRP; FC; and clinical, endoscopic, and histologic activities did not differ significantly between the CD and UC patients. Regarding clinical activity, 52.2% of patients with CD [HBI 5 (4-8; interquartile range (IQR))] and 64.7% of patients with UC [Mayo 5 (3-7; IQR)] were clinically active at the time of ileocolonoscopy. Notably, 77 (66.9%) patients with CD [SES-CD 5 (2-8; IQR)

and 35 (68.6%)] patients with UC [Mayo subscore 2 (1-3; IQR)] had endoscopic activity. Of the individuals who were regarded as endoscopically active, biopsy samples were obtained from 71/77 (92.2%) patients with CD, and from 29/35 (82.8%) patients with UC. Histological activity was detected in 33 of 71 (46.5%) patients with CD and 9 of 29 (31%) patients with UC. The median values for CRP, ESR, and FC were comparable in the CD and UC groups. The demographic and clinical characteristics of the patients and control group are provided in Table 1. A relatively weak but significant positive correlation was found between serum BG and FC concentrations (CC: 0.232; $P = 0.033$), but no correlation was detected between BG and CRP (CC: 0.106; $P = 0.221$), or between BG and ESR (CC: 0.062; $P = 0.547$). The concentrations of serum BG were significantly higher in the CD group than in the controls ($P < 0.001$). Compared with controls, a trend toward higher median serum BG was observed in the UC group, but the difference did not reach statistical significance ($P = 0.054$) (Figure 1).

Next, the levels of serum BG were evaluated among the groups of patients with IBD in relation to the clinical, endoscopic, and histologic disease activity. Although no difference was found in the BG levels regarding clinical activity, significant differences were shown for the endoscopic activity in the CD group and for histologic activity and combined endoscopic and histologic activity for both the CD and UC groups (Figure 2).

Determination of the BG cutoff and relationship with endoscopic and histologic results

Preliminary analyses were performed to estimate the optimal cutoff values to convert quantitative BG into a binary variable for the presence or absence of inflammatory activity based on standard scoring systems. The area under the curve (AUC) for the presence of clinical, endoscopic, histologic, and combined endoscopic and histologic activity and serum BG are shown in Figure 3 (CD) and Figure 4 (UC). The best cutoff value of serum BG in the CD group was 60 μg/mL for predicting endoscopic activity (AUC: 0.694; 95% confidence interval (CI): 0.60-0.79; $P = 0.001$), histologic activity (AUC: 0.860; 95%CI: 0.77-0.95; $P < 0.001$), and combined endoscopic and histologic activity (AUC: 0.847; 95%CI: 0.75-0.94; $P < 0.001$) (Figure 3). The best cutoff value of serum BG in the UC group was 40 μg/mL for predicting histologic activity (AUC: 0.786; 95%CI: 0.57-0.99; $P = 0.015$), and combined endoscopic and histologic activity (AUC: 0.741; 95%CI: 0.51-0.97; $P = 0.048$) (Figure 4). Next, the levels of serum BG were evaluated in relation to endoscopic and histologic activity compared with FC and CRP. The performance analysis of different cutoff values showed that the results with BG for predicting endoscopic activity were less sensitive, but more specific than FC, in both CD and UC groups. For predicting histologic activity, the sensitivity of BG was higher in CD and lower in UC, whereas the specificity of BG was remarkably higher in both CD and UC groups, compared with FC. In contrast to CRP, the sensitivity of BG for predicting endoscopic activity was comparable in CD and lower in UC, whereas the specificity of BG was higher in both CD and UC groups. For predicting histologic activity, the sensitivity of BG was higher in both CD and UC groups, whereas the specificity of BG was comparable in CD, but higher in the UC group, compared with CRP (Tables 2 and 3).

Comparison between serum BG and other potential serum biomarkers

In a consecutively selected subgroup of 28 patients with CD, 25 with UC and 15 controls, we performed another set of experiments to analyze the levels of BG in parallel with a series of potential serum biomarkers, including a panel of cytokines, LBP, and zonulin. Although patients with UC were more active in terms of clinical presentation, the disease activity based on endoscopic and histologic criteria were comparable. The levels of serum BG were significantly different among the groups, and pairwise comparison indicated the difference between CD and controls ($P = 0.003$). Of the other markers evaluated, only LBP showed levels significantly different among the groups, and pairwise comparisons revealed differences between the CD group and controls ($P = 0.015$), as well as between the UC group and controls (0.046) (Table 4). The assessment of potential associations between BG and the other serum markers revealed a significantly positive correlation only between BG and IL-17 (r: 0.576; $P < 0.0001$). However, analyzing the groups separately, we detected significant positive correlations between BG and IL-17 in the CD group (r: 0.737; $P = 0.001$) and the UC group (r: 0.574; $P = 0.005$) and between BG and IFN-gamma in the CD group (r: 0.597; $P = 0.015$).

Table 1 Patient demographics and medical characteristics

Parameters	Controls (*n* = 82)	Crohn's disease (*n* = 115)	Ulcerative colitis (*n* = 51)
Women	57 (69.5%)	58 (50.4%)	27 (52.9%)
Age (yr), median (IQR)	51.5 (36.5-60)	42 (28-58)	44 (37-56)
Disease duration (yr), median (IQR)		6 (3-11)	9 (3-14)
Age at diagnosis (yr)			
A1; A2; A3, *n* (%)		10 (8.7); 57 (49.6); 48 (41.7)	8 (15.7); 29 (56.9); 14 (27.5)
Disease behavior			
B1; B2; B3, *n* (%)		53 (46.1); 20 (17.4); 42 (36.5)	-
Disease location			
L1; L2; L3/E1; E2; E3, *n* (%)		16 (13.9); 39 (33.9); 60 (52.2)	14 (27.5); 17 (33.3); 20 (39.2)
Perianal disease		23 (20.0%)	-
EIM		12 (10.4%)	3 (5.9%)
PSC		6 (5.2%)	5 (9.8%)
Previous surgery		36 (31.3%)	2 (3.9%)
Medical therapy			
Biologicals		38 (33.0%)	5 (9.8%)
Immunosuppressants		66 (57.4%)	22 (43.1%)
Steroids		17 (14.8%)	3 (5.9%)
Salicylates		18 (15.7%)	26 (51.0%)
Clinically active		60 (52.2%)	33 (64.7%)
Endoscopically active		77 (66.9%)	35 (68.6%)
Histologically active		33/71 (46.5%)	9/29 (31.0%)
CRP, mg/L	1.65 (1.4-6.0)	2.8 (1.6-8.1)	4.3 (2.0-7.6)
ESR, mm/h	22 (10-30)	22 (10-42)	35 (19-67)
Fecal calprotectin, μg/g	122 (45-201)	199 (52-325)	218 (115-370)
Beta-glucan, μg/mL	17 (8-33)	35 (17-74)	23 (9-54)

The values are presented as numbers (percentages) or medians interquartile range; age at diagnosis: A1, < 17; A2, 17-40; A3, > 40; Disease behavior in Crohn's disease: B1: Inflammatory; B2: Fibrostenosing; B3: Penetrating; Disease location in Crohn's disease: L1: Ileal; L2: Colonic; L3: Ileo-colonic; Disease location in ulcerative colitis: E1: Proctitis; E2: Left sided colitis; E3: Extensive colitis; EIM: Extraintestinal manifestations; PSC: Primary sclerosing cholangitis; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

Serial analysis of serum BG during therapeutic intervention for disease activity

To analyze potential dynamic changes of serum BG in IBD, subgroup analysis was performed and included 29 patients with CD, 12 with UC and 12 controls (Supplementary Table 1). Patients with IBD were selected consecutively depending on the presence of active disease based on both clinical and endoscopic evaluation. Nevertheless, histologically active disease was detected in 81% of patients with CD and 50% of UC at baseline (T0). Selected patients regularly followed-up in the outpatient unit were submitted to changes in their respective therapeutic regimen, including the introduction of new medications, such as biological agents (anti-TNF: infliximab or adalimumab) (9 in the CD group and 3 in the UC group), immunosuppressants (azathioprine) (5 in the CD group and 3 in the UC group), and oral salicylates (3 in the UC group), with the association of a short course of steroids (less than 8 wk) (5 in the CD group and 4 in the UC group), or optimization of previous therapy (4 in the CD group and 3 in the UC group). After 12 to 16 wk of the therapeutic intervention (T1), all the patients were again evaluated based on the same clinical, ileocolonoscopic, and histologic criteria, and sera were collected to assess BG concentrations. Significant improvements in clinical (CD group, $P < 0.001$; UC group $P = 0.002$), endoscopic (CD group, $P < 0.001$; UC group, $P = 0.014$), and histologic (CD

Table 2 Performance analysis of different cutoff values of serum beta-glucan for patients with Crohn's disease in relation to endoscopic and histological criteria

Predicted outcome		Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy
Endoscopic inflammation	Beta-glucan	40	50.0 (39.0-61.0)	71.8 (56.2-83.5)	77.5 (64.1-87.0)	42.4 (31.2-54.4)	57.4 (48.3-66.0)
	(μg/mL)	60	34.2 (24.5-45.4)	87.2 (73.3-94.4)	83.9 (67.4-92.9)	40.5 (30.6-51.2)	52.2 (43.1-61.1)
	Calprotectin	100	75.0 (56.6-87.3)	53.3 (30.1-75.2)	75.0 (56.6-87.3)	53.3 (30.1-75.2)	67.4 (52.5-79.5)
	(μg/g)	200	53.6 (35.8-70.5)	66.7 (41.7-84.8)	75.0 (53.1-88.8)	43.5 (25.6-63.2)	58.1 (43.3-71.6)
	CRP	3	49.2 (37.1-61.4)	50.0 (31.4-68.6)	71.4 (56.4-82.8)	27.9 (16.7-42.7)	49.4 (39.0-59.8)
	(mg/L)	5	34.4 (23.7-46.9)	66.7 (46.7-82.0)	72.4 (54.3-85.3)	28.6 (18.4-41.5)	43.5 (33.5-54.1)
Histological inflammation	Beta-glucan	40	78.8 (62.2-89.3)	60.5 (44.7-74.4)	63.4 (48.1-76.4)	76.7 (59.1-88.2)	69.0 (57.5-78.6)
	(μg/mL)	60	57.6 (40.8-72.8)	76.3 (60.8-87.0)	67.9 (49.3-82.1)	67.5 (52.5-79.5)	67.6 (56.1-77.3)
	Calprotectin	100	69.2 (42.4-87.3)	41.7 (19.3-68.0)	56.2 (33.2-76.9)	55.6 (26.7-81.1)	56.0 (37.1-73.3)
	(μg/g)	200	46.1 (23.2-70.9)	50.0 (25.4-74.6)	50.0 (25.4-74.6)	46.1 (23.2-70.9)	48.0 (30.0-66.5)
	CRP	3	57.7 (38.9-74.5)	62.1 (44.0-77.3)	57.7 (38.9-74.5)	62.1 (44.0-77.3)	60.0 (46.8-71.9)
	(mg/L)	5	53.8 (35.5-71.2)	79.3 (61.6-90.1)	70.0 (48.1-85.4)	65.7 (49.1-79.2)	67.3 (54.1-78.2)

Endoscopic criteria based on the Simple Endoscopic Score-Crohn's disease endoscopic subscore; Histological criteria based on a modified Global Histologic disease activity score; Parentheses show lower-upper 95% confidence interval. CRP: C-reactive protein; PPV: Positive predictive value; NPV: Negative predictive value.

group, $P = 0.001$; UC group, $P = 0.023$) indexes were detected in the follow-up analysis. In parallel, the levels of serum BG decreased significantly from week 0 (T0) to week 12-16 (T1) in patients with CD ($P < 0.0001$) and UC ($P = 0.003$) but not in controls ($P = 0.407$) (Figure 5A). Considering the median values of serum BG in each group, the difference from T0 to T2 was 307% in the CD group, 141% in the UC group, and only 13% in controls.

Serum BG in relation to histologic and transmural inflammation in CD

To further investigate the potential use of BG to detect transmural inflammation in CD, we analyzed another subgroup of 62 consecutively selected patients concurrently evaluated with MRE. The levels of serum BG were significantly lower in patients with both histologic and transmural healing than in those with either histologic inflammation with transmural healing ($P = 0.047$) or combined histologic and transmural inflammation ($P = 0.041$) (Figure 5B). The AUC for the presence of transmural inflammation based on MRE findings and serum BG is shown in Supplementary Figure 1. Considering the cutoff values of 40 and 60 mg/mL, serum BG predicted deep healing (both histologic and transmural healing) in 68.4% and 84.2%, whereas no healing (both histologic and transmural inflammation) was predicted in 69.2% and 53.8% of the patients, respectively. Additionally, considering the cutoff values of 40 and 60 mg/mL serum BG detected 76.9% and 61.5% of patients in the context of histologic inflammation with transmural healing but only 41.2% and 35.3% of patients with transmural inflammation combined with histologic healing, respectively (Supplementary Table 2).

Table 3 Performance analysis of different cutoff values of serum beta-glucan for patients with ulcerative colitis in relation to endoscopic and histological criteria

Predicted outcome		Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy
Endoscopic inflammation	Beta-glucan	40	40.0 (25.6-56.4)	87.5 (64.0-96.5)	87.5 (64.0-96.5)	40.0 (25.6-56.4)	54.9 (41.4-67.7)
	(μg/mL)	60	22.9 (12.1-39.0)	87.5 (64.0-96.5)	80.0 (49.0-94.3)	34.1 (21.6-49.4)	43.1 (30.5-56.7)
	Calprotectin	100	81.2 (57.0-93.4)	20.0 (3.6-62.4)	76.5 (52.7-90.4)	25.0 (4.6-69.9)	66.7 (45.4-82.8)
	(μg/g)	200	62.5 (38.6-81.5)	80.0 (37.5-96.4)	90.9 (62.3-98.4)	40.0 (16.8-68.7)	66.7 (45.4-82.8)
	CRP	3	60.9 (40.8-77.8)	20.0 (5.7-51.0)	63.7 (42.9-80.3)	18.2 (5.1-47.7)	48.5 (32.5-64.8)
	(mg/L)	5	43.5 (25.6-63.2)	60.0 (31.3-83.2)	71.4 (45.3-88.3)	31.6 (15.4-54.0)	48.5 (32.5-64.8)
Histological inflammation	Beta-glucan	40	66.7 (35.4-87.9)	90.0 (69.9-97.2)	75.0 (40.9-92.8)	85.7 (65.4-95.0)	82.8 (65.5-92.4)
	(μg/mL)	60	55.6 (26.7-81.1)	95.0 (76.4-99.1)	83.3 (43.6-97.0)	82.6 (62.9-93.0)	82.8 (65.5-92.4)
	Calprotectin	100	100.0 (64.6-100.0)	25.0 (7.1-59.1)	53.8 (29.1-76.8)	100.0 (34.2-100.0)	60.0 (35.7-80.2)
	(μg/g)	200	85.7 (48.7-97.4)	50.0 (21.5-78.5)	60.0 (31.3-83.2)	80.0 (37.5-96.4)	66.7 (41.7-84.8)
	CRP	3	57.1 (25.0-84.2)	33.3 (13.8-60.9)	33.3 (13.8-60.9)	57.1 (25.0-84.2)	42.1 (23.1-63.7)
	(mg/L)	5	42.9 (15.8-74.9)	58.3 (31.9-80.7)	37.5 (13.7-69.4)	63.6 (35.4-84.8)	52.6 (31.7-72.7)

Endoscopic criteria based on the Mayo endoscopic subscore; Histological criteria based on the Geboes score. Parentheses show lower-upper 95% confidence interval. CRP: C-reactive protein; PPV: Positive predictive value; NPV: Negative predictive value.

DISCUSSION

In this study, we performed a prospective observational investigation examining the potential role of serum BG as a biomarker to predict inflammation in patients with IBD. Overall, the results indicated a favorable performance of serum BG compared with other routinely used biomarkers and a particularly strong association with histological inflammation. Moreover, the dramatic decrease in serum BG in the context of clinical, endoscopic, and histological improvements in the responders of therapeutic interventions, indicate that the relative serum levels may be even more important than the absolute values. Therefore, the results of this study support the idea that monitoring serum BG might be an important asset in the noninvasive follow-up of patients with IBD.

The cross-sectional analysis performed in this study showed that the concentrations of serum BG were higher in IBD, but a significant difference was noted only when comparing the CD group with controls. Although discrepancies in the number of individuals constituting each group may affect the analysis, the observed differences between CD and UC remain considerably large. However, in terms of clinical activity, patients with UC tended to be more symptomatic, while the other demographic and clinical parameters were similar. Regarding routine laboratory markers, FC, ESR, and CRP were all elevated but with comparable results in the CD and UC groups. The overall endoscopic activity, considered the gold standard for mucosal healing in IBD[3,47] was similar, but the histological analysis revealed more inflammatory activity among patients with CD in our series. A potential association of serum BG with histological analysis was further reinforced after categorizing patients as active or in remission, and statistical significance emerged for both CD and UC patients. Using the same strategy, serum BG was also significantly associated with endoscopic activity in CD patients. Considering combined endoscopic and histologic evaluations, significant associations were demonstrated for both CD and UC patients. These results indicate that the levels of serum BG are associated with inflammatory activity in IBD,

Table 4 Patient demographics and medical characteristics of the subgroup for the analysis of noninvasive markers

Parameters	Control group (*n* = 15)	Crohn's disease group (*n* = 28)	Ulcerative colitis group (*n* = 25)	*P* value
Women, *n* (%)	11 (73)	12 (43)	16 (64)	0.110
Age (yr), median (IQR)	57 (37-63)	36 (26-58)	41 (33-56)	0.158
Disease duration (yr), median (IQR)		6 (2-12.5)	9 (3-10.5)	0.521
Age at diagnosis (yr)				
A1; A2; A3, *n* (%)		3 (11); 14 (50); 11 (39)	5 (20); 15 (60); 5 (20)	0.269
Disease behavior				
B1; B2; B3, *n* (%)		16 (57); 4 (14); 8 (29)	-	-
Disease location				
L1; L2; L3/E1; E2; E3, *n* (%)		5 (18); 9 (32); 14 (50)	5 (20); 12 (48); 8 (32)	-
Perianal disease, *n* (%)		5 (18)	-	-
EIM, *n* (%)		4 (14)	2 (8)	0.391
PSC, *n* (%)		1 (3.6)	3 (12)	0.263
Previous surgery, *n* (%)		9 (32)	1 (4.0)	0.010
Medical therapy, *n* (%)				
Biologicals		10 (36)	2 (8.0)	0.017
Immunosuppressants		18 (64)	5 (20)	0.001
Steroids		8 (29)	1 (4.0)	0.019
Salicylates		6 (21)	14 (56)	0.010
Clinically active, *n* (%)		13 (46)	21 (84)	0.005
Endoscopically active, *n* (%)		23 (82)	17 (68)	0.191
Histologically active, *n* (%)		14/22 (64)	4/14 (29)	0.043
Beta-glucan, μg/mL	26 (8-43)	79 (30-183)	23 (9-69)	0.0003[a]
LBP, pg/mL	19.2 (16.4-22.7)	27.3 (24.3-28.6)	21.8 (19.8-24.6)	0.002[b]
Zonulin, pg/mL	16.4 (15.3-17.1)	17.6 (16.6-19.0)	17.2 (16.6-18.7)	0.09
IL-17 (10^2), pg/mL	0.04 (0.0-1.50)	0.08 (0.0-1.75)	0.00 (0.00-0.60)	0.2479
IFN-gamma, pg/mL	0.00 (0.00-0.690)	0.021 (0.00-0.66)	0.25 (0.001-0.92)	0.5276
TNF-alpha (10^2), pg/mL	0.00 (0.00-1.00)	0.10 (0.00-7.80)	0.00 (0.00-20.01)	0.9004
IL-10, pg/mL	0.00 (0.00-0.00)	0.00 (0.00-4.56)	1.35 (0.00-3.66)	0.4801
IL-6, pg/mL	3.27 (2.38-9.05)	5.70 (0.31-35.73)	4.35 (2.28-15.91)	0.4149
IL-4, pg/mL	0.055 (0.00-6.81)	0.00 (0.00-0.087)	0.00 (0.00-1.31)	0.2597
IL-2, pg/mL	0.00 (0.00-0.052)	0.00 (0.00-0.010)	0.00 (0.00-0.04)	0.9204

In pairwise comparisons:
[a,b]Crohn's disease > Control. Values are presented as number (percentage) or median (IQR: Interquartile range); age at diagnosis: A1, < 17; A2, 17-40; A3, > 40; Disease behavior in Crohn's disease: B1: Inflammatory; B2: Fibrostenosing; B3: Penetrating; Disease location in Crohn's disease: L1: Ileal; L2: Colonic; L3: Ileo-colonic; Disease location in ulcerative colitis: E1: Proctitis; E2: Left sided colitis; E3: Extensive colitis; EIM: Extraintestinal manifestations; PSC: Primary sclerosing cholangitis; LBP: Lipopolysaccharide-binding protein.

predominantly with CD, likely related to specific aspects of the inflammatory process and differences in the pathogenic mechanisms. Similar to FC, which is considered a reliable indicator of the presence of inflammatory activity in the gastrointestinal tract[48,49], the results from this study suggest that BG measurements can also be used to screen patients with suspected IBD.

After determining the optimal cutoff values to estimate inflammatory activity, comparisons with FC and CRP showed that serum BG was slightly less sensitive,

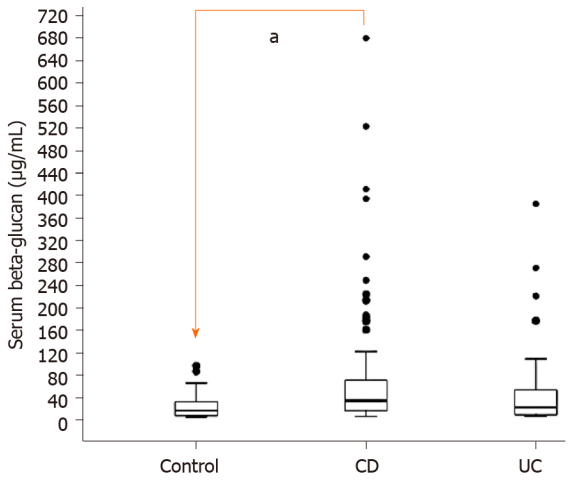


Figure 1 Serum beta-glucan concentrations in patients with Crohn's disease, ulcerative colitis, and controls. The analysis was performed by the Kruskal-Wallis on ranks test, in which multiple comparisons were performed using Dunn's test. The horizontal bars represent the medians, and the boxes represent the 25th and 75th percentiles. Significant results are depicted ($^{a}P < 0.0001$).

although more specific regarding endoscopic activity. However, the performance of serum BG in predicting histologic inflammation was remarkably better than that of FC and CRP. Comparative analysis appears to have unveiled differences regarding the nature of the proposed inflammatory markers. CRP is an acute-phase protein that often reflects systemic nonspecific inflammatory states, usually associated with the severity of underlying conditions, including IBD[50,51]. FC has been the most used noninvasive inflammatory biomarker for IBD in the last decade and indicates the presence of neutrophils in the intestinal mucosa[16,19]. However, serum BG can reflect fungal infection[52] but also may indicate the presence of ubiquitous circulating cell wall components of the gut microbiome[33]. Therefore, the presence of BG in serum may likely reflect the presence of active mucosal inflammation and an abnormal intestinal permeability, allowing the passage of components of the gut microbiome into the blood. This phenomenon may explain the stronger association of BG with histological rather than endoscopic disease activity. Therefore, we hypothesized that the levels of serum BG in patients with IBD could indicate more subtle changes, such as histological and, indirectly, epithelial permeability to luminal contents.

Currently, although histological assessment has not been recommended as a routine procedure in the follow-up of patients with IBD, persistent microscopic inflammation has been consistently associated with poor outcomes in UC[9,53]. However, the role of histological analysis is less clear in CD, and no consensus exists regarding scoring systems[54]. Nevertheless, recent data from studies with patients with CD suggest that histologic healing, more than endoscopic healing, is associated with a reduced risk of disease relapse[11,55]. Taken together, these study findings indicate that histologic assessment will become a major target in the near future for both forms of IBD; therefore, the need for invasive tests might continue and even increase.

In addition to reflecting an inflammatory state and abnormal intestinal permeability, the presence of high levels of BG per se could also promote additional inflammation. For example, BG was shown to activate macrophages through the dectin-1 receptor, increasing the production of pro-inflammatory cytokines such as IL-12 and TNF-alpha[56] and the release of arachidonic acid and eicosanoids[57], fueling inflammation. Moreover, the activation of NF-kappa B and the release of IL-6 and IL-23 by human macrophages induced by BG were shown to be enhanced by priming with LPS and interferons, suggesting a synergistic effect of an inflammatory microenvironment[58]. Nonetheless, in contrast to LPS, BG alone strongly induces the secretion of IL-1 beta from human macrophages, mediated by the NLRP3 inflammasome[59].

In subgroup analysis, we performed another set of experiments to compare serum BG with a series of other potential biomarkers. Of all candidate molecules investigated, only LBP, an endotoxin-related marker, showed significant differences among the groups. Similar to previous studies involving patients with IBD, increased serum levels of LBP correlated with disease activity[60], paralleled hs-CRP[61] and proinflammatory cytokines[62], and the recovery after treatment suggested its potential to predict clinical relapses. Although LBP demonstrated an association with the

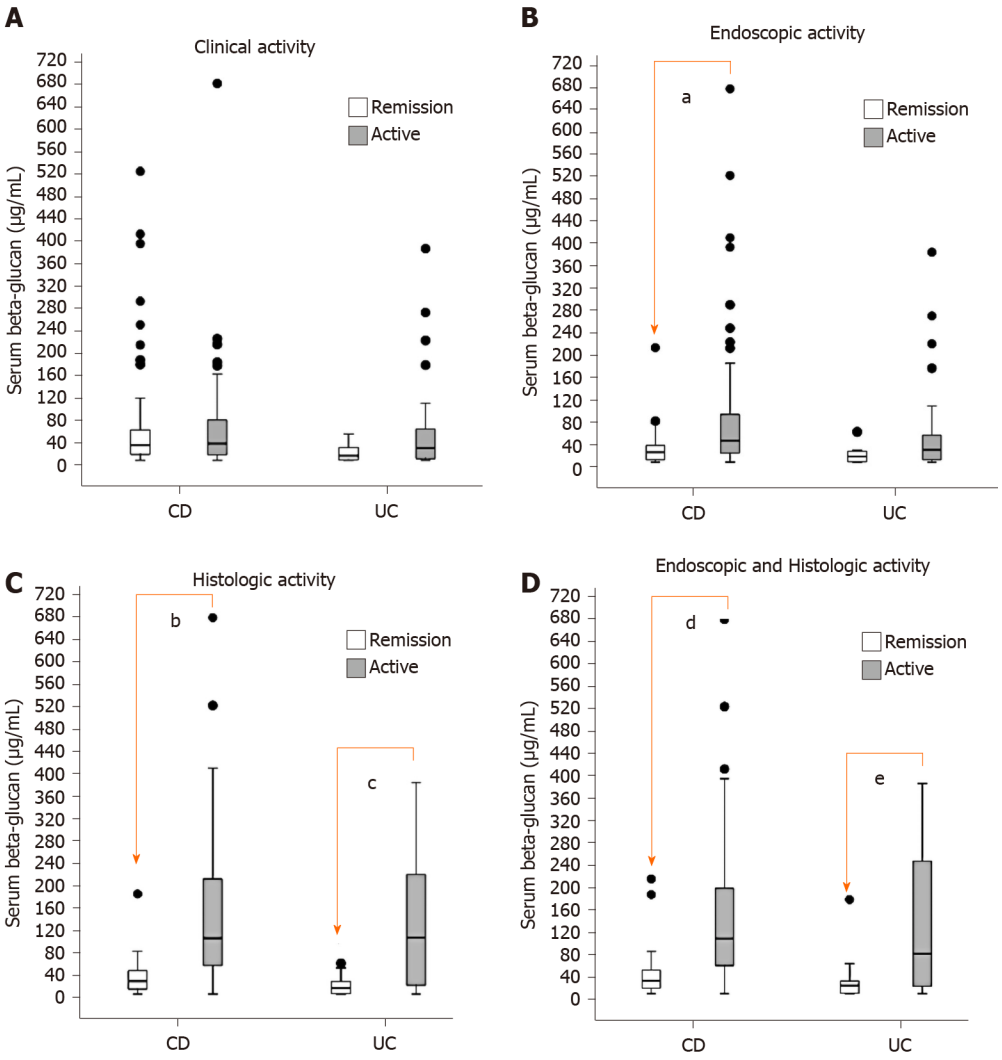


Figure 2 Although no difference was found in the beta-glucan levels regarding clinical activity, significant differences were shown for the endoscopic activity in the Crohn's disease group and for histologic activity and combined endoscopic and histologic activity for both the Crohn's disease and ulcerative colitis groups. Serum beta-glucan concentrations are stratified in Crohn's disease and ulcerative colitis according to (A) clinical; (B) endoscopic; (C) histologic; and (D) combined endoscopic and histologic indexes. The analysis was performed by the Mann-Whitney rank-sum test. The horizontal bars represent medians, and the boxes represent the 25th and 75th percentiles. Significant results are depicted ($^{a}P = 0.002$; $^{b}P < 0.001$; $^{c}P = 0.013$; $^{d}P < 0.001$; $^{e}P = 0.047$).

diagnosis of IBD in this study, no correlation was found with the levels of serum BG and inflammatory activity. Nevertheless, the finding of elevated LBP in IBD is conceptually important because it reflects the translocation of LPS into the systemic circulation, indicating intestinal barrier dysfunction[63]. Additionally, like serum BG, the increased levels of LPS/LBP support the idea of the intestinal microbiota fostering the inflammatory response. Among the cytokines investigated in this study, serum BG showed a strong positive correlation with IFN-gamma and IL-17A in CD and with IL-17A alone in UC. This finding agrees with previous studies showing that dectin-1 activates intracellular signals through CARD9, resulting in the production of pro-inflammatory cytokines and the promotion of immune responses based on Th17[64,65]. This finding may explain the stronger association of BG with CD, which typically displays marked mucosal infiltration of Th1 and Th17 cells[66,67].

In another subgroup analysis, the distinct time points analyzed aimed to determine the dynamic changes in serum BG and the potential association with the therapeutic intervention. In particular, the selection of patients with clinical and endoscopic activity allowed us to confirm that the relative levels of BG were probably more strongly correlated with disease activity than the absolute levels. Similar results were found in a previous study, showing that serum BG and LPS were increased in CD and were associated with disease activity[33]. However, in contrast to our study, the previous investigation involved a smaller number of patients, considered only the

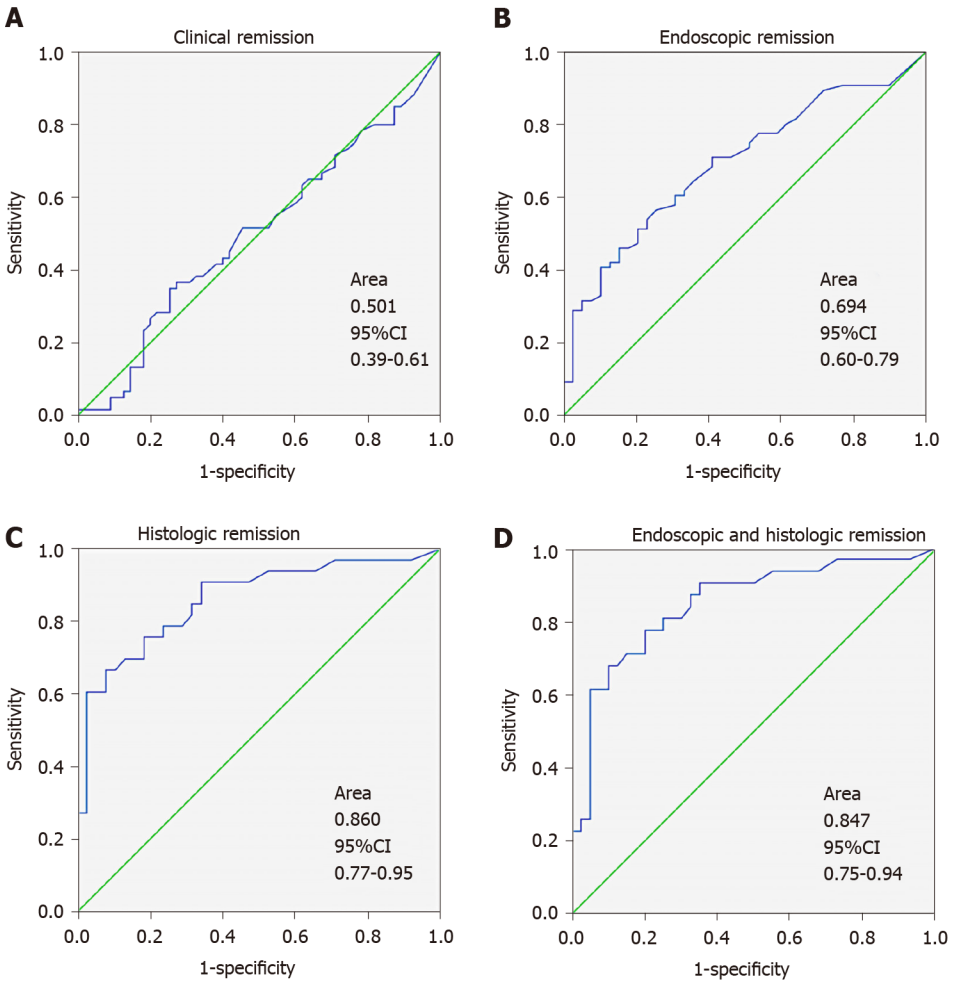


Figure 3 Receiver operating characteristic curves illustrating the diagnostic ability of serum beta-glucan for detecting disease activity in patients with Crohn's disease. Receiver operating characteristic curves are shown in relation to the (A) clinical; (B) endoscopic; (C) histologic; and (D) combined endoscopic and histologic criteria. Diagonal segments are produced by ties. The area under the curve with standard error and the 95% confidence interval (95%CI) are shown in each plot.

clinical status of the patients with CD, did not analyze patients with UC, and only healthy individuals constituted the control group. In the current study, the improvement achieved with the therapeutic intervention paralleled by the levels of serum BG strongly suggest that abnormalities in the microbiome and permeability to luminal contents probably represent crucial underlying pathogenic mechanisms in IBD, potentially modulated by the treatment.

Considering the stronger association with CD, particularly regarding histological activity, we attempted to investigate whether serum BG could also reflect the presence of transmural inflammation detected by MRE. MRE is a well-established modality for evaluating acute complications and the follow-up of patients with CD because it offers a detailed tridimensional view of the intestinal wall, can detect extraintestinal complications, and has been reported to detect mucosal inflammation, with results comparable to ileocolonoscopy[68,69]. In this subgroup analysis, the results continued to support the usefulness of serum BG for predicting histological inflammation but were relatively modest concerning transmural inflammation. Hence, we hypothesized that serum BG might serve to detect even minimal mucosal changes, probably involving permeability and dysbiosis, but probably not the transmural component of the inflammatory process underlying CD. Elevated serum BG levels may reflect preferentially acute and ongoing mucosal inflammation, but probably not damage accumulated over time due to the transmural involvement of CD. The current findings appear to corroborate previous studies suggesting that the objective assessment of lesions in CD may depend on a combination of histology and cross-sectional imaging[70].

Our study has limitations, including its predominant cross-sectional nature and the number of patients, particularly in the subgroup analyses. In view of the recognized

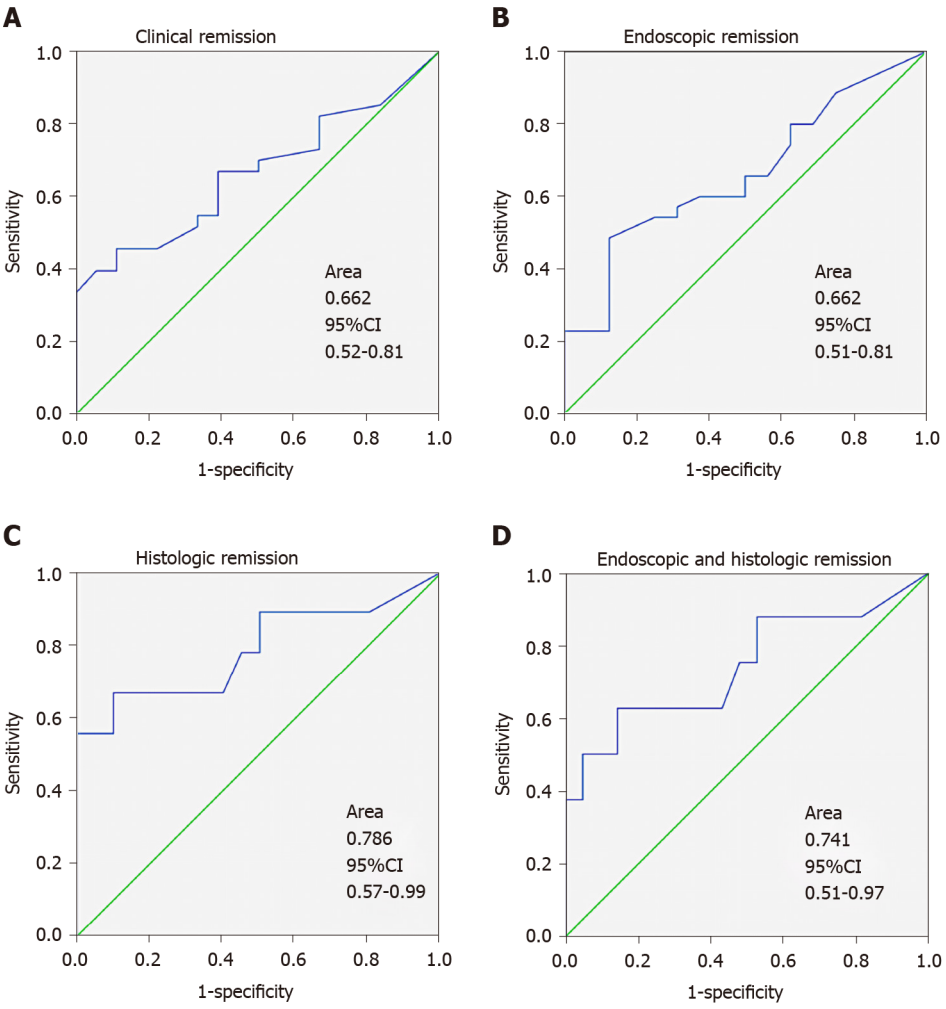


Figure 4 Receiver operating characteristic curves illustrating the diagnostic ability of serum beta-glucan for detecting disease activity in patients with ulcerative colitis. Receiver operating characteristic curves are shown in relation to the (A) clinical; (B) endoscopic; (C) histologic; and (D) combined endoscopic and histologic criteria. Diagonal segments are produced by ties. The area under the curve with standard error and the 95% confidence interval (95%CI) are shown in each plot.

disease heterogeneity in IBD, especially large in CD, including severity, age of onset, the predominant clinical aspects, localization and extent, behavior, previous surgical interventions, response to treatment, changes during the course of the disease, among other factors[71-73], it is expected that the more reliable evaluations will depend on the number of patients. In fact, a larger sample would probably allow a more precise estimation of the cut-off value for predicting disease activity. Additionally, a more detailed follow-up of the patients and more frequent serial BG measurements could have allowed an assessment of whether concentrations changes could predict early recurrence. Regarding subgroup analysis to investigate potential changes in the serial concentrations of BG, this study used a relatively small number of individuals and evaluated patients under different therapeutic regimens. However, technically, freshly collected blood for BG might result in more reliable readings than FC due to stability and conservation issues[74,75]. Moreover, the patients' acceptance of undergoing blood collection for BG would likely be greater than that of providing stool samples for FC, the compliance of which is low[76].

If elevations of serum BG detected among patients with IBD actually reflect an abnormal intestinal microbiome, it is likely that dysbiosis underlying other intestinal diseases could promote similar changes in serum BG. This could constitute a bias in this study, considering the composition of the control group. For example, several studies have shown that patients with IBS exhibit an abnormal intestinal microbiome[77-79], and treatments based on interventions in the intestinal microbiome tend to be effective, at least temporarily[80-82]. Nonetheless, the results of this study showed that the overall variability within the control group was very small compared to the dispersion seen among patients with IBD. In fact, the levels of serum BG among IBD patients in remission (both CD and UC) were not clearly distinguishable from the

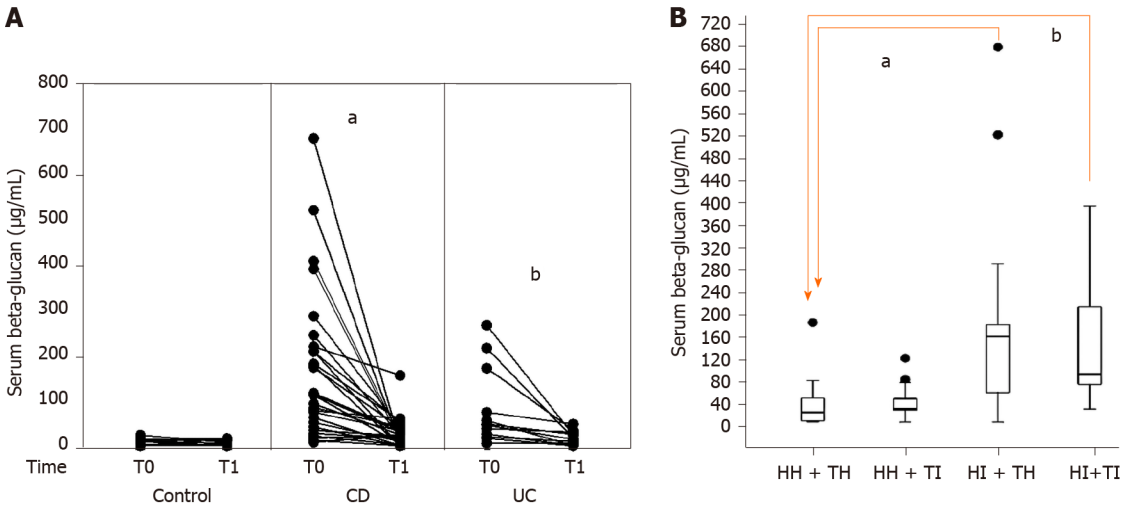


Figure 5 Analysis of serum beta-glucan in response to therapeutic intervention in inflammatory bowel diseases, and in relation to histologic and transmural inflammation in Crohn's disease. A: Serial concentrations of serum beta-glucan are shown at week 0 (T0) and between weeks 12 and 16 (T1) in controls and after therapeutic interventions in patients with Crohn's disease and ulcerative colitis. The analysis was performed by the pairwise Wilcoxon rank-sum test. Significant results are depicted ($^aP < 0.0001$; $^bP = 0.003$); B: Serum beta-glucan concentrations of patients with Crohn's disease are stratified according to histologic healing or inflammation, and transmural healing or inflammation, based on magnetic resonance enterography. The analysis was performed by the Kruskal-Wallis on ranks test, in which multiple comparisons were performed using Dunn's test. The horizontal bars represent medians, and the boxes represent the 25th and 75th percentiles. Significant results are depicted ($^aP = 0.0018$; $^bP = 0.011$).

levels detected in the control group. Therefore, although a combination of intestinal dysbiosis and abnormal permeability might be present in the context of IBD, the results of this study support the idea that the elevations of serum BG among IBD patients should be predominantly attributed to changes in the intestinal inflammatory activity.

CONCLUSION

In conclusion, serum BG concentrations are consistently associated with disease activity in IBD, particularly with histologic inflammation, the ultimate target of treatment. The stronger relationship of serum BG with CD than with UC appears to underlie differences in specific pathogenic mechanisms. The relative changes are even more tightly associated with disease activity, suggesting that repeated measurements of serum BG might become a useful resource in the follow-up of patients with IBD. Finally, serum BG might prove particularly useful in IBD because of its noninvasive nature, ease of performance and relative low cost. Further prospective studies will be necessary to determine the best time intervals for measuring serum BG routinely in patients with IBD.

ARTICLE HIGHLIGHTS

Research background

Currently, the approach for monitoring the most precise predictors of disease outcomes in inflammatory bowel diseases (IBD), mucosal or histologic remission, demands frequent endoscopic evaluations, which are costly and invasive procedures.

Research motivation

Finding novel non-invasive biomarkers to detect intestinal inflammation continues to represent a major challenge in the field of IBD research.

Research objectives

To determine whether the serum concentrations of beta-glucan (BG), a ubiquitous cell wall component of gut microorganisms, correlate with intestinal inflammation.

Research methods

A prospective observational study was performed in a tertiary referral center, from 2016 to 2019, in which serum BG was determined in patients with Crohn's disease (CD), ulcerative colitis (UC), and controls, using a photometric detection kit. The ability of BG to detect active *vs* inactive disease was assessed using the area under the receiver operating characteristic curve. Inflammatory activity was determined by ileocolonoscopy, histopathology, magnetic resonance enterography), and biomarkers, including fecal calprotectin, C-reactive protein, and a panel of cytokines. In subgroup analysis, serial BG was used to assess the response to therapeutic interventions.

Research results

The serum BG levels were higher in CD patients than in controls. The BG levels paralleled the endoscopic activity in CD patients and histologic activity and combined endoscopic and histologic activity in both CD and UC patients. Performance analysis showed that the BG results were remarkably better for predicting histologic inflammation than fecal calprotectin and C-reactive protein. Regarding the clinical, endoscopic, and histologic activities, the BG levels were reduced following therapeutic intervention in patients with CD and UC. Compared with and histologic healing, no significant correlation was found between serum BG and transmural healing based on magnetic resonance enterography, in CD patients. Positive correlations were detected between BG and interleukin-17 in the CD and the UC group, and between BG and interferon-gamma in the CD group.

Research conclusions

Serum BG concentrations are consistently associated with disease activity in IBD, particularly with histologic inflammation, the ultimate target of treatment.

Research perspectives

Serum BG emerges as an important novel noninvasive approach for detecting mucosal inflammation and therapeutically monitoring IBD.

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SYSTEMATIC REVIEWS

Predictive value of blood concentration of biologics on endoscopic inactivity in inflammatory bowel disease: A systematic review

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Abstract

BACKGROUND

Although blood concentration of biologics is an important composition of disease management in inflammatory bowel disease (IBD) patients, complexity and uncertainty of biological management encourage many disputes in predicting the outcome of IBD patients through blood concentration of biologics.

AIM

To verify the predictive value of blood concentration of biologics on endoscopic inactivity in IBD patients under different situations.

METHODS

We searched PubMed/MEDLINE, Embase, and Web of Science up to May 2020 and identified IBD patients as the research cohort as well as the correlations between blood concentration of biologics and endoscopic inactivity in IBD patients as the research direction.

RESULTS

A total of 23 articles with 30 clinical studies and 1939 IBD patients were included. The predictive cut-off value of blood concentration of infliximab on mucosal healing should be 2.7-10.6 μg/mL in IBD. Blood concentration of infliximab reaching 5.0-12.7 μg/mL or more increased the probability of fistula healing/closure in perianal fistulizing Crohn's disease. Blood concentration of adalimumab reaching 7.2-16.2 μg/mL or more could predict mucosal healing in IBD. The predictive cut-off value of blood concentration of adalimumab on fistula healing/closure should be 5.9-9.8 μg/mL in perianal fistulizing Crohn's disease.

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Blood concentration of vedolizumab surpassing 25.0 μg/mL indicated mucosal healing in ulcerative colitis patients under maintenance therapy and the predictive cut-off value of blood concentration on mucosal healing or endoscopic remission under induction therapy in IBD could be 8.0-28.9 μg/mL.

CONCLUSION
Blood concentration of biologics should not be utilized to predict endoscopic inactivity of IBD independently due to discrepancies in clinical studies, whereas conducting therapeutic drug monitoring intensively contributes to precise therapy.

Key Words: Inflammatory bowel disease; Biological blood concentration; Endoscopic inactivity; Infliximab; Adalimumab; Vedolizumab

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Core Tip: Deep remission is considered the primary endpoint of biological therapy in inflammatory bowel disease. However, it is still difficult to determine or predict whether inflammatory bowel disease (IBD) patients achieve deep remission or not. Although endoscopic examinations are widely accepted by gastroenterologists as the golden standard in evaluating disease states, the majority of IBD patients reject frequent invasive examinations. Hence, new methods for early prediction of therapeutic outcomes in IBD patients on biologics are brought forward by gastroenterologists. Blood concentration of biologics, one of the major monitoring indicators during biological therapy, exhibits enormous tendency to correlate with outcomes of IBD patients. Nonetheless, blood concentration of biologics is not a substitute for endoscopic examinations in detecting deep lesions. By contrast, the combination of blood concentration of biologics and endoscopic examinations is conducive to enhancing the accuracy of outcome prediction.

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a class of chronic and unspecific inflammatory intestinal diseases induced by immune-mediated disorder, which mainly destroys intestinal structure and impairs the intestinal function. The involvement of large intestinal mucosa, frequent spreading of inflammation to the whole digestive tract, and invasion of the mucosal muscular layer of the intestine may distinguish CD from UC.

Previously, the therapeutic goal for IBD focuses on the management of symptoms only, but changing the course of the disease is recognized as the therapeutic goal currently. Therefore, based on the traditional top-down therapy, several clinical trials have put forwarded two novel therapeutic strategies of accelerating top-down therapy and implementing step-up therapy in the early stage, during which biological management remains crucial[1-3]. Different responses of IBD patients to the same biologic due to individual variations and increment of biological therapy failure over time are still a nodus in the treatment. Monitoring blood concentration of biologics is regarded as the significant part of biological management, which assists in estimating the therapeutic effects in IBD patients sufficiently[4].

Endoscopic mucosal healing is considered the major therapeutic goal in IBD patients and it has been verified in several clinical trials, which showed that endoscopic healing is beneficial in decreasing the hospitalization rate, surgical risk, cancerous rate, and so on. Endoscopy remains the golden standard for mucosal healing evaluation, and is advantageous in reflecting gastrointestinal lesions directly

and disadvantageous due to its poor compliance, high cost, painful procedure, and subjective discrepancies in outcome evaluation among different physicians[5-9]. Hence, biomarkers that can temporarily substitute endoscopy or assist endoscopy in evaluating the prognosis of IBD have been focused on by current research studies[10,11]. Similar to the specific inflammatory biomarkers in IBD, blood concentration of biologics published in a series of clinical trials has the ability to predict the prognosis of IBD in patients under biological therapy, especially remission and relapse[4]. This review lays emphasis on the predictive value of blood concentration of biologics on endoscopic inactivity in IBD patients.

MATERIALS AND METHODS

Literature search

We searched PubMed/MEDLINE, Embase, and Web of Science till March 2020 for studies that met the following inclusion criteria: (1) Study cohorts containing adults or children diagnosed with IBD; (2) Studies that reported the definition of endoscopic inactivity outcome, and the therapeutic target of study cohort endoscopic inactivity; and (3) Studies that evaluated the correlation between biological blood concentration and endoscopic inactivity outcome and identified the best cut off values of blood concentration of biologics in predicting the endoscopic outcome. Studies were identified using the following key words: "UC", "Ulcerative colitis", "CD", "Crohn's disease", "IBD", "Inflammatory bowel disease", "IFX", "Infliximab", "ADA", "Adalimumab", "VDZ", "Vedolizumab", "anti-TNF-α drug", "anti-TNF-α agent", "anti-TNFα therapy", "anti-TNFα treatment", "Serum level", "Trough level", "Serum concentration", "Trough concentration", "Endoscopic remission", "Mucosal healing", "Histological healing", "Histological remission", "Endoscopic inactivity", and "Endoscopic healing". Literature search focused on full-texts and references of each article were carefully checked. Additionally, the literature search was not limited by country, language, and published date. First, two authors (Rong Huang and Xue-Hui Qiao) deleted repetitive studies according to the search results. Second, they included studies based on the inclusion criteria, after rigorous screening of the titles, abstracts, and key words of studies. Any disagreements during the screening process were judged by the third author (Ke-Fang Jiang).

Data extraction

The data collected from each study included author, year, and study type, and the data was divided into three major segments. The first part involves collection and systematic analysis of data including diagnosis, age, disease course, smoking, disease severity, disease location, previous medical therapy, previous surgery, previous biological therapy, and so on. The second part involves collection and systematic analysis of data that are relevant to biological management, including biological type, course of the research, therapeutic stage, injection dose, injection frequency, injection time, and so on. The third part includes collection and systematic analysis of data relevant to the predictive value of blood concentrations of biologics on endoscopic inactivity outcome, including the best predictive cut off values of blood concentration of biologics, sensitivity, specificity, positive predictive value, negative predictive value, area under the curve, the definition of endoscopic inactivity, and the number of patients achieving endoscopic inactivity.

RESULTS

Literature selection

Three researchers identified a total of 1086 studies based on the inclusion criteria. Of these, 455 duplicates or irrelevant studies or studies that did not report the original data were removed. Moreover, 604 studies were excluded after finishing rigorous check of the inclusion criteria, abstract, and key words. Additionally, another four studies were deleted due to the lack of best cut-off values of blood concentration after carefully reviewing the full-texts. Finally, 23 studies involving 30 clinical studies and 1939 IBD patients were included in the study. Two studies concentrated on pediatric CD, whereas the major study design and consequences were approximated to adults. Eight studies have focused on IBD patients and 15 studies distinguished CD patients from UC patients in study cohorts. The flow chart of study selection process is shown

in Figure 1 and the demographics and disease characteristics of study cohorts are shown in Tables 1 and 2.

Quality of literature

The Newcastle-Ottawa Scale (NOS) was adopted to evaluate the quality of studies included in this study. NOS mainly contains eight aspects: "The representativeness of the exposed cohorts", "the selective methods of unexposed cohorts", "identification of exposure", "the verification of concerned results absent in the start of study", "the comparability of cohorts based on design and analysis", "sufficient evaluation of study results", "long follow-up period after obtaining the study results", and "long follow-up period of cohorts". Quality evaluation of studies has ruled out two aspects by considering "the selection methods of unexposed cohorts" and "the comparability of cohorts based on design and analysis" that are irrelevant to the study. One star is assigned if the literature achieved any one of the six items and the highest point that can be achieved is six stars. However, the literature with not less than five stars is defined as high quality study. Nineteen studies[12-30] were considered as low quality and four studies[31-34] were considered as high quality (Table 3).

Biological management

Several discrepancies existed in the biological management put forwarded by different studies. Twenty-three studies showed clear records of biologicl type[12-34]; eleven studies involved adalimumab (ADA)[12,16,18-20,24,26-30], thirteen involved infliximab (IFX)[12,14,15,17,18,21-24,27,28,31,32], and four involved vedolizumab (VDZ)[13,25,33,34]. El-Matary *et al*[32] have failed to publish IFX therapeutic stage, nine studies referred to ADA maintenance therapy[12,16,18,19,24,26,27,29,30], three referred to ADA induction therapy[18,20,28], ten referred to IFX maintenance therapy[12,14,15,17,18,21,22,24,27,31], four referred to IFX induction therapy[18,23,28,31], three referred to VDZ induction therapy[13,33,34], and four referred to VDZ maintenance therapy[13,25,33,34]. Thirteen studies had the course of research[12-15,19,20,23,24,28,29,31,33,34]. The injection dose of ADA induction therapy recorded in the studies was 160 mg at week 0, 80 mg at week 2, and 40 mg at week 6[18,20,28]. ADA maintenance therapy adopted an injection dose of 40 mg every other week or 40 mg every week[16,18,19,24,27,29,30]. Injection dose of IFX induction therapy was 5 mg/kg at weeks 0, 2, and 6 in the published studies[18,31]. Nonetheless, the injection dose of IFX maintenance therapy varied in different studies. The standard dose of IFX maintenance therapy was 5 mg/kg every 8 wk whereas the interval time of injection was shortened to 6 wk or the dose of injection was strengthened to 7.5 mg/kg or 10 mg/kg in some patients[14,15,18,21,24,27,31]. Three studies recorded the injection dose of VDZ induction therapy as 300 mg at weeks 0, 2, and 6[13,33,34], while Dreesen *et al*[13] have implemented another VDZ injection dose of 300 mg at week 10 due to a low response rate. Injection dose of VDZ maintenance therapy adopted 300 mg every 4 wk or every 8 wk from week 14[13,25,33,34] (Table 4).

Endoscopic scores and definition of endoscopic inactivity

The definition of endoscopic inactivity varied in different studies. Ten studies did not utilize a standard criterion for endoscopic inactivity evaluation of CD or UC[13,14,24,26,27,29-32,34]. The definition of fistula healing/closure in three studies of perianal fistulizing Crohn's disease (pfCD) mainly concentrated on physical examination[24,27,32], and only Strik *et al*[27] utilized magnetic resonance imaging (MRI) examination to evaluate the endpoint with no detailed description. Seven studies have defined mucosal healing as a lack of inflammation or disappearance of ulcerations under endoscopy instead of endoscopic scoring systems[13,14,26,29-31,34]. Seventeen studies involved endoscopic scoring systems for outcome evaluation of CD or UC[12,13,15-23,25,26,28,31,33,34]. Four studies defined mucosal healing of CD as a Simple Endoscopic Score for Crohn's Disease (SES-CD) < 3[12,17,28] or SES-CD of 0[17] and endoscopic remission of CD as an SES-CD ≤ 4[33], Dreesen *et al*[31] selected a Crohn's Disease Endoscopic Index of Severity (CDEIS) < 3 to define endoscopic remission, and Morita *et al*[19] and Imaeda *et al*[15] selected a modified Rutgeerts' score of 0 (no lesions or scar) or 1 (5 apthous lesions) to define mucosal healing. Three studies selected a Rutgeerts' score of < 2 to define mucosal healing of CD in postoperative setting[12,16,21]. Ten studies defined mucosal healing of UC as a Mayo Endoscopic Score (MES) ≤ 1[12,13,16,20,22,23,26,28,33,34], Morita *et al*[18] utilized an Ulcerative Colitis Endoscopic Index of Severity (UCEIS) ≤ 1 (the bleeding descriptor and the erosions and ulcers descriptor were both 0, and the vascular pattern descriptor was 0 or 1) to define mucosal healing. Considering the inconsistency between intestinal inflammation under endoscopy and clinical symptoms, Hanžel *et al*[33] have included clinical remission and endoscopic

Table 1 Disease phenotype of study cohort

Ref.	CD, Location, *n* (%)				CD, Behavior, *n* (%)					UC, Location, *n* (%)			
	Ileal	Colonic	Ileocolonic	UGT	Inflammatory	Stricturing	Fistulizing	Perianal	Penetrating	Extensive	Left-side	Pancolitis	Proctitis
Chaparro *et al*[12], 2018	49 (38.6)	25 (19.7)	52 (40.9)	4 (3.1)	67 (52.8)	34 (26.8)	26 (20.5)	85 (66.9)		39 (30.7)	16 (12.6)		
Ungar *et al*[28], 2016	31 (27.9)	26 (23.4)	54 (48.6)		49 (44.1)	28 (25.2)			34 (30.6)		16 (14.4)	17 (15.3)	
Yarur *et al*[29], 2016	16 (27.9)	9 (15.6)	14 (24.1)	3 (5.2)		29 (50.0)		20 (34.4)	11 (19.0)		1 (14.3)	6 (86.7)	0
Roblin *et al*[26], 2014	13 (59.1)	4 (18.2)	5 (22.7)			4 (18.2)			4 (18.2)	14 (77.8)	4 (18.2)		
Morita *et al*[18], 2016											19 (29.7)	45 (70.3)	
Morita *et al*[19], 2016	14 (33.3)	4 (9.5)	24 (57.1)			50.0			6 (14.3)				
Strik *et al*[27], 2019													9 (13.6)
Plevris *et al*[24], 2020	11 (17.2)	26 (40.6)	27 (42.2)			11 (17.2)			16 (25.0)				
Zittan *et al*[30], 2016	12 (20.0)	14 (23.3)	34 (56.7)						18 (30.0)				
Juncadella *et al*[16], 2018	20 (27.8)	13 (18.1)	39 (54.2)	3 (4.2)		10 (13.9)		32 (44.4)	29 (40.3)	10 (38.5)	16 (61.5)		
Papamichael *et al*[23], 2016												63 (62.4)	
Papamichael *et al*[20], 2017										20 (46.5)			
Papamichael *et al*[22], 2018												29 (51.8)	
Feng *et al*[14], 2019	15 (10.6)	6 (4.3)	120 (85.1)			29 (20.6)			15 (10.6)				
Papamichael *et al*[21], 2018	20 (18.3)	25 (22.9)	59 (54.1)	5 (4.6)		18 (16.4)			38 (34.5)				
Dreesen *et al*[31], 2020													
Imaeda *et al*[15], 2014	14 (31.1)	4 (8.9)	27 (60.0)			16 (35.6)			4 (8.9)				
EI-Matary *et al*[32], 2019	5 (8.5)	17 (28.8)	27 (45.8)	10 (16.9)									
Kang *et al*[17], 2019	8 (7.6)	6 (5.7)	91 (86.7)	70 (66.7)		18 (17.1)			3 (2.9)				
Hanzel *et al*[33], 2019	0	4 (14.3)	24 (8.6)	6 (2.1)				4 (14.3)		16 (69.6)	7 (30.4)		
Dreesen *et al*[13], 2018													
Pouillon *et al*[25], 2019											13 (41.9)	15 (48.4)	3 (9.7)
Yacoub *et al*[34], 2018	14(35.9)	6 (15.4)	19 (48.7)			11 (28.2)			6 (15.4)	27 (62.8)	15 (34.9)		1 (2.3)

UGT: Upper gastroenterological tract; CD: Crohn's disease; UC: Ulcerative colitis.

remission into the primary endpoint. Additionally, five studies defined primary endpoint as histological healing or histological remission[16,21,22,25,29], whereas only Pouillon *et al*[25] have defined histological healing of UC as a Nancy Histological Index ≤ 1 rather than subjective description (Table 5 and Figure 2).

Predictive value of blood concentration of biologics on endoscopic inactivity

Study cohorts of inflammatory bowel disease: In the study by Ungar *et al*[28], IBD patients under IFX or ADA induction therapy had a 80%-90% probability of mucosal healing satisfying an IFX serum level ranging from 6 μg/mL to 10 μg/mL or ADA serum level ranging from 8-12 μg/mL. The combination of IFX serum level at week 2 during treatment and clinical symptoms demonstrated a great effect on the prognostic prediction as well as the meaningful option and evaluation of IFX continual or transformational or intensive therapy[35]. A cross-sectional study of IBD patients under IFX or ADA therapy using stable doses for at least 6 mo suggested the best cut off values of 3.4 μg/mL and 7.2 μg/mL of IFX and ADA trough levels in predicting mucosal healing, respectively[12]. Specifically, the ADA serum level that surpasses 7.5 μg/mL or 7.8 μg/mL was found to be predictive of mucosal healing or histological healing with a 61%-62% sensitivity and 83%-95% specificity in IBD patients under maintenance therapy[29]. Inversely, the possibility of the absence of mucosal healing was increased dramatically with an ADA trough level of lower than 4.9 μg/mL[26]. VDZ would be a better choice if anti-TNF-α agents failed to terminate inflammatory activity of IBD. Hanžel *et al*[33] have demonstrated that vedolizumab trough level (VTL) ≥ 8.0 μg/mL at week 22 or VTL ≥ 22.0 μg/mL at week 6 assisted in moderately predicting IBD remission (endoscopic remission and clinical remission) within the first year of VDZ treatment with a sensitivity and specificity of more than 70%.

Study cohorts of ulcerative colitis: In the study by Papamichael *et al*[23], IFX serum levels exceeding 15 μg/mL at week 6 or 2.1 μg/mL at week 14 during induction therapy in UC patients were shown to act as independent factors that affect the short term mucosal healing as defined by MES ≤ 1 from week 10 to week 14. Moreover, IFX trough level of more than 7.5 μg/mL or 10.5 μg/mL acts as a good predictor of endoscopic healing with a 77% sensitivity and 62% specificity or histological healing with a 54% sensitivity and 78% specificity, respectively, under maintenance therapy[22]. However, Morita *et al*[18] held the view that IFX trough level of ≥ 2.7 μg/mL and ADA trough level of ≥ 10.3 μg/mL were both capable of predicting UCEIS ≤ 1with a sensitivity and specificity exceeding 80% among UC patients under maintenance therapy. Differently, Juncadella *et al*[16] have considered ADA serum level exceeding 16.2 μg/mL as a good predictor of MES ≤ 1 with an 85% sensitivity and histological healing with a 100% sensitivity. However, under induction stage of therapy, ADA serum level of more than 9.4 μg/mL could predict short-term mucosal healing at weeks 8-14 with a 67% sensitivity and 77% specificity. Dreesen *et al*[13] have confirmed that VTL ≥ 28.9 μg/mL at week 2 and VTL ≥ 13.9 μg/mL at week 14 showed a moderate sensitivity and low specificity to predict MES ≤ 1 at week 14. Another study in UC patients under induction therapy with VDZ considered that VTL surpassed 18.0 μg/mL at week 6 predicted MES ≤ 1 in the first year treatment with a 100% sensitivity and 75% specificity[34]. Pouillon *et al*[25] have discovered that UC patients under maintenance therapy with VDZ had VTL ≥ 25.0 μg/mL, which showed a significant possibility of achieving histological healing (Nancy Histological Index ≤ 1).

Study cohorts of Crohn' S disease: The follow-up study of CD patients during the first year therapy of IFX demonstrated that IFX trough level exceeding 23.1 mg/L at week 2 or 10.0 mg/L at week 6 had a high sensitivity and low specificity in predicting endoscopic remission, which was defined as CDEIS < 3 or absence of ulcerations at week 12[31]. IFX serum levels of more than 4.85 μg/mL at week 14 and 2.85 μg/mL at week 30 could assist in moderately predicting the complete absence of any sign of the ulceration in CD[14]. Nonetheless, Papamichael *et al*[21] have verified that IFX serum level of ≥ 10 μg/mL might not be a good predictor of endoscopic remission or histological remission under maintenance stage with a low sensitivity and specificity. The study on CD patients with stable IFX or ADA infusions for at least 6 mo showed that IFX trough level of ≥ 4.0 ug/mL[15] or ADA ≥ 7.9 ug/mL[19] assisted in moderately predicting the modified Rutgeerts' Scoring System Score of ≤ 1. Similarly, a 91% sensitivity and 76% specificity acts as the predictive value of ADA trough level of ≥ 8.14 μg/mL on complete absence of ulceration in CD patients[30]. However, Juncadella *et al*[16] have suggested that ADA serum level of 12 μg/mL or more, much higher than trough level, assisted in predicting endoscopic remission or histological healing with a

Table 2 Demographics of study cohorts in inflammatory bowel disease

Ref.	Diagnostic	Biological	Number	Female, *n* (%)	Smoking, *n* (%)	Previous surgery, *n* (%)	Previous biological therapy, *n* (%)	Concomitant medications				
								CS, *n* (%)	IMMs, *n* (%)	5-ASA, *n* (%)	Surgical intervention, *n* (%)	Enteral nutrition, *n* (%)
Chaparro *et al*[12], 2018	IBD	ADA/IFX	182	90 (49.5)	50 (27.5)	49 (26.9)	48 (26.4)		63 (34.6)			
Ungar *et al*[28], 2016	IBD	ADA/IFX	145	64 (44.1)	19 (13.1)	33 (22.8)	37 (25.5)	27 (18.6)	79 (54.5)			
Yarur *et al*[29], 2016	IBD	ADA	66	27 (40.9)	6 (9.1)		42 (63.6)	14 (21.2)	21 (31.2			
Roblin *et al*[26], 2014	IBD	ADA	40	22 (55)					5 (12.5)			
Morita *et al*[18], 2016	UC	ADA/IFX	64	25 (39.1)			4 (6.3)	50 (78.1)	34 (53.1)	48 (75.0)		
Morita *et al*[19], 2016	CD	ADA	42	15 (35.7)		18 (42.9)	15 (35.7)	5 (11.9)	24 (57.1)	30 (71.4)		
Strik *et al*[27], 2019	PfCD	ADA	19	9 (47.4)	5 (26.3)		9 (47.4)				4 (21.1)	
Plevris *et al*[24], 2020	PfCD	ADA	35	17 (48.6)	3 (8.6)		21 (60.0)		15 (42.9)			
Zittan *et al*[30], 2016	CD	ADA	60	31 (51.7)	9 (15.0)		36 (60.0)	Total of concomitant therapy: 18 (30.0)				
Papamichael *et al*[20], 2017	UC	ADA	43	20 (46.5)	4/35 (11.4)		38 (88.4)	12 (27.9)	7 (16.3)			
Juncadella *et al*[16], 2018	IBD	ADA	98	60 (61.2)	34 (34.7)	28/72 (38.9)			26 (26.5)			
Feng *et al*[14], 2019	CD	IFX	141	51 (36.2)	5 (3.5)	22 (15.6)	8 (5.7)		20 (14.2)			
Papamichael *et al*[22], 2018	UC	IFX	56	24 (42.9)	11 (19.6)				18 (32.1)			
Papamichael *et al*[21], 2018	CD	IFX	110	51 (46.4)	19 (17.3)	20 (18.2)	8 (7.3)		28 (25.5)			
Strik *et al*[27], 2019	PfCD	IFX	47	29 (61.7)	7 (14.9)		15 (31.9)				13 (27.7)	
Plevris *et al*[24], 2020	PfCD	IFX	29	11 (37.9)	4 (13.8)		1 (3.4)		17 (58.6)			
Papamichael *et al*[23], 2016	UC	IFX	101	37 (36.6)	12 (12.0)		5 (4.9)	36 (35.6)	49 (48.5)			
Dreesen *et al*[31], 2020	CD	IFX	116	68 (58.6)								
Imaeda *et al*[15], 2014	CD	IFX	45	12 (26.7)				8 (17.8)	15 (33.3)	33 (73.3)		
EI-Matary *et al*[32], 2019	PCD	IFX	52	21 (40.4)				33 (63.5)	AZA 17 (32.7);MTX 30 (57.7)	7 (13.5)	15 (28.8)	10 (19.2)

Kang *et al*[17], 2019	PCD	IFX	105	31 (29.5)			6 (5.7)	6 (5.7)	95 (90.5)	
Dreesen *et al*[13], 2018	IBD	VDZ	179	106 (59.2)	32 (17.9)		153 (85.5)	73 (40.8)	20 (11.2)	
Yacoub *et al*[34], 2018	IBD	VDZ	82	44 (53.7)		18 (22.0)	67 (78.0)	6 (7.3)	13 (15.9)	14 (17.1)
Hanzel *et al*[33], 2019	IBD	VDZ	51	19 (37.3)	10 (19.6)		43 (84.3)	20 (39.2)	6 (11.8)	
Pouillon *et al*[25], 2019	UC	VDZ	31	13 (41.9)	7 (22.6)		28 (90.3)	12 (38.7)	7 (22.6)	14 (45.2)

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IFX: Infliximab; ADA: Adalimumab; VDZ: Vedolizumab; PFCD: Perianal fistula Crohn's disease; CS: Corticosteroid; IMM: Immunosuppressor; 5-ASA: 5-amino salicylic acid.

low specificity or sensitivity, respectively. IFX trough level surpassing 5 μg/mL had maximum possible opportunity to assist pediatric CD patients in attaining mucosal healing under maintenance therapy[17]. In CD patients under induction therapy, VDZ trough level of ≥ 18.0 μg/mL at week 6 predicted mucosal healing in the first year of treatment with an 80% sensitivity and 63% specificity[34]. VDZ trough level of ≥ 13.6 μg/mL at week 22 predicted mucosal healing with a 69% sensitivity and 71% specificity[13]. Although these two studies[13,34] did not adopt standard tools to evaluate mucosal healing, the former[34] utilized MRI to evaluate intestinal inflammation.

Study cohorts of perianal fistulating Crohn's disease: Yarur *et al*[36] have confirmed that the possibility of fistula healing would rise substantially among pfCD patients if the optimal trough level of IFX was increased to 10 μg/mL or more at week 4, while a minor change is needed in the optimal trough levels of IFX more than 20 μg/mL. Plevris *et al*[24] have demonstrated that pfCD patients under maintenance therapy attaining an IFX trough level > 7.1 μg/mL and ADA trough level > 9.8 μg/mL were highly probable to achieve fistula healing and an IFX trough level of over 7.1 μg/mL and an ADA trough level of over 6.8 μg/mL were the best serum levels in predicting the fistula closure. Strik *et al*[27] have suggested that IFX ≥ 5.0 μg/mL or ADA ≥ 5.9 μg/mL acts as a good predictor of fistula closure in pfCD patients. When compared to adults, pediatric pfCD patients should be cured with a higher IFX serum level for deeper remission. Moreover, the correlation between IFX trough level at week 24 and fistula healing in pediatric pfCD patients has been verified and the IFX trough level with an increment to more than 12.7 μg/mL increases the possibilities of fistula healing at week 24[32].

Table 3 Quality of articles

Ref.	Item 1	Item 2	Item 3	Item 4	Item 5	Item 6
Chaparro *et al*[12], 2018	★			★		
Ungar *et al*[28], 2016	★			★		
Yarur *et al*[29], 2016	★	★		★		
Roblin *et al*[26], 2014	★			★		
Morita *et al*[18], 2016	★	★		★		
Morita *et al*[19], 2016	★	★		★		
Strik *et al*[27], 2019	★	★		★		
Plevris *et al*[24], 2020	★	★		★		
Zittan *et al*[30], 2016	★	★		★		
Juncadella *et al*[16], 2018	★	★		★		
Papamichael *et al*[23], 2016	★	★	★	★		
Papamichael *et al*[20], 2017	★	★	★	★		
Papamichael *et al*[22], 2018	★		★	★		
Feng *et al*[14], 2019	★	★	★	★		
Papamichael *et al*[21], 2018	★	★		★		
Dreesen *et al*[31], 2020	★	★	★	★	★	★
Imaeda *et al*[15], 2014	★	★		★		
EI-Matary *et al*[32], 2019	★	★	★	★	★	★
Kang *et al*[17], 2019	★			★		
Hanzel *et al*[33], 2019	★	★	★	★	★	★
Dreesen *et al*[13], 2018	★	★		★		
Pouillon *et al*[25], 2019	★	★		★		
Yacoub *et al*[34], 2018	★	★	★	★	★	★

Item 1: The representativeness of the exposed cohorts; Item 2: Identification of exposure; Item 3: The verification of concerned results absent in the start of study; Item 4: The sufficient evaluation of study results; Item 5: Follow-up is long enough after study results take place; and Item 6: Follow-up of cohorts is long enough. ★: Contents of the literature in line with the item.

DISCUSSION

Pharmacological mechanism and clinical efficacy of biologics in IBD

Anti-TNF-α antagonists contain three anti-TNF-α biologics with integrated IgG1 antibody (IFX, ADA, and GOI), Certolizumab with fragment Fab modified by polyethylene glycol, and Etanercept with TNF-α extracellular domain including Receptor2/IgG1-Fc fusion protein[37-40]. As a matter of fact, the efficacy of inducing clinical or endoscopic remission in IBD is merely realized by the three anti-TNF-α biologics with integrated IgG1 antibody instead of Certolizumab or Etanercept. Anti-TNF-α biologics regulate immune response and maintain intestinal status by preventing TNF-α from inducing mucosal inflammation[41]. In IBD subtypes, IFX has better ability to induce mucosal healing of UC than ADA and is similar to that of ADA in inducing mucosal healing of CD[42].

VDZ is an integrin antagonist that combines with α4β7 integrin expressed on the surface of lymphocytes or monocytes. The crucial mechanism of intestinal lymphocyte migration to the intestinal mucosal layer attributes to the integration of α4β7 integrin and mucous membrane addressing cell adhesion molecule-1 expressed in the

Table 4 Biologic management in inflammatory bowel disease patients

Ref.	Biology	Diagnosis, *n*	Therapeutic stage	Therapeutic course	Injection dose	Injection frequency
Chaparro *et al*[12], 2018	ADA	IBD 94	Maintenance	At least 6 mo	/	/
Ungar *et al*[28], 2016	IFX	IBD 88	Maintenance	At least 6 mo	/	/
Yarur *et al*[29], 2016	ADA	IBD 67	Induction	Less than 1 mo	/	/
Roblin *et al*[26], 2014	IFX	IBD 78	Induction	Less than 2 mo	/	/
Chaparro *et al*[12], 2018	ADA	IBD 66	Maintenance	At least 12 wk	160 mg, 80 mg, 40 mg	Induction: 160 mg at week 0 and 80 mg at week 2; Maintenance: 2 weekly (47 patients), weekly (19 patients)
Ungar *et al*[28], 2016	ADA	IBD 40	Maintenance	/	/	2 weekly or weekly
Morita *et al*[18], 2016	ADA	UC 33	Induction and Maintenance	/	160 mg, 80 mg, 40 mg	160 mg at week 0, 80 mg at week 2, and 40 mg 2 weekly
	IFX	UC 31	Induction and maintenance	/	5 mg/kg	At weeks 0, 2, 6 and 8 weekly later
Morita *et al*[19], 2016	ADA	CD 42	Maintenance	At least 6 mo	40 mg	2 weekly
Strik *et al*[27], 2019	ADA	PfCD 19	Maintenance	/	40 mg	2 weekly (11 patients), Weekly (8 patients)
	IFX	PfCD 47	Maintenance	/	5 mg/kg, 10 mg/kg	5 mg/kg 8 weekly (27 patients), 5 mg/kg 6 weekly (10 patients)5 mg/kg 4 weekly (3 patients), 10 mg/kg 8 weekly (4 patients); 10 mg/kg 6 weekly (2 patients), 10 mg/kg 4 weekly (1 patients)
Plevris *et al*[24], 2020	ADA	PfCD 35	Maintenance	At least 24 wk	40 mg	Weekly (17 patients), Fortnightly (18 patients)
	IFX	PfCD 29	Maintenance	At least 24 wk	5 mg/kg, 10 mg/kg	5 mg/kg 8 weekly (16 patients), 5 mg/kg 6 weekly (7 patients); 10 mg/kg 8 weekly (3 patients), 10 mg/kg 6 weekly (3 patients)
Zittan *et al*[30], 2016	ADA	CD 60	Maintenance	/	40 mg or not	40 mg (53 patients), other than 40 mg (7 patients); 2 weekly (35 patients), other than 2 weekly (25 patients)
Juncadella *et al*[16], 2018	ADA	IBD 98	Maintenance	/	40 mg or not	40 mg 2 weekly (59 patients), other than 40 mg 2 weekly (36 patients)
Papamichael *et al*[20], 2017	ADA	UC 43	Induction	14 wk	160 mg, 80 mg, 40 mg	160 mg at week 0, 80 mg at week 2, 40 mg 2 weekly or weekly from week 4, or 80 mg 2 weekly or weekly from week 4
Feng *et al*[14], 2019	IFX	CD 141	Maintenance	At least 14 wk	5 mg/kg	5 mg/kg at weeks 0, 2, 6 and thereafter every 8 wk during the study period (week 30)
Papamichael *et al*[22], 2018	IFX	UC 56	Maintenance	/	/	/
Papamichael *et al*[21], 2018	IFX	CD 110	Maintenance	/	5 mg/kg or not	5 mg/kg 8 weekly (63 patients), other than 5 mg/kg 8 weekly (47 patients)
Papamichael *et al*[23], 2016	IFX	UC 101	Induction	14 wk	/	/
Dreesen *et al*[31], 2020	IFX	CD 116	Induction and maintenance	54 wk	5 mg/kg	Induction: 5 mg/kg at week 0, 2, 6; Maintenance: 5 mg/kg, 7.5 mg/kg, or 10 mg/kg at week 14 and later
Imaeda *et al*[15], 2014	IFX	CD 45	Maintenance	At least 6 mo	5 mg/kg, 10 mg/kg	5 mg/kg 8 weekly (37 patients), 10 mg/kg 8 weekly (8 patients)
El-Matary *et al*[32], 2019	IFX	PfCD 52	/	/	5 mg/kg or more	5 mg/kg/dose, often rounded up to the nearest 100 mg at weeks 0, 2, 6; Dose 4 was received at a median time interval following the third dose
Kang *et al*[17], 2019	IFX	PCD 105	Maintenance	/	/	/
Hanzel *et al*[33],	VDZ	IBD 51	Induction and	54 wk	300 mg	Induction: At weeks 0, 2, 6, 10; Maintenance: 4 weekly or 8

2019			maintenance			weekly from week 14 onwards
Dreesen *et al*[13], 2018	VDZ	UC 66; CD 113	Induction and maintenance	22 wk	300 mg	Induction: At weeks 0, 2, 6 (179 IBD patients), At week 10 (102 CD patients); Maintenance: 8 weekly (162 IBD patients), 4 weekly (4 UC and 13 CD patients)
Pouillon *et al*[25], 2019	VDZ	UC 31	Maintenance	/	300 mg	8 weekly (19 samples) or 4 weekly (16 samples)
Yacoub *et al*[34], 2018	VDZ	CD 39; UC43	Induction and maintenance	52 wk	300 mg	Induction: At weeks 0, 2, 6; Maintenance: 4 weekly or 8 weekly from week 14 onwards

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IFX: Infliximab; ADA: Adalimumab; VDZ: Vedolizumab; PFCD: Perianal fistula Crohn's disease.

gastrointestinal endotheliocytes. The mechanism of VDZ mainly involves inhibition of T lymphocytes from migrating and aggregation in the mucosal layer[43]. Recently, another viewpoint highlights the regulation of innate immunity and the interference of monocytes migrating and aggregating in the mucosal layer is considered the major mechanism of VDZ[44]. A meta-analysis including nine real-world studies verified the effectiveness of VDZ for IBD accompanied with adequate security[45]. Endoscopic healing, radiographic healing, and histological healing are achieved in IBD patients in long-time therapy of VDZ, whereas the risk of atypical hyperplasia also increases[46-48].

Effect of endpoint definition on blood concentration

MES is the most extensively used scoring system to evaluate disease severity of UC, and contains four grades, including no lesions (0), mild (1), moderate (2), and severe (3). Mucosal healing is normally defined as MES ≤ 1. However, it is a remarkable fact that UC patients with MES = 1 had extensive mucosal inflammation and higher probability of clinical relapse and colectomy in the future than UC patients with MES = 0[49,50]. What's more, even in UC patients with MES = 0, 30.4% had abnormal mucosal pattern and 73.9% had abnormal vascular pattern on high definition colonoscopy[51]. Similarly, 41.8% and 4.6% were classified as LCI-B (redness with visible vessels) and LCI-C (redness without visible vessels) based on color imaging[52]. Recently, UCEIS consisting of three major indicators (vascular pattern, bleeding, and erosions and ulcers) is divided into eight grades (0-8), and verifies the changes of symptoms and mucosal lesions more accurately than MES[53,54]. Considering that histological inflammation invisible under colonoscopy might persistently exist in UC patients with endoscopic mucosal healing, histopathological detection contributes to the direct reflection of mucosal microinflammation. Based on the histologic scoring system, histological healing is defined as Geboes score < 2 or Robarts Histological Index < 3 or Nancy Histological Index ≤ 1, but these histologic scoring systems should be simplified and verified again[50].

Different from UC, the definition of mucosal healing in CD is more complex due to extensive and deep lesions. CDEIS is the gold-standard for endoscopic mucosal healing in CD, and it consists of four major segments (deep ulcerations, superficial ulcerations, surface involved by ulcerations, and surface involved by disease). As SES-CD involves the same evaluative contents similar to that of CDEIS and is highly correlated with CDEIS, SES-CD has become more popular than CDEIS[55]. Nevertheless, both CDEIS and SES-CD focused only on colorectal lesion evaluation and ignored ileal lesions evaluation[56]. Hence, the modified Rutgeerts' Scoring System that paid much attention to mucosal lesions has been proposed in evaluating ileal lesions[57]. Capsule endoscopy and balloon-assisted endoscopy accompanied with the Lewis score system are utilized to evaluate small intestinal lesions while the Rutgeerts' scoring system is applied in CD patients with colectomy only. Therefore, definitions of primary endpoints involving only one endoscopic scoring system are unable to evaluate inflammation in CD completely. Additionally, different from mucosal lesions of UC, intestinal lesions of CD tend to invade the submucosa or muscular layer deeply or swollen lymph nodes so that deep healing has been proposed to be a part of expected outcome in CD.

In fact, by considering the segmental and transmural inflammation of the intestine in CD, diagnostic imaging tests have been put forwarded for detecting deep lesions whereas histological detection is not recommended due to tiny and shallow biopsies, especially in pfCD. Currently, gastroenterologists have opted deep remission as fistula healing or fistula closure under endoscopic examination or other radiological examinations to be the primary endpoint of pfCD[58]. T2-weighted MRI with fat-

Table 5 Correlation between endoscopic outcome and biologic blood concentration

Ref.	Patients	Definition of primary endpoint	Biology	Cut-off value	Clinical outcome (Yes/Not)	SE	SP	PPV	NPV	AUC
Chaparro *et al*[12], 2018	IBD	Mucosal healing: (1) CD: SES-CD < 3; (2) UC: MES ≤ 1; and (3) CD in Postoperative setting: Rutgeerts < 2	ADA	7.2 μg/mL (TL)	Mucosal healing (35/59)	0.65	0.56	0.46	0.72	0.60
			IFX	3.4 μg/mL (TL)	Mucosal healing (58/30)	0.60	0.60	0.73	0.42	0.63
Ungar *et al*[28], 2016	IBD	Mucosal healing: (1) CD: SES-CD < 3; (2) UC: MES ≤ 1	ADA	7.1 μg/mL (SL)	Mucosal healing	0.32	0.85	0.51	0.72	0.70
			IFX	6 μg/mL (SL)	Mucosal healing	0.39	0.85	0.70	0.62	0.75
Yarur *et al*[29], 2016	IBD	Mucosal healing: Lack of any inflammatory findings in the intestinal mucosa	ADA	7.8 μg/mL (SL)	Mucosal healing (19/47)	0.61	0.95			0.76
		Histological healing: Lack of histologic inflammation on biopsies obtained during colonoscopy	ADA	7.5 μg/mL (SL)	Histological healing (20/46)	0.62	0.83			0.73
Roblin *et al*[26], 2014	IBD	Mucosal healing: (1) CD: Disappearance of all ulcerations; (2) UC: MES < 2	ADA	4.9 μg/mL (TL)	Absence of mucosal healing (16/24)	0.66	0.85	0.88	0.51	0.77
Morita *et al*[18], 2016	UC	Mucosal healing: UCEIS: The bleeding descriptor and the erosions and ulcers descriptor were both 0, and the vascular pattern descriptor was 0 or 1	ADA	10.3 μg/mL (TL)	Mucosal healing (Absence)	0.82	0.80			0.87
			IFX	2.7 μg/mL (TL)	Mucosal healing (11/20)	0.83	0.89			0.93
Morita *et al*[19], 2016	CD	Mucosal healing: Endoscopic score based on the modified Rutgeerts' scoring system: 0 (No lesions or scar) or 1 (≤ 5 apthous lesions)	ADA	7.90 μg/mL (TL)	Mucosal healing (14/28)	0.69	0.86			0.79
Strik *et al*[27], 2019	PfCD	Fistula closure: Absence of active drainage at gentle finger compression and/or fistula healing on magnetic resonance imaging	ADA	5.9 μg/mL (SL)	Fistula closure (13/6)					0.89
			IFX	5.0 μg/mL (TL)	Fistula closure (32/15)					0.92
Plevris *et al*[24], 2020	PfCD	Perianal fistula healing: No spontaneous discharge or no discharge on palpation in the absence of seton drainage; Perianal fistula closure: Absence of an external skin opening	ADA	9.8 μg/mL (TL)	Fistula closure (15/20)	0.93	0.75			0.86
			ADA	6.8 μg/mL (TL)	Fistula healing (21/14)	1.00	0.79			0.90
			IFX	7.1 μg/mL (TL)	Fistula healing (18/11)	0.78	1.00			0.93
			IFX	7.1 μg/mL (TL)	Fistula closure (13/16)	0.64	1.00			0.97
Zittan *et al*[30], 2016	CD	Mucosal healing: Absence of any ulceration in all ileocolonic segments	ADA	8.14 μg/mL (TL)	Mucosal healing (35/25)	0.91	0.76	0.84	0.86	
Juncadella *et al*[16], 2018	CD	Endoscopic remission: Absence of a mucosal break for CD, a Rutgeerts score of ≤ 1 for CD with ileocolonic resection, a Mayo endoscopic score of ≤ 1 for UC. Histological healing: Absence of any sign of active inflammation including erosions, abscesses, or neutrophil infiltration	ADA	12 μg/mL (SL)	Endoscopic remission (20/25)	0.80	0.68			
			ADA	12.2 μg/mL (SL)	Histological healing (13/28)	0.57	0.85			
	UC		ADA	16.2 μg/mL	Endoscopic remission (7/20)	0.85	0.61			

				(SL)						
			ADA	16.2 μg/mL (SL)	Histological healing (3/23)	1.00	0.83			
Papamichael *et al*[23], 2016	UC	Short term mucosal healing: (1) MES ≤ 1 at weeks 10-14; (2) MES ≥ 2 at baseline	IFX	15 μg/mL week 6 (SL)	STMH at weeks 10-14 (54/47)	0.60	0.74	0.73	0.62	0.64
			IFX	2.1 μg/mL week 14 (SL)		0.84	0.62	0.78	0.71	0.64
Papamichael *et al*[20], 2017	UC	Short term mucosal healing: (1) MES ≤ 1 at weeks 8-14; (2) MES ≥ 2 at baseline	ADA	9.4 μg/mL week 4 (SL)	STMH at weeks 8-14 (12/31)	0.67	0.77	0.50	0.87	
			ADA	7.5 μg/mL week 4 (SL)	STMH at weeks 8-14	0.89	0.59	0.47	0.93	
Papamichael *et al*[22], 2018	UC	Endoscopic healing: a Mayo endoscopic subscore of ≤ 1. Histological healing: No or only focal mild active inflammation	IFX	7.5 μg/mL (TL)	Endoscopic healing (31/39)	0.77	0.62	0.62	0.77	
			IFX	10.5 μg/mL (TL)	Histological healing (28/41)	0.54	0.78	0.63	0.71	
Papamichael *et al*[21], 2018	CD	Endoscopic remission: Absence of any mucosal break, a Rutgeerts score of ≤ 1 for CD with ileocolonic resection. Histologic remission: Absence of active inflammation	IFX	9.7 μg/mL (SL)	Endoscopic remission (62/34)	0.57	0.73	0.80	0.48	
			IFX	9.8 μg/mL (SL)	Histological remission (43/44)	0.63	0.66	0.64	0.64	
Feng *et al*[14], 2019	CD	Mucosal healing: Complete absence of any sign of ulceration	IFX	4.85 μg/mL week 14 (SL)	Mucosal healing (82/59)	0.67	0.80			0.80
			IFX	2.85 μg/mL week 30 (SL)	Mucosal healing (59/50)	0.73	0.84			0.78
Dreesen *et al*[31], 2020	CD	Endoscopic remission: (1) CDEIS < 3 at weeks 12 and 54; (2) Absence of ulceration at weeks 12 and 54	IFX	23.1 mg/L week 2 (TL)	Endoscopic remission at week 12 (54/42)	0.56	0.80	0.72	0.65	0.67
			IFX	10.0 mg/L week 6 (TL)	Endoscopic Remission at week 12 (37/65)	0.37	0.89	0.76	0.59	0.64
			IFX	10.6 mg/L week 54 (TL)	Absence of ulceration at week 54 (59/24)	0.94	0.42	0.49	0.92	0.71
Imaeda *et al*[15], 2014	CD	Mucosal healing: Endoscopic score based on the modified Rutgeerts' scoring system: 0 (No lesions or scar) or 1 (≤ 5 apthous lesions)	IFX	4.0 μg/mL (TL)	Mucosal healing (20/58)	0.71	0.70			0.63
EI-Matary *et al*[32], 2019	PfCD	Healing perianal fistula: Decrease or cessation of fistula drainage, as reported by patients and confirmed by treating physicians; Healed fistula: Closure of a previously identified fistula opening, as reported by treating physicians	IFX	12.7 μg/mL (TL)	Fistula healing (14/13)	0.62	0.65			0.80
Kang *et al*[17], 2019	PCD	Mucosal healing: SES-CD 0	IFX	4.2 μg/mL (TL)	Mucosal healing	0.65	0.70	0.67	0.68	0.68
		Partial mucosal healing: SES-CD < 3	IFX	3.7 μg/mL (TL)	Partial mucosal healing	0.70	0.71	0.79	0.61	0.73

Hanzel *et al*[33], 2019	IBD	Endoscopic remission: (1) CD: SES-CD ≤ 4; (2) UC: MES ≤ 1; Clinical remission: (1) CD: mean daily stool frequency of ≤ 1.5, abdominal pain ≤ 1; (2) UC: a rectal bleeding score of 0, a stool frequency score of ≤ 1	VDZ	22.0 μg/mL week 6 (TL)	Combined Remission (Endoscopic Remission AND Clinical Remission) within the first year of treatment (16/35)	0.77	0.73	0.72	0.88	0.73
			VDZ	8.0 μg/mL week 22 (TL)		0.79	0.75	0.65	0.86	0.82
Dreesen *et al*[13], 2018	UC	Mucosal healing: MES ≤ 1	VDZ	28.9 μg/mL week 2 (TL)	Mucosal healing at week 14 (32/22)	0.73	0.62	0.59	0.75	0.70
			VDZ	13.9 μg/mL week 14 (TL)	Mucosal Healing at week 14 (32/22)	0.85	0.54	0.48	0.88	0.72
	CD	Mucosal healing: Complete absence of ulcerations	VDZ	13.6 μg/mL week 22 (TL)	Mucosal healing at week 22 (10/33)	0.69	0.71	0.83	0.52	0.70
Pouillon *et al*[25], 2019	UC	Histological healing: Nancy Histological Index ≤ 1	VDZ	25.0 μg/mL (TL)	Histological healing (18/17)	0.77	0.71	0.74	0.75	0.75
Yacoub *et al*[34], 2018	CD	Mucosal healing: (1) Absence of any ulcerations during endoscopy; (2) The absence of significant intestinal inflammation on MRI	VDZ	18.0 μg/mL week 6 (TL)	Mucosal healing within the first year of treatment (18/21)	0.80	0.63	0.73	0.71	0.70
	UC	Mucosal healing: (1) MES ≤ 1; (2) The absence of significant intestinal inflammation on MRI	VDZ	18.0 μg/mL week 6 (TL)	Mucosal healing within the first year of treatment (24/19)	1.00	0.75	0.88	1.00	0.75

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; PCD: pediatric Crohn's disease; IFX: Infliximab; ADA: Adalimumab; VDZ: Vedolizumab; SE: Sensitivity; SP: Specificity; PPV: Positive prospective value; NPV: Negative prospective value; AUC: Area under the curve; SL: Serum level; TL: Trough level.

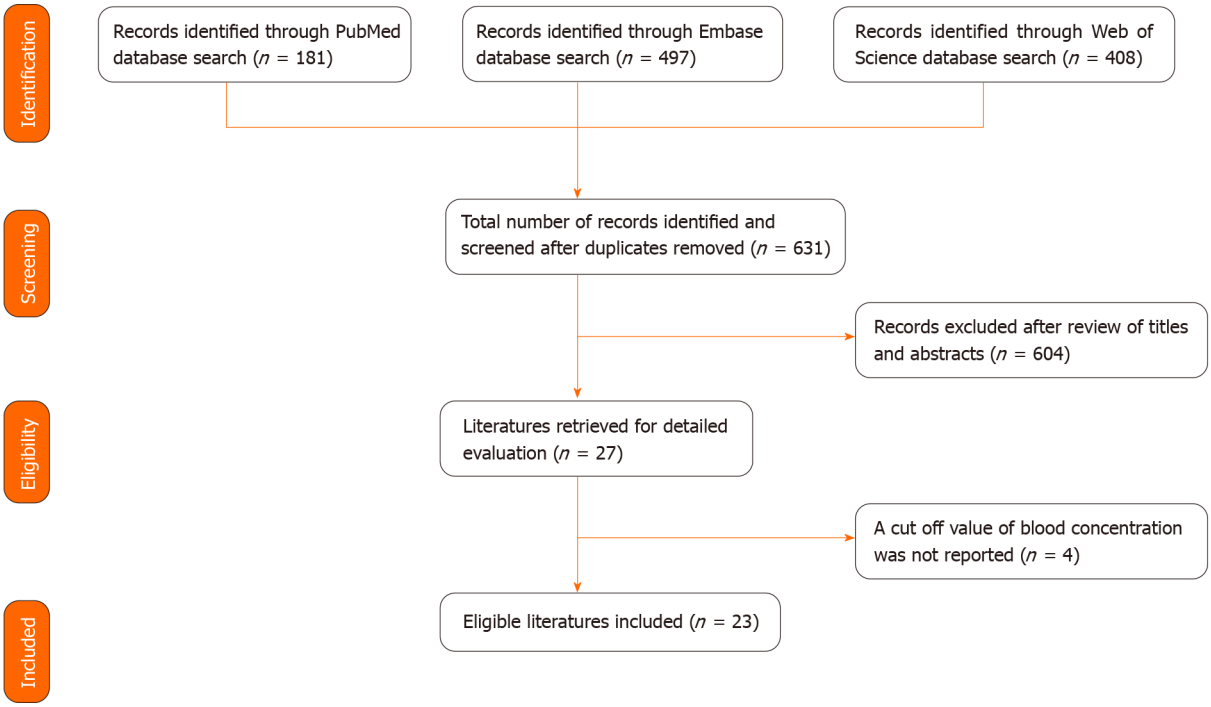


Figure 1 Literature selection.

suppression is considered the gold-standard for fistula imaging and an MRI-based score is currently available for defining disease activity[59]. Thomassin *et al*[60] have defined MRI healing as the disappearance of T2 hyperintensity and contrast enhancement after gadolinium injection. Nonetheless, the re-opening of "closed"

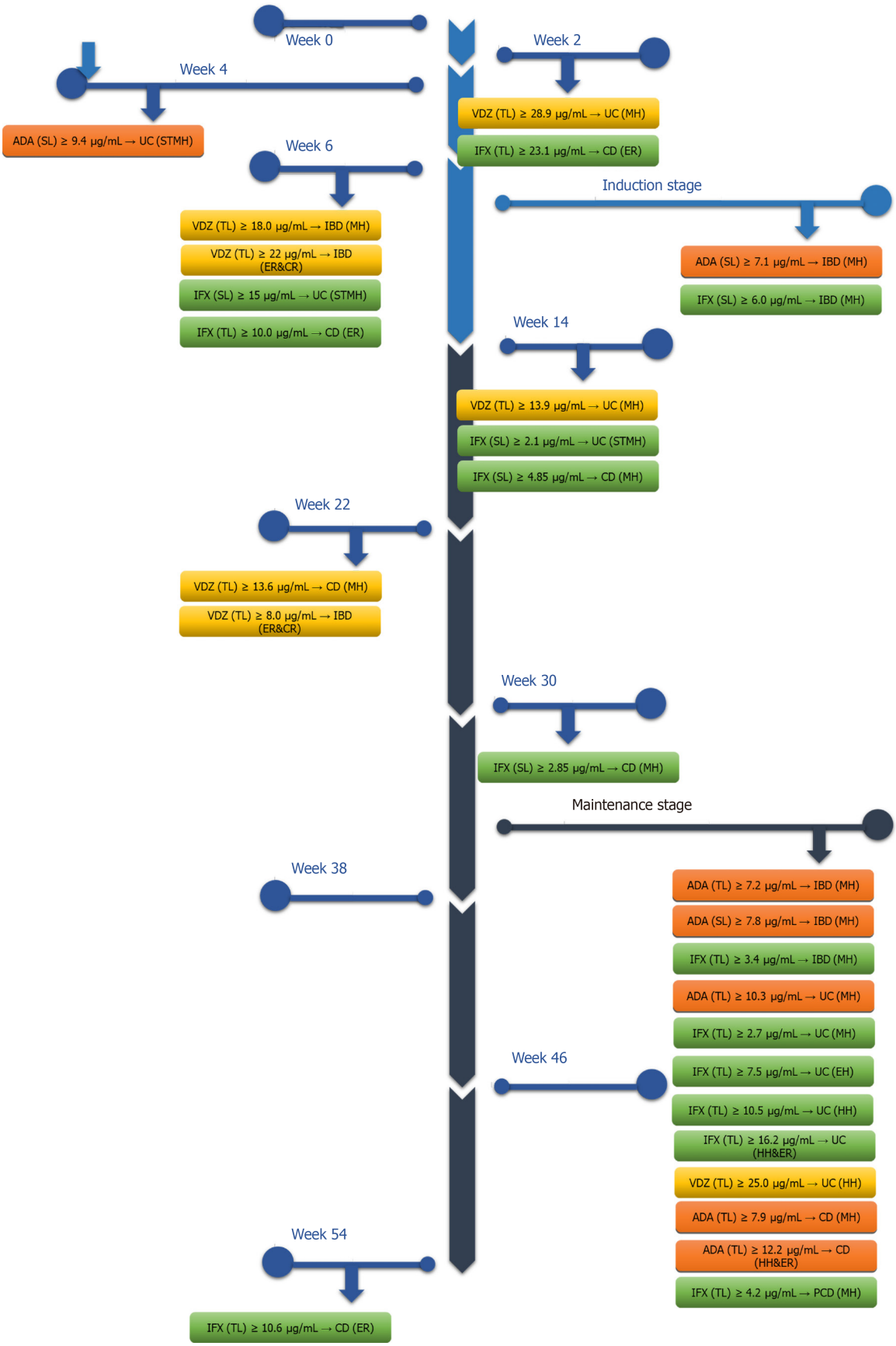


Figure 2 Target of blood concentration during different therapeutic stages of biologics. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; PCD: Pediatric Crohn's disease; IFX: Infliximab; ADA: Adalimumab; VDZ: Vedolizumab; MH: Mucosal healing; HH: Histological healing; EH: Endoscopic healing; ER: Endoscopic remission; CR: Clinical remission; STMH: Short term mucosal healing; SL: Serum level; TL: Trough level.

fistula tracts occurred more frequently in pfCD patients, which was diagnosed as fistula healing by MRI after the discontinuation of IFX maintenance therapy[61]. On one hand, radiologists without adequate experience in detecting perianal fistula and MRI itself were unable to discover activity around the anus, thus leading to inaccurate estimate of MRI. According to a recent review, examination under anaesthesia combined with MRI or endoanal ultrasound increased the accuracy of pfCD diagnosis to 100%[62]. On the other hand, half of pfCD patients relapse within 5 years after anti-TNF-α discontinuation.

Effect of biologic optimization on blood concentration

There is enormous discrepancy in the biological management of IBD patients due to complicated disease phenotypes and variable individual genes that contribute to the effect of blood concentration analysis. Above all, different timings for testing have been a major impact in analyzing optimal levels for biologic blood concentration that decreases with time and the incidence of biologic antibody that increases with time. Second, the primary or secondary onset of loss of response is universal in IBD patients under biological maintenance therapy, while dose optimization of biologics or shortening the interval time of injection contributes to regaining of response to biologics. Paul *et al*[63] have verified that optimization of therapeutic dose of IFX could enhance IFX serum level and ΔIFX serum level of > 0.5 μg/mL was confirmed as the only factor of IBD mucosal healing. Third, there is a growing tendency to combine biologics with other immune suppressants in medicine therapy for IBD with a high ratio of non-responders during biologic management, especially AZA or 6-mercaptopurine (6-MP). AZA is the precursor of 6-MP. 6-thioinosine 5′-monophosphate is a substance that is produced in the body as 6-MP is metabolized, ultimately producing 6-thioguanine nucleotides (6-TGN) and 6-methylthiopurine ribonucleotide through metabolism[64,65]. The clinical efficacy is dominated by adequate doses of 6-TGN, while myelosuppression is triggered by excessive doses of 6-TGN[66,67]. However, AZA or 6-MP in some CD patients produces 6-methylthiopurine ribonucleotide due to hepatotoxicity in preference to 6-TGN, resulting in the accumulation of hepatic toxin and suspension of this maintenance therapy finally. Yarur *et al*[68] have considered 6-TGN concentration of ≥ 125 pmol/8 × 10^8 red blood cells could assist IFX in facilitating mucosal healing of CD *via* increasing the blood concentration of IFX to more than 8.3 μg/mL. However, Yacoub *et al*[34] have demonstrated that the addition of immunosuppressants could neither enhance the blood concentration of VDZ nor improve the probability of deep remission in IBD patients by VDZ management because of low immunogenicity of VDZ[69]. Last but not the least, this review included study cohorts of VDZ, which mainly contained IBD patients previously exposed to anti-TNF-α agents or with inadequate response to anti-TNF-α agents that is considered one of the vital factors resulting in low blood concentration or failure of VDZ induction therapy[70,71].

Effect of patient demographics and characteristics on blood concentration

The demographics and characteristics of patients with IBD are another key point that impacts the biologic blood concentration. Above all, CD patients with perianal fistula require higher biologic blood concentrations than CD patients with luminal activity in order to achieve deep remission. Yarur *et al*[72] have discovered that the anti-TNF-to-TNF ratio in tissues remained higher in uninflammatory areas than in severely inflammatory areas as well as higher rate of serum to tissue drug level mismatch in patients with active disease than in those with remission. Hence, it can be postulated that obstruction of biologics to penetrate into the inflamed tissues surrounding the perianal fistula accounted for higher biological blood concentration as needed by pfCD patients[4,72]. Regrettably, the evidence of biological blood concentration on penetration, stenosis, or perianal disease besides fistula still requires in-depth research. Second, the morbidity associated with pediatric IBD has significantly increased in recent years and the efficacy of biologics on deep remission has also been verified. Nonetheless, Kelsen *et al*[73] showed a downward trend in the maintenance efficacy of IFX in children less than 5 years old. Third, smoking or duration of disease increases the probability of biological treatment failure or disease relapse after suspension of biologics, but according to Bond *et al*[74], neither smoking nor duration of disease showed an association with categorical trough levels of IFX or ADA, whereas the body mass index tended to decrease the trough level of ADA[74-76]. Last but not the least, females might be an adverse factor of IBD disease progression as estrogen signaling might play a role in local immune response and maintenance of epithelial homeostasis in a gender- and age-dependent manner[77]. Moreover, it has been confirmed that sex discrepancy influences the therapeutic target of pfCD when considering higher

incidence of perianal fistula and less therapeutic effect in females than in males[36,78,79].

CONCLUSION

In conclusion, several aspects of optimal serum levels demanded by IBD under biologic agents require deep investigation in the future. First, whether the discrepancy of optimal serum level in complicated phenotype or simple phenotype exists or not should be investigated. Second, how much maintenance time and serum levels of biologic agents are still needed to prevent IBD from disease flare after identification of deep remission. Furthermore, the achievement of deep remission in the prognosis of IBD patients should be evaluated in the future during the induction phase by combining with the serum level of biologics and patient characteristics. Finally, non-responders of IBD patients in the initial phase of biological treatment are considered appropriate to optimize the serum levels tentatively by increasing the injection dose, shortening the interval time of injection, or converting the type of biologics. Also the variation in serum levels during optimization period of biological therapy should be emphasized on therapeutic drug monitoring.

ARTICLE HIGHLIGHTS

Research background

Blood concentration has been proved to be an important predictor of outcomes in inflammatory bowel disease (IBD) patients during biological therapy. It has also been acknowledged that disparate therapeutic targets correspond to specific blood concentrations. The greater the therapeutic expectation required by IBD patients, the higher the value of blood concentration suggested by IBD specialists.

Research motivation

Given the invasive, painful, and expensive examinations, such as endoscopy, for disease evaluation in IBD patients, identification of biologic blood concentration for predicting endoscopic inactivity in IBD patients may contribute to better, less painful, less risky, less expensive treatments.

Research objectives

To identify the predictive value of biologic blood concentration on endoscopic inactivity in IBD patients and explore factors relevant to predictive value.

Research methods

A comprehensive search target was utilized to search PubMed/MEDLINE, Embase, and Web of Science systematically. Two authors screened and extracted the literature according to the inclusion and exclusion criteria. The quality of the included literature was assessed using the Newcastle Ottawa Scale. Authors assisted by a biostatistician extracted, synthesized, and reviewed the data in accordance with the research topic.

Research results

A total of 23 articles with 30 clinical studies and 1939 IBD patients were included. All studies verified the correlation between biologic blood concentration and endoscopic inactivity in IBD patients. Thirteen studies focused on infliximab and demonstrated that blood concentration reaching 4.0-10.6 μg/mL could predict the mucosal healing in Crohn's disease (CD) patients while ulcerative colitis (UC) patients with a blood concentration higher than 2.7-10.5 μg/mL were more likely to achieve mucosal healing under maintenance therapy. Whereas infliximab blood concentration of perianal fistulizing Crohn's disease (pfCD) patients reaching 5.0-12.7 μg/mL or more increased the probability of mucosal healing. Eleven studies focused on adalimumab and indicated that blood concentration reaching 7.2-16.2 μg/mL or more could predict mucosal healing in IBD patients while patients with a blood concentration lower than 4.9 μg/mL showed no mucosal healing under maintenance therapy. What's more, the predictive cut off value of adalimumab blood concentration on fistula healing/closed should be 5.9-9.8 μg/mL in pfCD. Four studies focused on vedolizumab and verified that blood concentration surpassing 25.0 μg/mL indicated mucosal healing in UC patients under maintenance therapy and the predictive cut off value of blood

concentration on mucosal healing or endoscopic remission under induction therapy in IBD could be 8.0-28.9 μg/mL. However, different studies had several discrepancies in the disease phenotype and demographics of study cohorts as well as the therapeutic stage, therapeutic course, injection dose, and injection frequency of biologic management. In addition, the definition of primary endpoints was not consistent in all studies. Fifteen studies considered mucosal healing as the main endpoint, three studies including pfCD patients adopted fistula healing/closure, and four adopted endoscopic remission alone or in combination with clinical remission as the main endpoint. Additionally, five studies identified the desirable endpoint as histological healing or histological remission.

Research conclusions

Considering the discrepancies in study design, study cohort, and biological management among different clinical studies, the best predictive cut-offs of biologic blood concentration on endoscopic inactivity published in 23 studies varied and the biological blood concentration might not be an appropriate predictor of endoscopic inactivity in IBD patients currently.

Research perspectives

In view of the fact that conduction of intensive monitoring for biological management plays a vital role in precise treatment of IBD patients, much larger and more stringent prospective studies are warranted to provide the best predictive cut-offs for biologic blood concentration as acknowledged globally in allusion to different types of IBD patients for distinguishing endoscopic inactivity from endoscopic activity.

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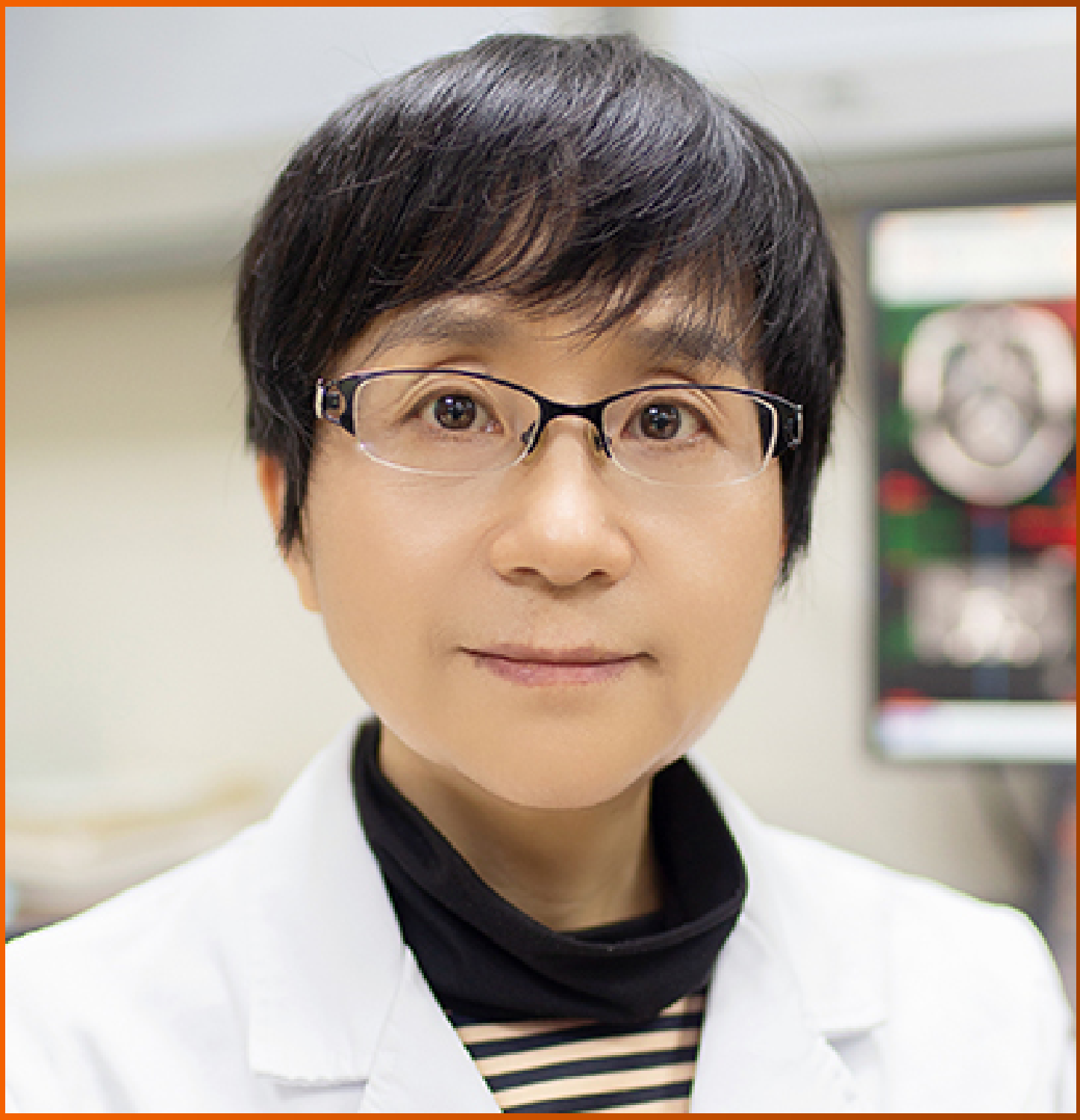
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OPINION REVIEW

Use of granulocyte/monocytapheresis in ulcerative colitis: A practical review from a European perspective

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Abstract

Half of the patients with ulcerative colitis require at least one course of systemic corticosteroids in their lifetime. Approximately 75% of these patients will also require immunosuppressive drugs (*i.e.*, thiopurines or biological agents) in the mid-term to avoid colectomy. Immunosuppressive drugs raise some concerns due to an increased risk of serious and opportunistic infections and cancer, particularly in elderly and co-morbid patients, underlining the unmet need for safer alternative therapies. Granulocyte/monocytapheresis (GMA), a CE-marked, non-pharmacological procedure for the treatment of ulcerative colitis (among other immune-mediated diseases), remains the only therapy targeting neutrophils, the hallmark of pathology in ulcerative colitis. GMA has proven its efficacy in different clinical scenarios and shows an excellent and unique safety profile. In spite of being a first line therapy in Japan, GMA use is still limited to a small number of centres and countries in Europe. In this article, we aim to give an overview from a European perspective of the mechanism of action, recent clinical data on efficacy and practical aspects for the use of GMA in ulcerative colitis.

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Core Tip: Approximately 30%-40% of patients with ulcerative colitis will require immunosuppressive therapies, including immunomodulators and biological agents. Unfortunately, none of these therapies achieve more than 40% of steroid-free clinical remission in the middle term; moreover, most immunosuppressive therapies increase the risk of infections and some malignancies, raising the unmet need for therapeutic alternatives in ulcerative colitis. Granulocyte/monocytapheresis (GMA) remains the only therapy targeting neutrophils, the hallmark of pathology in ulcerative colitis. GMA has proven its efficacy in different clinical scenarios and shows an excellent and unique safety profile.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory condition of unknown origin and has a relapsing-remitting clinical course. Approximately half of the patients present a mild course of the disease and are easily managed with aminosalicylates, while the other half require at least one course of oral or intravenous corticosteroids[1,2]. Among the latter, immunosuppressive drugs (including thiopurines and/or biological agents) are required in up to 75% of cases for steroid-refractoriness, steroid-dependency or chronic disease activity. Even with the more potent available drugs, only 30% to 40% of those patients achieve an appropriate disease control (*i.e.*, clinical remission) in the medium-term as assessed in randomized controlled trials (RCT)[3-5], and a large proportion of them lose the initial therapeutic benefit in the long-term[6]. In addition, immunosuppressive drugs increase the risk of serious and opportunistic infections and neoplasia, as well as other adverse outcomes, *e.g.*, worsening of chronic heart failure and thromboembolic complications[7,8], particularly in elderly patients with elderly-onset or long-lasting UC and those with other co-morbidities. For all these reasons, there is an unmet need for safe therapeutic alternatives in UC.

From a pathogenic point of view, neutrophils seem to play a key role in tissue damage. As in other chronic inflammatory conditions, dysregulation of neutrophil apoptosis has been observed in UC[9]. Neutrophilic infiltration is a hallmark of UC as emphasized by the fact that it is a crucial component of UC severity grading in several histological scores[10]. Moreover, the presence of neutrophils in mucosal colonic biopsies has been associated with a risk of clinical relapse and even dysplasia, leading some authors to pose histologic remission as the ultimate therapeutic goal in UC[11]. Accordingly, modulating the activity and numbers of mucosal neutrophils may be a viable therapeutic approach in UC[12], although no drug targeting these cells is available as yet.

The therapeutic effect of granulocyte/monocytapheresis (GMA) is based on the removal of the activated neutrophils from the bloodstream without increasing the risk of infections, given that immature cells are moved from the hematopoietic pools. In Japan, GMA has been covered by National Health Insurance for the treatment of active UC since April 2000 and it is widely used in clinical practice, even as a first line therapy for moderate-to-severe active UC in monotherapy; however, although GMA is CE-marked for the treatment of UC, Crohn's disease, pustular psoriasis and Behçet's disease in the European Union, it has hardly been used in Europe. Although other apheresis techniques focusing on lymphocyte removal have been studied in the past, they are currently not commercially available; thus, this review will focus on GMA. Given that a number of differences in genetic, epidemiologic and clinical features

between Western and Asian UC have been reported[13-16] and that these could lead to differences in response to therapies, we aim to give an overview of the mechanism of action, recent clinical data and practical aspects of GMA use in UC from a European perspective.

THE SELECTIVE MECHANISM OF ACTION OF GMA

GMA is an extracorporeal vein-to-vein apheresis technique. The available GMA device (Adacolumn™, JIMRO, Takasaki, Japan) is a column that contains 35000 beads of cellulose diacetate (2 mm diameter) as GMA carriers soaked in isotonic saline within a 335 mL polycarbonate housing. The blood flows into the column and returns to the patient from the column outflow, usually through two peripheral venous catheters. Many clinical, *in vitro* and *ex vivo* studies have been performed to ascertain the mechanisms of action of GMA (Table 1). The main effect is the selective removal of granulocytes, monocytes, along with a smaller number of platelets from the bloodstream, which can be seen by comparing their number in the inflow and outflow column lines. The cellulose beads adsorb circulating immunoglobulin (Ig) G and immunocomplexes and trigger the activation of complement fragments C3a and C5a, allowing for the removal of granulocytes and monocytes through the interaction of IgG and immunocomplexes with the Fc gamma receptor and the binding sites of leukocyte complement receptors (not present in lymphocytes). Hence, the GMA carriers selectively adsorb the leukocytes from peripheral blood, with the granulocytes exhibiting the highest affinity towards the carrier beads[17]. Despite granulocyte removal, the total number of circulating granulocytes after GMA remains stable. In fact, flow cytometry studies in the inflow and outflow column lines observed a decrease in CD10(+) (mature and activated) and an increase in CD10(-) (immature, naïve) granulocytes, indicating an increased turnover of these cells in the circulation[18]. Similarly, a significant reduction in peripheral CD14(+) CD16(+) monocytes (pro-inflammatory phenotype) has been observed in patients with inflammatory bowel disease treated with GMA[19]. Finally, a significant increase in circulating levels of the $CD^{4+}CD^{25high+/FoxP3}$ phenotype (functional regulatory T cells) following a course of GMA has also been reported in UC patients[20,21].

In addition to these changes in the phenotypical pattern of peripheral leukocytes, *in vitro* studies using human whole blood incubated with the GMA carriers measured significant amounts of interleukin (IL)-1 receptor antagonist, hepatocyte growth factor, and soluble tumor necrosis factor (TNF) receptors I and II released from granulocytes and monocytes that adhered to the carriers. Interestingly, the amounts of these anti-inflammatory cytokines were directly proportional to the number of cells that adhered to the carriers[17]. Some of these cytokines may reach the patient's circulation, exerting beneficial effects on the inflammatory process[22]. Finally, the exposure of neutrophils to cellulose beads results in the generation of apoptotic cells and over 40% of these apoptotic cells re-enter the patient's bloodstream[22] and can interact with B-cells, inducing IL-10 producing regulatory B-cells[23].

ROLE OF GMA IN THE MANAGEMENT OF UC IN EUROPE

The initial controlled European trials of GMA took place in the early 2000s (in the pre-biologic era of UC), yielding promising results, although most trials were too small to allow robust conclusions to be drawn. However, the negative results of an American double-blind, sham-controlled RCT for moderate-to-severe active UC[24] and the licensing of anti-TNF agent use for UC, led to a consequent loss of interest in GMA in Europe. However, safety concerns regarding anti-TNF agents and their lower efficiency in the medium-term (as compared to Crohn's disease), as well as an increasing concern for finding therapeutic alternatives for frail patients (elderly, comorbidities), reawakened interest in GMA[25].

The first systematic review on the efficacy of GMA in UC performed in 2010 concluded that, though it appeared to be of some benefit, high-quality RCTs comparing GMA with conventional medical therapy and sham procedures in Western populations with endoscopically confirmed disease activity were required[26]. Two European prospective controlled trials addressing the role of GMA in other clinical scenarios have recently been published. The ATTICA study, a RCT promoted by the Spanish Working Group in Inflammatory Bowel Disease (GETECCU) involving five European countries (Spain, Portugal, Italy, Germany and Austria), aimed to evaluate

Table 1 Main drivers of the mechanism of action of granulocyte/monocytapheresis

Within the apheresis filter	In the patient's bloodstream
(1) Absorption of circulating IgG and immunocomplexes by cellulose beads; (2) Activation of complement fragments (C3a, C5a); (3) Granulocyte and monocyte absorption *via* Fcγ receptors (IgG and immunocomplexes), and binding sites of leukocyte complement receptors (not in lymphocytes); and (4) Generation of apoptotic cells	(1) Reduction of activated neutrophils and pro-inflammatory monocytes (CD14+CD16+); (2) Apoptotic cells interact with regulatory B-cells, producing IL-10 and mature regulatory B-cells; and (3) Return of substances released by adsorbed cells: IL-1ra and HGF

IgG: Immunoglobulin G; IL: Interleukin; HGF: Hepatocyte growth factor.

the efficacy of GMA in steroid-dependent active UC (as defined by the inability to withdraw corticosteroids within three months after starting treatment or clinical relapse within three months after withdrawal)[27]. This study included 123 patients who were randomized to a 9-wk tapering schedule of oral prednisone alone or in combination with 7 GMA sessions. The main endpoint was steroid-free clinical and endoscopic remission at wk 24, which was achieved in 13% of the subjects (95% confidence interval: 6-24) in the GMA group and 7% [95% confidence interval: 2-16] in the control group (P = 0.11). The results were hampered by the lack of a final endoscopic assessment in two patients in the GMA group who were in steroid-free clinical remission, leading to a non-significant statistical difference due to the non-response imputation for missing values. However, time to relapse was significantly longer and steroid-related adverse events were significantly lower in the GMA group. Moreover, in patients naïve to thiopurines, GMA resulted in a significantly higher rate of steroid-free clinical and endoscopic remission as compared to the patients in the control group. The second study is the ART trial, a multicentre controlled trial for ultra-refractory UC conducted in three European countries (Germany, United Kingdom and France) that included patients with steroid-dependency or refractory moderate-to-severe active UC with insufficient response or intolerance to thiopurines and/or anti-TNF agents[28]. Of note, thiopurines had previously failed in 90% of the patients, anti-TNF agents as monotherapy in 40%, and combination therapy in 32%. Patients were treated with 5 to 10 GMA weekly sessions (at the discretion of the treating physician) and efficacy was assessed at 12, 24 and 48 wk. At wk 12, 47.8% of the patients had a steroid-free clinical response, of whom 28.7% were in steroid-free clinical remission; it is noteworthy that these figures remained almost identical at wk 24 and 48. Finally, the cumulative colectomy rate at wk 96 was 23.4%.

Given the excellent safety profile of GMA, its use in combination with biological agents in order to increase efficacy without increasing the risk of adverse effects seems appealing. To date, a number of small case-series reporting combination therapy with GMA have been published for patients showing primary non-response or secondary loss of response to biologicals[29,30]. Up to one third of those patients regained response and avoided dose-escalation, switching therapy and colectomy in the medium-term. Moreover, in a small study with patients treated for secondary loss of response, a decrease in anti-drug antibodies was observed in responders following GMA therapy[31].

The economic burden of GMA may also be taken into account in decision-making. In the early times of anti-TNF agents, a cost-effectiveness analysis using a decision tree model for patients with moderate-to-severe UC showed that incorporating GMA in the therapeutic management of UC was cost-effective and implied savings related to the reduction of adverse effects derived from corticosteroid use and to the decreased number of surgical interventions[32]. Of course, the availability of biosimilars has reduced the costs of anti-TNF agents, but GMA still has a cost slightly below the new biologicals (*i.e.*, ustekinumab and vedolizumab) with an even better safety profile.

In summary, recent controlled studies suggest a therapeutic effect of GMA on steroid-dependent UC and on patients with a previous failure of thiopurines or anti-TNF agents. Moreover, there are promising preliminary data using an add-on strategy with GMA in patients not responding or losing response mainly to anti-TNF agents. These data support considering the use of GMA, particularly in frail patients for whom safety has become a major issue or in those for whom colectomy seems to be the only available alternative.

SAFETY PROFILE OF GMA

One of the strengths of GMA is its safety profile. Beyond controlled trials, data from clinical practice have confirmed that no serious adverse events are observed in patients treated with GMA. In a post-marketing surveillance undertaken in Japan on 697 patients (for a total of 5287 GMA sessions) in 53 medical institutions over seven years (from 1999 to 2006), all reported events were of mild to moderate severity[33]. More than half of the reported events were related to the difficulty in performing blood access and adequate flow rate, elevation of venous pressure, coagulation and blood return problems. Among clinically adverse events, headache, fever and chills were the most common, occurring in less than 2% in each case. More recently, another Japanese study including a total of 894 GMA treatment courses in 593 patients, observed similar findings, with headache occurring in 13%, fever in 8%, and fatigue in 4%[34]. Importantly, in spite of being a therapy targeting neutrophils, concerns about an increased risk of infections have never been raised, strengthening the suitability of GMA for combination strategies. This excellent safety profile seemed to remain the same when it was assessed in a recent retrospective series including a subgroup of elderly patients, despite a higher baseline rate of co-morbidities[35].

Finally, patients' perceptions are also positive and agree with the convenience of the procedure. In a survey of patients treated with GMA in Spain, 82% of participants said they would agree to be treated with this technique again in the future, regardless of their response to the treatment[36].

PRACTICAL ASPECTS REGARDING THE USE OF GMA IN DAILY CLINICAL PRACTICE

Technical issues of GMA

GMA is usually performed in Blood Banks or haemodialysis units, but as the procedure is simple it can even be performed in dedicated GI units. The procedure is contraindicated when there is a suspicion of general infection, hypersensitivity to heparin, anaemia, hypercoagulability, the use of angiotensin-converting enzyme inhibitors (in this case, cancel the treatment 24 h before the process), pregnancy or lactation, paediatric patients of less than 30 kg body weight, and treatment with oral anticoagulants (unless switched to heparin for the duration of the apheresis procedures).

The procedure involves processing 1800 mL of blood that circulate through sterile non-reusable equipment (Adacircuit™), placed in a machine (Adamonitor™), to achieve an established flow rate of 30 mL/min with an overall process duration of 60 min, according to the specifications provided by the supplier (Figure 1). Higher flows might provoke a decrease in the column's adsorption capacity. In order to minimize the risk of severe hypovolaemia or anaemia in the patients undergoing the adsorption processes with Adacolumn®, it is recommended that the extracorporeal volume during any apheresis process should not surpass 13% of the volaemia; with Adacolumn® and Adacircuit®, the residual volume is 335 mL.

The guidelines for a correct apheresis procedure are based on the vein access through which the procedure is carried out, an appropriate previous count of the component to be adsorbed and good anticoagulation of the system. Antecubital vein access is recommended, as are 18-16G locking-handle needles with a side window or Abbocaths® of the same calibre. There is the option of inserting a tunnelled double-light central vein catheter, with lumens not inferior to 11F and Hickman®-type thick walls[37,38] *via* the subclavian for those patients with insufficient vein access, but a proper risk-benefit seems to be appropriate in this selected patient population. The manipulation and care of the central vein access must follow the established protocols in place at each centre. Currently, there is the also the possibility of carrying out the procedures through unipuncture[39,40].

The appropriate anticoagulation of Adacircuit® and Adacolumn® is obtained by an initial priming of both with 1 L of saline solution at a flow rate of 100 mL/min, and repeating the procedure with another saline solution of 1 L heparinized with 0.8 mL of 5% sodium heparin. This also allows for the removal of air from the column and promotes an increase in the surface area of the blood exposed to the Adacolumn® beads, favouring greater efficiency of the procedure. Additionally, intravenous low molecular weight heparin (at a dose of 0.8-1 mg/kg) must be administered between 10 to 30 min before the process or, alternatively, a continuous perfusion of 5000 IU of

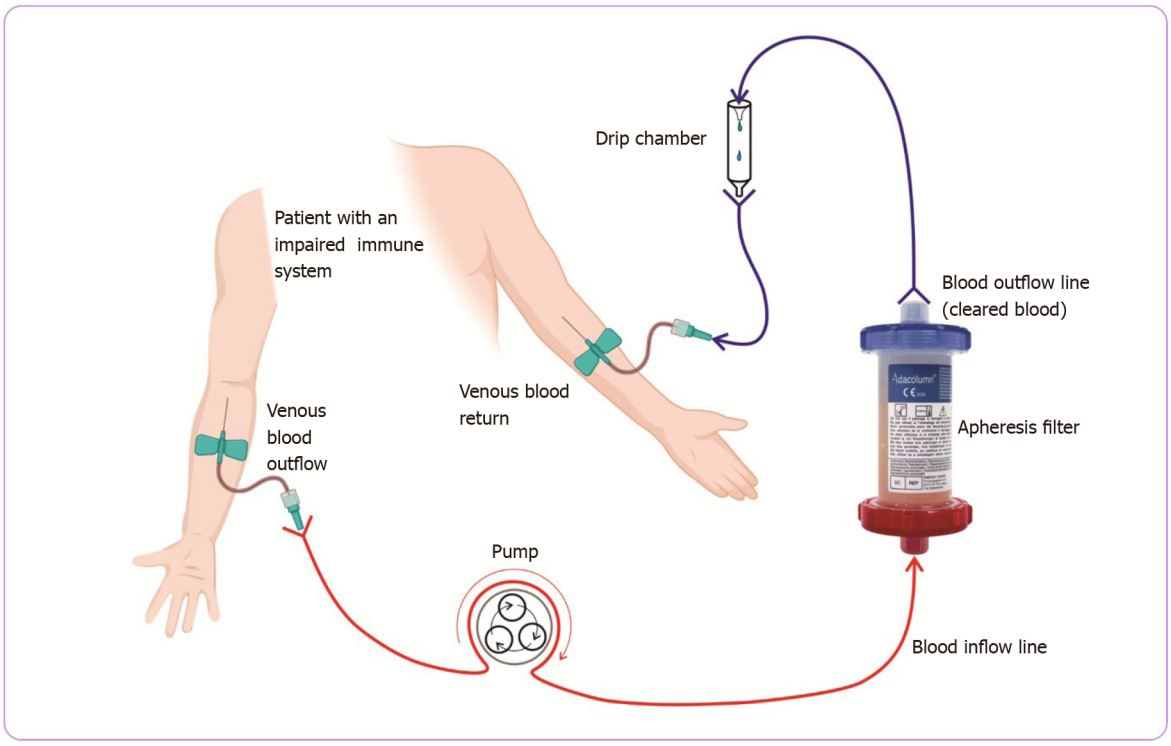


Figure 1 Basic scheme of the granulocyte/monocytapheresis procedure.

sodium heparin in 50 mL of physiological saline serum during the apheresis procedure. The anticoagulant effect of heparin disappears a few hours thereafter, its average life being one and a half hours, approximately. Using citrate as an anticoagulant solution is not advised, as it may interfere with the mechanism of action of GMA by acting as an ionic calcium chelator. Using nafamostat mesylate may be an alternative to heparin, although it is not available in some countries.

One of the most common procedural problems is the pressure problem in the access flow. The exposure of blood to the Adacircuit™ activates the coagulation cascade (a process that can be slowed by prior anticoagulation of the patient); for this reason, it is recommended to set in motion the blood retained in the Adacircuit™ by establishing a closed circuit.

At the end of the procedure, it is necessary to return the maximum amount of red blood cells retained in the Adacolumn® to ensure a minimum loss of blood. This is achieved by administering 300-500 mL of physiological saline to cause entrainment of the blood retained in the cartridge.

The monitoring of vital signs, blood pressure, pulse, breathing rate and temperature should be carried out at the beginning, midway through and at the end of the procedure. Before starting a round of treatment, it is necessary to analyze the clotting process and red blood cell and leukocyte counts. Although there are preliminary studies which indicate that better clinical results would be obtained if the procedure was carried out over 90 min, this has not been entirely proven. Therefore, the recommended flow rate is 30 mL/min. It must be noted that the complete recovery of the previous values of the different haematological cell lines takes place a few hours before the end of the procedure.

How many sessions should be performed and how often?

Most clinical trials involving GMA used 5 to 10 sessions, and this is the usual clinical practice. The choice to perform more than five sessions remains at the discretion of the treating physician; additional sessions are usually performed if the patient has not achieved clinical remission after the first five sessions. In the ATTICA study[27], patients in the GMA arm received a total of seven sessions. Interestingly, unlike what happened in the control group, in which a steady rate of relapse was observed, clinical relapses in the GMA group took place mainly once the scheduled regimen of GMA had finished, suggesting a therapeutic benefit of GMA and raising the question of whether a higher number of sessions would have resulted in greater efficacy.

Another controversial aspect regarding the GMA schedule is the number of sessions

per week. In 2009, Sakuraba *et al*[41] reported the results of a randomized, open study comparing the conventional (once per week) *vs* an intensive (twice per week) GMA regimen in 163 patients with mild-to-moderate active UC. Patients received at least five sessions with a maximum of 10 (once again, at the discretion of the treating physician). The intensive regimen resulted in a strikingly higher rate of clinical remission (71.2% *vs* 54%; $P = 0.029$) and a significantly shorter time to remission (14.9 ± 9.5 *vs* 28.1 ± 16.9 days; $P < 0.0001$). Although a similar trial in Crohn's disease found a significantly shorter time to clinical remission but a similar remission rate[42], no other controlled trial assessing the efficacy of intensive regimens has been performed for UC. Moreover, it remains to be assessed whether these differences would be the same in patients with a more severely active disease.

In summary, most centres use the schedule of five initial sessions. The decision to perform up to five additional sessions often relies on the clinical activity at the end of this schedule; additional sessions are usually added in cases of clinical response without remission. Similarly, the initial regimen is often intensive (two sessions per week) until clinical response or a decrease in inflammatory markers (C-reactive protein, faecal calprotectin) is achieved, moving thereafter to a conventional weekly schedule.

GMA also for maintenance therapy?

Whether or not GMA should be maintained once a patient has achieved clinical remission is still under debate. An initial, small-sized, randomized, sham-controlled trial reported promising, although confusing results on the usefulness of monthly GMA sessions for one year after achieving clinical remission with the same procedure[43]. Another pilot open-label, randomized study compared the efficacy of GMA every two weeks with mercaptopurine for two years to maintain remission of UC that was induced by GMA, corticosteroids or cyclosporine, and observed similar efficacy[44]. The results of the largest study assessing the efficacy of maintenance therapy with leukocytapheresis in UC have been published recently[45]. In this open, randomized trial, 163 UC patients in clinical remission induced by apheresis were randomized (stratified by the concomitant use of thiopurines) to receive two monthly sessions of leukocytapheresis or no additional treatment for one year. At the end of the study, the apheresis group showed a higher rate of endoscopic remission (42.5% *vs* 25.9%; $P = 0.048$) and a clear but non-significant trend towards a beneficial effect of apheresis was observed for clinical remission (47.5% *vs* 32.1%; $P = 0.054$) and complete endoscopic remission (33.8% *vs* 19.8%; $P = 0.051$). Unfortunately, in this trial two different leukocytapheresis devices (with supposed different mechanisms of action) were used with respect to the one that was used for inducing clinical remission.

Furthermore, the clinical outcomes of UC patients who respond to a first course of five to 10 GMA sessions suggest that a long-term therapeutic effect holds for most of them, as observed in the ART trial in which the rate of steroid-free clinical remission at 12 wk remained almost the same at 24 and 48 wk in spite of a lack of additional treatment[29]. Additionally, patients who responded once to GMA, seemed to regain response with GMA in the case of relapse[46,47]. Therefore, once remission has been achieved with GMA, patients are usually monitored and if relapse occurs, a new course of GMA is often successful. Given the mechanism of action of GMA and despite the lack of data on this, it makes sense to monitor patients by means of periodical faecal calprotectin. However, in patients at a high risk of relapse (*i.e.*, in clinical remission but increased faecal calprotectin levels or in patients intolerant or for whom no maintenance drug therapies are possible), monthly GMA sessions might be an option.

Prediction of response to GMA

The possibility of predicting the response to GMA could help the decision-making on when and how to treat UC patients with GMA and the positioning of this non-pharmacological therapy in UC. A retrospective study including 894 treatment courses in 593 patients treated with GMA for moderate-to-severe active UC from 2008 to 2017 at three Japanese referral centres aimed to identify the baseline factors that determined the efficacy of GMA (clinical remission)[33]. In the multivariate analysis, age below 60 years, and duration of UC less than one year were independently associated with clinical remission; endoscopic severity and prior exposure to steroids or biologics were independently associated with lower efficacy. Interestingly, the rate of clinical remission increased with the number of factors of good response to GMA. Short disease duration has been previously identified as a predictor of response to GMA in a large study[48]. However, this is unlikely to be practical for patient selection in Europe as GMA is not considered a first line therapy for UC. Previous corticosteroid exposure

has also been identified in other studies as a predictor of a worse response[48,49], and a European registry observed that the response rate was significantly higher among steroid-dependent UC as opposed to steroid-refractory patients[50]. Moreover, the ATTICA study also found that patients naïve to thiopurines had a significantly better response to GMA[27]. From this point of view, the worse response in patients with previous exposure to corticosteroids, thiopurines or biological agents should facilitate the use of GMA in frail patients, not only because of an expected higher efficacy but also because of its much better safety profile as compared to those drugs. Unfortunately, there is a lack of data on the usefulness for early detection of responders by means of the change or early normalization of faecal calprotectin levels, a potentially ideal biomarker for a therapy that targets the main source of this protein.

CONCLUSION

GMA is the only available therapy for UC directly targeting neutrophils. Two controlled, multicentre, European studies and a number of recent cases series found a potential therapeutic benefit of GMA in different clinical scenarios of UC with a still unmet need for optimal treatment. Moreover, GMA has an excellent safety profile and is perceived as a convenient procedure by patients, making this non-pharmacological therapy a suitable alternative or add-on therapy in UC, particularly for frail or co-morbid patients.

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MINIREVIEWS

Radiotherapy as an immune checkpoint blockade combination strategy for hepatocellular carcinoma

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Abstract

In the immune oncology era, the clinical efficacy of immune checkpoint inhibitors (ICIs) against most solid cancers is well known. In hepatocellular carcinoma, the recent success of combination therapy with targeting agents has accelerated the search for novel combination strategies. Radiotherapy (RT), an attractive modality, can be combined with ICIs, which act as strong modulators of the tumor immune microenvironment. Herein, we discuss immune modulation caused by radiation and the current trials of RT–ICI combination treatment as well as future perspectives.

Key Words: Hepatocellular carcinoma; Immune checkpoint inhibitor; Radiotherapy; Immune modulation; Tumor microenvironment; Immune oncology

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Core Tip: Immune modulatory effect of radiation is highlighted as a combination strategy with immune checkpoint inhibitors. This strategy has been actively adopted in most solid cancers. Although it is in relatively early stage for hepatocellular carcinoma, accumulated evidence drives clinical trials on testing its efficacy. Still, there remain several challenges to overcome for the best oncologic outcome.

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INTRODUCTION

As immune checkpoint inhibitors (ICIs) have demonstrated promising clinical outcomes, cancer immunotherapy has shifted the paradigm in cancer therapy. ICIs have shown efficacy against various types of cancers such as malignant melanoma and non-small cell lung cancer[1-3]. For hepatocellular carcinoma (HCC), ICI monotherapy has not been successful, with a response rate of no more than 20%, which suggests the need for a combination strategy. The recent IMbrave150 trial demonstrated superior progression-free survival (PFS) and better overall survival (OS) with combination treatment using atezolizumab [an anti-programmed death ligand-1 (PD-L1) agent] and bevacizumab (an anti-vascular endothelial growth factor-A agent) compared to those with sorafenib in HCC patients[4]. The IMbrave150 trial showed the potential of ICIs in combination with a tumor microenvironment (TME)-modulating agent for the treatment of HCC. Identification of the optimal combination treatment using ICIs as a novel therapy is gaining extensive attention.

Radiotherapy (RT), one of the major cancer treatments, promotes localized tumor cell killing and induces immune modulation in the TME[5,6]. Increasing evidence has demonstrated that radiation reinforces tumor-related immunity[7,8]. RT exerts synergistic effects with ICIs by increasing lymphocyte infiltration into tumors, inducing immunogenic cell death, and enhancing the performance of antigen-presenting cells (APCs)[9]. Herein, we discuss immune modulation by radiation, the rationale for RT–ICI combination treatment in preclinical settings, and future approaches to overcome the hurdles in combination therapy for HCC.

CHALLENGES IN CURRENT ICIs

Although ICIs show promising treatment outcomes, challenges remain in their application. One recent study reported that the tumor immune cell composition plays a key role in the response to immunotherapy[10-12]. Although the initial T-cell population mainly comprised effective "transitional" cells, a substantial number of infiltrating CD8 T-cells transformed gradually into dysfunctional T-cells. CD8 T-cells with cytotoxic functions were rare among intratumoral immune cells, while dysfunctional T-cells were the major immune cells in tumors. Furthermore, the proportion of dysfunctional T-cells was associated with tumor proliferation. These findings suggest that ICIs alone might be insufficient for achieving an adequate clinical response.

The infiltrating dysfunctional T cells by immunosuppressive mechanisms in TME is one of the reasons for failed ICI[13,14]. The exhausted T cells can explain the lack of response in ICI. To elevate the efficacy of ICI response, converting the dysfunctional T cell into functional T cell is important. The reinvigorating exhausted T cell is expected to improve the outcome of ICI. The successful reinvigoration of T cell function would recover the antitumor activity[15].

Currently, the reinvigoration of T-cells appears to be a key outcome in immune oncologic therapy. Huang *et al*[16] reported that the reinvigoration of T-cells is closely related to tumor burden, and this association was also correlated with the clinical response to ICIs[16-18]. The authors reported that the ratio of T-cell reinvigoration to the tumor burden was the key predictive factor of the clinical response to ICIs, which explains the heterogeneous and unsustainable clinical benefit in patients[16-18]. Therefore, reducing the tumor burden before administering ICIs seems important for improving clinical outcomes. In this regard, RT may be effective in reducing the tumor burden[19]. In addition, RT has been known for its modulation effect on the immune TME.

IMMUNE MODULATION EFFECT OF RADIATION

Besides cell killing, RT induces an immune-mediated antitumor response. Its effect in terms of immune modulation is summarized in Table 1. First, RT induces antigen release and immunogenic cell death. Radiation upregulates the expression of major histocompatibility complex (MHC) class I, thus enhancing the immune response and efficacy of ICIs[20,21]. Naturally, MHC expression is downregulated in tumors to allow immune evasion. Expression of MHC class I enables CD8 T-cells to recognize tumor cells and trigger a major cell-mediated cytotoxic response. Enhanced antigen presentation with upregulated MHC class I expression is one mechanism by which

Table 1 Four key steps of a radiation-induced immune response

Major steps	Ref.
Induction of antigen release and immunogenic cell death	[20-22]
Induction of antigen-presenting cell maturation and antigen presentation	[23,24]
Induction of T-cell recruitment and infiltration	[21,29-32]
Induction of tumor cell sensitization to immune-mediated cell death	[27,28,33]

radiation induces immune-mediated cell death[20]. Radiation promotes the expression of not only MHC class I in tumor cells but also damage-associated molecular patterns, such as HMGB1, and the release of prophagocytic signals, such as calreticulin[20,22].

Second, RT mediates the release of tumor antigens, which leads to the activation and transfer of dendritic cells to draining lymph nodes, resulting in tumor-specific T-cell activation and proliferation. After RT, antigens are released from dying tumor cells, and antigens are taken up by APCs such as macrophages, dendritic cells, and B-cells. Antigen uptake by APCs is an important step in priming adaptive immunity. Dendritic cells are activated after antigen uptake, and the activated dendritic cells migrate to lymph nodes. In tumor-draining lymph nodes, dendritic cells present antigens to either T-cells or B-cells[23]. HMGB1, a radiation-induced damage-associated molecular pattern, enhances dendritic cell maturation[24].

Several studies have demonstrated that RT increases the number of tumor-infiltrating lymphocytes (TILs), indicating that RT aids in overcoming the physical barriers of the tumor, which facilitates a substantial response by the adaptive immune system[25-28]. Two mechanisms have been proposed to explain the increased number of TILs after RT[21,29-31]. One mechanism involves the modification of the vascular endothelium, enabling the extravasation of immune cells. The expression of E-selectin and intercellular adhesion molecule (ICAM)-1, one of the cell adhesion molecules on the vascular endothelium, has been shown to increase after RT[32]. These molecules help the leukocytes migrate from vessels, which is a key step in enhancing the immune response against the tumor. Another mechanism involves the promotion of the expression of chemokines, increasing immune cell migration and invasion. Radiation increases the expression of CXCL16, the ligand for CXCR6[31]. CD8 T-cells expressing CXCR6 are recruited toward tumor cells as radiation exposure increases the expression of CXCL16. In summary, radiation promotes the migration of TILs into the TME, ultimately generating an immunogenic environment.

Lastly, RT induces the sensitization of tumor cells to immune-mediated cell death. As previously described, radiation increases MHC class I expression, together with the immunogenic release of damage-associated molecular patterns and prophagocytic signals. This mechanism induces immune-mediated cell death[28]. Along with the enhanced expression of MHC class I and HMGB1, radiation exposure induces FAS expression. FAS is a cell surface molecule that induces programmed cell death. FAS expression is upregulated in human tumor cell lines after radiation[33]. Upregulated FAS expression on tumor cells enhanced binding to nearby immune cells expressing the FAS ligand[27,33]. Radiation-induced upregulation of FAS expression is one of the important mechanisms by which the immune system can trigger tumor cell death. Taken together, RT can change the immunogenicity of tumors from low to high through these key mechanisms. The mechanisms that occurred when combining the ICI and radiation are summarized in Figure 1.

These immune-modifying mechanisms were also observed in a murine HCC model[34]. MHC class I expression was upregulated after RT in the HCC model. The expression level of MHC class I was significantly higher in the RT group than in the control group. Concordant with the expression of MHC class I, upregulated expression of HMGB1 and ICAM-1 was observed after RT. The upregulated expression of HMGB1 is expected to lead to dendritic cell maturation, and increased ICAM-1 expression is thought to induce leukocyte outflow. These molecules, induced by radiation, alter the TME to an immunogenic environment in HCC.

COMBINATION OF RADIATION WITH ICIS

The notion of combination treatment is now generally accepted with respect to the clinical application of ICIs. It is known that multiple co-stimulatory and inhibitory

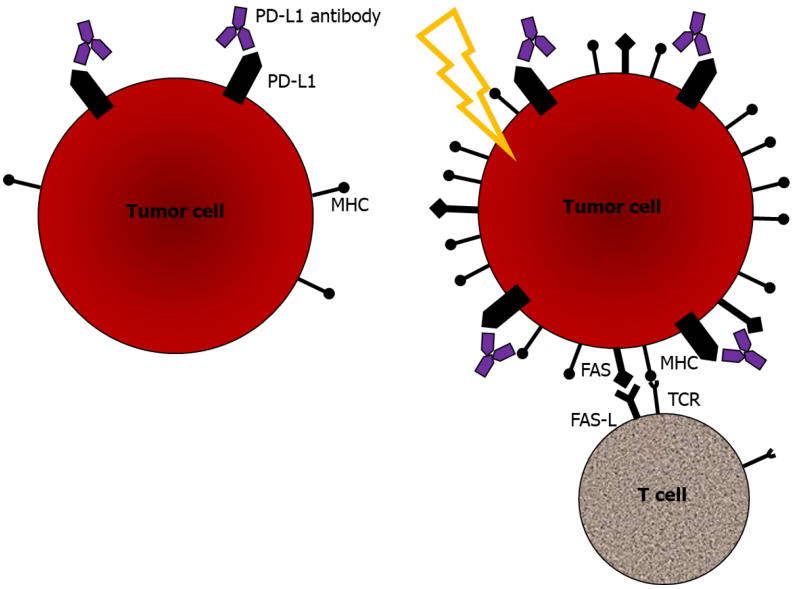


Figure 1 Combination of immune checkpoint inhibitor and radiation enhances immune-mediated cell death. PD-L1: Programmed cell death ligand 1; MHC: Major histocompatibility complex; TCR: T cell receptor.

signals regulate T-cell activation[35,36]. These co-stimulatory and inhibitory signals play an important role in immune resistance. ICIs, which block these inhibitory signals, eliminate immune resistance mechanisms. Interestingly, these co-stimulatory and inhibitory signals are modulated by radiation[29,37]. Based on these findings, the combination of RT and ICIs is thought to have a synergistic effect, and some preclinical data support its use against HCC.

Our group demonstrated that RT induced PD-L1 expression in tumor cells and showed the potential antitumor effect of anti-PD-L1 agents against HCC[38]. The expression of PD-L1 is induced maximally between 24 and 48 h after RT. RT with up to 10 Gy was administered, and PD-L1 expression was upregulated in a dose-dependent manner. The antitumor effect was also examined for the anti-PD-L1 agent–RT combination *in vivo*. Tumor growth suppression and survival improvement were significantly superior in the combination group than in the anti-PD-L1 agent alone or RT alone group. Furthermore, the combination of an anti-PD-L1 agent and RT significantly increased cytotoxicity and the proliferation of CD8 T-cells compared to RT alone or the anti-PD-L1 agent alone.

T-cell immunoglobulin and mucin-domain-containing molecule-3 (TIM3) is an inhibitory molecule present on T-cells. TIM-3-expressing T-cells showed dysfunction or "exhaustion"[39]. It has been reported that TIM-3 expression is higher in HCC patients than in those with other liver diseases, such as chronic hepatitis and liver cirrhosis[40]. TIM-3-positive T-cells showed high expression in HCC cells, in contrast to that in normal cells present in adjacent tissues[41]. Although TIM-3 blockade can modulate the immune response *via* several cell types[42-44], there are limited studies regarding the effect of anti-TIM3 agents in HCC patients. RT upregulated TIM-3 expression in HCC cell lines, and the combination of an anti-TIM-3 agent and radiation promoted cytotoxicity and the proliferation of CD8 T-cells[45]. Furthermore, the combination of an anti-TIM-3 agent and radiation significantly suppressed tumor growth compared to radiation or anti-TIM-3 agent alone. Concordant with the results of tumor growth, the combination group demonstrated a significant improvement in survival.

Despite these promising preclinical data regarding the combination of RT and ICIs against HCC, clinical studies are severely limited. One study investigated the clinical implications of PD-L1 levels in patients with HCC undergoing RT[46]. The level of soluble PD-L1 (sPD-L1) was quantified in patients who underwent RT for HCC. The initial sPD-L1 level was significantly associated with tumor aggressiveness (tumor size and stage). A high initial sPD-L1 level was related to poorer OS than a low initial sPD-L1 level. The sPD-L1 levels increased significantly after both conventional RT and stereotactic body RT (SBRT), but the pattern of sPD-L1 change was different depending on the dose scheme. The sPD-L1 level increased immediately after RT but decreased at 1 mo after conventional RT, while a continuous increase was observed in those undergoing SBRT. In the SBRT group, the median sPD-L1 level at 1 mo increased

to approximately 3-times the initial sPD-L1 level. Therefore, the combination of ICIs and RT may be a promising treatment in patients with HCC, and efficacy might be better with SBRT. This notion needs clinical validation to evaluate the efficacy of combined treatment with RT and ICIs for HCC. Several prospective trials registered at www.ClinicalTrials.gov are ongoing to investigate the combination of ICIs and RT (Table 2).

CHALLENGES WITH THE COMBINATION OF RADIATION AND ICIS

Radiation fractionation

There is no established dose or fractionation regimen that optimizes the therapeutic effect of RT plus ICIs. It is clear that the immunologic response differs depending on the RT dose as *per* fractionation. With conventional fractionation, RT promotes the recovery of tumor vessels *via* the migration of immune cells through the endothelium[47] and induction of M1-type macrophages[47,48]. These actions have a positive effect on immunity. With hypofractionation, treatment-related lymphopenia occurs less frequently[49], and Tregs are activated, shifting the balance of T-cells toward the immunosuppressive state[50,51]. Additionally, preclinical data have shown that hypofractionation regimens favor an antitumor response and induce a strong lymphoid response[52,53].

In contrast, a high fractional dose led to a different immune response. As *per* preclinical data, fractional doses higher than 12 Gy induce the production of 3′ repair exonuclease 1 (TREX1), which degrades cytosolic DNA after RT[54]. Consequently, TREX1 inactivates the cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS)–stimulator of interferon genes (STING) pathway. With conventional fractionation, the cytosolic DNA after radiation binds to cGAS and activates STING[55,56]. Consequently, cGAS and STING induce the production of type I interferon and activate an antitumor immune response[57,58]. As a fractional dose of more than 12 Gy induces TREX1, the fractional dose of more than 12 Gy results in the inhibition of antitumor immune responses. Furthermore, doses greater than 10 Gy *per* fraction enhance vascular damage, leading to less effective T-cell recruitment because of reduced vascularity[59]. The different types of radiation-induced immune modulation by different fractionation scheme of radiation are summarized in Figure 2. For an optimal combination of RT and ICIs, it is important to gain an understanding of the immune response depending on different fractionations to improve therapy efficacy and administer personalized medicine[19].

Treatment sequence

The optimal timing of administering ICIs in combination with RT has not yet been defined. To determine the optimal timing of RT and ICI treatment, several preclinical studies have been performed. One report showed that administering ICIs 7 d after RT was less effective in enhancing OS than administering ICIs concurrently with RT[37]. In the PACIFIC trial, durvalumab started within 14 d of completing RT resulted in better PFS than durvalumab started after 14 d[2]. A recent study showed that OS was longer in patients who received ICIs concurrently with RT[60]. Among the patients who received concurrent treatment, induction immunotherapy administered more than 30 d before RT led to longer OS than that of administered within 30 d before RT. Scheduling of RT and immunotherapy must be considered with caution in the context of clinical trials.

CONCLUSION

ICIs have emerged as a promising therapy for various malignancies including HCC. T-cell reinvigoration by activating dysfunctional T-cells into cytotoxic T-cells is a key factor in the novel therapeutic effect of ICIs. However, ICI monotherapy has some limitations in circumstances such as T-cell dysfunction and high tumor burden. Meanwhile, RT has been shown to cause high immunogenicity in tumors through various mechanisms of immune modulation. The combination of ICIs and RT is being studied as a promising treatment for HCC to take advantage of the synergistic effect. Further studies are necessary to determine the appropriate treatment regimen for achieving optimal clinical benefit.

Table 2 Ongoing clinical trials investigating the combination of radiotherapy and immune checkpoint inhibitors against hepatocellular carcinoma

NCT number	Institution	Disease	Estimated enrollment	Phase	Primary endpoint	Intervention
NCT04167293	China (Sun Yat-sen University Cancer Center)	HCC with portal vein invasion	116	II/III	6-mo PFS	SBRT + sintilimab *vs* SBRT
NCT03817736	Hong Kong (Queen Mary Hospital)	HCC	33	II	Number of patients eligible for curative surgical interventions	TACE/SBRT + ICI
NCT03203304	United States (University of Chicago)	Unresectable HCC	50	I	Number of participants with adverse events	Nivolumab + SBRT *vs* nivolumab and ipilimumab + SBRT
NCT04611165	South Korea (NCC)	HCC with major vascular invasion	50	II	PFS	Nivolumab + EBRT
NCT03482102	United States (MGH)	Locally advanced/unresectable or metastatic HCC or biliary tract cancer	70	II	ORR	Tremelimumab + durvalumab + RT
NCT03316872	Canada (UHN)	HCC progression after sorafenib	30	II	ORR	Pembrolizumab + SBRT
NCT04547452	China (West China Hospital)	Metastatic HCC	84	II	PFS	SBRT + sintilimab *vs* sintilimab
NCT04193696	China (Guangxi Medical University)	Advanced HCC	39	II	ORR	RT+ anti-PD-1 agent

HCC: Hepatocellular carcinoma; PFS: Progression-free survival; SBRT: Stereotactic body radiation therapy; TACE: Transcatheter arterial chemoembolization; ICI: Immune checkpoint inhibitor; NCC: National Cancer Center; EBRT: External beam radiotherapy; MGH: Massachusetts General Hospital; ORR: Overall response rate; RT: Radiotherapy; UHN: University Health Network; PD-L1: Programmed cell death ligand 1.

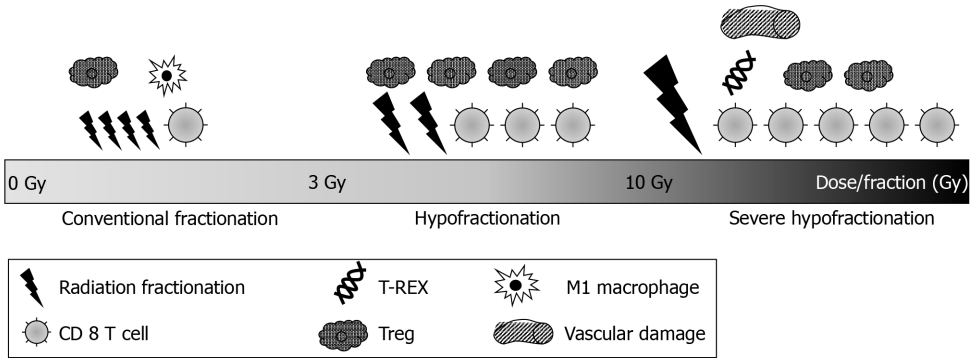


Figure 2 Types of radiation-induced immune modulation by different fractionation scheme of radiation.

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MINIREVIEWS

Impact of the COVID-19 pandemic on liver donation and transplantation: A review of the literature

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic has upended healthcare systems worldwide and led to an inevitable decrease in liver transplantation (LT) activity. During the first pandemic wave, administrators and clinicians were obliged to make the difficult decision of whether to suspend or continue a life-saving procedure based on the scarce available evidence regarding the risk of transmission and mortality in immunosuppressed patients. Those centers where the activity continued or was heavily restricted were obliged to screen donors and recipients, design COVID-safe clinical pathways, and promote telehealth to prevent nosocomial transmission. Despite the ever-growing literature on COVID-19, the amount of high-quality literature on LT remains limited. This review will provide an updated view of the impact of the pandemic on LT programs worldwide. Donor and recipient screening, strategies for waitlist prioritization, and posttransplant risk of infection and mortality are discussed. Moreover, a particular focus is given to the possibility of donor-to-recipient transmission and immunosuppression management in COVID-positive recipients.

Key Words: Severe acute respiratory syndrome coronavirus type 2; Liver cirrhosis; Donor and recipient screening; Donor-to-recipient transmission; Immunosuppression; Resource allocation in transplantation

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Core Tip: The coronavirus disease 2019 (COVID-19) pandemic has reduced liver transplantation (LT) activity worldwide at different rates in different regions. Testing for COVID-19 has been included in routine donor and recipient evaluations. LT recipients are likely at increased risk of infection, but COVID-related mortality appears to be comparable with the general population if corrected for concurrent risk factors. Immunosuppression could exert a protective effect against the most severe forms of COVID-19, and its complete withdrawal or reduction may not be useful. Transplant centers and administrators should allocate resources considering the actual burden of the infection, waitlist priority, risk of posttransplant infection, and mortality.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) has upended healthcare systems worldwide. During the first pandemic wave, overwhelming hospitals rapidly reallocated their resources and increased their capacity to attend to an exponential increase in the number of critically ill patients, leading to the limitation of oncological and elective surgery[1]. Organ donation and transplantation have also suffered an inevitable decrease[2,3]. The second pandemic wave in most European countries has posed similar issues regarding resource allocation, although most patients are asymptomatic or present with less severe symptomatology[4].

In the field of liver transplantation (LT), administrators and clinicians were forced to make difficult decisions regarding whether to suspend or continue a life-saving procedure during the pandemic. Therefore, the main ethical question was whether it was riskier to accept a patient for LT or to wait until the peak of the infection had decreased. The pandemic has also led to additional complexities regarding donor and recipient testing, obliged to design COVID-safe clinical pathways, and promoted telehealth to prevent nosocomial transmission.

An ever-growing number of papers on COVID-19 have been published since its initial outbreak, making it difficult to keep up with the most recent evidence on this topic. However, the amount of high-quality literature on LT remains limited, and particular care should be taken in drawing any conclusions[5]. Indeed, preliminary data during the first pandemic wave were often obtained in difficult conditions, and the race to publication has led, in some cases, to corrections and retractations[6,7]. Aware of these limitations, in this review, we aim to analyze the impact of the pandemic on LT programs worldwide, mainly focusing on donor and recipient screening, waitlist prioritization, and immunosuppression management in COVID-positive recipients.

TRENDS IN ORGAN DONATION AND TRANSPLANT ACTIVITY DURING THE PANDEMIC

The first pandemic wave between February and March 2020 led to a reduction in organ donation compared to the same period in 2019, varying between 0–30% in different countries (Table 1). For example, early data from Italy – one of the first Western countries dealing with the COVID-19 outbreak – reported a 25% decrease in procured organs during the first month of the outbreak, which paralleled the progressive rise of patients admitted to the intensive care unit (ICU)[8]. Particularly regarding LT, the United Network for Organ Sharing (UNOS) data revealed a more than 25% decrease between February and April 2020, with an inactivation rate of waitlisted patients between 5%-10%[3]. This trend is consistent with the 29% decrease registered in the Eurotransplant network in approximately the same period, which inevitably led to increased death and dropout from the waitlist[9]. Moreover, in most

Table 1 Reduction in LT activity around the world during the pandemic

Ref.	Country	Reduction in organ donations	Reduction in LT activity	Period
Putzer *et al*[9], 2020	Europe[1]	N/A	-29%	mid-March – mid-June, 2020 *vs* 2015 – 2019
Agopian *et al*[18], 2020	United States	N/A	-24%	February – March, 2020 *vs* 2019
Turco *et al*[19], 2020	France	-28%	-22%	January 1 – May 31, 2020 *vs* 2019
Domínguez-Gil *et al*[4], 2020	Spain	N/A	-75.8% LT/wk	March 13 – April 23 *vs* weekly mean 2019
Angelico *et al*[8], 2020	Italy	-30% (North)-9% (South)	-17%	February 24 – March 22, 2020 *vs* 2015-2019
Lee *et al*[20], 2020	South Korea	No difference	No difference	January – March, 2020 *vs* 2000-2019

[1]Eurotransplant data. N/A: Not applicable; LT: Liver transplantation.

countries, LT activity slowly recovered in the following months from this abrupt reduction due to a second pandemic wave[4,9].

Three different scenarios have been observed during the pandemic among centers in both Europe and the United States:

Complete shutdown of activity

This was a last-resort measure in situations where no ICU or healthcare personnel were available[10]. It was estimated that 6% of centers in Europe temporarily halted LT activity due to the lack of donors and logistical problems correlated to the first pandemic wave[2].

The limitation of transplant activity favors a "sickest-first" approach

LT activity continued even in some highly stressed hospitals without being stopped a priori, but evaluating each organ offer based on the resources available at the moment[11]. Two-thirds of centers in Europe have adopted the policy of selecting only urgent recipients[2]. However, this sickest-first approach poses the risk of prolonged hospital stays for such patients, thus conversely increasing the ICU length of stay[12]. For this reason, some centers have temporarily suspended treating aging patients with comorbidities and surgical complexities to minimize the chance of complications[10,13,14]. Likewise, different centers have reduced the use of marginal grafts and those from donation after circulatory death for both fear of poor transplant outcomes and heavier commitment on ICU personnel[3,15,16].

Continuation of routine transplant activity

Other centers in areas where COVID-19 prevalence was low – or the infection reached its peak slowly – have continued their activity at a routine or even increased rate compared to 2019, being able in some cases to shunt COVID-19 affected patients elsewhere[17-20].

Significant heterogeneity has been noted in the three aforementioned levels of activity across centers within the same country or even the same region. These differences were unrelated to the local prevalence of COVID-19 but more likely reflected a different perception of risk and prioritization of hospital resources[18,19,21]. In this context, a phased approach has been proposed to decrease transplant activity based on risk tolerance, hospital capacity, and degree of virus activity in different areas[10]. Nevertheless, it is essential to note that the decision of whether to continue LT activity was made in an emergent situation and based on the very limited evidence available at the moment on the risk of transmission and mortality in LT recipients[5]. These aspects will be analyzed in the following sections.

LIVING DONOR LIVER TRANSPLANTATION ACTIVITY DURING THE PANDEMIC

Living donor LT (LDLT) poses additional issues during the pandemic due to the risk of nosocomial transmission to donors as they recover from major surgery. Given the mainly elective nature of the procedure, most programs have considered postponing LDLT an ethically appropriate action during the pandemic peak[2,3]. In April 2020,

LDLT activity nearly stopped in Spain[4]. According to UNOS data, LDLT has been performed seldomly, comprising only 32 cases throughout April 2020[3]. However, some centers have continued this practice even in high COVID-19 prevalence regions by creating COVID minimal-exposure pathways and reported favorable outcomes[3,22,23]. Surprisingly, data from South Korea showed that LDLT – the main type of transplantation performed in this county – did not significantly decrease even during the peak of the epidemic, thanks to a strict screening and tracing policy based on the experience of the previous Middle Eastern Respiratory Syndrome Coronavirus (MERS-CoV) infection[20].

TRANSPLANT-RELATED TRANSMISSION AND UTILIZATION OF COVID-POSITIVE DONORS

Most scientific societies worldwide have strongly recommended nucleic acid testing for SARS-CoV-2 to be part of the routine evaluation of both donors and recipients (Table 2). Significant variability in the false-negative rate has been reported in testing for SARS-CoV-2 by nasopharyngeal swabs (NFSs)[3,24]. For this reason, the results should be interpreted alongside clinical history and chest computed tomography when available. Molecular testing on NFS is the recommended screening method for living donors in centers where LDLT has not been halted[24]. Conversely, bronchoalveolar lavage (BAL) should be preferred for deceased donor screening, as it has a very low false-negative rate[25]. However, it should be considered that BAL may not be logistically feasible and has the greatest sensitivity later in the course of the infection, whereas NPS has the greatest sensitivity during the early period[3,10]. Donor and recipient testing can significantly extend the timing and complicate the logistics of transplantation. In this context, many local medical centers are now beginning to obtain consistent access to rapid reverse transcription-polymerase chain reaction (RT-PCR) diagnostics[3,15]. Moreover, machine perfusion has been used to extend preservation times of the liver graft while awaiting SARS-CoV-2 test results to be available[26]. As no molecular test is perfectly sensitive or specific, an additional epidemiological and clinical screening (which includes travel to a high-risk area, contact with a confirmed case, or onset of symptoms) of each case has been suggested[24]. The organs of donors who are positive for the epidemiological and clinical screening are considered high risk and should not be used for transplantation.

There is no consensus on time and testing requirements before recovering organs from donors who had previously documented COVID-19 infection. Waiting periods after the resolution of symptoms vary between 14-28 d, and most groups specifically recommend repeating a single or two negative tests before proceeding to donation[10,27]. The use of liver grafts from donors testing positive for COVID-19 is widely considered unacceptable[4,10,24]. However, their use is hotly debated for super-urgent patients. Some authors have suggested that for selected patients with imminently life-threatening organ failure, transplants from deceased donors with asymptomatic or mild SARS-CoV-2 infection may offer a favorable risk-benefit balance[14,28,29]. The two main arguments in favor of this position were as follows:

There is little evidence that SARS-CoV-2 can directly infect the liver in initial reports

SARS-CoV-2 was initially thought to be less likely to infect the liver due to lower expression in the hepatocytes of angiotensin-converting enzyme 2 (ACE-2), the cell surface receptor for SARS-CoV-2. This was considered consistent with the limited initial autopsy studies, which failed to demonstrate SARS-CoV-2 in the liver[28,29].

There was no transmission after blood transfusions or transplantation in initial reports

To date, there have been no reports of recognized transmissions of SARS-CoV-2 following blood transfusion, even in immunosuppressed patients[28,30]. Moreover, Hong *et al* reported the case of a patient who underwent LDLT without knowing that the donor was infected with COVID-19 at the time of the procedure. Donor-derived transmission to the recipient was not identified, and retrospective RT-PCR for SARS-CoV-2 from the liver biopsy confirmed no evidence of viral infection in the liver[22].

Conversely, other authors advise against the use of livers from COVID-19-positive donors in any case[31]. In this context, the main arguments are as follows:

Table 2 Recommendations of international societies

	AASLD[58]	EASL[51]	APASL[32]
Donor screening	RT-PCR for SARS-CoV-2. Screen for exposure and clinical symptoms/fever compatible with COVID-19. Additionally, consider chest X-ray	RT-PCR for SARS-CoV-2	SARS-COV-2 RNA on NPS or BAL. Exclude any evidence of COVID-19 infection on chest CT scan
Recipient testing	Screen for exposure and clinical symptoms/fever compatible with COVID-19. RT-PCR for SARS-CoV-2	Evaluation of clinical history, chest radiology, and SARS-CoV-2 testing. Screening before admission	Assess recipients for COVID-19 infection, particularly in the presence of symptoms or contact with a known COVID-19 case
Liver allocation policy	High MELD scores. HCC based on their risk of drop-out and disease progression	Acute liver failure. ACLF. High MELD score. HCC at the upper limits of the Milan criteria	Acute liver failure. High MELD. High risk of HCC progression
Living donation	Consider suspending, except for pediatric patients with acute liver failure	Should be considered on a case-by-case basis	Not specified (avoid if evidence of COVID-19 infection)
Immunosuppression in COVID-19 positive recipients	Standard immunosuppression protocol. Reduction of immunosuppression may be considered in the setting of lymphopenia, fever, or worsening pulmonary status	Standard immunosuppression protocol. Reduction should only be considered under special circumstances	Standard immunosuppression protocol. Reduction of immunosuppression may be considered in patients diagnosed with moderate COVID-19 infection

AASLD: American Association for the Study of Liver Diseases; ACLF: Acute on chronic liver failure; APASL: Asian-Pacific Association for the Study of the Liver; BAL: Bronchoalveolar lavage; CT: Computerized tomography; EASL: European Association for the Study of the Liver; HCC: Hepatocellular carcinoma; MELD: Model for end stage liver disease; NAT: Nucleic acid test; RT-PCR: Reverse transcription-polymerase chain reaction; SARS-CoV-2: Severe acute respiratory syndrome coronavirus type 2; COVID-19: Coronavirus disease 2019.

More recent evidence indicates that SARS-CoV-2 can directly infect the liver

The expression of ACE-2 in cholangiocytes was found to be comparable to that in alveolar type 2 cells[32]. Wang *et al* recently demonstrated that SARS-CoV-2 can directly infect hepatocytes, most likely through alternative extra-ACE-2 receptors, causing conspicuous cytopathy[33]. Moreover, Lagana *et al*[34] reported a case of likely COVID-related hepatitis in a pediatric recipient whose living donor subsequently tested positive for COVID-19, although no RT-PCR for SARS-CoV-2 was performed on liver biopsy.

There is an unclear understanding of the risk associated with transmission

The impossibility of clearly defining the risk of transmission prevents the recipient from providing adequate informed consent. This is further accentuated by the absence of effective prophylaxis[31].

Exposure of healthcare workers during organ procurement is a concern

Staff members contracting the disease or in quarantine can result in the shutdown of a program for weeks. Organ recovery teams moving across centers and regions are exposed to the risk of transmission, and they must take appropriate respiratory and contact precautions throughout the procedure, even if the donor tested negative[15,20]. To reduce the exposure of recovery teams, many transplant centers have started relying on local teams and information sharing between centers *via* secure digital platforms[3].

Theoretically, the possibility exists to transplant these organs in patients who recovered from COVID-19 infection or received the vaccine, thus having developed a protective antibody titer[35]. Nevertheless, cases of reinfection with different SARS-CoV-2 clades have been documented, and this practice still entails healthcare workers' exposure[36].

RISK OF INFECTION AND COVID-ASSOCIATED MORTALITY AMONG LT RECIPIENTS

Qin *et al*[37] first reported the case of an LT recipient who experienced COVID-19 infection in the perioperative period and was discharged without sequelae after 56 days. Two reports from the same high-prevalence Italian region reported extremely low infection rates of 1.25% and 1.5% among 640 adult and 200 pediatric LT recipients, respectively[38,39]. The apparently lower risk of COVID-19 infection among LT recipients

compared to the general population could be explained partly by the higher degree of surveillance[38]. A survey from Germany has shown that during the pandemic, LT recipients used personnel protective equipment and practiced social distancing significantly more frequently than waitlist candidates[40]. Moreover, many transplant centers preferred suspending family visits during the posttransplant course and providing postdischarge follow-up care *via* telehealth[41]. However, the incidence of infection among LT recipients might also be underestimated due to the milder disease expression in these patients[38]. Therefore, short-term LT recipients seem to present fewer atypical symptoms, frequently of the gastrointestinal type, most likely because higher immunosuppression attenuates typical COVID-19 presentation[42,43]. In fact, a nationwide experience in Spain revealed that LT patients had almost double the incidence rates of COVID-19 compared with the age- and gender-matched general population[44].

Rabiee *et al*[45] recently reported a liver injury rate of 34.6% among COVID-19-positive LT recipients, which was higher than that of the general population but lower than that of nontransplant patients with chronic liver disease. Likewise, mortality among LT recipients testing positive for COVID-19 varies between 0%-23% in most studies (Table 3) and seems higher than that in the general population but lower than that in cirrhotic patients, where it was estimated to be between 34%-40%[15,41,46]. Male sex, advanced age, and metabolic comorbidities, which are known to increase COVID-19 severity, are more frequent among LT recipients than among the general population and could account for this difference[2,47]. However, risk factors that may increase mortality among LT recipients are not completely clear. Preliminary data from Italy and the European Liver Transplant Registry (ELTR) suggested that mortality could be higher among recipients with a longer time since transplantation, which was not confirmed by the analysis of the COVID-hep and SECURE-Cirrhosis registries[42,48,49]. It seems, however, that biological age and comorbidities rather than time from transplantation are most strongly correlated with death[42]. Different studies have confirmed the association between age and mortality among LT recipients with COVID-19[42,50]. Bhoori *et al*[49] first reported increased mortality among LTs who tested positive for COVID-19 with metabolic-related comorbidities, such as hypertension, chronic kidney disease, and diabetes. While the analysis of the COVID-hep and SECURE-Cirrhosis registries confirmed this finding, Becchetti *et al*[43] found no correlation between comorbidities and mortality in their series.

IMMUNOSUPPRESSION MANAGEMENT DURING THE PANDEMIC

Clinicians should be aware of the high reported rates of fear and anxiety regarding COVID-19 in LT recipients and the consequent risk of scarce compliance with immunosuppressive medication[40,51]. The immunocompromised status seemed not to affect mortality during previous coronavirus-related infections, such as SARS-CoV in 2003 and MERS-CoV in 2015[39]. Moreover, the pathogenesis of severe COVID-19 was found to be mainly explained by the immune-mediated inflammatory reaction rather than direct cellular damage[52]. Thus, immunosuppression could theoretically have a protective role in LT patients[49,53].

There is limited guidance related to immunosuppression management during the pandemic. Societies advise against the reduction of maintenance immunosuppressive therapy to prevent SARS-CoV-2 infection. Some authors have initially suggested a reduction in the use of lymphocyte depletion therapy for induction immunosuppression during the pandemic to reduce the risk of nosocomial infection[5,51].

The management of immunosuppression in LT recipients testing positive for COVID-19 is currently under debate. In the aforementioned report by Qin *et al*[37], tacrolimus and steroids were gradually titrated and then increased due to the suspicion of acute rejection. The retrospective analysis of different series during the first pandemic wave showed that immunosuppression was modified in nearly half of the cases, most frequently among patients with moderate and severe COVID-19, but rarely discontinued. Frequently reported changes were mycophenolate withdrawal, steroid dosing increase, and calcineurin inhibitor reduction[2,54,55]. However, the target trough levels of the immunosuppressants were rarely reported in these studies[56].

An analysis of data from the COVID-Hep and SECURE-Cirrhosis registries has shown that the immunosuppressive regimen used had no impact on the outcome of COVID-19[42]. Moreover, Becchetti *et al*[43] reported no impact on the disease course whether immunosuppression was decreased or left unchanged. Therefore, most transplant societies have recommended the continuation of immunosuppression at

Table 3 Mortality, hospitalization, intensive care unit admission, and risk factors among liver transplantation recipients

Ref.	Registry	*n*	Mortality (%)	Hospital admission (%)	ICU admission (%)[1]	Major correlations with mortality
Polak *et al*[2], 2020	ELTR	272	15	N/A	14	sexage
Rabiee *et al*[45], 2020	COLD	112	22.3	72.3	26.8	N/A
Colmenero *et al*[44], 2020	SETH	111	18	86.5	10.8	Charlson comorbidity index; Male sex; Dyspnea at diagnosis; Immunosuppression with mycophenolate
Bhoori *et al*[49], 2020	–	111 (long term); 40 (short term)	30	N/A	N/A	N/A
Belli *et al*[48], 2020	ELTR/ELITA	103	16	66	15	N/A
Becchetti *et al*[43], 2020	–	57	12	72	7	N/A
Webb *et al*[42], 2020	COVID-hep and SECURE-cirrhosis	39	23	N/A	N/A	N/A
Patrono *et al*[41], 2020	–	10	10	N/A	N/A	N/A

[1]Calculated as proportion of the total cohort. N/A: Not applicable; COLD: Consortium of investigators to study COVID-19 in chronic liver disease; ELITA: European Liver and Intestine Transplantation Association; ELTR: European Liver Transplant Registry; SETH: Spanish Society of Liver Transplantation; COVID: Coronavirus disease.

stable doses for asymptomatic or mildly symptomatic patients[45,55].

A Spanish nationwide prospective study recently showed that mycophenolate was an independent predictor of severe COVID-19, particularly at doses higher than 1000 mg/d, and complete withdrawal of mycophenolate at COVID-19 diagnosis ameliorated the risk of severe COVID-19[44]. This datum was, however, not confirmed in another study[42].

Belli *et al*[50] showed that the use of tacrolimus was independently associated with reduced mortality among LT recipients testing positive for SARS-CoV-2. The biological explanation of this effect is still unknown but may be due to the inhibition of viral replication and interaction with the inflammatory cascade triggered by the infection. Based on this background, a Spanish randomized clinical trial has been started to test the effect of tacrolimus plus steroids in the management of COVID-19 occurring in immunocompetent patients (ClinicalTrials.gov Identifier: NCT04341038).

RISK ASSESSMENT AND DECISION-MAKING

In light of the aforementioned data on transmission and mortality among LT patients, some authors have attempted a quantitative approach to the question of whether to continue transplant activity during the pandemic. Chew *et al*[16] proposed a score to guide the regulation of LT activity despite the competing needs of the pandemic through the 3 aforementioned scenarios based on 4 main ethical instances: waiting list mortality, donor and graft safety, recipient outcome, and healthcare resources available. Likewise, in the field of kidney transplantation, a machine learning algorithm has recently been developed to quantify the benefit-to-harm ratio of immediate transplant *vs* delay until after the pandemic[57]. COVID-19, like other pandemics, tends to come in several waves over a protracted period until hopefully subsiding through vaccination and herd immunity. These quantitative tools are potentially helpful in decision-making during pandemic peaks but still need to be fully validated. During the interpeak phases, every effort should be made to restore LT activity to regular rates and indications to prevent accumulated death and dropout from the waitlist over the long term.

CONCLUSION

The COVID-19 pandemic has heavily affected health care systems worldwide and, despite some exceptions, has generally led to a reduction in LT activity, with different rates in different countries. Donor and recipient molecular screening for SARS-CoV-2 has become a routine practice in LT to prevent infection in the peritransplant period, although the possibility of using positive donors in super-urgent patients is currently debated. LT recipients seem to have an increased risk of being infected with SARS-CoV-2 but a milder and frequently atypical presentation. Mortality, in particular, seems comparable with the general population if corrected for concurrent risk factors. Immunosuppression could exert a protective effect against the most severe forms of COVID-19, and complete withdrawal or significant reduction in immunosuppression may not be useful, thus increasing the risk of acute rejection. The decision of whether to continue or suspend LT activity during the pandemic should be based on the actual strain on the healthcare system, waitlist priority, risk of posttransplant infection, and mortality.

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ORIGINAL ARTICLE

Basic Study

Huanglian decoction suppresses the growth of hepatocellular carcinoma cells by reducing *CCNB1* expression

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers in human populations worldwide. Huanglian decoction is one of the most important Chinese medicine formulas, with the potential to treat cancer.

AIM

To investigate the role and mechanism of Huanglian decoction on HCC cells.

METHODS

To identify differentially expressed genes (DEGs), we downloaded gene expression profile data from The Cancer Genome Atlas Liver Hepatocellular Carcinoma and Gene Expression Omnibus (GSE45436) databases. We obtained phytochemicals of the four herbs of Huanglian decoction from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform. We also established a regulatory network of DEGs and drug target genes and subsequently analyzed key genes using bioinformatics approaches. Furthermore, we conducted *in vitro* experiments to explore the effect of Huanglian decoction and to verify the predictions. In particular, the *CCNB1* gene was knocked down to verify the primary target of this decoction. Through the identification of the expression levels of key proteins, we determined the primary mechanism of Huanglian decoction in HCC.

RESULTS

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Based on the results of the network pharmacological analysis, we revealed 5 bioactive compounds in Huanglian decoction that act on HCC. In addition, a protein-protein interaction network analysis of the target genes of these five compounds as well as expression and prognosis analyses were performed in tumors. *CCNB1* was confirmed to be the primary gene that may be highly expressed in tumors and was significantly associated with a worse prognosis. We also noted that *CCNB1* may serve as an independent prognostic indicator in HCC. Moreover, *in vitro* experiments demonstrated that Huanglian decoction significantly inhibited the growth, migration, and invasiveness of HCC cells and induced cell apoptosis and G2/M phase arrest. Further analysis showed that the decoction may inhibit the growth of HCC cells by downregulating the *CCNB1* expression level. After Huanglian decoction treatment, the expression levels of Bax, caspase 3, caspase 9, p21 and p53 in HCC cells were increased, while the expression of *CDK1* and *CCNB1* was significantly decreased. The p53 signaling pathway was also found to play an important role in this process.

CONCLUSION

Huanglian decoction has a significant inhibitory effect on HCC cells. *CCNB1* is a potential therapeutic target in HCC. Further analysis showed that Huanglian decoction can inhibit HCC cell growth by downregulating the expression of *CCNB1* to activate the p53 signaling pathway.

Key Words: Huanglian decoction (*Coptidis Rhizoma*, *Zingiberis Rhizoma*, *Folium Artemisiae Argyi*, *Mume Fructus*); Hepatocellular carcinoma; The Cancer Genome Atlas; Gene Expression Omnibus; P53 pathway; Cell cycle; Apoptosis

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Core Tip: The purpose of this study was to investigate the role and mechanism of Huanglian decoction on hepatocellular carcinoma (HCC) cells. The results showed that Huanglian decoction has a significant inhibitory effect on the growth of HCC cells. Huanglian decoction can inhibit HCC cell growth by downregulating the expression of *CCNB1* to activate the p53 signaling pathway. *CCNB1* is a potential therapeutic target for liver cancer.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and the third leading cause of cancer mortality worldwide[1-3]. Current treatment options for liver cancer primarily include surgery, local treatment, biological treatment, and various other methods. Recently, molecular targeted therapy has been used to treat some patients with advanced liver cancer, but because targeted drugs are relatively expensive and induce side effects, they have not been fully utilized[4-6]. In the past few years, there has been growing research attention on traditional Chinese medicine (TCM). Previous studies have established that TCM can improve the clinical symptoms of patients, especially for those with advanced liver cancer by prolonging their survival period, and the cost is relatively inexpensive. In particular, TCM formulation contains a variety of chemical components that act on multiple targets and diseases[7-9]. However, due to the complexity and diversity of TCM components, our understanding of the specific molecular mechanisms of Chinese medicine is still largely unknown. This, therefore, limits the development of Chinese medicine. Nevertheless, the recent development of computer technology has allowed us to

analyze the components of these medicines and systematically analyze their effects on diseases through network pharmacology, which has greatly promoted our understanding of the molecular network of Chinese medicines and their nature[10-12].

Huanglian decoction is a classical Chinese medicine formula that has the effect of clearing heat and treating abdominal pain[13,14]. This formulation was originally recorded in the classic Chinese medicine book "Treatise on Febrile Diseases" (Chinese name: Shang-Han Lun). In this study, we obtained the formula of Huanglian decoction from the ancient Chinese medicine book (Sheng Ji Zong Lu), which contains one of the original recipes. The formula consists of four Chinese herbal medicines: *Coptidis Rhizoma*, *Zingiberis Rhizoma*, *Folium Artemisiae Argyi*, and *Mume Fructus*. In addition, this decoction plays a pivotal role in the field of TCM due to its various effects, such as treating colds, white diarrhea, and abdominal pain[15-17]. In some Asian-Pacific regions, this decoction is used as an auxiliary medicine for the treatment of liver cancer. Research has demonstrated that this decoction can exert its effects on the liver[18-20]. However, very few studies have investigated the treatment of HCC with this decoction. Thus, in this respect, we aimed to investigate the therapeutic potential of Huanglian decoction as an HCC treatment.

CCNB1 plays a key role in regulating and forming a complex with CDK1 to promote the transition from the G2 phase of the cell cycle to mitosis[21]. Increasing evidence has shown that *CCNB1* is overexpressed in certain human cancers, including colorectal, bladder and stomach cancers[22-24]. In addition, the p53 tumor suppressor pathway also plays a key role in mediating the responses of commonly used cancer treatments, and *CCNB1* is significantly correlated with the p53 signaling pathway[25-29]. In this study, we explored the main targets of Huanglian decoction in HCC cells. By reducing *CCNB1* expression, we studied the effects of Huanglian decoction on the cell cycle and apoptosis, and the potential functional pathways were also explored. The detailed flow chart of this study is depicted in Figure 1. This study provides novel insights into the mechanism of Huanglian decoction treatment in HCC.

MATERIALS AND METHODS

Data collection and cell culture

For this study, we downloaded the gene expression profile data of HCC patients from the Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). This dataset includes 347 tumors and 50 non-tumor samples. Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) is a public functional genomics data repository. The GSE45436 dataset contained 93 HCC tissue samples and 41 non-cancerous samples. We retrieved data from publicly available databases, hence it was not applicable for additional ethical approval. Then, we selected PLC/PRF/5 and HepG2 cells for the following experiments. Specifically, the cells were purchased from the Chinese National Infrastructure of Cell Line Resource. The culture media used was Dulbecco's Modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, United States), supplemented with 10% fetal bovine serum (FBS). Finally, the cells were placed in an incubator (37 °C; 5% CO_2) as previously described.

Identification of differentially expressed genes

Here, we screened differentially expressed genes (DEGs) between HCC and noncancerous samples using the "limma" R package. The log2FC (fold change) > 1 or < -1 and adjusted *P* values < 0.05 were considered statistically significant. To identify the DEGs shared by the TCGA and GEO databases, we used TCGA data in the experimental group, while GEO data were applied in the verification group. Afterward, the intersections were obtained and visualized using the website (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Identification and screening of bioactive components

For this experiment, we retrieved all phytochemicals of the four components of Huanglian decoction from the TCM System Pharmacology Database and Analysis Platform (TCMSP) (http://tcmspw.com/tcmsp.php). Based on the standards recommended by the TCMSP, we selected an oral bioavailability (OB) index ≥ 30% and drug-likeness (DL) index ≥ 0.18 to determine the pharmacological properties of the compound. Notably, compounds that met all these criteria were considered biologically active ingredients[30-32].

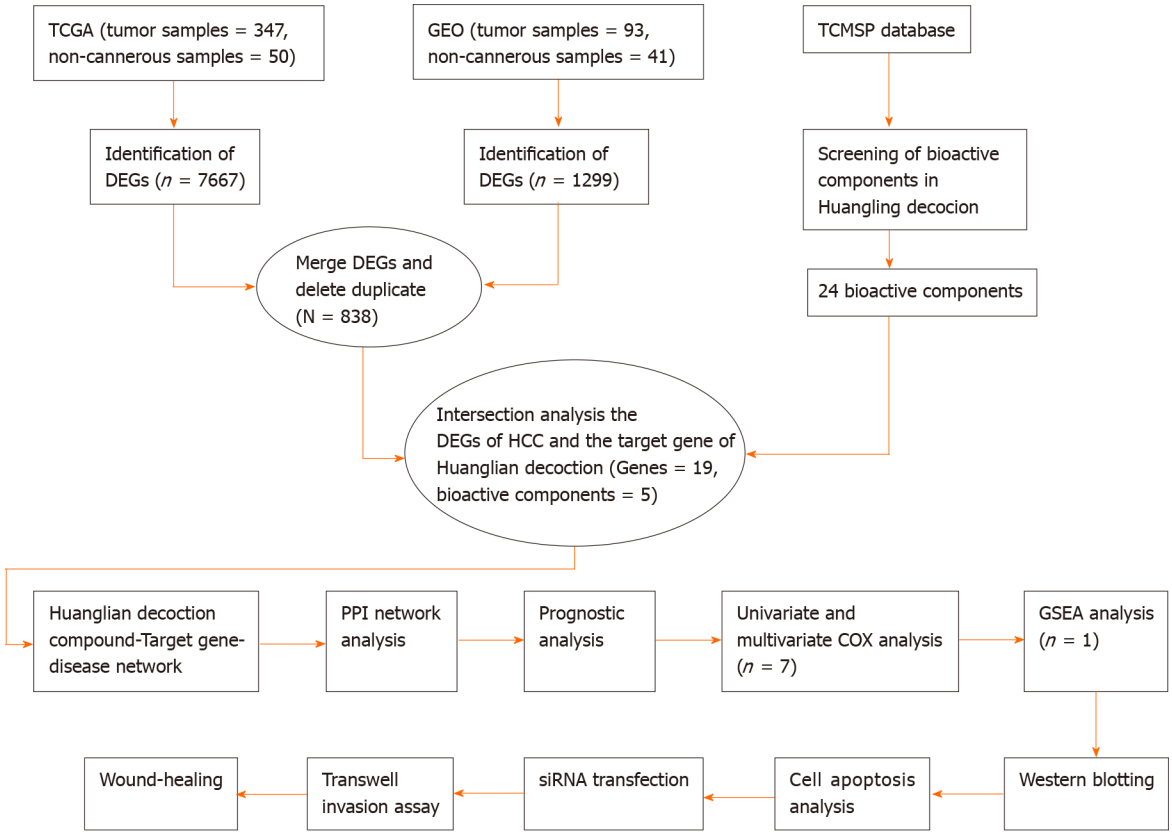


Figure 1 Flow chart. TCGA: The Cancer Genome Atlas; GEO: Gene Expression Omnibus; TCMSP: Traditional Chinese Medicine System Pharmacology Database and Analysis Platform; DEGs: Differentially expressed genes; HCC: Hepatocellular carcinoma; PPI: Protein-protein interaction; GSEA: Gene set enrichment analysis.

Construction of the network

To comprehensively understand the molecular mechanisms of Huanglian decoction, we collectively analyzed DEGs and active ingredients of the drug. Using Cytoscape version 3.7.2, we constructed Huanglian decoction-compound, compound-target genes, and target gene-disease networks[33-35]. In addition, to further understand the relationship between target genes, we constructed a protein-protein interaction (PPI) network according to the information retrieved from the Search Tool for the Retrieval of Interacting Genes (STRING; https://string-db.org/) online database.

Prognostic analysis of key genes

Key genes in the network were extracted, and survival analyses were performed using R software ("survival" packages) to identify genes associated with prognosis, followed by univariate and multivariate Cox analyses of genes associated with prognosis.

Gene set enrichment analyses

To explore the potential molecular mechanisms of key genes, we performed a Gene Set Enrichment Analysis (GSEA) using the GSEA software version 4.0.3. The number of random sample permutations was set at 1000, while the significance threshold was set at $P < 0.05$[36-39].

Preparation of Huanglian decoction aqueous extract

The formula of Huanglian decoction used in this study is different from the previously reported formula. This formulation consisted of 50 g of *Coptidis Rhizoma*, *Zingiberis Rhizoma* (25 g), *Folium Artemisiae Argyi* (25 g), and *Mume Fructus* (10 g). All the above-mentioned herbs were acquired from the Chinese TongRenTang pharmacy. Briefly, all herbs were mixed and soaked for 30 min, then boiled in a casserole (1000 mL of distilled water) for 2 h. Subsequently, the supernatant was centrifuged at 8000 rpm (4293 × g) for 30 min, at which point the whole procedure was repeated twice and the supernatants were mixed together and then evaporated to dryness. Finally, the precipitate was redissolved in distilled water to a concentration of 10 mg/mL and filtered using a 0.22 μm pore-size filter and stored at 4 °C. The active compound of

Huanglian Decoction (berberine hydrochloride) was approximately 0.26 mg/mL, which was analyzed by high-performance liquid chromatography (Alliance 2695, Waters, Milford, MA, United States) on an Inertsil ODS-2 C18 analytical column (4.6 mm × 250 mm, 5 μm) with a detection wavelength of 345 nm. It was eluted with acetonitrile-0.05 moL/L phosphoric acid aqueous solution (50:50), the flow rate was 1.0 mL/min, and the column temperature was 30 °C.

Wound-healing and transwell invasion assay

For the wound healing assay, we treated cells with different concentrations of Huanglian decoction (50 and 100 μg/mL for PLC/PRF/5 cells, 100 and 200 μg/mL for HepG2 cells) and then analyzed wound healing[40-42]. On the other hand, for transwell invasion assay, we treated HCC cells (1×10^5 cells/well) under different conditions, then fixed them with 4% paraformaldehyde for 2 h, followed by staining the invaded cells with crystal violet solution for 30 min. Finally, we counted the stained cells under an optical microscope.

SiRNA transfection

SiRNA targeting CCNB1 (CCNB1-siRNA, GAAUUCUGCACUAGUUCAA), was designed and synthesized by Nanjing InvivoGene Biotechnology Co., Ltd. (Nanjing, China). When the cells reached 70%-80% confluence, we performed siRNA transfection using Lipofectamine 2000 (Invitrogen, United States) according to the manufacturer's instructions.

Cell apoptosis analysis

To identify apoptotic cells, HCC cells were treated under different conditions. Using an Annexin V-fluorescein isothiocyanate Apoptosis Detection kit (Thermo Fisher Scientific, Waltham, MA, United States) after 48 h, we performed Annexin V and PI staining. Cell apoptosis was detected using a flow cytometer (arachidonyl-2'-chloroethylamide, NovoCyteTM).

Cell cycle analysis

Cells were seeded (4×10^5 cells/well) in 60 mm dishes with 10% FBS and incubated overnight at 37 °C in a 5% CO_2 incubator. The HCC cells were then treated under different conditions (48 h). The cells were collected, incubated in ice-cold 70% ethanol, and fixed overnight at 4 °C. The cells were then washed twice with poly (butylene succinate) (PBS) and incubated with 20 mg/mL RNase A and 200 mg/mL propidium iodide in PBS at room temperature for 30 min in the dark. Finally, flow cytometry was used for the analysis.

Western blotting analysis

HCC cells in the logarithmic phase were seeded in a 10 cm dish. When the cells occupied 80% of the dish, they were treated with Huanglian decoction for 24 h in culture medium containing 10% FBS. The harvested cells were then disrupted, and the protein concentration was determined using a dye-binding protein assay kit according to the manufacturer's instruction manual. After centrifugation at 12000 rpm (9660 × *g*) for 30 min, the supernatants were collected for protein measurement. After the samples were immersed in water at a temperature of 95 °C for 10 min, the proteins were separated using gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Thereafter, the membranes were incubated overnight at 4 °C with the primary antibody [Rabbit monoclonal (Y106) to Cyclin B1, ab32053, 1/30000 dilution, Abcam, United States] and then further incubated with the secondary antibody (Goat Anti-Rabbit immunoglobulin G H&L (horseradish peroxidase), ab205718, 1/5000 dilution, Abcam, United States). The results were then observed after X-ray exposure, development, and fixation of membranes in a dark environment.

Statistical analysis

Based on three independent experiments, data are expressed as mean ± SEM. All statistical analyses were implemented using R software version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 8.0.1 (GraphPad Software Inc., United States). If not specified above, a level of $P < 0.05$ was considered statistically significant.

RESULTS

Identification of DEGs in HCC

We conducted this study as illustrated in the flow chart (Figure 1). To identify the common DEGs in the TCGA and GEO datasets, we performed differential gene analysis on the two datasets. We identified a total of 7667 DEGs at the mRNA level in tumor tissues (n = 374) compared with normal tissues (n = 50) based on the analysis of the TCGA dataset (Figure 2A). On the other hand, we identified a total of 1299 DEGs at the mRNA level in tumor tissues (n = 93) compared with normal tissues (n = 41) according to the GEO dataset analysis (Figure 2B). In summary, these results show an overlap of 838 DEGs (Figure 2C), between the TCGA and GEO datasets.

Identification of bioactive compounds in Huanglian decoction

The four main active ingredients in Huanglian decoction are *Coptidis Rhizoma*, *Zingiberis Rhizoma*, *Folium Artemisiae Argyi*, and *Mume Fructus*. We retrieved the main chemical constituents of the four components from the TCMSP analytical platform and selected OB ≥ 30% and DL index ≥ 0.18 to determine the druggability of the compounds. Overall, we identified 765 bioactive compounds of the four main herbs, which were reduced to 24 after deleting duplicates (Supplementary Table 1).

Huanglian decoction network analysis and PPI network analysis

To further understand the target genes of Huanglian decoction, we constructed a regulatory network. First, the DEGs of disease and the target gene of Huanglian decoction were subjected to intersection analysis, and 19 common genes were found (Figure 3A and Supplementary Table 2). Then, we constructed the Huanglian decoction-compound, compound-target genes, and target gene-disease networks. As shown in Figure 3B, we uncovered 5 biologically active substances (Quercetin, Stigmasterol, beta-sitosterol, Worenine, and kaempferol) and 19 target genes (*HSPB1*, *CCNB1*, *CYP1A2*, *BIRC5*, *NQO1*, *CDK1*, *FOS*, *CHEK1*, *TOP2A*, *E2F1*, *CHEK2*, *MAP2*, *SERPINE1*, *ADH1C*, *AKR1C3*, *IGFBP3*, *CYP3A4*, *MMP9*, and *PARP1*). Finally, we constructed a protein interaction network for these 19 genes (Figure 3C) and screened the top 10 genes according to the number of nodes (Figure 3D).

Analysis of the expression levels of hub genes and survival analysis

In all, 10 genes were identified as hub genes. The survival analysis of the hub genes was evaluated using a Kaplan-Meier curve (Figure 4). Overall, we identified 7 genes that were significantly associated with prognosis [*E2F1* (P = 0.005), *MMP9* (P = 0.014), *CDK1* (P = 1.36e-04), *CHEK1* (P = 7.838e-04), *CCNB1* (P = 0.002), *BIRC5* (P = 4.06e-05), and *TOP2A* (P = 0.001)]. We then analyzed the expression of these genes in tumors and normal tissues and found that all except *FOS* were highly expressed in tumors.

Identification of key prognostic genes in HCC

To further examine the role of key genes in prognosis, we performed univariate and multivariate Cox analyses of these genes. From the results of the univariate Cox analysis, we noted that *CCNB1* [hazard ratio (HR) = 1.467, 95%CI: 1.252-1.719; P < 0.001], *CDK1* (HR = 1.421, 95%CI: 1.207-1.673; P < 0.001), *CHEK1* (HR = 1.919, 95%CI: 1.436-2.564; P < 0.001), *E2F1* (HR = 1.134, 95%CI: 1.083-1.418; P = 0.002), *MMP9* (HR = 1.134, 95%CI: 1.023-1.256; P = 0.017), *TOP2A* (HR = 1.293, 95%CI: 1.133-1.476; P < 0.001), and *BIRC5* (HR = 1.342, 95%CI: 1.171-1.538; P < 0.001) were all associated with poorer outcomes (Figure 5A). From the multivariate Cox analysis, we found that only *CCNB1* (HR = 1.040, 95%CI: 1.011-1.070; P = 0.007) was associated with a worse prognosis (Figure 5B). These findings revealed that only *CCNB1* can serve as an independent prognostic gene. Subsequently, we noted that *CCNB1* was associated with tumor development (Figure 5C) and with multiple biological pathways (Table 1 and Figure 5D). Finally, we performed GO (Supplementary Figure 1) and KEGG (Supplementary Figure 2) enrichment analyses on *CCNB1* using R software.

Huanglian decoction suppressed HCC cell growth in vitro

Here, we performed cell viability assays on liver cancer cells, the outcomes of which are depicted in Figure 6A. These findings demonstrate that Huanglian decoction exhibited time- and dose-dependent effects on HCC cell viability. The IC_{50} value of Huanglian decoction after 48 h of treatment was approximately 100 μg/mL for PLC/PRF/5 and 200 μg/mL for HepG2 cells. Moreover, the apoptosis analysis indicated that this decoction can induce apoptosis in both PLC/PRF/5 and HepG2 cells (Figure 6B). Interestingly, further analysis showed that this decoction can reduce

Table 1 Gene set enrichment analysis

Name	ES	NES	NOM *P* value	FDR *q*-value
KEGG_CELL_CYCLE	0.78	2.24	0.000	0.000
KEGG_BASE_EXCISION_REPAIR	0.82	2.23	0.000	0.000
KEGG_RNA_DEGRADATION	0.76	2.17	0.000	0.000
KEGG_SPLICEOSOME	0.81	2.16	0.000	0.000
KEGG_OOCYTE_MEIOSIS	0.66	2.14	0.000	0.000
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	-0.80	-2.27	0.000	0.000
KEGG_DRUG_METABOLISM_CYTOCHROME_P450	-0.72	-2.17	0.000	0.000
KEGG_RETINOL_METABOLISM	-0.73	-2.16	0.000	0.000
KEGG_PRIMARY_BILE_ACID_BIOSYNTHESIS	-0.92	-2.09	0.000	0.000
KEGG_TRYPTOPHAN_METABOLISM	0.73	-2.09	0.000	0.000

NOM *P* value < 0.01 was statistically significant. FDR: False discovery rate.

the migration and invasion activities of PLC/PRF/5 and HepG2 cells (Figure 6C and D).

Huanglian decoction suppressed HCC cell growth in vitro by inhibiting the expression level of CCNB1 protein

To explore the potential mechanism of Huanglian decoction on HCC cells, we first identified the protein level. Our results highlighted that this decoction can reduce the CCNB1 protein expression levels in both PLC/PRF/5 and HepG2 cells. Furthermore, we noted that after adding this decoction (100 μg/mL for PLC/PRF/5 cells and 200 μg/mL for HepG2 cells) to the si-CCNB1 group, the protein level did not change significantly (Figure 7A and B). Notably, the results of cell proliferation showed that the growth rate of cells was significantly reduced after transfection. Another important finding was that when this decoction was added to the transfected cells, the growth rate of the cells did not change significantly (Figure 7C and D). Remarkably, the findings of cell apoptosis analysis revealed that inhibiting the expression of *CCNB1* can promote apoptosis of both PLC/PRF/5 and HepG2 cells, whereas adding Huanglian decoction to the cells after transfection did not significantly promote apoptosis (Figure 7E and F). The cell cycle analysis revealed that Huanglian decoction treatment caused a significant accumulation of cells in G2/M phase in both cell lines (Figure 7G and H), which may be regulated by CCNB1.

Protein expression of CCNB1, Bax, caspase 3, caspase 9, CDK1, p53, and p21 in the different groups by western blot

To further explore the effect of Huanglian decoction on HCC cells, we analyzed the expression levels of several key proteins following treatment of PLC/PRF/5 cells with Huanglian decoction for 48 h (Figure 8A). From the results shown in Figure 8B, we found that the expression levels of apoptosis-related proteins (Bax, caspase 3 and caspase 9) in the Huanglian decoction treatment group and the si-CCNB1 group were upregulated.

Western blotting was performed to examine the expression levels of key regulators responsible for cell cycle regulation (G2/M), including CCNB1, CDK1, and p21. The complex CyclinB1/CDK1 formation is the essential player in G2/M transition, and the decrease of CyclinB1/CDK1 formation will induce G2/M arrest. As shown in Figure 8B, the expression levels of CDK1 and CCNB1 in the Huanglian decoction treatment group and the si-CCNB1 group were both decreased, while the expression level of p21 was significantly increased.

It is well known that the p53 pathway plays an important role in the process of cancer treatment. Jin *et al*[43] noted that CCNB1 silencing significantly promoted activation of the p53 pathway in HCC cells[43]. We have shown that Huanglian decoction works mainly by inhibiting *CCNB1*, and thus, we speculate that Huanglian decoction may affect the p53 pathway. We first verified the expression of CDK1, p53, and p21 in the p53 pathway and found that in the Huanglian decoction treatment

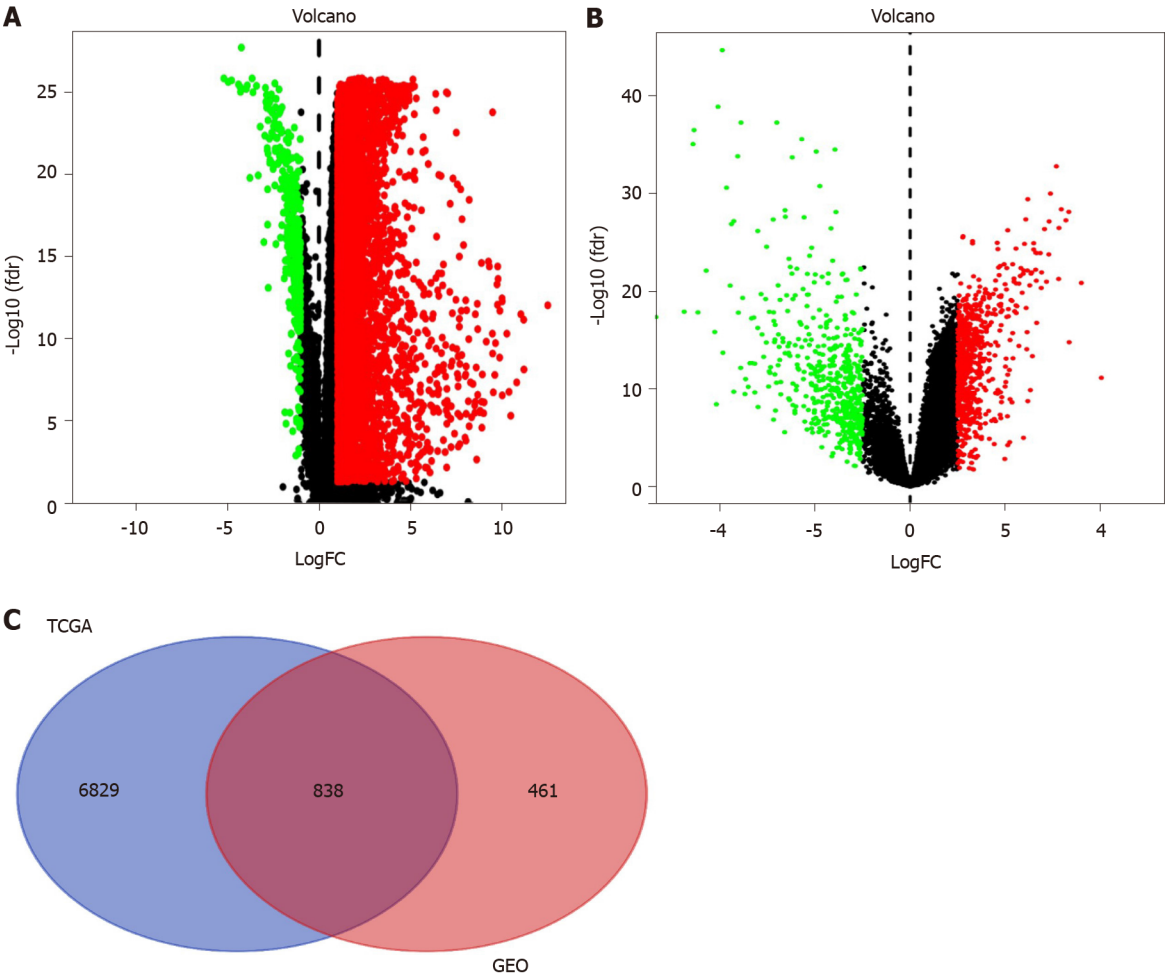


Figure 2 Volcano and Venn diagrams of differentially expressed genes. A: Differential gene analysis of The Cancer Genome Atlas (TCGA) datasets; B: Differential gene analysis of Gene Expression Omnibus (GEO) datasets; C: The TCGA and GEO datasets show an overlap of 838 genes. Upregulated genes are marked in red; downregulated genes are marked in light green. TCGA: The Cancer Genome Atlas; GEO: Gene Expression Omnibus.

group and the si-CCNB1 group, the expression of CDK1 was significantly reduced, while the expression of p53 and p21 was greatly increased (Figure 8B).

DISCUSSION

In this study, we found that Huanglian decoction exhibits an effective therapeutic effect on HCC, primarily by inhibiting the expression level of *CCNB1*. Based on the analysis of TCGA and GEO databases, we identified DEGs in HCC. Subsequently, we also uncovered the main bioactive compounds associated with HCC in Huanglian decoction and key target genes using network pharmacological analysis. Simultaneously, we further screened key genes through PPI network and survival analyses and ultimately, selected 7 genes (*E2F1*, *MMP9*, *CDK1*, *CHEK1*, *CCNB1*, *BIRC5*, and *TOP2A*) that met the conditions. We then performed univariate and multivariate Cox analyses on these 7 genes. The outcomes indicated that *CCNB1* can be used as an independent prognostic indicator. Another important finding is that *CCNB1* is involved in the cell cycle, base excision repair, RNA degradation, spliceosome, oocyte meiosis, complement and coagulation cascades, drug metabolism-cytochrome P450, retinol metabolism, primary bile acid biosynthesis, and the tryptophan metabolism signaling pathway of HCC (Figure 5D). Previous studies established that the normal process of cell division occurs through the cell cycle. However, dysregulation of the cell cycle would result in sustained unscheduled cell growth, proliferation, and eventually a hallmark of cancer. Base excision repair is a key genomic maintenance pathway that possesses a tumor-suppressing effect. Therefore, *CCNB1* may regulate the development of HCC through these pathways. We also established an *in vitro* experimental model. Our results show that Huanglian decoction

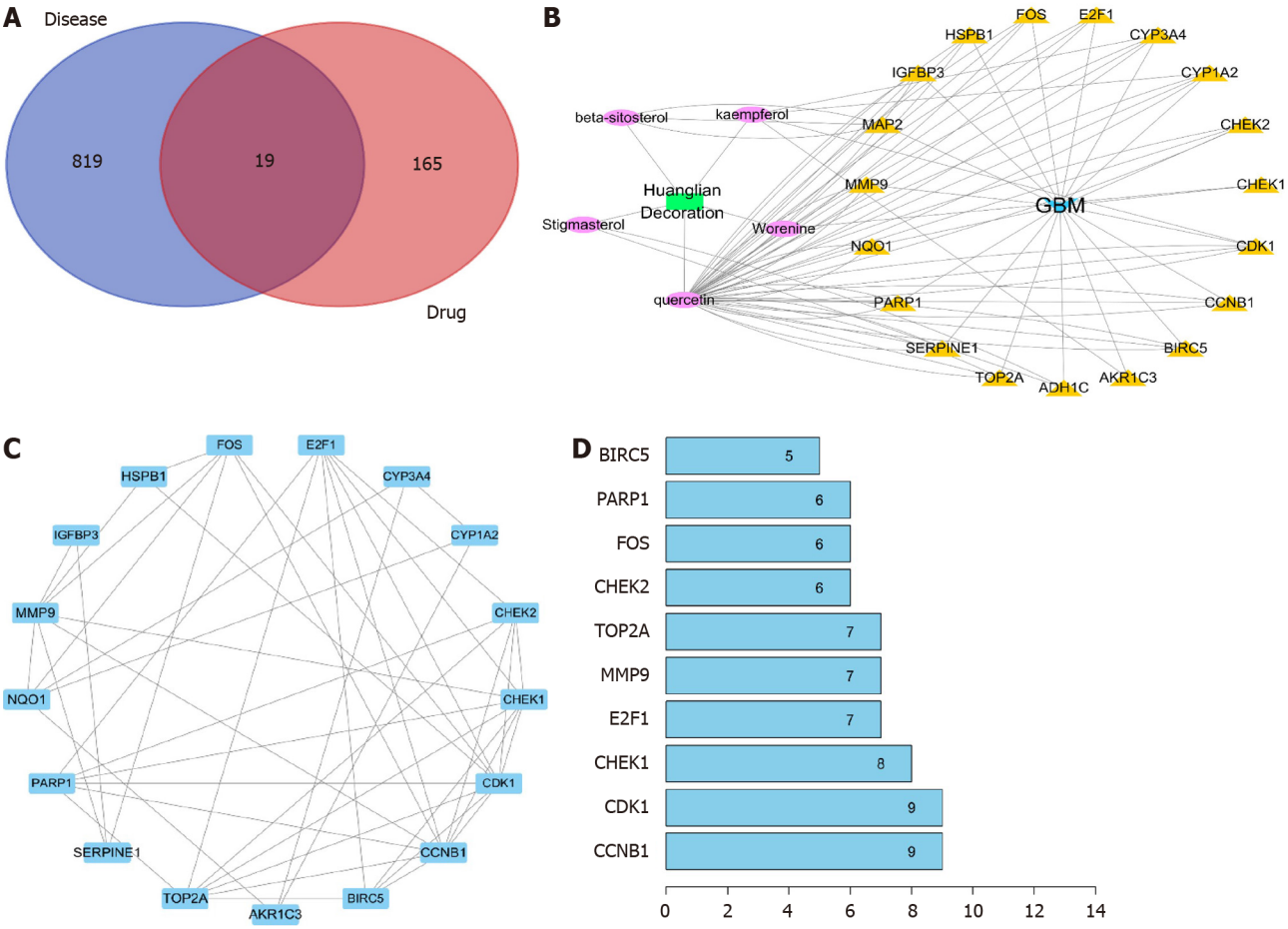
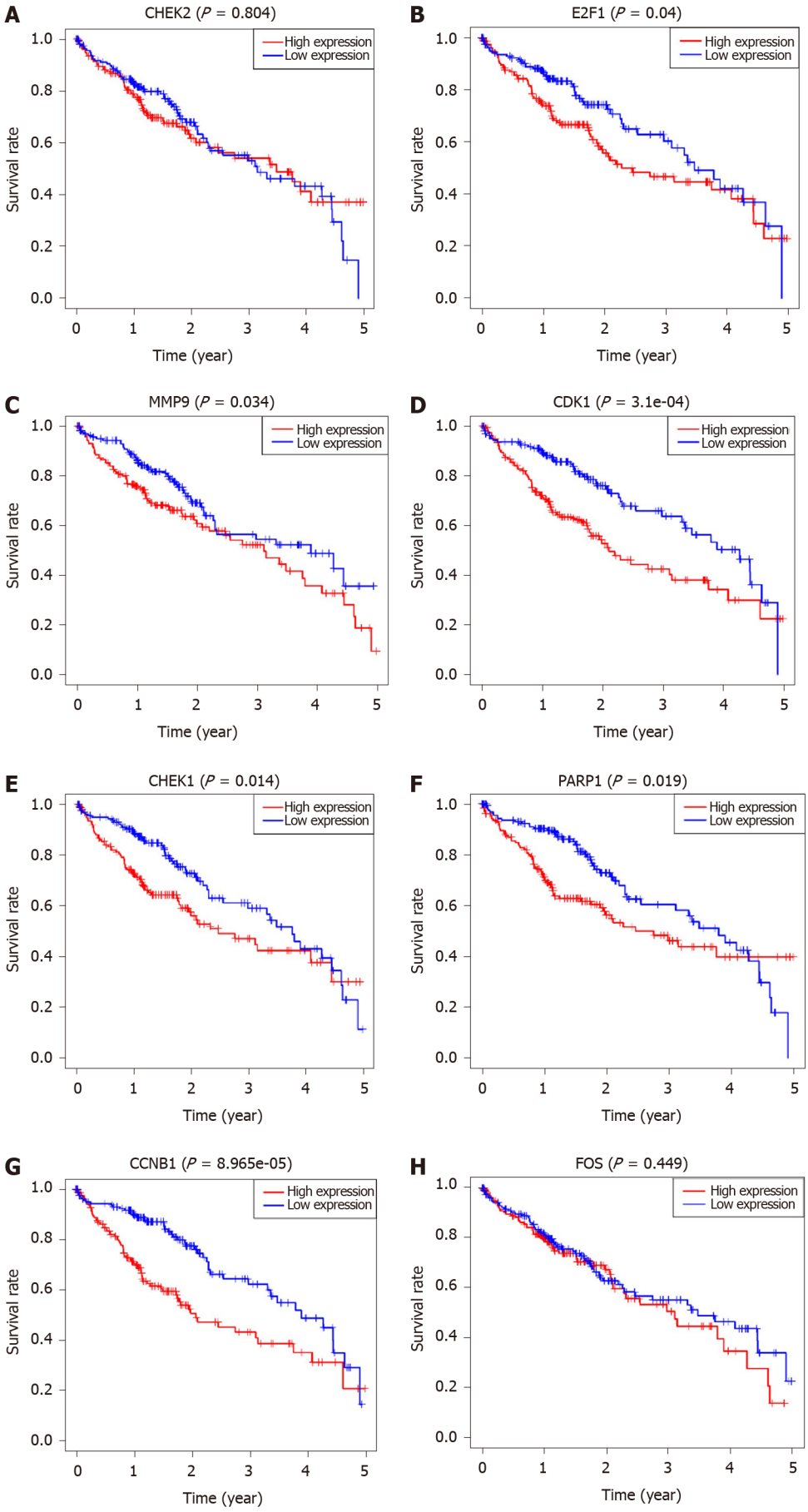


Figure 3 Identification of key genes. A: 19 genes overlap between differentially expressed genes of disease and drug target genes; B: Herb-Compound network of potential bioactive constituents for Huanglian decoction. The circles on the left are the drugs and their bioactive compounds, while the circles on the right are the target genes; C: Protein-protein interaction network for target genes. The edges represent the interaction between them; D: The top 10 genes according to the number of nodes.

can inhibit the growth of HCC cells *in vitro*. Further analysis elucidated that this decoction exhibited no obvious inhibitory ability on HCC cells (si-CCNB1). Thus, we speculated that this decoction might inhibit the growth of HCC cells by suppressing the expression of *CCNB1*.

Induction of apoptosis in cancer cells is a key therapeutic strategy for cancer treatment. Following Huanglian decoction treatment, we found that apoptosis of HCC cells was significantly increased and that the expression of apoptosis-related proteins was increased. The results of the si-CCNB1 group and the si-CCNB1 + Huanglian decoction group showed that Huanglian decoction mainly affected cell apoptosis through *CCNB1*. The cell cycle is a complex and complicated process. We found that Huanglian decoction can induce G2/M phase arrest. Cyclin B1 plays an important role in G2/M transition and during M phase[43,44]. The cyclin-dependent kinase inhibitor p21 protein plays an important role in G2 phase arrest[45] and has been shown to contribute to cell cycle arrest through transcriptional repression of cell cycle regulatory genes. Therefore, Huanglian decoction may regulate G2/M cell cycle arrest through p21. Our study showed that CCNB1 silencing suppressed the expression of *CCNB1* and *CDK1* but increased the expression of Bax, caspase 3, caspase 9, p53 and p21. Bax has been reported to be directly activated by p53 in the absence of other proteins to permeabilize mitochondria and initiate the apoptotic program[46-48]. Moreover, p53-induced apoptosis involves triggering the caspase-9 initiator and its downstream caspase-3 executioner. Previous studies have shown that CCNB1 silencing can inhibit cell proliferation and promote cell senescence by activating the p53 signaling pathway in pancreatic cancer[21]. We found that the same phenomenon exists in HCC. Therefore, we speculate that Huanglian decoction can prevent HCC cell progression by inhibiting *CCNB1* expression and activating the p53 signaling pathway.

Multiple reports have emphasized that Chinese medicine formula plays an essential role in the treatment of tumors. For instance, Ze-Qi-Tang (ZQT) can inhibit the growth


A
CHEK2 (P = 0.804)
B
E2F1 (P = 0.04)
C
MMP9 (P = 0.034)
D
CDK1 (P = 3.1e-04)
E
CHEK1 (P = 0.014)
F
PARP1 (P = 0.019)
G
CCNB1 (P = 8.965e-05)
H
FOS (P = 0.449)
High expression
Low expression
Survival rate
Time (year)

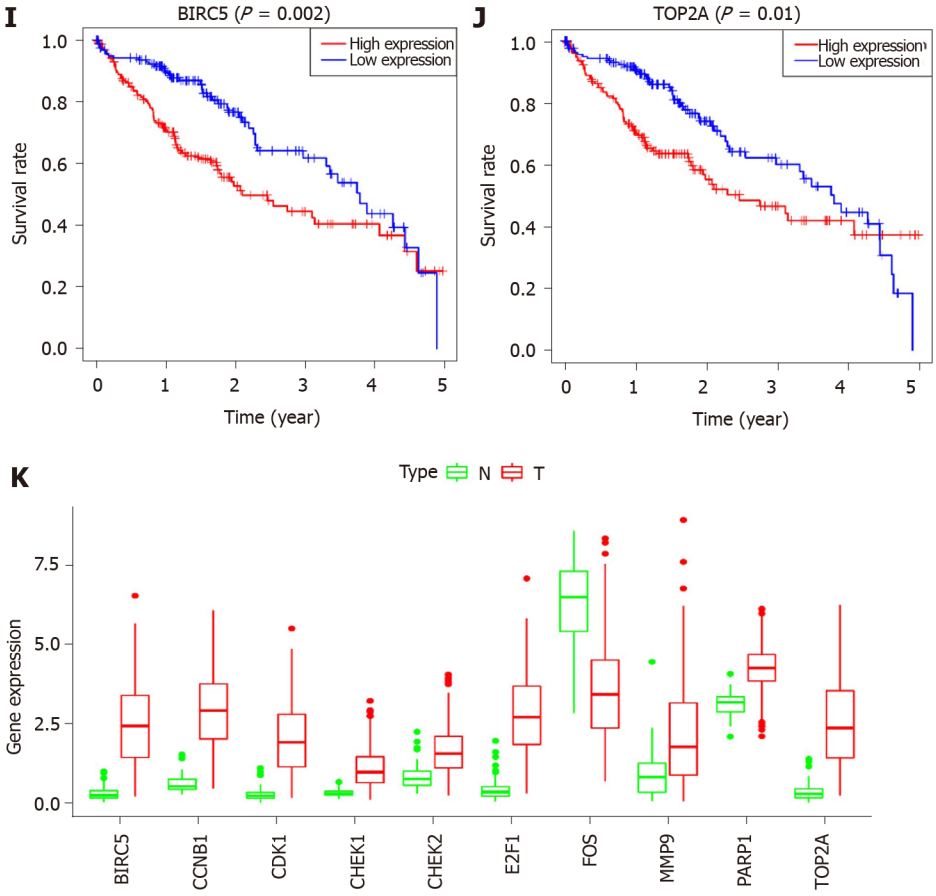


Figure 4 Survival and expression analyses. A-J: Survival analysis of the hub genes; K: Expression analysis of the hub genes. A level of $P < 0.05$ was considered statistically significant.

of non-small cell lung cancer cells[49], whereas Jianpi Jiedu decoction can inhibit the tumorigenesis, metastasis, and angiogenesis of colorectal cancer[50]. In addition, Huanglian Jiedu decoction can induce apoptosis and cell cycle arrest and inhibit the migratory and invasive properties of HCC cells[19,51]. These results further support the idea that TCM formulas are potential complementary and alternative treatments for cancer. Nonetheless, our literature search found that most of the TCM formulas related to Huanglian include Huanglian Jiedu, Huanglian-Wendan, and Huanglian-Renshen decoctions and that studies on Huanglian decoction are scarce. Interestingly, a recent article reported that Huanglian decoction exerts a certain therapeutic effect on diabetes, but the formula source of this decoction is somewhat different from the formula used in this study, and thus, it may not have reference value. However, it is important to note that exploration of the differences among various formulas and their potential medicinal value may be the next step. This is a critical area that should be addressed in future research.

Admittedly, our study had some limitations. First, we did not investigate the mechanism of *CCNB1* in HCC. Moreover, it would be better if *in vivo* experiments were performed to validate our findings.

CONCLUSION

In summary, this study demonstrates that *CCNB1* is an independent prognostic indicator in HCC and is significantly associated with a worse prognosis. We confirmed that Huanglian decoction suppressed HCC cell growth *in vitro* by inhibiting the expression level of the CCNB1 protein. We also explored the mechanism of Huanglian decoction in HCC cells. The results showed that Huanglian decoction can inhibit HCC cell growth by downregulating the expression of *CCNB1* to activate the p53 signaling pathway. *CCNB1* is a potential therapeutic target for liver cancer. Therefore, this information may help in the development of novel HCC treatments.

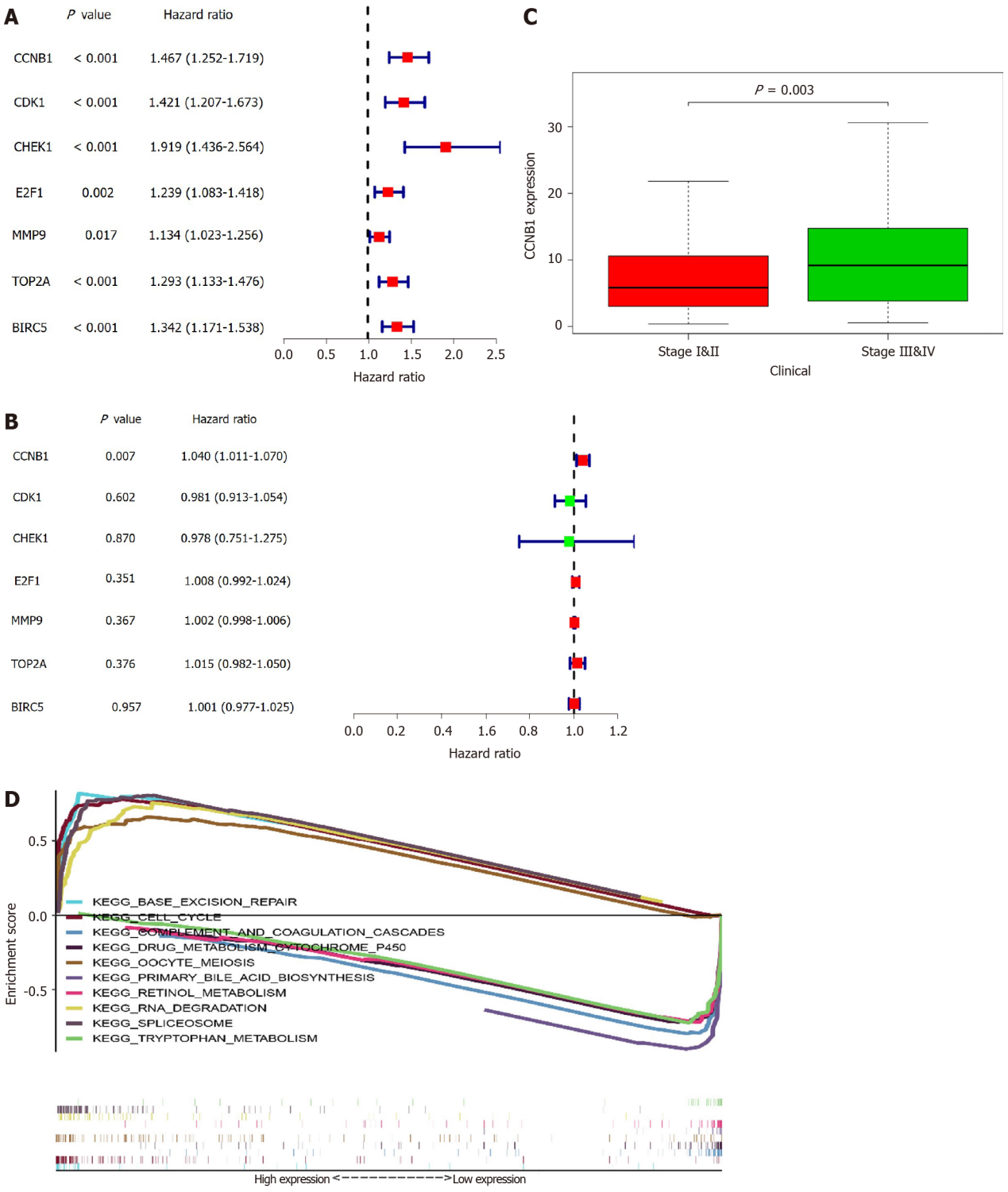
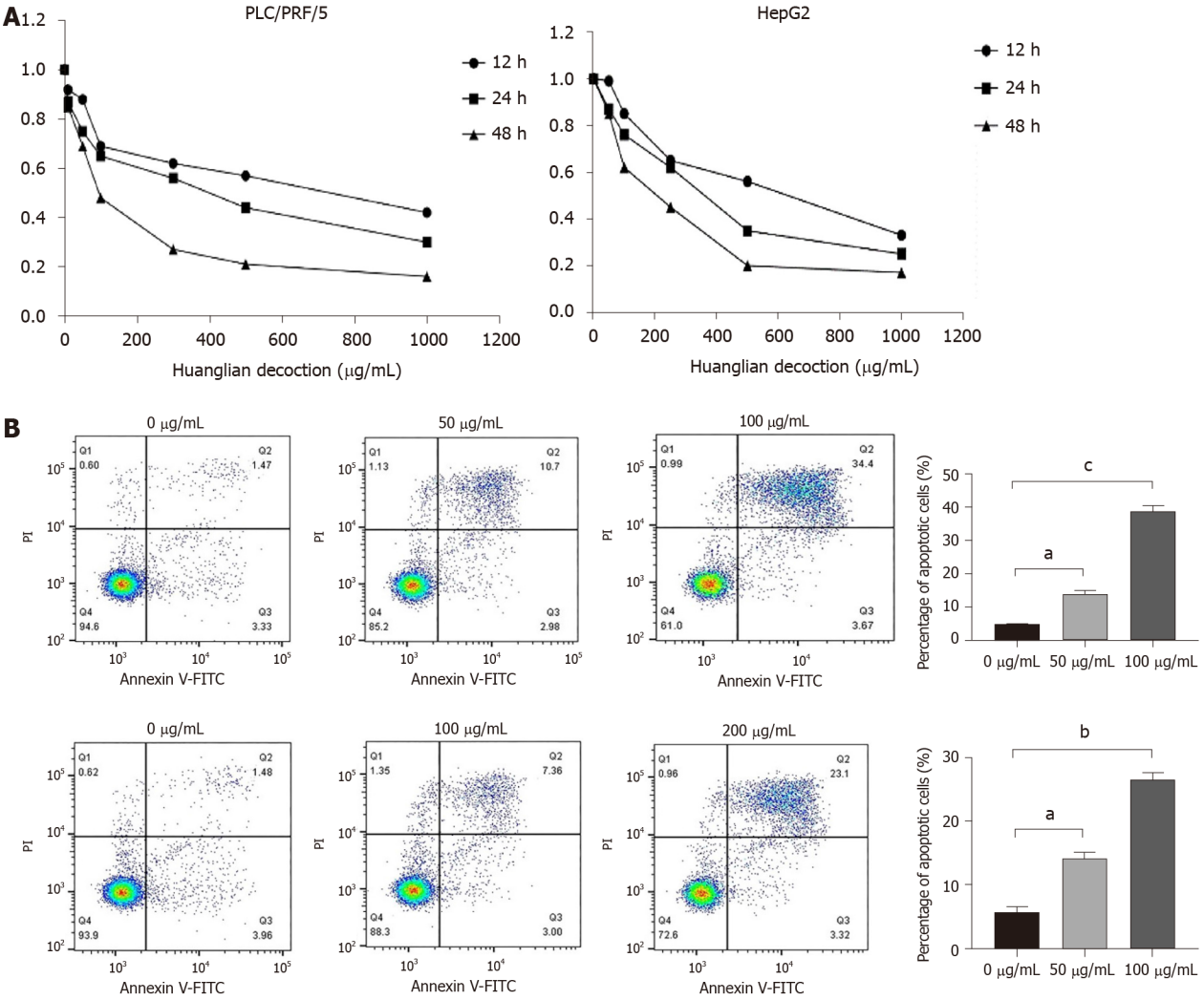


Figure 5 Identification of key prognostic genes in hepatocellular carcinoma. A: Univariate Cox analysis; B: Multivariate Cox analysis; C: *CCNB1* found to promote tumor development; D: The Gene Set Enrichment Analysis reveals the biological pathways and processes associated with *CCNB1*.


A
PLC/PRF/5
HepG2
12 h
24 h
48 h
Huanglian decoction (μg/mL)
B
0 μg/mL
50 μg/mL
100 μg/mL
200 μg/mL
PI
Annexin V-FITC
Q1 0.60
Q2 1.47
Q4 94.6
Q3 3.33
Q1 1.13
Q2 10.7
Q4 85.2
Q3 2.98
Q1 0.99
Q2 34.4
Q4 61.0
Q3 3.67
Q1 0.62
Q2 1.48
Q4 93.9
Q3 3.96
Q1 1.35
Q2 7.36
Q4 88.3
Q3 3.00
Q1 0.96
Q2 23.1
Q4 72.6
Q3 3.32
Percentage of apoptotic cells (%)
a
b
c
0 μg/mL
50 μg/mL
100 μg/mL

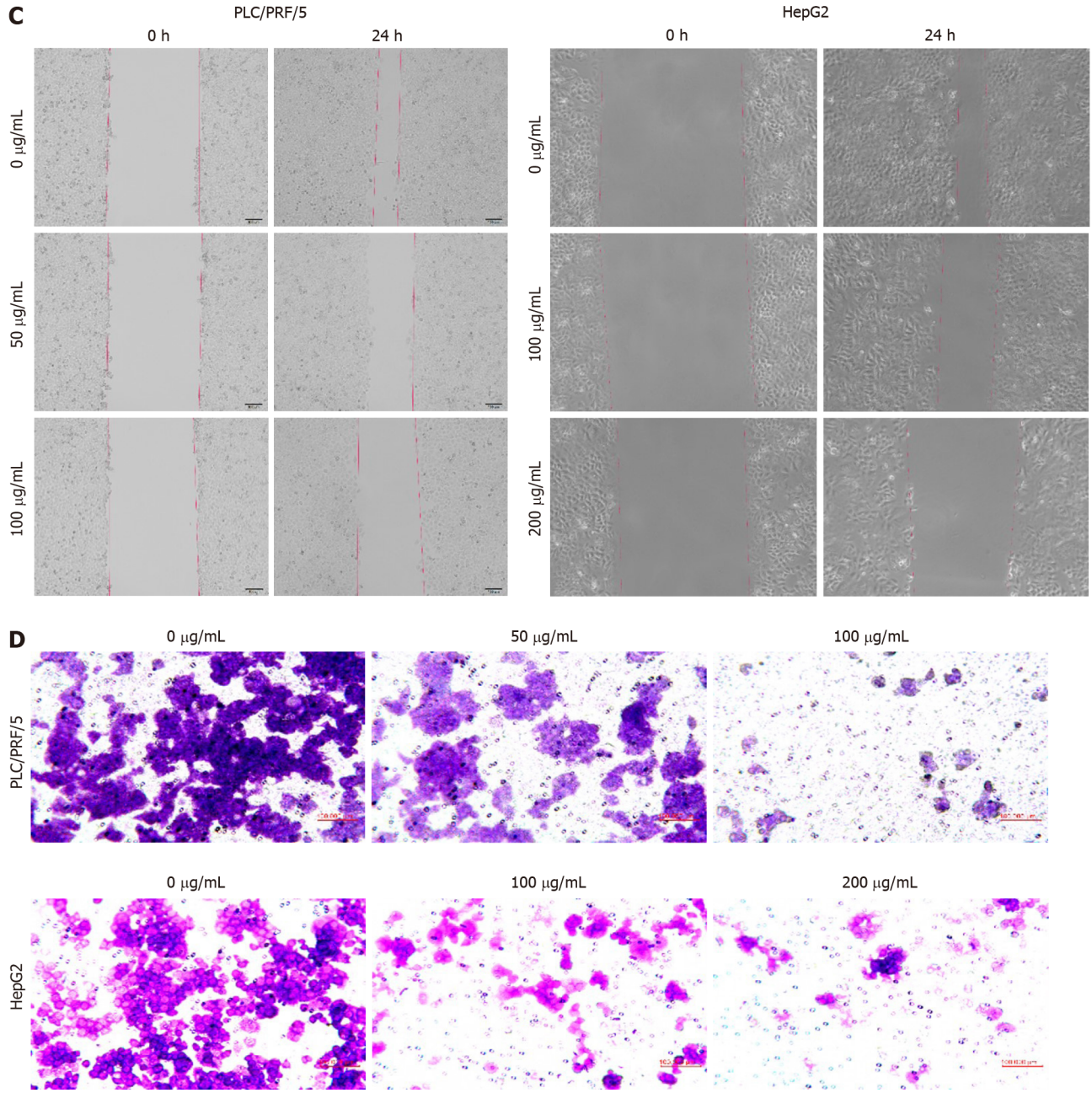
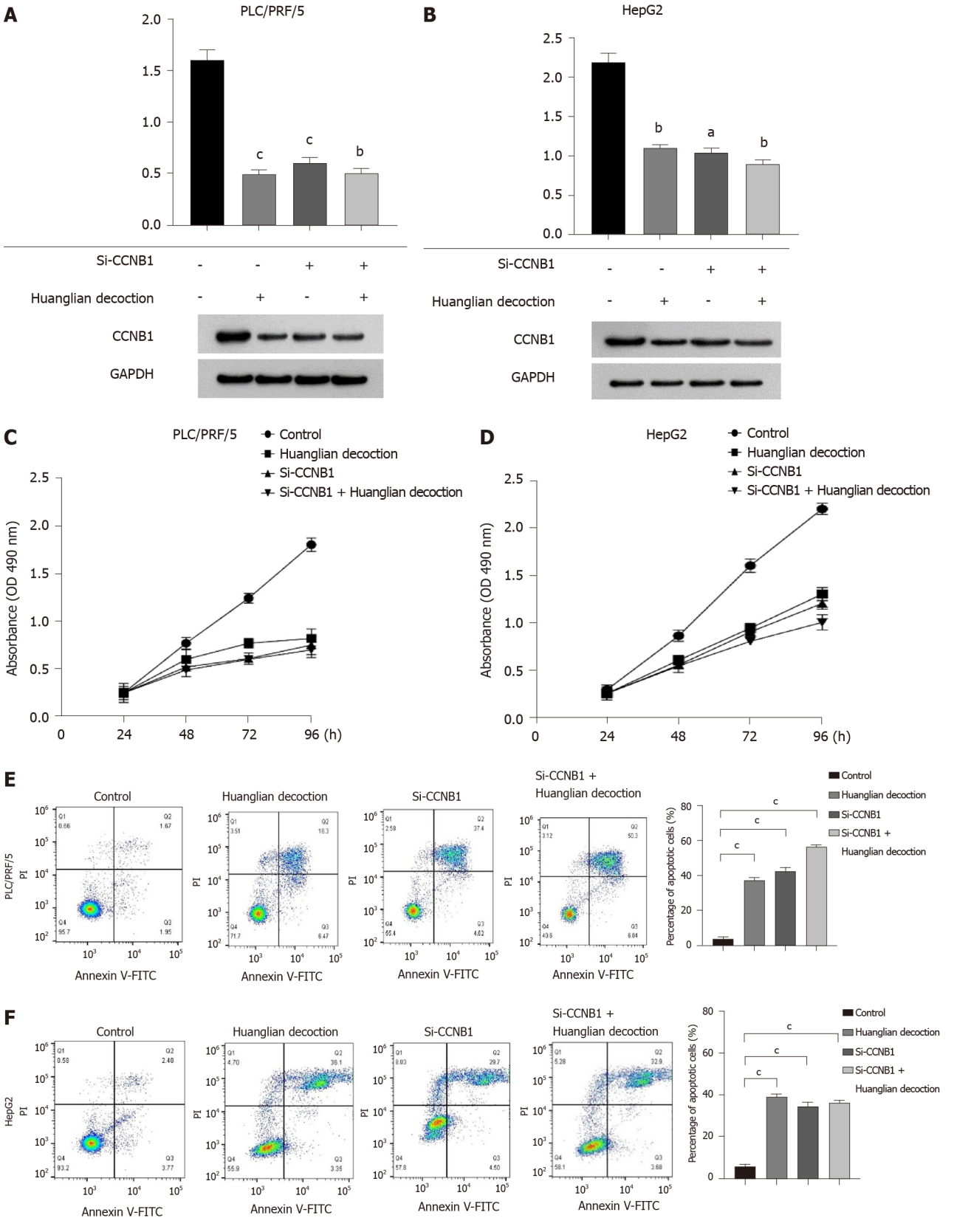


Figure 6 Huanglian decoction suppressed hepatocellular carcinoma cell growth *in vitro*. A: Time- and dose-dependent effects of Huanglian decoction treatment on the viability of both PLC/PRF/5 and HepG2 cells; B: Huanglian decoction can induce apoptosis of both PLC/PRF/5 and HepG2 cells; C: Huanglian decoction can reduce the migration activity of both PLC/PRF/5 and HepG2 cells; D: Huanglian decoction can reduce the invasion activity of both PLC/PRF/5 and HepG2 cells. $^{a}P < 0.05$ *vs* negative control (NC) group; $^{b}P < 0.01$ *vs* NC group; $^{c}P < 0.001$ *vs* NC group. FITC: Fluorescein isothiocyanate.


A
PLC/PRF/5
2.0
1.5
1.0
0.5
0.0
c
c
b
Si-CCNB1 - - + +
Huanglian decoction - + - +
CCNB1
GAPDH
B
HepG2
2.5
2.0
1.5
1.0
0.5
0.0
b
a
b
Si-CCNB1 - - + +
Huanglian decoction - + - +
CCNB1
GAPDH
C
PLC/PRF/5
Control
Huanglian decoction
Si-CCNB1
Si-CCNB1 + Huanglian decoction
Absorbance (OD 490 nm)
0 24 48 72 96 (h)
D
HepG2
Control
Huanglian decoction
Si-CCNB1
Si-CCNB1 + Huanglian decoction
Absorbance (OD 490 nm)
0 24 48 72 96 (h)
E
Control
Huanglian decoction
Si-CCNB1
Si-CCNB1 + Huanglian decoction
PLC/PRF/5
PI
Annexin V-FITC
Percentage of apoptotic cells (%)
Control
Huanglian decoction
Si-CCNB1
Si-CCNB1 + Huanglian decoction
F
Control
Huanglian decoction
Si-CCNB1
Si-CCNB1 + Huanglian decoction
HepG2
PI
Annexin V-FITC
Percentage of apoptotic cells (%)
Control
Huanglian decoction
Si-CCNB1
Si-CCNB1 + Huanglian decoction

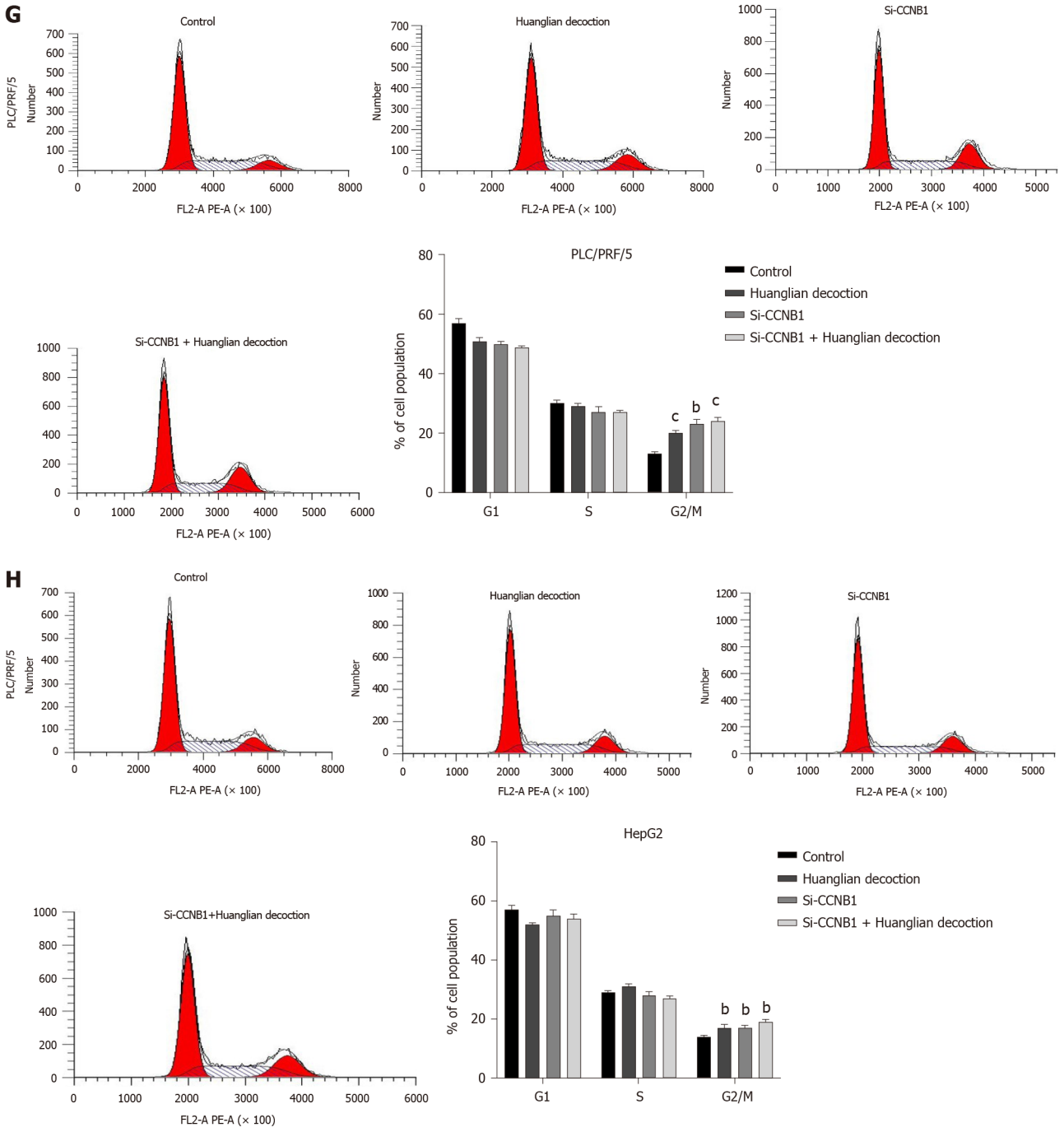


Figure 7 Huanglian decoction suppressed hepatocellular carcinoma cell growth *in vitro* by inhibiting the expression level of CCNB1 protein. A and B: Huanglian decoction can reduce the expression level of CCNB1 protein in hepatocellular carcinoma cells, but has no significant effect on the transfected cells; C and D: At 24, 48, 72, and 96 h after transfection, cell proliferation was determined using the MTT assay; E and F: The level of cell apoptosis increased significantly after transfection, and Huanglian decoction had no significant effect on the cells after transfection; G and H: Huanglian decoction can induce G2/M phase arrest of PLC/PRF/5 and HepG2 cells. $^{a}P < 0.05$ *vs* negative control (NC) group; $^{b}P < 0.01$ *vs* NC group; $^{c}P < 0.001$ *vs* NC group. FITC: Fluorescein isothiocyanate.

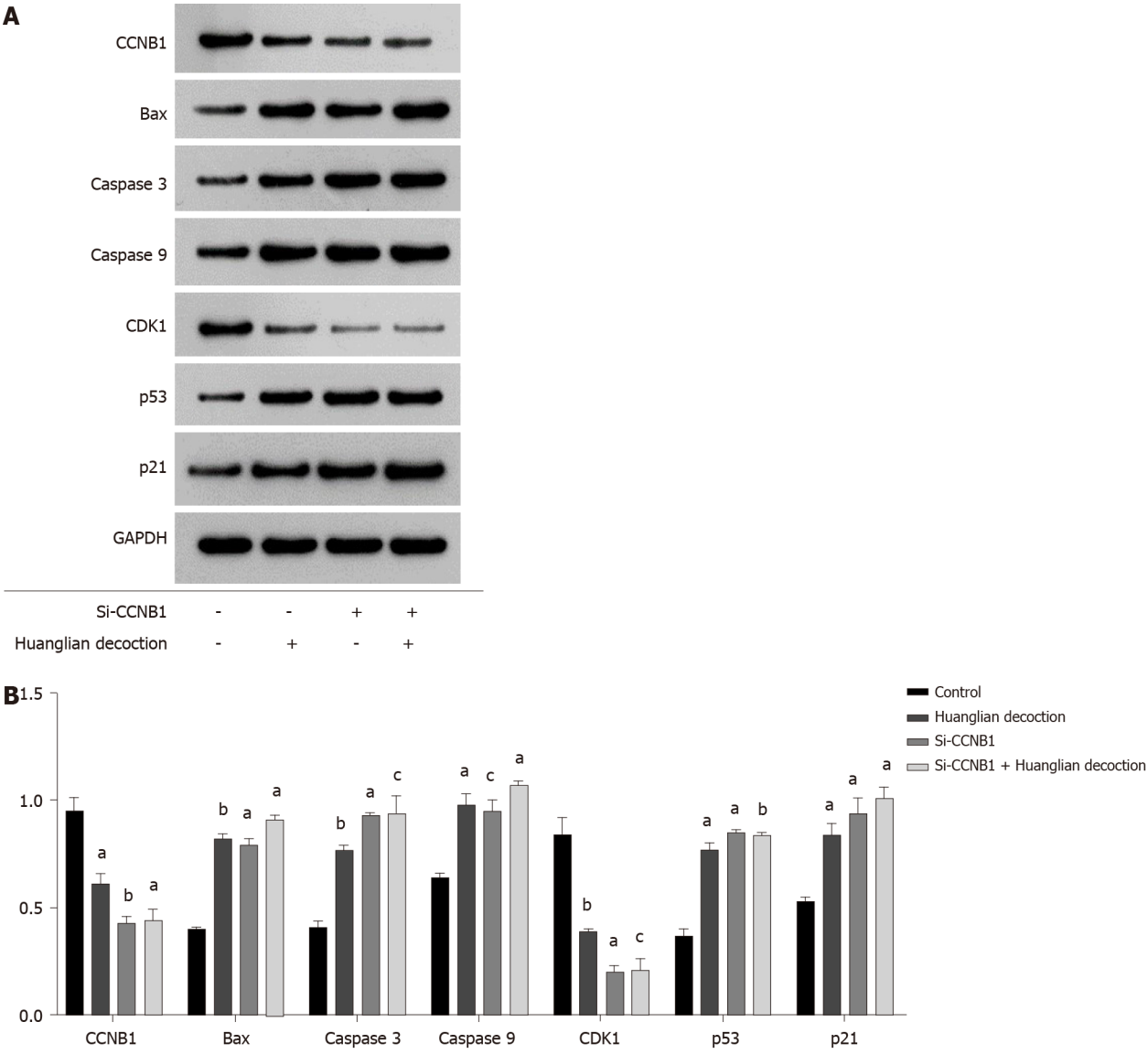


Figure 8 Protein expression of CCNB1, Bax, caspase 3, caspase 9, CDK1, p53, and p21 in the different groups by western blot. A: Western blot analysis results; B: Histogram of protein expression levels. $^{a}P < 0.05$ *vs* negative control (NC) group; $^{b}P < 0.01$ *vs* NC group; $^{c}P < 0.001$ *vs* NC group.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and is the third leading cause of cancer-related mortality worldwide. Huanglian decoction is one of the most important Chinese medicine formulas, with the potential to treat cancer.

Research motivation

In recent years, the application of traditional Chinese medicine in the field of cancer treatment has increased. However, research on Huanglian decoction is still insufficient. We believe that Huanglian decoction may have the potential to treat HCC.

Research objectives

Our research aimed to clarify the effect of Huanglian decoction on HCC cells and to analyze its primary targets and potential functional pathways.

Research methods

In our study, we analyzed the regulatory network of Huanglian decoction and HCC through bioinformatics and network pharmacology and identified key genes. *In vitro* experiments were performed to verify the effect of Huanglian decoction on HCC cells,

and the *CCNB1* gene was knocked down to confirm that it is the main target of Huanglian decoction. Determination of the expression levels of key proteins verified the primary mechanism of Huanglian decoction.

Research results

Huanglian decoction can significantly inhibit the growth, migration and invasiveness of HCC cells and can also induce apoptosis and G2/M phase arrest. The results of the network pharmacological analysis showed that the main target of Huanglian decoction in HCC is *CCNB1*. We verified that Huanglian decoction had an inhibitory effect on liver cancer cells mainly *via CCNB1* downregulation. Following Huanglian decoction treatment, the expression levels of Bax, caspase 3, caspase 9, p21 and p53 in HCC cells were increased, while the expression of *CDK1* and *CCNB1* was significantly decreased. Finally, the p53 signaling pathway plays an important role in this process.

Research conclusions

Huanglian decoction has a significant inhibitory effect on HCC cells. Further analysis showed that Huanglian decoction can inhibit HCC cell growth by downregulating *CCNB1* expression to activate the p53 signaling pathway.

Research perspectives

Huanglian decoction has the potential to treat HCC, and *CCNB1* is a key therapeutic target in HCC.

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ORIGINAL ARTICLE

Clinical Trials Study

Ursodeoxycholic acid as a means of preventing atherosclerosis, steatosis and liver fibrosis in patients with nonalcoholic fatty liver disease

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Institutional review board statement: The study was

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Abstract

BACKGROUND

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of mortality in patients with nonalcoholic fatty liver disease (NAFLD). Weight loss is a key factor for successful NAFLD and CVD therapy. Ursodeoxycholic acid (UDCA), which is one of the first-line therapeutic agents for treatment of NAFLD, is reported to have a beneficial effect on dyslipidemia and ASCVD risk because of antioxidant properties.

AIM

To evaluate the effects of 6 mo of UDCA treatment on hepatic function tests, lipid profile, hepatic steatosis and fibrosis, atherogenesis, and ASCVD risk in men and women with NAFLD, as well as to assess the impact of > 5% weight reduction on these parameters.

METHODS

An open-label, multicenter, international noncomparative trial was carried out at primary health care settings and included 174 patients with ultrasound-diagnosed NAFLD who received 15 mg/kg/d UDCA for 6 mo and were prescribed lifestyle modification with diet and exercise. The efficacy criteria were liver enzymes, lipid profile, fatty liver index (FLI), noninvasive liver fibrosis tests (nonalcoholic fatty liver disease fibrosis score and liver fibrosis index), carotid intima-media thickness (CIMT), and ASCVD risk score. To test statistical hypotheses, the Wilcoxon test, paired *t*-test, Fisher's exact test, and Pearson's chi-squared test were used.

RESULTS

The alanine aminotransferase (ALT) level changed by -14.1 U/L (-31.0; -5.3) from baseline to 3 mo and by -6.5 U/L (-14.0; 0.1) from 3 to 6 mo. The magnitude of ALT, aspartate transaminase, and glutamyltransferase decrease was greater during the first 3 mo of treatment compared to the subsequent 3 mo ($P < 0.001$, $P < 0.01$, $P < 0.001$, respectively). At 6 mo, in the total sample, we observed a statistically significant decrease in body weight and levels of FLI: 84.9 ± 10.4 *vs* 72.3 ± 17.6, $P < 0.001$, total cholesterol: 6.03 ± 1.36 *vs* 5.76 ± 1.21, $P < 0.001$, low-density lipoprotein: 3.86 ± 1.01 *vs* 3.66 ± 0.91, $P < 0.001$, and triglyceride: 3.18 (2.00; 4.29) *vs* 2.04 (1.40; 3.16), $P < 0.001$. No effect on nonalcoholic fatty liver disease fibrosis score or liver fibrosis index was found. The CIMT decreased significantly in the total sample (0.985 ± 0.243 *vs* 0.968 ± 0.237, $P = 0.013$), whereas the high-density lipoprotein ($P = 0.036$) and 10-year ASCVD risk ($P = 0.003$) improved significantly only in women. Fifty-four patients (31%) achieved > 5% weight loss. At the end of the study, the FLI decreased significantly in patients with (88.3 ± 10.2 *vs* 71.4 ± 19.6, $P < 0.001$) and without > 5% weight loss (83.5 ± 10.3 *vs* 72.8 ± 16.7, $P < 0.001$). The changes in ALT, aspartate transaminase, glutamyltransferase, total cholesterol, and low-density lipoprotein levels were similar between the subgroups.

CONCLUSION

UDCA normalizes liver enzymes greatly within the first 3 mo of treatment, improves lipid profile and hepatic steatosis independent of weight loss, and has a positive effect on CIMT in the total sample and 10-year ASCVD risk in women after 6 mo of treatment.

Key Words: Ursodeoxycholic acid; Nonalcoholic fatty liver disease; Liver function tests; Fatty liver index; Carotid intima-media thickness; Atherosclerotic cardiovascular disease

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Core Tip: An open-label, multicenter, international noncomparative trial demonstrated the effect of ursodeoxycholic acid (UDCA) on hepatic steatosis and fibrosis,

reviewed and approved by the Russian Scientific Liver Society Review Board. Approval No. 003.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at liver.orc@mail.ru. Informed consent was not obtained but the presented data are anonymized and risk of identification is low.

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atherogenesis, and atherosclerotic cardiovascular disease risk in 174 ultrasound-diagnosed nonalcoholic fatty liver disease patients who received 15 mg/kg/d UDCA for 6 mo and were prescribed lifestyle modifications with diet and exercise. UDCA had a bidirectional positive effect on the liver and cardiovascular system in patients with and without > 5% weight loss: It improved liver enzymes, lipid profiles, and hepatic steatosis in addition to improving carotid intima-media thickness and atherosclerotic cardiovascular disease risk.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is currently the leading cause of liver disease and liver transplantation in developed countries; the number of people with this pathology is steadily growing[1,2]. NAFLD-related morbidity in the world is approximately 25%, being the highest in Middle Eastern and South American countries, while in Europe and Asia, the morbidity is approximately the same[3].

According to the DIREG 1, DIREG_L_01903, and DIREG 2 studies, up to 27% of the population suffers from NAFLD in Russia[4]. In Kazakhstan, the morbidity is almost 30%[5], and in Uzbekistan, it reaches 37%[6]. Among the NAFLD risk factors, obesity is considered to be one of the key factors: a body mass index (BMI) increase of 1 kg/m^2 is associated with a 13%-38% increased risk of NAFLD development[7]. Such a high morbidity puts this disease among the most important diseases in primary health care.

NAFLD is often associated with cardiovascular diseases (CVDs), which have become the main cause of decreased life expectancy in patients[8,9]. According to one prospective study with over almost three decades of follow-up, 30% of deaths in patients with NAFLD were due to CVD, and 19% were due to liver disease[10].

The gold standard methods for the diagnosis of NAFLD are liver biopsy, proton magnetic resonance spectroscopy, and quantitative fat/water selective magnetic resonance imaging.

An ultrasound examination (US), a less expensive and more accessible method, is preferred for hepatic steatosis detection in primary health care settings. NAFLD is diagnosed after steatosis secondary causes are excluded. At all stages of the disease, the following validated scales are used: the fatty liver index (FLI), nonalcoholic fatty liver disease fibrosis score (NFS), and liver fibrosis index (FIB-4)[11].

Validated scales are also used to assess the CVD risk in patients with NAFLD. To accomplish this task, the Atherosclerotic Cardiovascular Disease (ASCVD) 2013 Risk Calculator and the Framingham Risk Score (2008) are used, the results of which, according to a recent study, correlate with the severity of steatosis according to US data and fibrosis severity, as assessed by the NFS and FIB-4[12].

In a number of studies, it has been noted that hepatic steatosis is an early predictor of coronary atherosclerosis and is associated with an increase in carotid intima-media thickness (CIMT)[13,14] as well as with uncalcified atherosclerotic plaque formation[15].

There is currently no approved standard therapy for NAFLD. The main factor in successful treatment is weight loss, which is a key link in both NAFLD itself and CVD pathogenesis[16]. Among the NAFLD drug treatments, those that affect various disease pathogenesis links may be considered, including increased sensitivity to insulin (pioglitazone, rosiglitazone and liraglutide), lipid-lowering agents (statins), and antioxidant and cytoprotective drugs such as ursodeoxycholic acid (UDCA), vitamin E, obeticholic acid, and omega-3 fatty acid-polyunsaturated fatty acids[17-24].

Several antioxidant mechanisms may explain the beneficial effect of UDCA on dyslipidemia and CVD risk. UDCA treatment seems to protect against iron-dependent and hydroxyl radical-dependent oxidative damage, inhibit lipid peroxidation products, and prevent reactive oxygen species-induced oxidative stress by activation of the PI3K/Akt/Nrf2 pathway in hepatocytes[25-33]. Recent studies demonstrated that

the oxidative stress products increase significantly along with the thickening of artery intima, and elevated peroxidative glutathione redox status is associated with atherosclerosis progressing[34,35].

Study Purpose: To evaluate the effects of 6 mo of UDCA treatment on liver function tests (LFT), lipid profile, hepatic steatosis and fibrosis, atherogenesis, and CVD risk in men and women with NAFLD, as well as to assess the impact of > 5% weight reduction on these parameters.

MATERIALS AND METHODS

An open international noncomparative study with the code USPEH –Ursodeoxycholic acid as a means of preventing atherosclerosis, steatosis and liver fibrosis in patients with nonalcoholic fatty liver disease – was carried out at primary health care settings.

The study protocol passed the review procedure and was approved at a meeting of an independent interdisciplinary committee on the ethical review of clinical trials in each center of the Russian Federation, Kazakhstan and Uzbekistan. The study took place from November 1, 2017, until August 31, 2018.

Inclusion criteria

Age over 18; proven NAFLD case based on US abdominal data; FLI index value > 60; the physician's decision to prescribe UDCA regardless of the patient's inclusion in the study; and availability of the patient's written informed consent to participate in the program and use of personal data in accordance with the legislation of the participating countries.

Non-inclusion criteria

Pregnancy; hepatic decompensation (serum albumin ≤ 35 g/L, international normalized ratio ≥ 1.2, platelets < 150 × 10^9/L); UDCA allergy in past medical history; presence of atherosclerosis complications; use of statins; use of other drugs that potentially affect the studied parameters; use of medications in past medical history associated with secondary hepatic steatosis development (amiodarone, methotrexate, tamoxifen, glucocorticoids, valproic acid, and antiretroviral drugs); unhealthy alcohol use (40 g ethanol per day for men and 20 g ethanol per day for women); scores on the AUDIT (Alcohol Use Disorders Identification Test) questionnaire > 8 for both men and women; type 1 diabetes mellitus; parenteral nutrition; fasting; and presence of a secondary etiology of liver disease (viral, metabolic, autoimmune, cholestatic, or drug etiology).

Exclusion criteria

A patient's decision to discontinue participation in the study at any stage; acute hepatocellular or cholestatic injury that occurred during the study: Increased alanine aminotransferase (ALT), aspartate transaminase (AST), glutamyltransferase (GGT), or alkaline phosphatase levels by two or more times; and the need to take other drugs that potentially affect the studied parameters, arising during the study.

To select study participants, a total of 207 Caucasian patients from regional registries of patients with NAFLD were screened as follows: 150 people from 3 centers in the Russian Federation (Moscow, Tyumen, Chelyabinsk), 47 people from 3 centers in Kazakhstan (Almaty, Nur-Sultan, Shymkent), and 10 people from 1 center in Uzbekistan (Tashkent).

The inclusion criteria were met by 183 patients who had been receiving daily UDCA (Ursosan®) therapy at a dose of 15 mg/kg body weight for 6 mo. In addition, total sample were given standard recommendations to modify their lifestyle and diet: strength or aerobic exercise for at least 150 min per week, Mediterranean diet, and consumption of no more than 1500 kcal/d[36].

Each patient was assessed according to the following parameters: weight, height, BMI (weight, kg/height, m^2), waist circumference (WC), smoking, alcohol consumption, metabolic syndrome criteria according to National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III)[37], complete blood count and LFT, and lipid profile.

The criteria for the treatment effectiveness evaluation were as follows: LFT [ALT, AST, and GGT upper limit of normal (ULN) = 40 U/L]; lipid profile [total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglyceride (TG)]; noninvasive hepatic steatosis assessment by the FLI; noninvasive fibrosis assessment by the NFS and FIB-4; 10-year and lifetime ASCVD risk; and CIMT.

FLI was calculated using the formula FLI = e^y /(1 + e^y) × 100, where y = 0.953 × ln (TG, mg/dL) + 0.139 × BMI, kg/m^2 + 0.718 × ln (GGT, U/L) + 0.053 × WC, cm - 15.745).

FLI index values that exceeded a value of 60 corresponded to a high probability of hepatic steatosis, indicators from 30 to 59 constituted the "gray zone", and a result less than 30 excluded the likelihood of hepatic steatosis[38].

The noninvasive liver fibrosis assessment was carried out using the NFS score, which was calculated by the formula NFS = -1.675 + [0.037 × age (years)] + [0.094 × BMI (kg/m^2)] + [1.13 × Impaired fasting glucose/diabetes (yes = 1, no = 0)] + (0.99 × AST/ALT ratio) – [0.013 × platelet count (× 10^9/L)] – [0.66 × albumin (g/dL)] URL: https://www.mdcalc.com/nafld-non-alcoholic-fatty-liver-disease-fibrosis-score. Fibrosis index values of -1.455 and below have made it possible to exclude the presence of advanced liver fibrosis, and a value of more than 0.675 testified in favor of the F3-F4 fibrosis stages[39].

Another method for liver fibrosis assessment was the calculation of the fibrosis index FIB-4 according to the formula FIB-4 = age (years) × AST (U/L)/platelets (10^9/L) × √ ALT (U/L). A FIB-4 value less than 1.45 excluded the presence of severe liver fibrosis, and a value greater than 3.25 indicated the presence of advanced fibrosis.

The risk of developing complications from the cardiovascular system was assessed using the ASCVD 2013 calculator. In accordance with the age limits, the CVD risk over 10 years in patients over 40 years of age and the risk over a lifetime in those under 59 years of age were calculated. A program presented in the public domain was used for calculation[40].

The CIMT was assessed with a standard method in accordance with the European Society of Cardiology recommendations. CIMT was measured from longitudinal images of both the right and left common carotid arteries during B-mode ultrasonography as the distance from the echogenic lumen-intima interface to the echogenic media-adventitia interface; the highest value for each patient was used for analysis[41]. Values of the 95th percentile for the corresponding age and sex were used as the ULN[42].

All data were entered into the patient's Case Report Form. After 3 mo of the study, 9 patients were excluded due to acute hepatocellular or cholestatic injury associated with nonsteroidal anti-inflammatory drug intake and alcohol consumption during the study. The study was completed by 174 patients who were included in the final analysis (Figure 1).

To assess treatment effectiveness, the comparisons were made for the total sample and for men and women separately. The body weight, BMI, WC, and LFT were compared between baseline and 3 mo as well as between 3 and 6 mo of the treatment. The lipid profile, FLI, NFS, FIB-4, 10-year and lifetime ASCVD risk, and CIMT were compared between baseline and the end of the follow-up (Figure 2).

Considering that the patients were given dietary and lifestyle recommendations, it was assumed that some patients would lose weight by the end of the study, which in itself could affect the parameters studied. Based on this assumption, a subgroup analysis with and without weight loss > 5% from baseline was planned.

Statistical processing

For the statistical processing of the study results, all data from the case report forms were entered by two researchers (M.N., Kh.K.) independently into a database created on the basis of Excel spreadsheets (Microsoft, United States). In case of data discrepancies, the indicators were manually checked from the case report forms. The missing data for the studied parameters were 2.1% ± 0.8%. Data are presented as absolute and relative indicators, mean ± SD for normally distributed values and for the rest of the values, as median (Me) and the 25th and 75th percentiles-interquartile range (IQR). To test statistical hypotheses, the Wilcoxon test, paired *t*-test, Fisher's exact test, and Pearson's chi-squared test were used. The critical value of the statistical significance level, when testing the null hypotheses, was equal to 0.05. Statistical analysis was performed using SPSS Statistics v.23.0 (IBM Corporation, United States).

RESULTS

The study involved 121 (69.5%) men and 53 (30.5%) women aged 24 to 68 years. The patients' baseline data are presented in Table 1. Among the concomitant diseases, type 2 diabetes mellitus (T2DM) was diagnosed in 54 (31.0%) patients, and arterial hypertension (AH) was diagnosed in 41 (23.6%) patients. Tobacco smoking was noted

Table 1 Baseline characteristics of the study patients

Parameter	Total sample (*n* = 174)	Men (*n* = 121)	Women (*n* = 53)	*P* value
Age, years, Me (IQR)	45.2 ± 10.1	42.9 ± 8.7	50.7 ± 10.5	< 0.001
T2DM	54 (31.0)	35 (28.9)	19 (35.8)	0.466
AH	41 (23.6)	27 (22.3)	14 (26.4)	0.695
Smoking	58 (33.3)	45 (37.2)	13 (24.5)	0.146
Obesity, classes I-III	121 (69.5)	79 (65.3)	42 (79.2)	0.097
Metabolic syndrome, NCEP ATP III	79 (45.4)	45 (37.2)	34 (64.2)	0.002

Data are presented as mean ± SD or *n* (%). Me: Median; IQR: Interquartile range; T2DM: Type 2 diabetes mellitus; AH: Arterial hypertension; NCEP ATP III: National cholesterol education program adult treatment panel III.

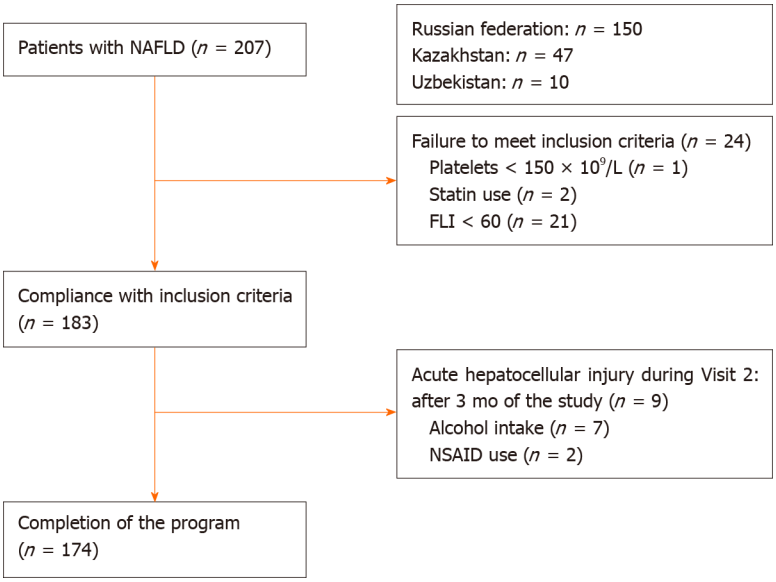


Figure 1 Patient enrollment flowchart. NAFLD: Nonalcoholic fatty liver disease; FLI: Fatty liver index; NSAID: Nonsteroidal anti-inflammatory drug.

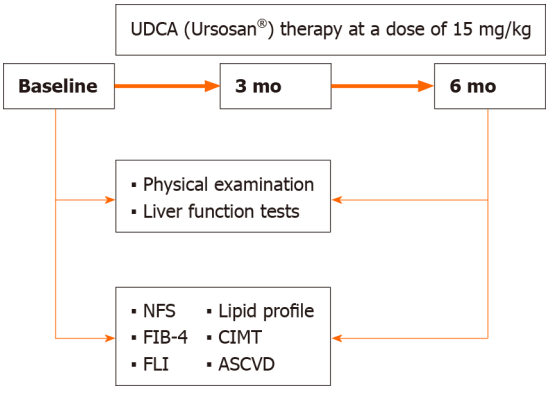


Figure 2 Scheme of the study. UDCA: Ursodeoxycholic acid; NFS: Nonalcoholic fatty liver disease fibrosis score; FIB-4: Liver fibrosis index; FLI: Fatty liver index; CIMT: Carotid intima-media thickness: ASCVD: Atherosclerotic Cardiovascular Disease.

in every third patient. Obesity (Classes I-III) was observed in 121 (69.5%) patients, and the remaining patients were overweight. Seventy-nine (45.4%) patients met the NCEP ATP III criteria for metabolic syndrome. The mean age of women was significantly higher than that of men ($P < 0.001$); the metabolic syndrome was more common in women compared with men ($P < 0.002$). Men and women did not differ in frequency of T2DM, AH, tobacco smoking, and obesity (classes I-III) (Table 1).

Analysis within the total sample and by sex

During the study, smoking status, need for AH treatment, and the number of T2DM cases did not change.

As compared with their initial levels, body weight, BMI, and WC decreased significantly ($P < 0.001$) in men and women after 3 mo of treatment and continued to reduce between 3 and 6 mo ($P < 0.001$) (Table 2). No significant difference was reported in body weight change between the 0- to 3-mo and 3- to 6-mo intervals, whereas the WC was greatly reduced between baseline and 3 mo of treatment ($P < 0.001$).

After 3 mo of treatment, 11 (6.3%) patients lost > 5% weight, whereas at the end of treatment, 54 (31%) patients had > 5% weight reduction, a 3-mo difference being statistically significant ($P < 0.001$) (Table 2).

The levels of ALT, AST, and GGT decreased significantly from baseline to 3 mo and from 3 to 6 mo. The ALT level initially exceeded 39.9 U/L in 116 (66.7%) of patients but decreased to 64 (36.8%) by the 3rd mo and to 40 (23%) by the end of the study. Low-normal ALT values (0-19.9) were noted in 5 (2.8%) at baseline, in 15 (8.6%) at 3 mo, and in 26 (14.9%) after treatment. The ALT level changed by -14.1 U/L (IQR = -31.0 U/L to -5.3 U/L) from baseline to 3 mo and by -6.5 U/L (IQR = -14.0 U/L to 0.1 U/L) from 3 to 6 mo. Similar changes were seen with AST and GGT levels. From baseline to 3 mo, the levels of AST and GGT decreased by -4.0 U/L (IQR = -12.0 U/L to 1.0 U/L) and -5.0 U/L (IQR = -15.0 U/L to 2.0 U/L), respectively. From 3 to 6 mo, AST and GGT levels dropped by -2.0 U/L (IQR = -7.0 U/L to 2.2 U/L) and -3.0 U/L (IQR = -8.0 U/L to 2.0 U/L), respectively.

The magnitude of ALT, AST, and GGT decrease was greater during the first 3 mo of treatment compared to the subsequent 3 mo ($P < 0.001$, $P < 0.01$, $P < 0.001$, respectively). The baseline ALT, AST, and GGT levels did not differ between the sexes, in contrast to the rates of change of these parameters over the trial. Men had a significant decrease in ALT, AST, and GGT levels both from baseline to 3 mo and from 3 to 6 mo, whereas women had the only significant decrease in ALT over the both periods. The AST and GGT levels decreased to normal levels during the first 3 mo of treatment in most women (Table 2).

During the study, there was a statistically significant change in lipid profile: a decrease in the TC, TG, and LDL levels in the total sample and by sex. The HDL level did not change significantly in men, but significantly increased in women (Table 3).

By the end of the study, the FLI had decreased to a value of < 30 in one men, and in another 40 patients [24 (20%) men 16 (30%) women], it had gone into the "gray zone." No significant changes in fibrosis were noted according to the NFS and FIB-4.

The CIMT decreased significantly by the end of 6 mo in the total sample. When men and women were analyzed separately, the rate of CIMT change remained close to significant with P values of 0.073 and 0.052, respectively. At the same time, no statistically significant changes in the proportion of persons with reference values exceeding the ULN for the corresponding sex and age were established (Table 3).

A significant decrease in the 10-year ASCVD risk score was observed only in women ($P = 0.003$). The same patient's 10-year risk with optimal risk factors was 1.5 (0.9; 2.9) in both sexes.

The lifetime ASCVD risk did not change during the study and remained significantly increased relative to lifetime risk with an optimal risk factor of 5.0 (5.0; 8.0).

Analysis within the subgroup

Weight loss of more than 5% of the initial weight was observed in 54 patients (31%); in 54 patients (31%), the weight decreased to 5% of the initial weight; in 35 patients (20%), it did not change; and in 31 patients (18%), it increased by an average of 3%. A comparison was made between subgroups with a weight loss of more than 5% and all remaining patients.

The delta of the studied parameters ALT, AST, GGT, TC, and LDL did not differ between the subgroups (Table 4). In the subgroup with more than 5% weight loss, the FLI baseline was higher, and during the study, there was a more pronounced decrease than in the other group. By the end of the study, the subgroups with and without weight loss of more than 5% did not differ in FLI. Additionally, in the subgroup with a weight loss of more than 5%, there was a tendency toward a more pronounced decrease in TG (P was close to statistically significant).

Table 2 Changes in clinical variables and liver function tests from baseline during the trial

Parameter	Baseline	3 mo	6 mo
Weight (total sample), kg	94.8 (86.7; 102.1)	93.0 (86.0; 100.0)[c]	92.9 (85.0; 98.3)[c]
Men	95.0 (92.0; 104.2)	94.0 (90; 101.1)[c]	93.7 (88.7; 100.0)[c]
Women	86.7 (82.0; 96.0)	85.0 (80.0; 94.8)[c]	85.0 (79.0; 95.0)[b]
Δ total sample, kg		-1.0 (-2.4; 0.0)	-1.0 (-2.4; 0.0)
Weight loss > 5% (total sample)		11 (6.3)	54 (31)[c]
Men		8 (6.6)	39 (32.2)[c]
Women		3 (5.6)	15 (28.3)[b]
BMI (total sample), kg/m^2	31.2 (29.4; 34.0)	30.9 (29.3; 33.3)[c]	30.6 (29.0; 33.1)[c]
Men	30.7 (29.0; 33.3)	30.4 (29.0; 32.6)[c]	30.1 (28.4; 32)[c]
Women	32.0 (30.7; 36.2)	31.5 (30.1; 34.6)[c]	31.6 (29.7; 34.4)[b]
Δ total sample, kg/m^2		-0.4 (-0.9; 0.0)	-0.3 (-0.8; 0.0)
Waist circumference (total sample), cm	102.0 (97.0; 111.8)	100.0 (96.0; 108.0)[c]	100.0 (94.0; 106.0)[c]
Men	103.0 (98.0; 112.0)	102.0 (97.0; 110.0)[c]	100 (97.0; 106.0)[c]
Women	99.0 (93.0; 106.0)	98.0 (92.0; 105.0)[c]	97.0 (90.0; 102.0)[c]
Δ total sample, cm		-2.0 (-3.0; 0.0)	0.0 (-2.0; 0.0)[c]
ALT (total sample), U/L	53.0 (34.0; 78.0)	35.0 (26.0; 45.0)[c]	29.0 (24.0; 38.0)[c]
Men	55.8 (37.0; 78.0)	38.0 (29.0; 46.0)[c]	32.0 (25.9; 41.0)[c]
Women	44.0 (30.8; 69.0)	28.5 (21.6; 38.0)[c]	26.0 (16.5; 33.2)[c]
Δ total sample, U/L		-14.1 (-31.0; -5.3)	-6.5 (-14.0; 0.1)[c]
ALT (total sample) 10-19.9/20-39.9/≥ 40 U/L	5 (2.8)/53 (30.5)/116 (66.7)	15 (8.6)/95 (54.6)/64(36.8)[b]	26 (14.9)/108 (62.1)/40 (23.0)[c]
Men	1 (0.8)/35 (28.9)/85 (70.2)	5 (4.1)/64 (52.9)/52 (43)[c]	10 (8.3)/79 (65.3)/32 (26.4)[a]
Women	4 (7.5)/18 (34)/31 (58.5)	10 (18.9)/31 (58.5)/12 (22.6)[c]	16 (30.2)/29 (54.7)/8 (15.1)
AST (total sample), U/L	31.0 (24.0; 42.0)	26.5 (21.0; 34.0)[c]	25.0 (21.0; 29.0)[c]
Men	29.1 (23.5; 42.0)	26.0 (21.0; 33.0)[c]	25.0 (21.0; 28.0)[c]
Women	32.0 (24.0; 42.0)	28.0 (22.0; 35.0)[c]	26.0 (23.0; 32.0)
Δ total sample, U/L		-4.0 (-12.0; 1.0)	-2.0 (-7.0; 2.2)[b]
GGT (total sample), U/L	36.0 (26.0; 48.0)	28.0 (21.0; 38.0)[c]	26.0 (19.0; 35.0)[c]
Men	36.0 (28.0; 46.0)	31.0 (24.0; 41.0)[c]	26.0 (21.0; 35.0)[c]
Women	35.0 (23.0; 53.0)	23.0 (18.0; 30.0)[c]	21.0 (12.0; 34.0)
Δ total sample, U/L		-5.0 (-15.0; 2.0)	-3.0 (-8.0; 2.0)[c]

[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$. Data are presented as median (IQR) or *n* (%). BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate transaminase; GGT: Glutamyltransferase.

DISCUSSION

The NAFLD prevalence rate in the population, as well as associated complications and risk factors, is growing steadily. Thus, a 10-fold increase in hepatocellular carcinoma related to NAFLD has been reported over the past decade. Moreover, it has been shown that patients with more advanced NAFLD are 4 times more likely to have a fatal CVD event and twice as likely to suffer a nonfatal CVD event[43]. These data demonstrate the urgency of NAFLD-related problems and the need to find a modern drug to treat it effectively.

Male sex is one of the nonmodifiable risk factors for NAFLD and ASCVD. In our study, the number of men with NAFLD was 2 times higher than the number of

Table 3 Changes in laboratory and imaging variables from baseline during the trial

Parameter	Baseline	6 mo	*P* value
TC (total sample), mmol/L	6.03 ± 1.36	5.76 ± 1.21	< 0.001
Men	5.99 ± 1.39	5.77 ± 1.24	0.003
Women	6.11 ± 1.32	5.73 ± 1.16	0.019
LDL (total sample), mmol/L	3.86 ± 1.01	3.66 ± 0.91	< 0.001
Men	3.81 ± 1.04	3.67 ± 0.90	0.033
Women	4.0 ± 0.94	3.65 ± 0.93	0.006
HDL (total sample), mmol/L	1.24 ± 0.32	1.24 ± 0.27	0.910
Men	1.24 ± 0.33	1.21 ± 0.22	0.160
Women	1.23 ± 0.29	1.31 ± 0.34	0.036
TG (total sample), mmol/L	3.18 (2.00; 4.29)	2.04 (1.40; 3.16)	< 0.001
Men	3.13 (2.10; 4.29)	2.04 (1.4; 2.79)	< 0.001
Women	3.45 (1.80; 4.36)	2.26 (1.35; 3.62)	< 0.001
FLI (total sample)	84.9 ± 10.4	72.3 ± 17.6	< 0.001
Men	86.3 ± 9.0	73.6 ± 17.2	< 0.001
Women	81.9 ± 12.7	69.4 ± 18.4	< 0.001
FLI (total sample) ≥ 60/30-59/< 30	174 (100)/-/-	133 (76.4)/40 (23.0)/1 (0.6)	< 0.001
Men	121 (100)/-/-	96 (79)/24 (20)/1 (1)	< 0.001
Women	53 (100)/-/-	37 (70)/16 (30)/-	< 0.001
NFS (total sample) < -1.455 (F0-F2), *n* (%)	141 (81.0)	148 (85.0)	0.353
Men	102 (84.3)	112 (92.6)	0.071
Women	39 (76.6)	34 (64.2)	0.402
FIB 4 (total sample) < 1.45 (F0-F1), *n* (%)	157 (90.2)	163 (93.7)	0.256
Men	111 (91.7)	114 (94.2)	0.615
Women	46 (86.8)	49 (92.5)	0.402
CIMT (total sample), mm	0.985 ± 0.243	0.968 ± 0.237	0.013
Men	0.993 ± 0.224	0.977 ± 0.217	0.073
Women	0.967 ± 0.284	0.947 ± 0.276	0.052
CIMT (total sample) exceeding ULN for the corresponding age and sex	143 (84)	139 (82)	0.549
Men	103 (85.1)	101 (83.5)	0.860
Women	40 (75.5)	38 (72)	0.826
ASCVD (total sample, *n* = 112) 10-year risk	5.1 (2.9; 9.1)	4.8 (2.6; 8.0)	0.053
Men (*n* = 71)	6.0 (3.6; 11.1)	5.8 (3.4; 8.8)	0.720
Women (*n* = 41)	3.5 (2.3; 7.8)	3.2 (2.0; 6.0)	0.003
ASCVD (total sample, *n* = 152) lifetime risk	50 (39; 60)	50 (39; 64)	0.370
Men (*n* = 112)	50 (46; 69)	50 (46; 69)	0.870
Women (*n* = 40)	39 (39; 50)	39 (39; 50)	0.160

Data are presented as median (IQR) or *n* (%). TC: Total cholesterol; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglyceride; FLI: Fatty liver index; NFS: Nonalcoholic fatty liver disease fibrosis score; FIB-4: Liver fibrosis index; ULN: Upper limit of normal; ASCVD: Atherosclerotic Cardiovascular Disease.

women, which is consistent with the NAFLD prevalence data in other populations[44-46]. The patients' average age in our study was 45 years, which generally corresponds to the NAFLD epidemiological study data. Thus, according to individual studies, in Europe, the average age of patients with NAFLD is 56 years in Italy and 58 years in Spain[47,48]. In Asia, this parameter is lower; in the Chinese population, the average age is 45 years, and in the Japanese population, it is 51 years[49,50]. In a NAFLD study conducted in Kazakhstan, the patients' average age was 55 years[5].

It is well known that NAFLD and CVD share common modifiable risk factors[43]. In our study, one-third of patients had T2DM, most (69.5%) of the observed participants were obese (Classes I-III obesity), and the rest were overweight. A total of 45.4% of patients met the criteria for metabolic syndrome based on the NCEP ATP III, which is consistent with a large-scale epidemiological study data conducted by Younossi *et al*[3], in which the number of patients with metabolic syndrome was 42.5%.

Factors released from fatty liver that could link NAFLD to CVD are proinflammatory and proatherogenic cytokines (IL-6, tumor necrosis factor-alpha), procoagulant factors (fibrinogen, factors VII and VIII, plasminogen activator type 1 inhibitor), and pro-oxidant factors (reactive oxygen species); other pathogenic mechanisms include progression of insulin resistance and dyslipidemia[10,13].

In this study, patients were given lifestyle and dietary modifications, and UDCA was prescribed. For 6 mo of the study, there was a statistically significant decrease in weight, BMI, and WC, which is associated with the adherence of a certain proportion of the subjects to the recommendations on lifestyle modification and diet, which were discussed with patients prior to the UDCA administration and three months later.

In a randomized study by Wong *et al*[51], the participation of patients with NAFLD in lifestyle and diet modification programs contributed to a decrease in body weight, liver TG content and disease remission achievement. At the same time, in a study by Dudekula *et al*[52], it has been shown that in a primary care setting, prescribing only lifestyle recommendations for significant (> 5%) weight loss in NAFLD patients is insufficient. In this study, the degree of weight loss was correlated with the frequency of outpatient visits.

During the study, a statistically significant decrease in ALT level was achieved; the number of patients whose ALT level exceeded 40 U/L decreased almost 3-fold. The Me ALT reduction was 45.3%. A decrease in ALT levels in NAFLD patients during treatment with UDCA has been demonstrated in previous studies. For example, in a randomized controlled trial by Ratziu *et al*[19], high-dose UDCA therapy for one year was associated with a decrease in ALT level by 28.3% and ALT level normalization in a quarter of the patients, regardless of weight loss. An even more pronounced decrease in ALT level during a 6-mo course of UDCA at a dose of 15 mg/kg/d, as in our study, was noted in a prospective study by Ozel Coskun *et al*[20]: The ALT level decreased after therapy by 54% in patients with histologically confirmed NAFLD.

In addition, during the study, there was a significant decrease in AST and GGT levels by 6 and 10 U/L, respectively, which was repeatedly noted in other similar studies[21-24]. The percentage of AST reduction was 19.4% and 27.8% for GGT, which is consistent with previously mentioned publications: a decrease in AST of 18.9% and GGT of 62% in a study by Ratziu *et al*[19], and a decrease in AST of 43% and GGT of 41% in a study by Ozel Coskun *et al*[20]. A statistically significant decrease in AST levels of 8.5 U/L and GGT of 16.5 U/L was also demonstrated after a relatively short (3-wk) course of UDCA 20 mg/kg/d[53].

In comparison with other studies, we also analyzed the rates of LFT change during the first and subsequent 3-mo intervals. The magnitude of ALT, AST, and GGT decrease was significantly higher during the first 3 mo of treatment, whereas the rates of weight and BMI change were the same in both periods.

Improvement in LFT is probably associated with the antioxidant effect of UDCA, the ability to suppress the production of tumor necrosis factor-alpha, which is associated with an improvement in LFT and histological findings[54,55].

Positive changes have also been observed in relation to lipid profile. The Me TC during follow-up decreased significantly by 0.27 mmol/L, which is consistent with the results of a recently published meta-analysis, which, based on an analysis of 20 studies, demonstrated a significant decrease in TC with UDCA therapy by 0.36 mmol/L[56]. The Me of other lipid parameters (LDL and TG) also decreased during the study. The HDL level increased significantly only in women.

Administration of UDCA improves the lipid spectrum by decreasing cholesterol absorption and synthesis and the activity of the farnesoid X-receptor and increasing the synthesis of bile acids[56].

The effect of UDCA therapy on the FLI has not been previously shown. In our study, the FLI, a noninvasive assessment of steatosis, decreased significantly. This is

Table 4 Comparison of the parameter decrease degree in subgroups with weight loss of more and less than 5% from baseline to the end of treatment

Parameter	Weight loss > 5% (*n* = 54)	Weight loss ≤ 5% (*n* = 120)	*P* value
ΔALT, U/L	-21.1 (-45.0; -6.0)	-25.0 (-42.5; -6.0)	0.683
ΔALT, %	-46 (-63; -18)	-43 (-54; -23)	0.281
ΔAST, U/L	-6.0 (-15.0; 1.6)	-5.0 (-18.0; 1.0)	0.673
ΔAST, %	22 (-41; 0)	-17 (-42; 0)	0.446
ΔGGT, U/L	-5.0 (-17.0; 0)	-8.0 (-22.5; 0)	0.492
ΔGGT, %	-19 (-41; 0)	-32 (-50; 0)	0.354
ΔTC, mmol/L	-0.10 (-0.66; 0.30)	-0.10 (-0.57; 0.2)	0.666
ΔTG, mmol/L	-1.04 (-1.80; -0.23)	-0.70 (-1.33; -0.11)	0.079
ΔLDL, mmol/L	-0.20 (-0.7; 0.2)	-0.14 (-0.58; 0.3)	0.401
FLI			
Initially	88.3 ± 10.2	83.5 ± 10.3	0.002
After 6 mo	71.4 ± 19.6[c]	72.8 ± 16.7[c]	0.916
ΔFLI	-14.6 (-21.8; -6.3)	-8.45 (-17.9; -2)	0.003

[c]$P < 0.001$. Δ is the difference in indicators after 6 mo and at baseline. The minus sign indicates a decrease in the parameter by the end of the study.

due to a decrease in the parameters included in the FLI algorithm, including BMI, WC, GGT, and TG. At the end of the program, 1/4 of the patients moved to the "gray zone". Similarly, Laurin *et al*[22] previously reported a histologic improvement in steatosis after UDCA treatment.

The literature contains a limited number of clinical studies evaluating the effects of drug therapy on fibrosis in patients with NAFLD. In our work, liver fibrosis was assessed using the NFS and FIB-4 scales, which have similar diagnostic value according to the Sun meta-analysis[57]. We observed no significant effect on fibrosis, which is consistent with a 52-wk study by Parikh *et al*[58] using the NFS scale to compare the fibrosis severity before and after UDCA therapy. Notably, in another study, there was a decrease in fibrosis severity when assessed using the FibroTest, but the study duration and the UDCA dose were higher than those in our trial[19].

NAFLD is currently known to be associated with increased CIMT, an early predictor of atherosclerosis and CVD. In our study, patients achieved a statistically significant decrease in CIMT with UDCA treatment. Similar data have been demonstrated in the prospective Coscun study (6 mo of UDCA at a dose of 15 mg/kg/d). The mechanism for reducing CIMT appears to be associated with the antioxidant, anti-inflammatory, and hypolipidemic effects of UDCA, as well as its effect on insulin resistance[20]. In turn, CIMT reduction plays an important role in CVD prevention. Notably, CIMT, exceeding the ULN for the corresponding sex and age, remained unchanged during the study, which can probably be explained by the short duration. The duration of cardiac studies, which showed a more pronounced decrease in CIMT, was 24 and 36 mo[59].

In our study, the impact of UDCA on CVD risk was assessed using the ASCVD risk calculator for the first time. We found a statistically significant decrease in the 10-year ASCVD risk only in women. Of all the parameters assessed in ASCVD, this one was due to a decrease in the TC level in both sexes and the HDL level only in women.

At the same time, in the present study, the predicted 10-year risk of CVD at the end of 6 mo was 3 times higher than the same patient's 10-year risk with optimal risk factors. There was no reduction in the predicted lifetime CVD risk. Indeed, it was 10 times higher than the same individual's lifetime risk with optimal risk factors.

Achieving more significant reductions in CIMT and ASCVD risk appears to take longer and requires control of other modifiable risk factors such as sedentary lifestyle, T2DM, AH, and smoking. Cardiological and endocrinological studies have shown that regular physical activity for 1 year or more, smoking cessation, blood pressure control, and glycemic control using new-generation hypoglycemic agents such as sodium–glucose cotransporter 2 inhibitor (dapagliflozin) or inhibitor of dipeptidyl peptidase-4 (sitagliptin) contribute to CIMT and ASCVD risk decrease[60-65].

Weight loss is believed to be a key factor in successful NAFLD treatment and in reducing the risk of CVD. A recent meta-analysis has shown clinical and laboratory improvements in NAFLD patients with 5%-10% weight loss[66]. To assess the effect of weight loss on the studied efficacy parameters in our patients, we divided them into two subgroups: weight loss of more than 5% and weight loss of 5% or less. The cutoff point of 5% was chosen due to the short 6-mo study period.

When comparing groups, there was no statistically significant difference in absolute and relative reductions in ALT, AST, GGT, TC, and LDL levels. At the same time, in the group with a weight loss of more than 5%, we observed a close to significant decrease in TG level and a significant decrease in FLI steatosis. Since the FLI formula includes the GGT, TG, BMI, and WC values, it is likely that the steatosis improvement in the group of patients with a decrease in body weight of more than 5% is associated with a greater change in the values of the last three parameters. However, even in patients who did not achieve more than 5% weight loss, a statistically significant decrease in the FLI index was observed.

A recent meta-analysis[66], which assessed the relationship between weight loss and LFT, found that weight reduction resulted in a decrease in ALT by –9.81 U/L; 95%CI: -13.12; -6.50. In our study, patients with a weight loss of more than 5% had a decrease in ALT levels by an average of 21.1 U/L; this reduction is greater than that in the meta-analysis, demonstrating the UDCA effectiveness. Moreover, in the current study, both the subgroups with and without a loss of > 5% body weight achieved significant improvement in FLI, while the above mentioned meta-analysis did not show any differences in FLI under UDCA therapy.

In addition, in contrast to the meta-analysis data, our study achieved a statistically significant decrease in FLI both in the subgroup with a weight loss of more than 5% and in the subgroup without weight loss; in the meta-analysis, the weight loss effect on FLI was not established. The study was conducted in a primary health care setting, which expands the possibilities of its transfer to regular practice. Some limitations of our study should also be noted, namely, the open design, the absence of a control group, and the relatively short follow-up period.

CONCLUSION

The study suggests that 6-mo UDCA therapy at a dose of 15 mg/kg/d may improve ALT, AST, GGT, TC, and TG levels and FLI in the primary care setting independent of weight loss. A positive effect of UDCA on ALT, AST, and GGT levels is greater during the first 3 mo of treatment. Our study also demonstrates a beneficial effect of UDCA on the progression of CIMT in both sexes and reduction in 10-year CVD risk in women. Further long-term studies with control groups are needed to confirm these findings.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) is currently the leading cause of liver disease and liver transplantation in developed countries. Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of mortality in patients with NAFLD. Weight loss is a key factor for successful NAFLD and cardiovascular disease therapy. Ursodeoxycholic acid (UDCA), which is one of the first-line therapeutic agents for treatment of NAFLD, is reported to have a beneficial effect on dyslipidemia and ASCVD risk because of antioxidant properties.

Research motivation

The main motivation was our wish to improve the treatment outcomes of patients with NAFLD. Each author of our international study had a personal positive experience of treating NAFLD with UDCA. We sincerely wanted to combine our efforts to gain a new knowledge.

Research objectives

To evaluate the effects of 6-mo administration of UDCA on liver function tests (LFT), lipid profile, hepatic steatosis and fibrosis, carotid intima-media thickness (CIMT), and ASCVD risk in total sample with NAFLD, separately in men and women, in patients

with and without > 5% weight loss.

Research methods

An open international noncomparative study was carried out at primary health care settings. The study was completed by 174 patients who were included in the final analysis and received UDCA (Ursosan®) therapy at a dose of 15 mg/kg body weight daily for 6 mo. In addition, total sample was given recommendations to modify lifestyle behaviors and diet. The body mass index, waist circumference, and LFT were compared between baseline and 3 mo as well as between 3 and 6 mo of the treatment. The lipid profile, fatty liver index (FLI), nonalcoholic fatty liver disease fibrosis score, liver fibrosis index, 10-year and lifetime ASCVD risk, and CIMT were compared between baseline and the end of the follow-up.

Research results

The magnitude of LFT decrease was greater during the first 3 mo of treatment compared to the subsequent 3 mo. At 6 mo, in the total sample, we observed a statistically significant decrease in body mass index, waist circumference, and levels of FLI ($P < 0.001$), total cholesterol (TC) ($P < 0.001$), low-density lipoprotein (LDL) ($P < 0.001$), triglyceride (TG) ($P < 0.001$), and the CIMT ($P = 0.013$), whereas the high-density lipoprotein (HDL) ($P = 0.036$) and 10-year ASCVD risk improved significantly only in women ($P = 0.003$). No effect on nonalcoholic fatty liver disease fibrosis score, and liver fibrosis index was found. At the end of the study, the FLI decreased significantly in patients with and without > 5% weight loss; the changes in LFT, TC, TG, and LDL levels were similar between the subgroups.

Research conclusions

The study suggests that 6-mo UDCA therapy at a dose of 15 mg/kg/d may improve LFT, TC, LDL, and TG levels, FLI, and CIMT in total sample with NAFLD. These changes are observed independent of sex and weight loss. UDCA has also a beneficial effect on HDL and 10-year ASCVD risk in women.

Research perspectives

Further long-term studies with control groups are needed to confirm these findings. For further research, we suggest to compare lipid profile and hepatic steatosis between baseline to 3 mo and 3 to 6 mo; evaluate the effects of long-term UDCA therapy (during 1-year follow-up or more) on HDL and 10-year and lifetime risk ASCVD in men and women.

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ORIGINAL ARTICLE

Observational Study

Advanced small-bowel well-differentiated neuroendocrine tumours: An international survey of practice on 3rd-line treatment

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Abstract

BACKGROUND

Somatostatin analogues are an established first-line therapy for well differentiated small bowel neuroendocrine tumours (Wd-SBNETs), while and peptide receptor radionuclide therapy (PRRT) is frequently used as a second-line therapy. Adequate treatment selection of third-line treatment remains challenging due to

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the limited prospective data currently available on the best therapeutic sequence.

AIM

To understand current practice and rationale for decision-making by physicians in the 3rd-line setting by building an online survey.

METHODS

Weighted average (WA) of likelihood of usage between responders (1 very unlikely; 4 very likely) was used to reflect the relevance of factors explored.

RESULTS

Replies from representatives of 28 centers were received (5/8/2020-21/9/2020); medical oncologist (53.6%), gastroenterologist (17.9%); United Kingdom (21.4%), Spain (17.9%), Italy (14.3%). Majority from European Neuroendocrine Tumor Society (ENETS) Centres of Excellence (57.1%), who followed ENETS guidelines (82.1%). Generally speaking, 3rd-line treatment for Wd-SBNETs was: everolimus (EVE) (66.7%), PRRT (18.5%), liver embolization (LE) (7.4%) and interferon-alpha (IFN) (3.7%); chemotherapy (0%); decision was based on clinical trial data (59.3%), or personal experience (22.2%). EVE was most likely used if Ki-67 < 10% (WA 3.27/4) or age < 70 years (WA 3.23/4), in the 3rd-line setting (WA 3.23/4); regardless of presence/absence of carcinoid syndrome (CS), rate of progression or extent of disease. Chemotherapy was mainly utilised only if rapid progression (within 6 mo) (WA 3.35/4), Ki-67 10%-20% (WA 2.77/4), negative somatostatin receptor imaging (WA 2.65/4) or high tumour burden (WA 2.77/4); temozolomide or streptozocin was used with capecitabine or 5-fluorouracil (5-FU) (57.7%), FOLFOX (5-FU combined with oxaliplatin) (23.1%). LE was selected if presence of CS (WA 3.24/4) or Ki-67 < 10% (WA 2.8/4), after progression to other treatments (WA 2.8/4). IFN was rarely used (WA 1.3/4).

CONCLUSION

Everolimus was the most frequently used therapeutic option in the third-line setting. The most important factors for decision-making included Ki-67, rate of progression, functionality and tumour burden; since this decision is based on multiple factors, it highlights the need for a multidisciplinary assessment.

Key Words: Neuroendocrine tumour; Small bowel; Survey; Third-line; Advanced; Practice

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Core Tip: Our survey delineates a possible treatment algorithm in patients with advanced small bowel neuroendocrine tumour (SBNET). While somatostatin analogues (SSAs), peptide receptor radionuclide therapy (PRRT) and everolimus are usually considered preferred first, second and third-line options respectively, chemotherapy is generally used when all other available treatments have failed. Locoregional therapies appear particularly useful when facing patients with functioning tumours, but their use is mainly limited to after at least two prior lines of treatment. We were also able to identify relevant unanswered questions in the field of advanced SBNET treatment, mainly in regards to the role of maintenance SSA after PRRT for non-functioning tumours. Multiple factors were identified as relevant at time of decision making; among them, Ki-67, rate of progression, tumour functionality and tumour burden may have a key role in helping physicians tailoring the treatment. Based on this, we would encourage for treatment decisions to be made within a multidisciplinary setting.

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INTRODUCTION

Neuroendocrine neoplasms are rare and heterogeneous[1,2]. One of the main primary sites of development of well-differentiated neuroendocrine tumours (NETs) is the small bowel (SBNETs)[1]. These are characterised by the production of certain bioactive compounds (such as serotonin) which may derive on specific symptoms, such as carcinoid syndrome (CS)[3].

Nearly half of SBNETs are diagnosed at a non-resectable, advanced stage, being only suitable for palliative therapeutic approaches[4]. For treatment decision making, assessment of hormonal syndrome, disease stage, burden of disease, somatostatin receptor (SSTR) expression and tumour grade are crucial[5,6], with available guidelines informing on treatment options[7-11].

In recent years, management of SBNETs has changed significantly, with development of new anti-tumour treatment options. There is now high-level evidence phase III clinical trial data supporting the use of somatostatin analogues (SSAs)[12,13], which have shown both anti-tumour and anti-secretory effects. Above-label dosages of SSAs may also provide antitumour activity after progression on standard doses of SSAs, particularly in patients with slow-growing tumours[14]. After progression to SSA, the results of the NETTER-1 trial support the use of peptide-receptor radionuclide therapy (PRRT) with ^{177}Lu-DOTATATE in SBNETs with high SSTRs expression (expected to account for above 80% of cases)[15]. In addition, the RADIANT-4 trial supported the use of everolimus over placebo for non-functioning SBNETs after progression to one prior line of treatment[16].

SBNETs have a better prognosis than other gastrointestinal malignancies, with estimated median survival from time of identification of metastatic disease of above 5 years[1,2]. In view of this, many patients are fit enough for multiple lines of treatment. In view of increasing treatment options[4], and despite available guidelines[7,8], selection of the best treatment strategy remains challenging. The use of SSAs in the first-line setting for patients diagnosed with SBNETs is supported by randomised clinical trial results and reflected in multiple guidelines. As mentioned above, options for second-line treatment could include PRRT or everolimus, with most recent guidelines supporting the use of PRRT over everolimus in the second-line setting especially if tumour expresses SSTR[8].

Thus, it is expected that majority of patients will receive first-line SSA and second-line PRRT as the predominant treatment strategy. However, the right choice for third-line treatment remains unclear and practice regarding the use of everolimus, chemotherapy and other therapy options is likely to vary significantly between different institutions. Such therapeutic options may include everolimus[16] (approved for patients with non-functioning SBNETs), liver embolization[17], chemotherapy (mainly alkylating-based or oxaliplatin-based and generally combined with fluoropyrimidine)[18,19] and interferon-alpha[20,21].

This survey of practice aims to provide an understanding of current practice and main factors used for treatment selection in advanced SBNETs, with special focus on third-line treatment strategies.

MATERIALS AND METHODS

An online survey was built and shared with European Neuroendocrine Tumor Society (ENETS) Centres of Excellence (CoE)[22] and other health care professionals with expertise in NETs (*via* NETConnect group[23]). The survey contained questions regarding current practice, with special focus on factors currently influencing treatment decision making in SBNETs and especially on third-line treatment options. Even though baseline characteristics of responders (*i.e.*, country, specialty, whether they practiced in an ENETS CoE or not) were collected, participation was anonymous and aimed to reflect their personal views.

The primary aim of this survey was to provide information on the patterns of use of third-line treatments in SBNET patients. The secondary aim was to define the factors associated with treatment selection in the third-line setting. Replies were collected between August 5, 2020 until September 21, 2020 and all data was analysed together. Duplicate replies from an individual centre were excluded, if all provided replies concurred. In order to assess for likelihood of prescribing a specific treatment in a specific scenario, respondents were asked to determine how likely they were to do so and replies collected in numbers ranging between 1 (very unlikely) to 4 (very likely). Joined analysis was performed by calculating weighted average (WA) of likelihood of

usage between responders for each scenario explored, to reflect the relevance of each factor in the decision making; reported WA ranged between 1 and 4 (1 very unlikely; 4 very likely).

RESULTS

Answers from 28 health care providers were collected. Characteristics of responders are summarized in Table 1. Most responders were medical oncologist (53.6%) or gastroenterologist (17.9%), from United Kingdom (21.4%), Spain (17.9%) or Italy (14.3%). Majority were completed by healthcare professionals working in ENETS CoEs (57.1%) who reported using ENETs guidelines for informing their practice (82.1%).

First-line and second-line treatments

The most frequently used first- and second-line therapies for advanced SBNET were SSAs (88.9%) and PRRT (70.4%), respectively (Figure 1). Rationale for such choice was based on clinical trial evidence in 96.3% and 74.1% of responses, respectively. A proportion of responders selected alternative second-line options after SSA, such as high-dose SSA (3/24; 12.5%) or everolimus (2/24; 8.3%) (Figure 1).

The likelihood of using SSAs for all SBNET patients (regardless of baseline characteristics) in the first-line setting had a weighted average of 3.48 (out of a maximum on 4) (Figure 2A; full details in Supplementary Figure 1A). The patients who were most likely to receive SSA in the first-line setting were those with functioning tumours (CS) (weighted average 3.93/4) and/or Ki-67 < 10% (weighted average 3.85/4), among others. Patients with rapid progression (<6 mo) (weighted average 2.33/4), negative SSTR imaging (weighted average 2.19/4) and/or Ki-67 10%-20% (weighted average 2.85/4) were the least likely to receive this treatment.

In terms of the use of PRRT in the second-line setting, the patients who were most likely to receive PRRT (Figure 2B; full details in Supplementary Figure 1B) were those with positive SSTR imaging (weighted average 3.81/4) and slow progression (> 1 year) (weighted average 3.62/4), among others. Patients with negative (weighted average 1.12/4) or heterogenous (weighted average 2.19/4) SSTR imaging were the least likely to be offered this treatment, followed by patients with low tumour burden (weighted average 3.04/4) and rapid progression (< 6 mo) (weighted average 3.00/4).

Regarding the use of PRRT, most considered this only after clinical or radiological progression on SSA (76.9%); however, there was no consensus regarding the use of concomitant SSA during/after PRRT (Figure 3): 38.5% always used SSA during/after PRRT (regardless presence/absence of CS), 26.9% used SSA during PRRT and maintain after PRRT only if evidence of CS and 30.8% used SSA during/after PRRT only in the presence of CS. One respondent (3.9%) specified that SSA was given during PRRT and individualised decision thereafter (details of factors involved not provided).

Third-line treatment

Third-line treatment choice was as follows: everolimus (66.7%), PRRT (18.5%), liver embolization (7.4%) and interferon-alpha (3.7%) (Figure 1); none of the responders selected chemotherapy as their preferred third-line therapy (0%). These replies were mainly based on clinical trial evidence (59.3%) and personal experience (22.2%). All five responders who selected PRRT as a third-line treatment did so following second-line treatment with high-dose SSA (2 responders), everolimus (2 responders) or liver embolization (1 responder); none of the responders considered rechallenge with PRRT as a predominant strategy.

Half of the responders (51.9%) would consider a trial at any point during patient's pathway; 11.1%, 11.1%, 14.8% and 11.1% would do so in the first-, second-, third- and beyond third-line setting, respectively.

Factors associated with third-line treatment choices

Everolimus was mainly used in the third-line setting (weighted average 3.23/4) or after progression to all other available treatment options (weighted average 3.15/4). The patients who were most likely to receive everolimus (Figure 2C; full details in Supplementary Figure 1C) were those with Ki-67 < 10% (weighted average 3.27/4) and/or age < 70 years (weighted average 3.23/4), within others. Choice of everolimus did not seem to vary depending on presence (weighted average 2.81/4) or absence (weighted average 3.19/4) of CS, rate of progression, extent of disease or Ki-67 (weighted average 3.27/4 if Ki-67 < 10% *vs* weighted average 3.15/4 if Ki67 10%-20%); full details Figure 2C; full details in Supplementary Figure 1C.

Table 1 Baseline characteristics of responders

		Number of responses	Percentage (%)
Specialty	Medical oncology	15	53.6%
	Clinical oncology	3	10.7%
	Gastroenterology	5	17.7%
	Endocrinology	3	10.7%
	Surgery	2	7.1%
Country of practice	Belgium	2	7.1%
	Germany	1	3.6%
	France	2	7.1%
	Italy	4	14.3%
	Netherlands	2	7.1%
	Spain	5	17.9%
	Sweden	2	7.1%
	Switzerland	2	7.1%
	United Kingdom	6	21.4%
	Other	2	7.1%
Practice at ENETS Centre of Excellence	Yes	16	57.1%
	No	12	42.9%
Use of guidelines to inform management of SBNETs	Yes	28	100.0%
	No	0	0.0%
Guidelines used	ENETS guidelines	23	82.1%
	ESMO guidelines	0	0.0%
	NCCN guidelines	0	0.0%
	NANETS guidelines	0	0.0%
	Other	5	17.7%

ENETS: European Society of Neuroendocrine Tumours; SBNETs: Well-differentiated small bowel neuroendocrine tumours; NANETS: North American Neuroendocrine Tumor Society; ESMO: European Society for Medical Oncology; NCCN: National Comprehensive Cancer Network.

None of the responders selected chemotherapy as the preferred main third-line treatment option. Despite these, factors associated with its use were explored. Similar to the scenario described with everolimus, chemotherapy was mainly used predominantly after progression on all other available treatment options (weighted average 3.31/4) (Figure 2D; full details in Supplementary Figure 1D). In addition, chemotherapy was mainly chosen for patients with rapid progression to prior treatment (within 6 mo) (weighted average 3.35/4), Ki-67 10%-20% (weighted average 2.77/4), negative SSTR (weighted average 2.65/4) and high tumour burden (weighted average 2.77/4). Combination of temozolomide or streptozocine with capecitabine or 5-fluorouracil (5-FU) was the most popular choice of chemotherapy (57.7%), followed by FOLFOX (5-FU combined with oxaliplatin) (23.1%).

The use of liver-directed therapies, such as transarterial liver embolization, was also limited to second-line (weighted average 2.20/4) and third-line scenarios (weighted average 2.6/4); with a predominance of use after progression to all other available treatment options (weighted average 2.80/4) (Figure 2E; full details in Supplementary Figure 1E). The patients who were most likely to receive liver embolization were those with presence of CS (weighted average 3.24/4) and Ki-67 < 10% (weighted average 2.8/4), within others. Patients with extensive skeletal metastases were the least likely to be offered liver embolization (weighted average 1.64/4).

Use of interferon-alpha was rarely reported, with overall weighted average 1.3/4 (Figure 2F; full details in Supplementary Figure 1F). According to responses collected,

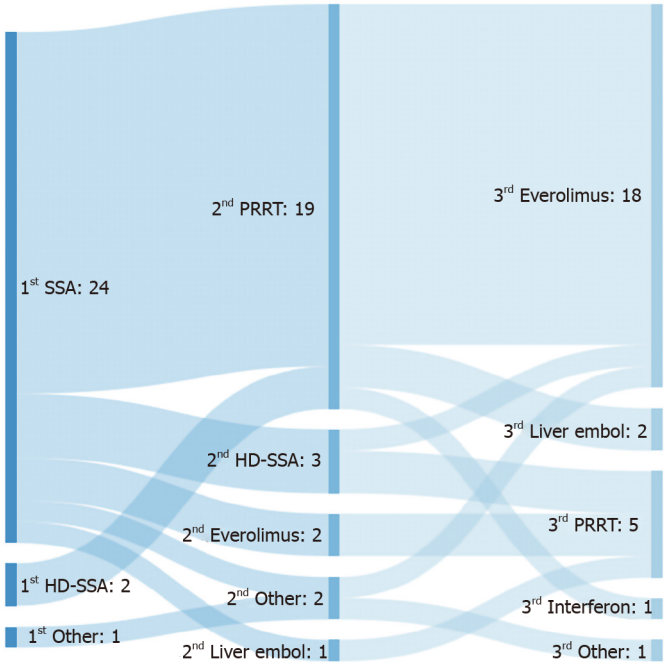


Figure 1 Summary of treatment predominant flow by line of therapy. SSA: Somatostatin analogues; PRRT: Peptide receptor radionuclide therapy; Liver embol: Liver embolization; HD-SSA: High-dose of somatostatin analogues.

no specific factors were associated with its use.

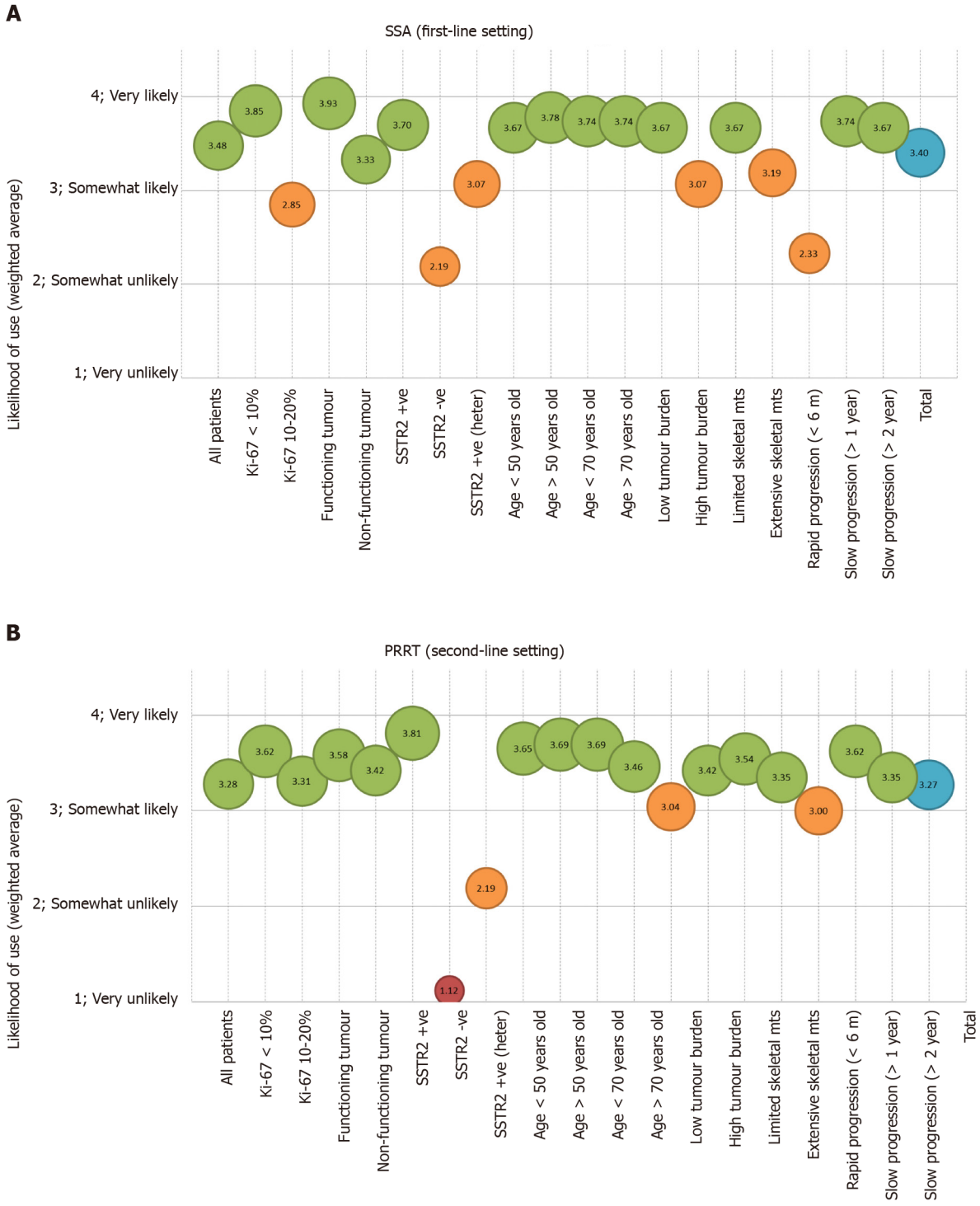
DISCUSSION

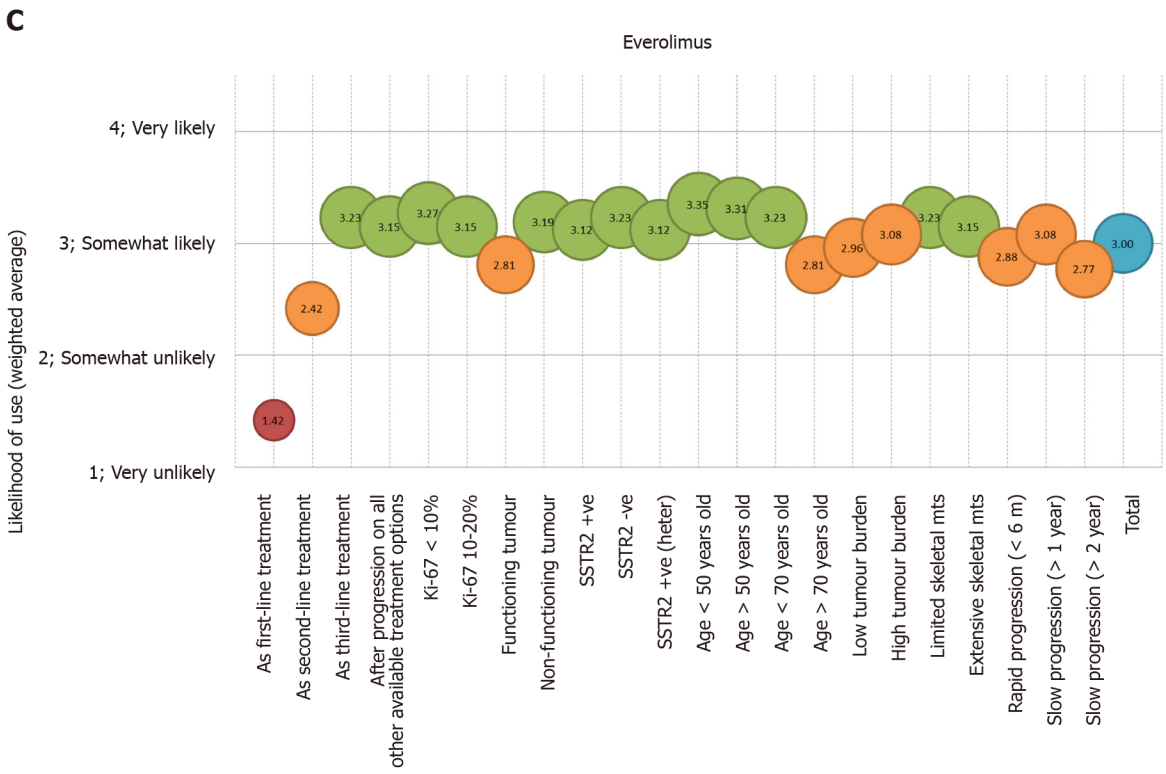
This survey aimed to address one of the main caveats in the management of SBNET: treatment decision making in terms of therapy sequencing, with special interest in the third-line setting. Our results identified everolimus as the preferred option of third-line therapy, with multiple factors identified as relevant at time of decision making, such as Ki-67, rate of progression, tumour functionality (CS) and tumour burden. The data underline that treatment decisions should be made within a multidisciplinary setting, where all available therapeutic options can be considered.

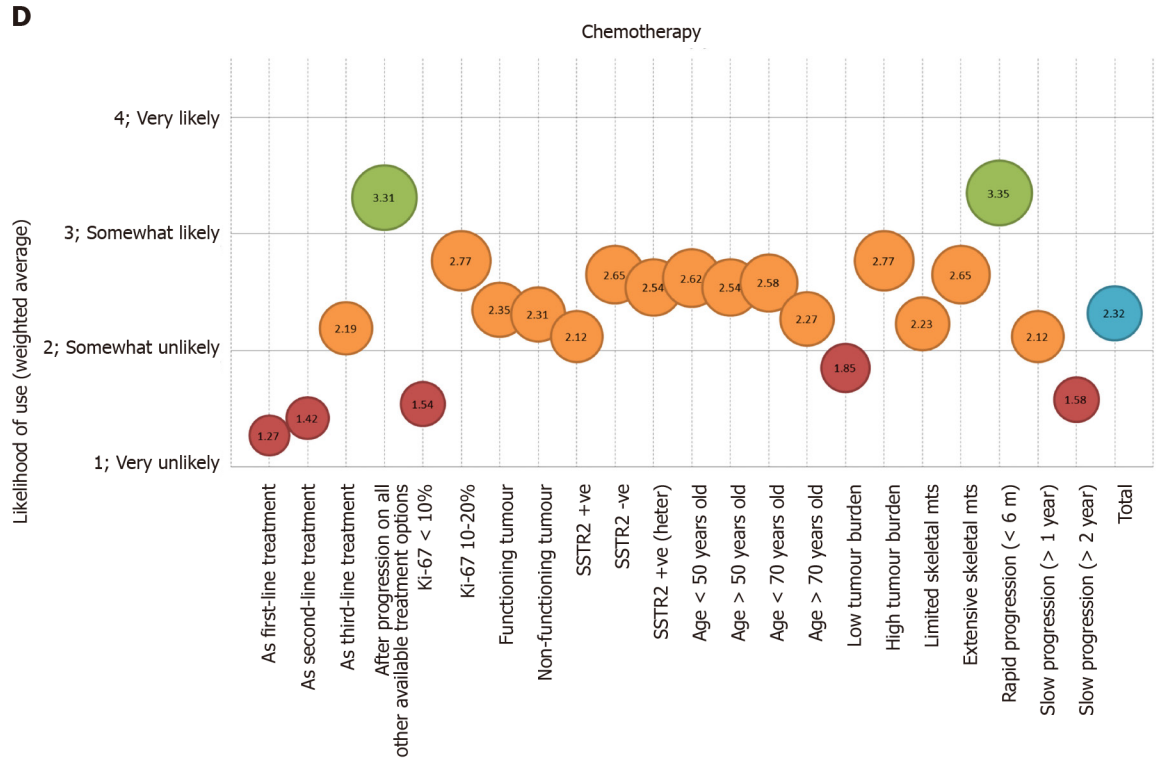
Choice of SSAs and PRRT as predominant first- and second-line treatments was expected. This is in keeping with current available guidelines[7,8] and can be accepted as current standard of care as supported by phase III clinical trial evidence[12,13,15]. The only exception would be those patients with SSTR2 negative disease who would not be eligible for PRRT and had been excluded from the NETTER-1 clinical trial[15]. Results of our survey do reflect this as well (Figure 2B; full details in Supplementary Figure 1B).

As shown in Figure 1, a small proportion of responders chose treatment with high-dose SSAs following progression to standard-dose SSAs. While the specific factors involved in this choice were not explored in the present survey, it is worth highlighting that this therapeutic option is suggested by guidelines mainly for patients with slowly-progressive SBNET and refractory functioning syndrome[7,8]. Results of the recently reported CLARINET-FORTE trial would support its anti-proliferative use following progression to SSA in selected scenarios, especially in case of Ki-67 ≤ 10%[14]. This phase II study included a cohort of 51 patients diagnosed with SBNET, who following progression to standard 4-weekly 120 mg lanreotide, received an increased dose of this same drug (120 mg 2-weekly); median duration of prior lanreotide exposure was 1.3 years (95%CI: 1.0-1.9) and 82.4% of patients had low-burden liver disease (affecting < 25% of liver parenchyma). Among the 51 patients with advanced SBNET included, median progression-free survival (PFS) was 8.3 (95%CI: 5.6-11.1) mo, with median PFS of 8.6 mo (95%CI: 5.6-13.8) and 5.5 mo (95%CI: 2.6-nr) for Ki-67 ≤ 10% and > 10% tumours, respectively. In view of this, authors concluded that high-dose SSAs could be recommended in selected patients, "before switching to an alternative, more toxic treatment".

Our survey highlights three main unanswered questions around the use of PRRT in SBNETs.


A
SSA (first-line setting)
Likelihood of use (weighted average)
4; Very likely
3; Somewhat likely
2; Somewhat unlikely
1; Very unlikely
3.48
3.85
3.93
3.33
3.70
2.85
3.07
2.19
3.67
3.78
3.74
3.74
3.67
3.07
3.67
3.19
2.33
3.74
3.67
3.40
All patients
Ki-67 < 10%
Ki-67 10-20%
Functioning tumour
Non-functioning tumour
SSTR2 +ve
SSTR2 -ve
SSTR2 +ve (heter)
Age < 50 years old
Age > 50 years old
Age < 70 years old
Age > 70 years old
Low tumour burden
High tumour burden
Limited skeletal mts
Extensive skeletal mts
Rapid progression (< 6 m)
Slow progression (> 1 year)
Slow progression (> 2 year)
Total
B
PRRT (second-line setting)
Likelihood of use (weighted average)
4; Very likely
3; Somewhat likely
2; Somewhat unlikely
1; Very unlikely
3.28
3.62
3.31
3.58
3.42
3.81
1.12
2.19
3.65
3.69
3.69
3.46
3.04
3.42
3.54
3.35
3.00
3.62
3.35
3.27
All patients
Ki-67 < 10%
Ki-67 10-20%
Functioning tumour
Non-functioning tumour
SSTR2 +ve
SSTR2 -ve
SSTR2 +ve (heter)
Age < 50 years old
Age > 50 years old
Age < 70 years old
Age > 70 years old
Low tumour burden
High tumour burden
Limited skeletal mts
Extensive skeletal mts
Rapid progression (< 6 m)
Slow progression (> 1 year)
Slow progression (> 2 year)
Total


C
Everolimus
Likelihood of use (weighted average)
4; Very likely
3; Somewhat likely
2; Somewhat unlikely
1; Very unlikely
1.42
2.42
3.23
3.15
3.27
3.15
2.81
3.19
3.12
3.23
3.12
3.35
3.31
3.23
2.81
2.96
3.08
3.23
3.15
2.88
3.08
2.77
3.00
As first-line treatment
As second-line treatment
As third-line treatment
After progression on all other available treatment options
Ki-67 < 10%
Ki-67 10-20%
Functioning tumour
Non-functioning tumour
SSTR2 +ve
SSTR2 -ve
SSTR2 +ve (heter)
Age < 50 years old
Age > 50 years old
Age < 70 years old
Age > 70 years old
Low tumour burden
High tumour burden
Limited skeletal mts
Extensive skeletal mts
Rapid progression (< 6 m)
Slow progression (> 1 year)
Slow progression (> 2 year)
Total


D
Chemotherapy
Likelihood of use (weighted average)
4; Very likely
3; Somewhat likely
2; Somewhat unlikely
1; Very unlikely
1.27
1.42
2.19
3.31
1.54
2.77
2.35
2.31
2.12
2.65
2.54
2.62
2.54
2.58
2.27
1.85
2.77
2.23
2.65
3.35
2.12
1.58
2.32
As first-line treatment
As second-line treatment
As third-line treatment
After progression on all other available treatment options
Ki-67 < 10%
Ki-67 10-20%
Functioning tumour
Non-functioning tumour
SSTR2 +ve
SSTR2 -ve
SSTR2 +ve (heter)
Age < 50 years old
Age > 50 years old
Age < 70 years old
Age > 70 years old
Low tumour burden
High tumour burden
Limited skeletal mts
Extensive skeletal mts
Rapid progression (< 6 m)
Slow progression (> 1 year)
Slow progression (> 2 year)
Total

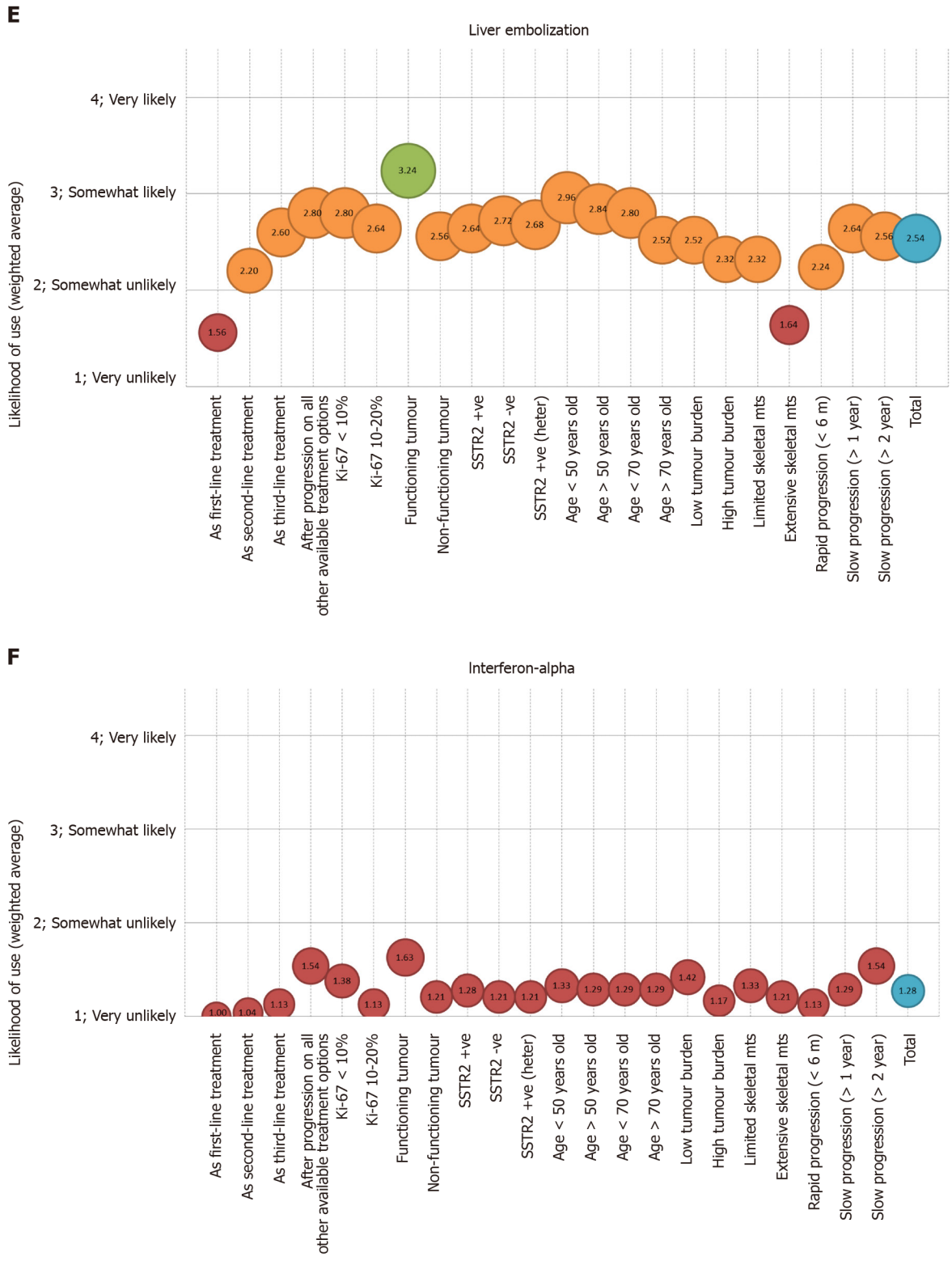


Figure 2 Factors associated with treatment decision making process. A: Factors associated with use of somatostatin analogues in the first-line setting; B: Factors associated with use of peptide receptor radionuclide therapy in the second-line setting; C: Factors associated with use of everolimus; D: Factors associated with use of chemotherapy; E: Factors associated with use of liver embolization; F: Factors associated with use of Interferon-alpha. SSA: Somatostatin analogues; SSTR2 –ve: Somatostatin receptor positive disease (imaging based); SSTR2 +ve: Somatostatin receptor negative disease (imaging based); heter: Heterogeneous; mts: Metastases; m: months; TOTAL: Represents the average of all factors; PRRT: Peptide receptor radionuclide therapy.

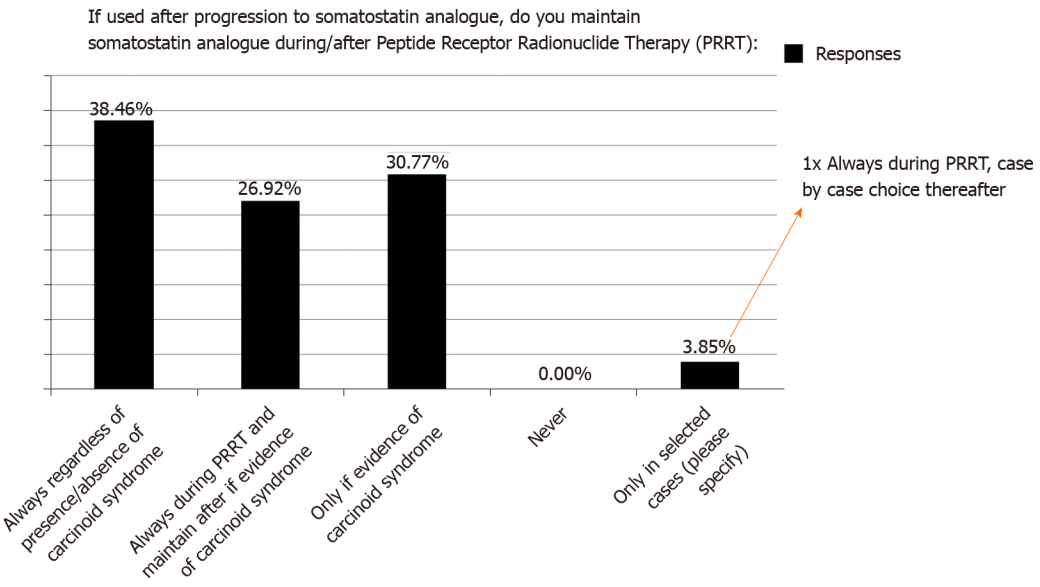


Figure 3 Ongoing controversies around use of peptide receptor radionuclide therapy in current practice.

First, there was discrepancy in terms of the current use of concomitant SSAs during and after PRRT (especially in non-functioning tumours). The NETTER-1 clinical trial recruited patients with SBNETs and as part of the study protocol, all patients (regardless of presence or absence of CS) received concomitant 4-weekly long-acting octreotide injection[15]. Thus, strictly speaking, NETTER-1 trial showed that PRRT was superior to high dose SSA when PRRT was given concomitantly with SSA (during and after PRRT). There is some evidence suggesting that combination of "hot" (PRRT) and "cold" (SSA) somatostatin analogue therapy could impact biodistribution of PRRT and may enable higher doses of PRRT to be delivered to tumour sites[24]. However, the benefit of maintenance SSA following PRRT completion is of much controversy. Some retrospective studies have suggested a benefit in terms of prolonged PFS[25], but selection bias (due to retrospective design) may confound findings. Thus, in the absence of CS, there is still disagreement regarding the benefit derived from maintenance SSAs after completion of PRRT and further studies are required to inform practice.

Second, the potential role of rechallenge with PRRT remains unclear. None of the responders chose rechallenge with PRRT in this survey as part of the main treatment pathway. However, it is likely that this is still a valid option of treatment for selected patients. There are data supporting the safety and efficacy of rechallenge with PRRT, mainly for patients with prolonged PFS to prior PRRT. In a series of 35 patients (23 SBNET), with median PFS of 33 mo (95%CI: 30-36) to prior PRRT, rechallenge with PRRT was shown to be safe (both in terms of kidney toxicity and myelo-suppression)[26]. Main benefit was in the form of disease stabilisation (identified in 81.3% of patients), with partial response identified in 1 patient (3.1%). Median PFS reported with rechallenge PRRT was 6 mo (95%CI: 0-16). Other series also identified a possible association between initial response (in terms of prolonged PFS) to PRRTon first use and PFS benefit on PRRT rechallenge[27]. In view of these findings, and despite absence of prospective trial data, rechallenge PRRT may be a treatment option in selected patients (mainly patients with prolonged PFS to prior PRRT), despite low partial response rate and limited median PFS.

Third, the potential role of PRRT earlier in patients' pathway remains undefined. There is currently no clinical trial data supporting this and available guidelines do consider PRRT after SSA for SBNETs[7,8]. This was reflected in our survey results, in which none of the responders selected PRRT as a first-line treatment, highlighting that further clinical trials would be required prior to any changes in current clinical practice. In this scenario, ongoing studies such as COMPETE (NCT03049189) and NETTER-2 (NCT03972488) are likely to provide useful insight to inform future practice.

There was a clear trend in favour of everolimus as the preferred choice of treatment in the third-line setting and this is likely based on vast clinical experience with everolimus. It is worth noting that the presence or absence of CS did not play a significant role on the likelihood of everolimus being considered. The fact that the

weighted average was higher for non-functioning tumours (3.19/4) was not really surprising, in view of the benefit from everolimus over placebo in the setting of non-functioning SBNETs from the RADIANT-4 trial results[16]. However, the weighted average for functioning tumours SBNETs was high (2.81/4). This was despite the fact that everolimus is approved only in the setting of non-functioning SBNETs[28], which is reflected in current guidelines[7,8] and derived from the negative results of the RADIANT-2 study[29].

Based on our findings, interferon-alpha seems to have a minor role in the management of SBNETs and to be rarely used in clinical practice. Liver embolization was a preferred treatment option for patients with functioning tumours, in keeping with data from recent systematic review and meta-analysis[17], which reported a pooled symptomatic response of 55.2%. Chemotherapy was mainly used as a rescue treatment when progression to other available treatments was shown, and was mainly suggested for patients with rapid progression. These findings were expected and in keeping with current guideline recommendations[7,8]. As shown in our survey, most suitable choice of chemotherapy remains unclear, and despite a preference for temozolomide and capecitabine-based combinations, many responders reported to use other schedules. Collaborative work and trials in this setting are urgently needed.

Our results identified that many factors are involved in treatment choices, not only in second-line but also in the third-line setting, when choosing between a variety of treatment options such as everolimus, chemotherapy, liver embolization and interferon-alpha. The main factors identified as relevant for decision making were Ki-67, rate of progression, tumour functionality and tumour burden. It is therefore clear that "one size does not fit all" when selecting treatment options, especially in the third-line setting. Based on this, discussion within multidisciplinary meetings when disease progression to prior therapies is documented is recommended, to secure that the best treatment choice is made.

The main strength of this work is the fact that responders were clinicians with wide experience in the management of NETs, thus including the insights of healthcare professionals with adequate expertise to answer the proposed questions and dilemmas. Unfortunately, the number of replies was limited, which is the main limitation of our work. In addition, variability in drug access between different countries was likely to impact on some of the decision making steps, although it was not interrogated in the present work. Since treatment choices are based on approval and accessibility to drugs as well as reimbursement it remains unclear how much our findings are applicable to other countries including outside Europe.

CONCLUSION

In conclusion, our survey delineates a possible treatment algorithm in patients with advanced SBNET. While SSAs, PRRT and everolimus are usually considered preferred first, second and third-line options respectively, chemotherapy is generally used when all other available treatments have failed. Locoregional therapies appear particularly useful when facing patients with functioning tumours, but their use is mainly limited to after at least two prior lines of treatment. We were also able to identify relevant unanswered questions in the field of advanced SBNET treatment, mainly in regards to the role of maintenance SSA after PRRT for non-functioning tumours. Multiple factors were identified as relevant at time of decision making; among them, Ki-67, rate of progression, tumour functionality and tumour burden may have a key role in helping physicians tailoring the treatment. Based on this, we would encourage for treatment decisions to be made within a multidisciplinary setting.

ARTICLE HIGHLIGHTS

Research background

Adequate sequencing of treatment for small bowel neuroendocrine tumour (SBNET) is unclear and likely to rely on multiple factors.

Research motivation

We aimed to understand current practice for advanced SBNET.

Research objectives
This international survey aimed to collect data on current algorithms and main factors involved in decision making process.

Research methods
An international survey was completed by many health care professionals with an expertise in neuroendocrine tumours.

Research results
Our survey delineates a possible treatment algorithm in patients with advanced SBNET. While somatostatin analogues (SSAs), peptide receptor radionuclide therapy (PRRT) and everolimus are usually considered preferred first, second and third-line options respectively, chemotherapy is generally used when all other available treatments have been used. Locoregional therapies appear particularly useful when facing patients with functioning tumours, but their use commonly follows the first two lines of treatment.

Research conclusions
We were also able to identify relevant unanswered questions in the field of advanced SBNET treatment, mainly in regards to the role of maintenance SSA after PRRT for non-functioning tumours. Multiple factors were identified as relevant at time of decision making; among them, Ki-67, rate of progression, tumour functionality and tumour burden may have a key role in helping physicians tailoring the treatment.

Research perspectives
Based on this, we would encourage for treatment decisions to be made within a multidisciplinary setting.

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FRONTIER

Chronic renal dysfunction in cirrhosis: A new frontier in hepatology

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Abstract

Chronic kidney disease (CKD) in patients with liver cirrhosis has become a new frontier in hepatology. In recent years, a sharp increase in the diagnosis of CKD has been observed among patients with cirrhosis. The rising prevalence of risk factors, such as diabetes, hypertension and nonalcoholic fatty liver disease, appears to have contributed significantly to the high prevalence of CKD. Moreover, the diagnosis of CKD in cirrhosis is now based on a reduction in the estimated glomerular filtration rate of < 60 mL/min over more than 3 mo. This definition has resulted in a better differentiation of CKD from acute kidney injury (AKI), leading to its greater recognition. It has also been noted that a significant proportion of AKI transforms into CKD in patients with decompensated cirrhosis. CKD in cirrhosis can be structural CKD due to kidney injury or functional CKD secondary to circulatory and neurohormonal imbalances. The available literature on combined cirrhosis-CKD is extremely limited, as most attempts to assess renal dysfunction in cirrhosis have so far concentrated on AKI. Due to problems related to glomerular filtration rate estimation in cirrhosis, the absence of reliable biomarkers of CKD and technical difficulties in performing renal biopsy in advanced cirrhosis, CKD in cirrhosis can present many challenges for clinicians. With combined hepatorenal dysfunctions, fluid mobilization becomes problematic, and there may be difficulties with drug tolerance, hemodialysis and decision-making regarding the need for liver *vs* simultaneous liver and kidney transplantation. This paper offers a thorough overview of the increasingly known CKD in patients with cirrhosis, with clinical consequences and difficulties occurring in the diagnosis and treatment of such patients.

Key Words: Acute kidney injury; Cirrhosis; Chronic kidney disease; Renal failure; Hepatorenal syndrome; Renal function

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Core Tip: The current definition of chronic kidney disease (CKD) in patients with liver cirrhosis is based on a 3-mo decline in the glomerular filtration rate of < 60 mL/min, and it may be structural or functional CKD, depending on the presence or absence of kidney injury. Emerging data show that the incidence of CKD has risen dramatically in patients with cirrhosis over the last decade. The main reasons behind the increased prevalence of CKD appear to be a growing recognition of this condition and a rising trend in the prevalence of diabetes, hypertension and nonalcoholic fatty liver disease. This paper offers a detailed overview of CKD in patients with cirrhosis, including the clinical implications and difficulties clinicians may face with regard to diagnosis and treatment.

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INTRODUCTION

Most attempts to assess renal impairment in cirrhosis have so far concentrated on acute kidney injury (AKI), and as a result, detailed knowledge of AKI in cirrhosis is now available[1]. However, there is still scarce evidence on the prevalence, clinical impact and treatment of chronic kidney disease (CKD) in cirrhosis. A sharp rise in the diagnosis of CKD among patients with cirrhosis has been observed in recent years. The prevalence of CKD in hospitalized patients with cirrhosis, which used to be approximately 1% in 2005, has now risen to as high as 46.8% in 2019[2,3]. The growing prevalence of CKD in patients with cirrhosis may represent the convergence of several important epidemiological patterns: the continuing increase in the prevalence of metabolic risk factors such as obesity, hypertension and Medicine degree (DM); the increasing prevalence of nonalcoholic fatty liver disease (NAFLD) as a major contributor to the burden of cirrhosis; and the aging cohort of cirrhosis[4-6]. Moreover, some emerging evidence indicates that the risk of developing de novo CKD remains high for AKI survivors[7]. Liver cirrhosis patients are susceptible to developing AKI due to circulatory abnormalities, neurohormonal changes and the involvement of risk factors such as bacterial infection, gastrointestinal bleeding, medication and paracentesis[1,8]. Depending on the severity, length and frequency, AKI increases the risk of developing incident CKD due to decreases in renal mass and nephron number, vascular insufficiency, and maladaptive repair mechanisms[9]. Therefore, rather than separate entities, AKI and CKD may represent a continuum. The term CKD now encompasses both structural CKD due to structural damage to the kidney and functional CKD due to circulatory and neurohormonal imbalances in cirrhosis. The differentiation between various forms of renal dysfunction in cirrhosis is crucial, as each requires a different treatment plan.

DEFINITION AND CLASSIFICATION

The definition of CKD in cirrhosis was originally based on a serum creatinine level of > 1.5 mg/dL until 2011, when an updated definition was introduced by a working group composed of experts from various disciplines[10]. The definition endorsed by kidney disease: Improving global outcomes was largely adopted by this group, and CKD was defined as an estimated glomerular filtration rate (eGFR) of < 60 mL/min for more than 3 mo, measured using the Modification of Diet in Renal Disease-6 (MDRD-6) equation. While the group further agreed that the MDRD-6 equation was not perfect for estimating GFR in patients with cirrhosis, it may still be adopted until better alternatives become available. Currently, the diagnosis of CKD does not require corroborating evidence of kidney damage, such as proteinuria, hematuria, abnormal renal imaging or pathology.

Kidney Disease: Improving Global Outcomes has classified CKD into structural and functional CKD on the basis of the presence or absence of kidney injury. The old entity, type 2 hepatorenal syndrome (HRS), now referred to as HRS-CKD, is essentially a functional CKD[11]. While functional CKD is considered potentially reversible, since biomarkers of renal tubular damage have been found in patients with HRS, this may not exactly be the case[12-14]. Patients with cirrhosis may have several risk factors for developing structural CKD per se, such as DM, NAFLD, and atherosclerosis[4-6]. In addition, persistent renal vasoconstriction in functional CKD can lead to structural changes, transforming it into structural CKD.

THE GROWING PREVALENCE OF CHRONIC KIDNEY DISEASE IN CIRRHOSIS

In recent years, not only has the prevalence of CKD increased significantly in the general population, but an increasing rise in the prevalence of CKD has also been reported in patients with cirrhosis (Table 1)[3,15-17]. The prevalence rates of CKD in cirrhosis, however, vary significantly across studies due to variations in parameters used to describe CKD and differences in the severity of patients with chronic liver disease (CLD).

In a study by Rustgi *et al*[18], although the prevalence of CKD among 94431 patients with cirrhosis collected from the insurance claim database was 3.37%, the proportion of patients with decompensated cirrhosis was higher in the combined CLD-CKD group (27.2% *vs* 11.8%), suggesting that the prevalence of CKD increases with the severity of CLD. In a recent retrospective analysis of a large cohort of patients with cirrhosis (n = 78640) awaiting liver transplantation (LT), while the prevalence of CKD was 7.8% in 2002, it increased to 14.6% in 2017. This is a documented increase in the CKD prevalence rate of 187% in just 15 years. Moreover, among 39719 LT recipients, 6269 (16%) patients met the CKD criteria at the time of last transplant[4]. Another study evaluating the prospectively managed database of the North American Consortium for the End-Stage Liver Disease Study reported a 46.8% prevalence of CKD among 2346 admitted patients with cirrhosis[3]. In an Indian study, the occurrence of CKD was observed in 32.8% of a large prospective cohort (n = 818) of patients with cirrhosis[16]. Functional CKD is considered to be relatively uncommon and accounts for only approximately 3.9% to 15.8% of renal impairments among hospitalized cirrhotic patients; however, new data need to be developed in light of the updated concept of CKD in cirrhosis[2,19].

DETERMINANTS OF INCREASING CHRONIC KIDNEY DISEASE IN CIRRHOSIS

Apart from the aging and the high incidence of AKI in cirrhosis, a rising recognition of this condition and a rising trend in the prevalence of DM, hypertension and NAFLD seem to be the key factors behind the increased prevalence of CKD in cirrhosis (Figure 1).

AKI to CKD transition

AKI is an independent risk factor for developing CKD in the general population. AKI to CKD transition appears to reflect a continuum. In a meta-analysis of 13 studies, the pooled adjusted hazard ratio for developing CKD among patients with AKI was 8.8 (95% confidence interval: 3.1-25.5)[20]. AKI occurs very frequently in patients with decompensated cirrhosis because of pre-existing circulatory abnormalities, neurohormonal changes and the involvement of risk factors such as DM, bacterial infection, gastrointestinal bleeding, medications and therapeutic paracentesis[1,7,10,11]. Emerging data suggest that in patients with decompensated cirrhosis, a large proportion of AKI progresses to CKD. In a recent study, 25% of patients with decompensated cirrhosis with AKI who survived for at least 3 mo developed CKD, compared with only 1% of those without AKI[7]. Moreover, the odds of developing CKD in patients with decompensated cirrhosis with AKI were 31, suggesting that they are more prone to CKD development than general AKI patients. A higher transition from AKI to CKD was seen when the severity of AKI was higher and when it developed after hospitalization. In another study published in India, 32.8% of 818 patients with cirrhosis developed CKD, approximately 80% of patients with CKD had

Table 1 Incidence and prevalence of chronic kidney disease in cirrhosis patients

Ref.	Study subjects (*n*)	Criteria for CKD diagnosis	Incidence or prevalence of CKD
Cullaro *et al*[4], 2020	39719 LT recipient cirrhosis patients	CKD-EPI equation based eGFR < 60 mL/min for 90 d or ≥ 42 d of hemodialysis	16% patients (*n* = 6269) met CKD criteria at the time of LT
Bassegoda *et al*[7], 2020	409 hospitalized cirrhosis patients, 168 with AKI	MDRD-4 equation based eGFR < 60 mL/min > 3 mo	Among survived patients at 3 mo, 9.1% (26/285) developed CKD, Incidence of CKD among cirrhosis-AKI patients was 25% (24/97)
Rustgi *et al*[18], 2020	598455 CLD patients, including 94431 patients with cirrhosis	As per the record, based on International Classification of Disease (ICD-9) code	Among 94431 cirrhosis patients, prevalence of CKD was 3.37%
Wong *et al*[3], 2019	2346 non-electively admitted patients with cirrhosis	MDRD-4 equation based eGFR < 60 mL/min for > 3 mo	Prevalence of CKD was 46.8% (*n* = 1099)
Maiwall *et al*[16], 2020	818 cohort of both hospitalized and outdoor cirrhosis patients	MDRD-6 equation based eGFR < 60 mL/min for > 3 mo and abnormal urine microscopy > 12 wk	Incidence of CKD was 32.8% (*n* = 269)
Chen *et al*[17], 2018	7440 adult patients with cirrhosis	MDRD-6, CKD-EPI and MDRD-4 equation based eGFR < 60 mL/min for > 3 mo	CKD was present in 46.0%, 45.7% and 45.6% of patients using the MDRD-6, CKD-EPI and MDRD-4 equations, respectively
Choi *et al*[19], 2014	643 hospitalized cirrhosis patients	MDRD-6 equation based eGFR < 60 mL/min for > 3 mo	Prevalence of CKD was 3.4% (*n* = 22)
Martín-Llahí *et al*[32] , 2011	463 hospitalized cirrhosis patients with renal impairment	Old criteria for HRS based on serum creatinine: > 1.5 mg/dL	Proportion of CKD-HRS was 3.9% (*n* = 22)
Salerno *et al*[33], 2011	263 hospitalized cirrhosis patients with renal impairment	Old criteria for HRS based on serum creatinine: > 1.5 mg/dL	Proportion of CKD-HRS was 18.5% (*n* = 40)
Péron *et al*[2], 2005	932 hospitalized cirrhosis patients with renal impairment	Old criteria for HRS based on serum creatinine: > 1.5 mg/dL	Proportion of CKD was 10 (1.07%), Proportion of CKD-HRS was 07 (0.75%)

CKD: Chronic kidney disease; LT: Liver transplantation; CKD-EPK: Chronic kidney disease epidemiology collaboration; eGFR: Estimated glomerular filtration rate; AKI: Acute kidney injury; MDRD: Modification of diet and renal disease; CLD: Chronic liver disease; HRS: Hepatorenal syndrome.

at least one episode of AKI, and one-third of patients with AKI had progression to CKD[16]. The mechanisms underlying AKI-CKD progression are still poorly understood. In general, it is believed to be a result of maladaptive repair in the interstitial, vascular and tubular structures of the kidney[9]. However, it is not clear whether the same mechanisms contribute to the development of CKD in cirrhosis. Patients with higher baseline levels of serum creatinine are more likely to develop AKI and less likely to recover from such AKI episodes[21]. Because DM and hypertension patients are more likely to have intrinsic renal disease, such as diabetic nephropathy or hypertensive nephrosclerosis, they are not only at higher risk of developing AKI episodes but also less likely to recover from these episodes due to a lower renal reserve.

Increase in the multiple risk factors

There has been a substantial increase in the multiple shared risk factors for cirrhosis and CKD over the years. NAFLD is independently and significantly associated with an increased incidence and prevalence of CKD[22,23]. In a recent meta-analysis that included nearly 64000 subjects, NAFLD was associated with an approximately 2-fold increased risk of both prevalent and incident CKD[23]. Multiple factors, such as the proinflammatory environment, insulin resistance, oxidative stress, and the activated renin-angiotensin system, may account for the accelerated development and progression of CKD in NAFLD subjects, apart from the common occurrence of DM and hypertension[24]. In addition, NAFLD has been strongly associated with atherosclerosis, as shown by increased intima media thickness or atherosclerotic plaques in the carotid arteries, and atherosclerosis has been associated with glomerulosclerosis, a process that can lead to CKD[25]. In one study, approximately 20%-25% of LT candidates were found to have severe coronary artery disease, suggesting that atherosclerosis in cirrhosis is not uncommon[26]. Over the last 2 decades, the prevalence of NAFLD and NAFLD-related cirrhosis has also increased considerably[27]. In a study, NAFLD accounted for a substantial rise in simultaneous

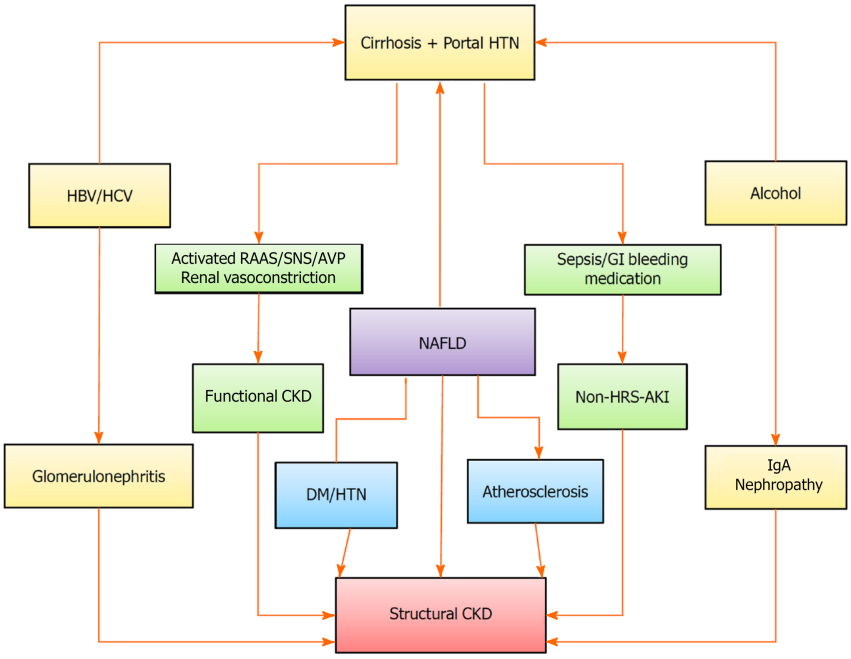


Figure 1 Risk factors associated with chronic kidney disease in patients with liver cirrhosis. A rising trend in the prevalence of medicine degree, hypertension and non-alcoholic fatty liver disease seem to be the key factors behind the increased prevalence of chronic kidney disease in cirrhosis. The risk of developing de-novo chronic kidney disease remains high for acute kidney injury survivors. CKD: Chronic kidney disease; HTN: Hypertension; HBV: Hepatis B virus; HCV: Hepatitis C virus; RAAS: Renin-angiotensin-aldosterone system; SNS: Sympathetic nervous system; AVP: Arginine vasopressin; GI: Gastrointestinal; NAFLD: Non-alcoholic fatty liver disease; HRS: Hepatorenal syndrome; AKI: Acute kidney injury.

liver and kidney transplantation (SLKT), from 8.2% in 2002 to 22% in 2011[28]. In cirrhosis, DM is highly prevalent, with recorded prevalence rates ranging from 35% to 71%, which is far higher than in the general population[29]. Glomerulopathy, which can progress to CKD, may be associated with certain specific causes of cirrhosis, such as hepatitis B virus (HBV) or hepatitis C virus (HCV). Glomerular involvement in patients with viral hepatitis occurs *via* an immune pathogenic mechanism. Circulating immune complexes containing viral antigens have been found in the kidney[30].

CLINICAL IMPLICATIONS OF CHRONIC KIDNEY DISEASE IN CIRRHOSIS

In several ways, CKD can affect the clinical manifestations, complications, therapeutic decisions, and outcomes of patients with cirrhosis.

Impact on clinical manifestations

Anorexia, anemia, ascites, bleeding tendency and encephalopathy can be independently due to both hepatic and renal diseases, so the contributions from individual diseases are often difficult to determine in patients with cirrhosis with CKD. This may create uncertainty about optimal therapeutic choices, such as requirements of renal replacement therapy. In patients with cirrhosis, CKD may contribute to ascites and edema in various ways, such as nephrogenic ascites, chronic fluid overload, hypoproteinemia, and cardiomyopathy[31]. Refractory ascites is almost universal in patients with functional CKD[32,33]. Due to multiple and complex hemostasis abnormalities, patients with concurrent hepatorenal dysfunction may have a higher tendency to bleed. On the other hand, even thrombotic complications are not unusual in such patients[34]. CKD is an independent cardiovascular mortality risk factor, and it can worsen anemia due to cirrhosis[35,36]. Both CKD and cirrhosis can cause immunodepression, leading to an increased risk of infection[37]. Patients with cirrhosis and CKD appear to have an increased risk of developing malignancy[27,38]. In recent years, NAFLD, which is significantly linked to CKD, has also emerged as one of the leading causes of hepatocellular carcinoma (HCC)[27]. Alcohol, HBV infection and HCV infection are other shared risk factors that can be associated with both HCC and CKD. HCC has been found to be associated with a higher prevalence of CKD than any other cancer[39]. After adjustment for many possible confounders, a lower GFR has been

shown to be independently associated with a higher risk of incident renal cell and urothelial cancer[40]. CKD has been shown to be associated with increased mortality from liver, kidney, and urothelial cancers[41].

Impact on complications and outcomes

CKD in cirrhosis is associated with poor outcomes and an increased frequency of complications[3,7]. Wong *et al*[3] found that patients with cirrhosis with CKD had higher rates of superimposed AKI (68% *vs* 21%), need for dialysis (11% *vs* 2%) and 30-d mortality rates (16% *vs* 7%) than patients with cirrhosis without CKD. A 10 mL/min decrease in eGFR was found to be associated with a 13% increase in 30-d mortality in patients with cirrhosis. In a study by Bassegoda *et al*[7], patients with cirrhosis with CKD had a higher frequency of AKI (75% *vs* 45%), refractory ascites (25% *vs* 7%), bacterial infections (58% *vs* 34%) and LT requirement (25% *vs* 10%) compared with those without CKD[7]. In addition, the involvement of cirrhosis is independently related to a poor outcome in patients with CKD[42]. CKD impacts not only waitlist mortality but also worsens post-LT survival. Cullaro *et al*[4] reported that the one-year post-LT mortality rate in patients with CKD was 12%, compared with 9% in those without CKD. In addition, posttransplant renal outcomes may also be affected by the presence of CKD[13].

Impact on health care utilization

Wong *et al*[3] found that cirrhosis patients with CKD had higher rates of hospitalization during the preceding 6 mo (70% *vs* 63%) than those without CKD. Similarly, Bassegoda *et al*[7] reported a higher 3-mo readmission rate (67% *vs* 37%) in cirrhosis-CKD patients compared to cirrhosis alone. A recent study analyzed the usage of health care services and the cost burden associated with CKD in patients with CLD (n = 9869) compared to patients with CLD alone (n = 588586) by using real-world insurance claims data. In a propensity-matched cohort analysis, patients with combined CLD-CKD were found to have substantially greater annual per-person all-cause health care costs than patients with CLD alone[18].

DIAGNOSTIC EVALUATION

The diagnosis of CKD in cirrhosis is based on GFR. Abnormal urine analysis and/or abnormal findings on renal ultrasonography are usually found in advanced CKD and hence are not required for diagnosis. Cirrhosis can be diagnosed in patients with CKD by histopathology or hepatic ultrasound, as well as by clinical manifestations of portal hypertension and/or hepatic decompensation.

Since CKD diagnosis in cirrhosis requires a decrease in GFR to < 60 mL/min for 12 wk, a reliable and reproducible method is required to estimate GFR. The direct iothalamate clearance test is the gold standard for GFR measurement; however, the cumbersome technique and lack of widespread availability limit its usage in clinical practice[43]. Several indirect methods are available to calculate eGFR in clinical practice.

Creatinine-based eGFR

For the assessment of renal function, an eGFR based on the serum creatinine level is commonly used in clinical practice. In patients with cirrhosis, the most commonly used creatinine-based equation is the MDRD. However, serum creatinine levels in patients with cirrhosis may be unreliable due to hepatic dysfunction causing decreased production of creatine, reduced skeletal muscle mass causing decreased creatine-to-creatinine conversion, increased tubular secretion of creatinine, and underestimation of the serum creatinine level by hyperbilirubinemia[44-46]. Therefore, GFR in cirrhosis is typically overestimated by the creatinine-based equation, where a normal serum creatinine level cannot rule out renal dysfunction. In a meta-analysis, the formula based on creatinine was found to overestimate GFR by 18 mL/min[47]. However, despite limitations and until better substitutes become available, the latest creatinine-based MDRD equation (MDRD-6) has been recommended by expert panels to be used in patients with cirrhosis[4,44]. The MDRD-6 equation includes 6 variables: Age, sex, race, serum creatinine, serum albumin, and blood urea nitrogen.

Cystatin-based eGFR

Cystatin C is a protein produced by all nucleated cells in the body that is exclusively removed by glomerular filtration. Hepatic function, muscle mass, sex, hyperbilirubinemia and tubular secretion do not affect the level of cystatin C. Therefore,

cystatin C-based eGFR may be a better alternative to the serum creatinine-based equation for patients with cirrhosis[48-50]. However, the level of cystatin C is affected by hypoalbuminemia, elevated C-reactive protein and leukocytosis, which may limit its role in estimating GFR in cirrhosis[50,51]. Additionally, in patients with cirrhosis, the diagnostic performance of all cystatin C-based GFR equations has been found to be lower than in those without cirrhosis[44]. Combining serum creatinine and cystatin C in an equation appears to predict GFR more accurately than either alone[52]. However, eGFR measurement based on cystatin C has not yet been approved for routine use in patients with cirrhosis.

Biomarkers of kidney damage

The role of conventional urinary markers such as albuminuria is very limited in patients with cirrhosis, which may be because of hypoalbuminemia and relatively increased capillary permeability[44]. In addition, a normal proteinuria or urine examination may not exclude parenchymal changes in the kidney. The recent identification of many urinary biomarkers of renal tubular injury, such as urinary neutrophil gelatinase-associated lipocalin (uNGAL), interleukin-18, liver-type fatty acid-binding protein and kidney injury molecule-1, has revolutionized research into organic renal dysfunction[44,53-55]. These biomarkers have, however, been studied primarily in the context of AKI, and their role in the assessment of CKD is not yet clear. The most extensively assessed biomarker in cirrhotic patients has been uNGAL, an inflammatory biomarker produced by damaged renal tubular cells. The uNGAL levels can help distinguish organic from functional AKI; however, cutoff values for such discrimination lack specificity. In addition, the existence of concomitant infections or prolonged renal vasoconstriction in patients with HRS may significantly increase uNGAL levels, thereby limiting its discriminatory function in patients with cirrhosis[14,53]. In general, uNGAL has a positive correlation with the severity of renal dysfunction in patients with CKD, which indicates its prognostic significance for CKD[53]. However, the prognostic value of uNGAL in patients with cirrhosis with CKD is not known.

The profiles of urinary microRNAs may be an attractive noninvasive tool for future kidney damage assessment[54]. Other biomarkers, such as osteopontin and metalloproteinase-1 tissue inhibitor, are usually elevated in patients with CKD, but their clinical significance has not yet been established[55].

Role of duplex Doppler ultrasonography

Renal duplex Doppler ultrasound is a simple, noninvasive and efficient method that can be used in patients with cirrhosis to study intrarenal hemodynamics (Figure 2). It is a test to assess renal vascular resistance as a vasoconstriction marker, and the renal resistive index (RRI) can be used to detect early renal dysfunction in patients with cirrhosis[56]. In general, there is a progressive increase in RRI as cirrhosis patients move from without ascites to with ascites and then to HRS[57]. Since severe renal vasoconstriction is a feature of HRS, duplex ultrasonography can play a potential role in the assessment of functional CKD in patients with cirrhosis. Furthermore, the RRI can predict CKD progression as it correlates with renal histopathological changes such as glomerular sclerosis, interstitial fibrosis, and arteriolosclerosis[58].

Differentiation between functional and structural CKD

It is important to determine what proportion of CKD in cirrhosis is functional due to HRS and what proportion is associated with structural renal damage. Such differentiation has important therapeutic and prognostic implications. This would assist the clinician in deciding on the use of diuretics, vasoconstrictor treatment and the recommendation of LT *vs* SLKT. Structural CKD patients are more likely to be indolent and have higher survival rates than functional CKD patients[32]. However, in the absence of a renal biopsy, it is often difficult to differentiate a functional CKD from a structural CKD. Abnormal urine analysis (proteinuria > 500 mg/d or hematuria > 50/high power field) and/or abnormal findings on renal ultrasonography (reduced cortical thickness, increased cortical echogenicity and scarring) are features of advanced structural CKD (Figure 2). Currently, no accurate biomarkers are available that can diagnose subclinical renal parenchymal injury or differentiate between reversible and permanent renal injury. Importantly, prolonged renal vasoconstriction in patients with functional CKD may lead to irreversible structural changes in the kidney[32,33]. Studies on the outcome of LT in patients with type 2 HRS have found that 50%-60% of patients develop stage 3 CKD during the posttransplant period, even when HRS reverses[13,59]. Therefore, essentially a long-standing functional CKD can be

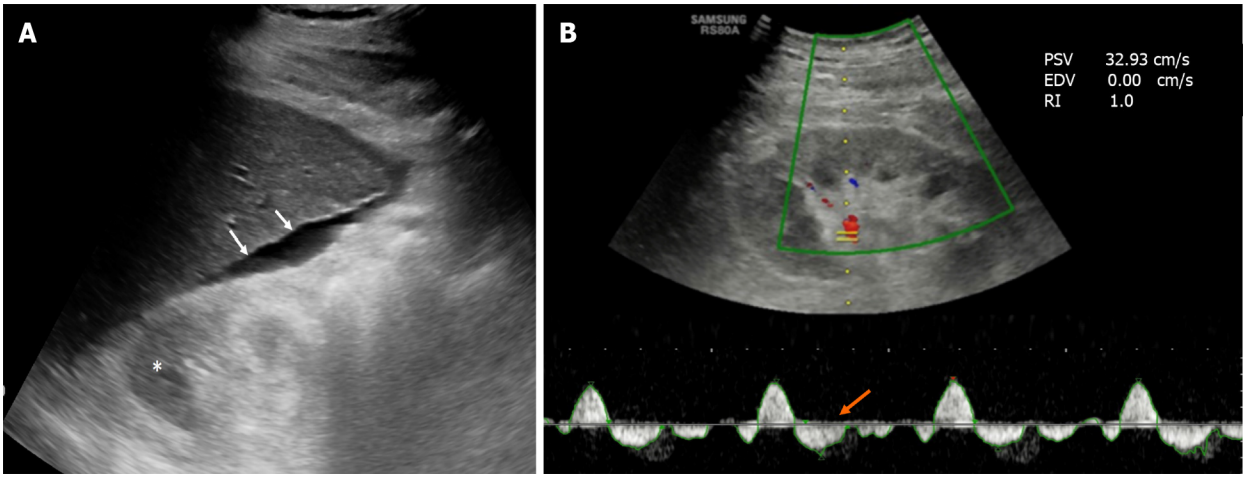


Figure 2 Ultrasonographic image of a 65-year-old diabetic patient with liver cirrhosis and chronic kidney disease. A: The liver outline is irregular (white arrows) and there is ascites around it. The right kidney is small and the parenchymal echogenicity is increased with loss of corticomedullary differentiation (asterisk), suggesting chronic kidney disease; B: Doppler sonogram of the same kidney showed reversal of diastolic flow (orange arrow) with absent end-diastolic velocity, indicating very high resistance vessels.

regarded as structural CKD. In the absence of abnormal early imaging features and reliable biomarkers of CKD, renal biopsy remains the only choice to diagnose and further characterize CKD. However, due to coagulopathy, thrombocytopenia, and the presence of large ascites in cirrhosis, as well as scarred kidneys due to CKD, percutaneous renal biopsy may be technically challenging in patients with decompensated cirrhosis.

Pitfalls in the diagnosis of CKD

A dramatic rise in the diagnosis of CKD in patients with cirrhosis raises some concerns as to whether, in the absence of any corroborating evidence of renal injury, the dependence solely on eGFR leads to overdiagnosis of CKD[60]. An arbitrary single threshold of eGFR < 60 mL/min might have a high propensity to cause overestimation of CKD in elderly subjects. There is a natural steady decrease in GFR with increasing age, and eGFR levels between 50 and 60 mL/min can be insignificant for older individuals, with very little propensity to progress to symptomatic kidney disease[61]. The risks of overdiagnosis of CKD may be significant in patients with cirrhosis since many of them belong to the old age group. In addition, a varying degree of deterioration in GFR may occur in patients with decompensated cirrhosis due to neurohormonal alterations and circulatory dysfunction well before the detection of overt renal disease, which may lead to overdiagnosis of CKD. Therefore, on the basis of a single eGFR threshold and in the absence of any corroborating evidence of kidney damage, caution before labeling CKD in elderly patients with cirrhosis may be needed. Since creatinine-based equations tend to overestimate the GFR, they can underestimate CKD and thus may provide clinicians with false reassurance. Studies need to be performed to determine whether the diagnosis of CKD in patients with decompensated cirrhosis requires a different GFR cutoff.

MANAGEMENT IMPLICATIONS

The presence of CKD in patients with cirrhosis presents many challenges to clinicians with regard to medical care. In particular, fluid mobilization to control ascites and edema becomes a real challenge. The use of diuretic therapy has several limitations. As ascites often reaccumulates rapidly, patients require repeated large volume paracentesis. This puts them at risk of multiple complications, such as worsening circulatory dysfunction, infection and bleeding, in addition to causing discomfort to the patients.

Diuretic therapy

Diuretic therapy is often not prescribed in patients with functional CKD because of the concern that it may further worsen renal failure by causing intravascular volume loss and may precipitate electrolyte imbalance[33]. In patients with structural CKD, the use

of diuretics seems appropriate to manage ascites and edema. However, a varying degree of diuretic resistance is usually present in patients with CKD. This occurs primarily because of decreased renal blood flow, hyperuricemia and organic anion accumulation[62]. The organic anions, uric acid and hypoalbuminemia interfere with the function of loop diuretics. A higher dose of diuretics is therefore required to overcome diuretic resistance in the presence of CKD.

There is a lack of evidence to guide clinicians as to which single or combination diuretic agent is most appropriate for these patients. Furosemide is primarily eliminated by the kidney, while torsemide has predominant hepatic clearance[63]. Therefore, if kidney dysfunction is a predominant issue, torsemide might be preferred over furosemide, while in the case of severe hepatic dysfunction, furosemide may be preferred over torsemide. A recent meta-analysis found that coadministration of albumin with furosemide had a modest effect on overcoming diuretic resistance in hypoalbuminemic patients[64]. Correction of metabolic acidosis and hyperuricemia, adequate restriction of fluid and salt intake, and avoidance of medications that interfere with peritubular diuretic uptake, such as nonsteroidal anti-inflammatory drugs and beta-lactam antibiotics, may be other measures to enhance the diuretic response[62]. Spironolactone should be better avoided in advanced CKD to prevent hyperkalemia.

Vaptans

Vaptan, an antagonist of vasopressin 2 receptor, may be considered in patients with CKD with cirrhosis who are intolerant to or poorly responsive to diuretics. Tolvaptan has been found to be potentially safe with an efficacy rate of 77% for the treatment of refractory ascites in decompensated cirrhosis patients with coexisting type 2 HRS[65]. Tolvaptan significantly increases urine volume in patients with CKD with liver cirrhosis without worsening renal dysfunction[66]. However, its diuretic response gradually diminishes with progression of the CKD stage[67]. Due to the possible risks of hepatocellular damage identified during a clinical trial involving patients with autosomal dominant polycystic kidney disease, the United States Food and Drug Administration issued a warning for tolvaptan use in 2013. However, very high doses of tolvaptan had been used for a long period of time in this study (120 mg/d for 3 years), and no such adverse effects have been reported from the study on patients with cirrhosis where the recommended dose was much lower.

Vasoconstrictor therapy

Midodrine is an orally available α1-agonist that serves as a vasoconstrictor and has been found to have an effect on the systemic hemodynamics of cirrhotic patients. However, midodrine trials in patients with cirrhosis have shown contradictory results. There is insufficient evidence about its use in patients with CKD. In patients with type 2 HRS, midodrine has only a slight beneficial effect on systemic hemodynamics, with no effect on renal hemodynamics[68]. Additionally, treatment with terlipressin or noradrenaline along with albumin appears to have a limited role in patients with CKD. While there are several cases of reversal of type 2 HRS, recurrence after withdrawal of therapy is very common. In addition, evidence on the impact of this treatment on the outcomes of patients is controversial. Few studies have assessed the efficacy of terlipressin in a limited number of patients with type 2 HRS, and the findings have been equivocal[69,70]. In a recent study, 46% of treated patients demonstrated reversal of type 2 HRS; however, nearly half of responders experienced relapse[71]. Furthermore, reversal of type 2 HRS before LT does not appear to provide a major benefit over patients who are untreated or who have failed treatment before LT[13]. Therefore, most of the current guidelines do not recommend vasoconstrictor treatment in functional CKD.

Transjugular intrahepatic portosystemic shunt

Transjugular intrahepatic portosystemic shunt (TIPS) decreases portal pressure, improves kidney function and relieves ascites. While it is being used increasingly to treat patients with refractory ascites and functional renal failure, there is limited evidence on its use in patients with advanced CKD. In a study on TIPS in 17 patients with cirrhosis with CKD, Lakhoo *et al*[72] found that ascites control occurred in 83% of patients but at the expense of a high incidence (47%) of new or worsening hepatic encephalopathy (HE). Michl *et al*[73] reported improvement in renal function and a decrease in the frequency of paracentesis following TIPS in 10 patients with cirrhosis, including three with structural kidney disease. A recent systematic review and meta-analysis found a potential survival benefit of TIPS in patients with HRS but with a

high (49%) incidence of HE. In type 2 HRS, the pooled short-term and 1-year survival rates after TIPS were 86% and 64%, respectively. Moreover, 83% of patients with HRS experienced improvement in renal function after TIPS[74]. In summary, TIPS appears to be very effective in patients with functional CKD, and limited data indicate its effectiveness in structural CKD as well. However, TIPS may increase the incidence of HE, so it should be avoided in patients with encephalopathy, cardiopulmonary disease, and significant hepatic dysfunction.

Cell-free and concentrated ascites reinfusion therapy

Cell-free and concentrated ascites reinfusion therapy (CART) is an apheresis therapy in which ascitic fluid is filtered to remove unwanted cells, sterilely concentrated, and then intravenously reinfused[75]. It was introduced in Japan as a novel treatment for refractory ascites in patients with cirrhosis. The potential advantages of CART include its ability to maintain nutritional status, control ascites, and improve quality of life. Unlike large volume paracentesis, CART is not associated with the risk of hypoproteinemia, hemodynamic instability, renal dysfunction or fatigue. It has been used for both cirrhotic and malignant ascites; however, its safety and efficacy need to be assessed in CLD-CKD patients with refractory ascites. CART has been found to be equally as effective as large volume paracentesis plus albumin infusion[76]. However, its routine use can be limited by the high cost of the CART apparatus[77].

Concerns related to medications

Since the majority of drugs are metabolized and/or excreted by the hepatorenal system, it is a challenging task to prescribe medicines in patients with advanced cirrhosis with renal impairment[78]. There are no evidence-based guidelines for the use of medicines in such patients. Drugs with significant hepatotoxic or nephrotoxic potential or both need to be avoided in such patients. In addition, the dosage of several antibiotics needs to be modified in accordance with GFR. Nonselective β-blockers can increase mortality in patients with advanced cirrhosis with renal dysfunction due to their adverse impact on cardiac compensation[79]. Treatment with hepatitis B nucleos(t)ide analogues can also raise the risk of lactic acidosis if renal dysfunction is present[80].

Treatment modifications according to the etiology of cirrhosis

There are no large controlled studies available to direct appropriate antiviral therapy for patients with CKD with HBV cirrhosis. The use of tenofovir disoproxil fumarate has been associated with a mild risk of CKD progression, but a recent meta-analysis has shown that such a decrease in renal function is inappreciable compared with entecavir[81,82]. Nevertheless, entecavir tends to be the most preferred drug for these patients. However, entecavir therapy may not be as effective in patients with lamivudine resistance, so tenofovir alafenamide, an orally bioavailable tenofovir prodrug with a lower risk of renal toxicity, may be considered in such patients. In patients with HCV-cirrhosis and CKD, high sustained virologic response rates with glecaprevir/pibrentasvir combination in all genotypes and with elbasvir/grazoprevir in genotypes 1 and 4 can be achieved. However, these drugs are largely metabolized in the liver and are therefore not safe in advanced cirrhosis[83]. Patients with advanced decompensated cirrhosis and renal dysfunction are a difficult-to-treat category for which there are no guidelines for treatment; therefore, a treatment decision should be made on a case-by-case basis. While a treatment based on sofosbuvir is not recommended for patients with severe renal impairment, it may be used in patients with mild renal impairment[84]. In patients with nonalcoholic steatohepatitis-CKD, statins have been shown to decrease cardiovascular disease mortality, and sodium-glucose cotransporter-2 inhibitors have been found to slow the progression of CKD and minimize all-cause mortality[85]. However, it is important to determine the role of these drugs in nonalcoholic steatohepatitis-cirrhosis patients with CKD.

Renal replacement therapy

The shared symptoms between the two diseases and overestimation of eGFR make it difficult for patients with cirrhosis with CKD to determine the ideal time for commencing renal replacement therapy. The hemodynamic alterations of cirrhosis pose a challenge to maintaining hemodynamic stability during dialysis, where a sudden decrease in intravascular volume due to ultrafiltration may cause hypotension. A sharp change in blood osmolarity and electrolyte levels increases the risk of developing HE. In addition, thrombocytopenia, platelet dysfunction, and coagulopathy due to combined CKD-cirrhosis may increase the risk of bleeding

complications. In these patients, peritoneal dialysis may be a better choice because it will not only resolve many problems associated with intermittent HD but will also allow ascitic fluid to be regularly evacuated[86,87]. Studies evaluating the survival of cirrhotic patients on peritoneal dialysis have reported a modest survival rate of 8 to 66 mo[88].

LT vs SLKT

Increased post-LT mortality is associated with any form of renal dysfunction. However, most studies evaluating the impact of renal function on post-LT survival do not differentiate between CKD and AKI[4,89,90]. Few studies have reported the progression of CKD, including the development of end-stage renal disease and increased post-LT mortality in cirrhotic patients with CKD receiving LT alone[90-92]. In a recent study, the presence of CKD at the time of LT increased the risk of post-LT mortality by 16%[4]. Thus, there has been a drive to perform more SLKT in patients with combined cirrhosis-CKD. However, in patients with cirrhosis, predicting renal recovery post-LT is difficult, and the degree or severity of CKD that warrants SLKT *vs* LT remains undefined. The 2012 SLKT summit guidelines indicate that SLKT should be considered for cirrhosis-CKD patients with an eGFR of ≤ 40 mL/min measured by the MDRD-6 equation[93]. Once again, however, the use of SLKT is highly variable, and the role of kidney transplantation in nondialysis CKD is controversial. Singh *et al*[94] recently reported the outcome of LT only in nine CKD-cirrhosis patients who had persistently low eGFR < 40 mL/min for ≥ 12 wk but relatively normal kidney biopsy findings. Post-LT, eGFR increased in all nine patients within a week and remained stable afterwards; one patient progressed to ESRD 9 years post-LT, and another patient expired 7 years after LT. While no definite conclusions can be drawn from this small study, there is an indication that, in the absence of other indicators of renal injury, low eGFR alone below an arbitrary cutoff value does not constitute an absolute requirement for SLKT in patients with liver cirrhosis. Functional CKD is potentially reversible after LT[13,52]. However, prolonged renal ischemia can cause permanent tubular or glomerular damage that may not recover with LT, leading to post-LT CKD progression. Because the assessment of renal function may be difficult in patients with advanced cirrhosis, a renal biopsy should be considered whenever possible for identifying parenchymal changes and to decide between LT and SLKT. In patients with low eGFR and kidney biopsy showing > 30% glomerulosclerosis and/or interstitial fibrosis, SLKT should be considered[93]. Future prediction models to assist in decision-making between SLKT and LT should consider integrating kidney injury markers, including new CKD biomarkers.

CONCLUSION

In conclusion, the incidence of CKD in patients with cirrhosis has increased significantly as a result of a rise in risk factors and a change in the diagnostic criteria from a fixed level of serum creatine to a dynamic change in GFR. The available data on this condition are extremely limited. Future studies on this subject are required to explain several contentious issues. Taking into account the problems related to the calculation of GFR in patients with cirrhosis, the main issue to be addressed would be the refining of diagnostic criteria. The other areas that require future research are the identification of reliable biomarkers of chronic kidney damage, the formulation of management strategies based on phenotypic features of CKD in cirrhosis, and the development of prediction models to assist in decision-making between SLKT and LT.

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REVIEW

Genotype 3-hepatitis C virus' last line of defense

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Abstract

Chronic infection with hepatitis C virus (HCV) is one of the leading causes of liver disease globally, affecting approximately 71 million people. The majority of them are infected with genotype (GT) 1 but infections with GT3 are second in frequency. For many years, GT3 was considered to be less pathogenic compared to other GTs in the HCV family due to its favorable response to interferon (IFN)-based regimen. However, the growing evidence of a higher rate of steatosis, more rapid progression of liver fibrosis, and lower efficacy of antiviral treatment compared to infection with other HCV GTs has changed this conviction. This review presents the specifics of the course of GT3 infection and the development of therapeutic options for GT3-infected patients in the era of direct-acting antivirals (DAA). The way from a standard of care therapy with pegylated IFN-alpha (pegIFNα) and ribavirin (RBV) through a triple combination of pegIFNα + RBV and DAA to the highly potent IFN-free pangenotypic DAA regimens is discussed along with some treatment options which appeared to be dead ends. Although the implementation of highly effective pangenotypic regimens is the most recent stage of revolution in the treatment of GT3 infection, there is still room for improvement, especially in patients with liver cirrhosis and those who fail to respond to DAA therapies, particularly those containing inhibitors of HCV nonstructural protein 5A.

Key Words: Hepatitis C virus; Genotype 3; Antiviral treatment; Interferon; Direct-acting antivirals; Pangenotypic

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Core Tip: Genotype 3 which is second in frequency worldwide, is unique among genotypes of hepatitis C virus in its higher rate of steatosis, accelerated fibrosis progression, and lower cure rates. This paper describes the genotype-specific mechanisms of liver injury and provides an overview of therapeutic options. Currently,

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available highly potent pangenotypic regimens have revolutionized the treatment of genotype 3 infection, however, patients with liver cirrhosis and those who fail to response to direct-acting antiviral therapy still present a therapeutic challenge.

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INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is assumed to be one of the leading causes of liver disease globally, affecting approximately 71 million people[1]. Due to the high genetic diversity of the viral nucleic acid sequence, six major genotypes (GT), differing from each other by 30% at the nucleotide level, comprising multiple subtypes of HCV, have been identified[2]. The majority of patients worldwide are infected with GT1, but infections of GT3 are also common in some regions. GT3 is defined by a higher rate of steatosis, increased risk of liver cirrhosis, and different response to antiviral drugs compared to other GTs. In the era of treatment with pegylated interferon alpha (IFNα) and ribavirin (RBV), patients infected with GT3 were considered "easy to treat" due to an efficacy rate of 70%, compared to less than 50% in GT1 and GT4 infected patients. The introduction of direct-acting antivirals (DAA) has changed the management of HCV infection, allowing a substantial increase in the treatment efficacy, however, the improvement for GT3 infection was not as pronounced as for other GTs, particularly in treatment-experienced patients and those with liver cirrhosis.

THE GLOBAL SPREAD OF GT3

GT3 infections are widely distributed worldwide and are estimated to be the second in frequency accounting for approximately 25%-30% of all HCV cases globally[2]. The distribution of GT3 varies between different countries and continents. The highest global prevalence (exceeding 70%) is found in South Asian countries, while a rate of approximately 20% is reported in Central and Southeast Asia, a few percent in East Asia, and only 0.4% in the Asia-Pacific region. A relatively high frequency of 36% is documented in Australia[3]. GT3 infection accounts for 14.2% of HCV cases in South America, 2.1% in Central America, and 15.7% in North America, ranging from 8.9% in the United States to 22.3% in Canada[3]. In Europe, the distribution of GT3 infections is also heterogeneous with the highest frequency exceeding 40% in Scandinavia and England, over 30% in Ireland, Greece Russia, and Slovenia, and more than 20% of infections in Germany, Switzerland, Montenegro, Belgium, Bosnia, and France[4-6]. The rate of a dozen percent is reported from Spain, Poland, Portugal, Bulgaria, and Croatia, whereas the lowest prevalence, below 10%, are documented in Italy, Albania, Hungary, and Romania[7-10]. The lowest proportional frequency of GT3 infections is found in Africa at an average of 5.3%, with the highest frequency at 7.4% in East Africa through 6.3% in North Africa to 0.8% in the central part of the continent[3].

GOOD OR BAD IN THE HCV FAMILY

For many years, GT3 was considered to be less pathogenic compared to other GTs in the HCV family due to its favorable response to an IFN-based regimen. However, the growing evidence of a higher rate of steatosis and more rapid progression of liver fibrosis compared to infection with other HCV GTs has changed this conviction.

Liver steatosis is a frequent histological finding in patients with chronic hepatitis C (CHC). Although this feature is common among all HCV infected individuals, with an average rate of 50%, the highest prevalence exceeding 70% is observed in patients infected with GT3[11-14]. In in-vitro studies, GT3 is also demonstrated to be much more

likely to induce liver steatosis than other HCV GTs[15]. The pathogenesis of hepatic steatosis is complex and related to host and viral factors, as well as to alcohol consumption. Metabolic steatosis, which is associated with host risk factors including high body mass index, obesity, dyslipidemia, metabolic syndrome, insulin resistance and type 2 diabetes, is commonly found in patients infected with non-GT3 HCV, whereas in the GT3-infected individuals, virus-related hepatic steatosis is described as predominantly being induced by the direct cytopathic effect of the HCV[16-20]. Although the exact mechanism remains unknown, several pathways are linked to the pathogenesis of GT3-induced steatosis. A central role is played by the inhibition of microsomal triglyceride transfer protein function by HCV core protein, resulting in overall decreased hepatocyte lipid export with intracellular triglycerides accumulation. This effect is documented to be amplified with HCV-3 core proteins[21]. Another mechanism through which the virus modulates the host lipid metabolic pathways is inhibition of the peroxisome proliferator associated receptor-α (PPAR-α), a transcription factor inducing hepatic fatty acid oxidation and ketogenesis. A decrease in PPAR-α level leads to hepatic lipid collection. *In vitro* studies document inhibition of PPAR-α observed in the GT3 infections as being more efficient than in infections with GT1 HCV[22]. Viral-induced hepatic steatosis results not only from the reduction of the lipid excretion with subsequent intracellular lipid accumulation but also from the promotion of the neolipogenesis with fatty acid synthesis. This activity is proposed to be a consequence of an increase in function of sterol regulatory element-binding protein-1c activated by the HCV-3 core protein, however, the exact mode of activation is unknown[23]. The hypothesis of a pathogenic link between GT3 infection and steatosis is supported by a significant correlation of the steatosis score and intrahepatic titer of HCV RNA only in patients infected with GT3[16]. The improvement in liver steatosis in GT3-infected patients after successful antiviral therapy, which is not observed in patients infected with GT1, seems to indirectly confirm this association[24,25]. GT3 HCV was also identified as an independent predictor for the accelerated progression of liver fibrosis in addition to established risk factors including the age of infection, male gender, coinfection with hepatitis B virus and human immunodeficiency virus, insulin resistance, iron overload, alcohol and drugs intake[26]. Precise analysis of the influence of HCV GT on the more advanced liver disease is difficult due to the coexistence of the aforementioned predictors and the previously discussed higher prevalence of liver steatosis in GT3 infection, which contributes to more rapid progression of hepatic fibrosis[12,27,28]. However, the pooled analysis confirmed a significantly more severe liver disease in single-biopsy studies and a trend towards the faster progression of fibrosis in GT3 patients compared with the other GTs[29]. The strong association between GT3 infection and end-stage liver disease was documented in HCV-infected drug abusers in France and confirmed by a population-based study in a cohort of native Alaskans with CHC[30,31].

An increased risk not only of liver cirrhosis but also of hepatocellular carcinoma (HCC) among GT3 infected individuals compared to those infected with other GTs was reported in a large cohort (> 110000) of American patients from the Veterans Affairs Registry[32,33]. Consistent results of significantly higher incidence of HCC in GT3 patients were also obtained in French and Korean populations[34,35]. Nevertheless, the effect of HCV GT3 infection on the higher prevalence of liver cancer remains controversial because of the data demonstrated for GT1b as a major risk factor for HCC development[36,37].

IFN AND RBV COUPLE

The standard of care therapy of pegylated (peg) IFNα and RBV established in 2000 has resulted in a sustained virologic response (SVR) of approximately 70% in GT3-infected patients[38-42]. Such a high effectiveness compared to the SVR below 50% achieved by patients with GT1 and GT4 infection was the basis for the GT3 being deemed "easy to treat" and has led to attempts to shorten the treatment course to 16, 14, and even 12 wk. However, the reduction in the SVR rate was reported in patients who did not achieve the so-called rapid virologic response (RVR) defined as undetectability of HCV RNA after 4 wk of therapy[38,39,42-44]. The meta-analysis of twelve clinical trials performed by Andriulli *et al*[45] documented a wide variance in response to pegIFNα and RBV in GT3-infected patients depending on the baseline viral load. Individuals with a high baseline level of HCV RNA demonstrated a significantly lower SVR rate of 58% compared to 75% in those with a low baseline viral load. The strongest predictive factor for treatment response was RVR and this finding provided the basis for the

conclusion that patients without RVR may need a longer therapy duration. The negative impact of cirrhosis on treatment response among subjects infected with GT3, leading to poor antiviral effectiveness was documented by Powis *et al*[46], suggesting that such patients also require an alternative management strategy. However, the extension of the treatment course did not result in higher effectiveness, nor was there an improvement in the SVR rate on increasing the dose of RBV[47-49].

THE COUPLE WITH A LITTLE HELP OF DAA

The registration of the first DAAs in 2011, which were inhibitors of the HCV serine protease (nonstructural protein 3/4A, NS3/4A), started a revolution in the treatment of CHC. The combination of telaprevir or boceprevir with pegIFNα and RBV significantly increased the SVR rate, but only in patients infected with GT1[50,51]. Those infected with other GTs, including GT3, were still treated with pegIFNα and RBV because no significant improvement was demonstrated after the addition of telaprevir or boceprevir[52,53]. Therefore, at the beginning of the DAA era GT3 emerged as a "difficult-to-treat" GT. New hopes for higher effectiveness were raised with the introduction of the next-generation DAAs for possible combination with pegIFNα and RBV. Unfortunately, clinical trials demonstrated that simeprevir, a second-wave protease inhibitor with documented in-vitro pangenotypic activity has limited efficacy in GT3-infected patients, and the effectiveness of daclatasvir (DCV), acting through inhibition of the HCV nonstructural protein 5A (NS5A), has proven to be also disappointing, with SVR rates of 45% and 74% in GT3 patients with and without liver cirrhosis, respectively[54,55]. However, the expectations of a higher response rate among GT3-infected patients have been met by sofosbuvir (SOF), a new DAA class representative, HCV polymerase (NS5B) inhibitor. The addition of SOF to pegIFNα and RBV (SPR) leads to better outcomes when compared to standard of care therapy, regardless of liver fibrosis and history of previous antiviral therapy. Phase 2 clinical trials documented a response of 83% among treatment-experienced patients with liver cirrhosis participating in the LONESTAR-2 study, while non-cirrhotic, treatment-naïve individuals treated in the QUANTUM study responded in 92% of cases[56,57].

Patients included in the BOSON phase 3 clinical trial achieved an SVR of 93%, specifically 88% among individuals with liver cirrhosis and 95% in those without; the lowest efficacy of 86% was demonstrated for patients with liver cirrhosis who failed to respond to previous antiviral therapy[58]. An open-label clinical study evaluating the outcome of SOF-containing treatments reported 100% efficacy among GT3-infected treatment naïve patients without cirrhosis treated with triple therapy[59].

Those results from clinical trials were supported by real-world experience (RWE) data that documented an SVR rate higher than that following dual therapy. The best response of 93% was reported for non-cirrhotics among Americans treated in the Veterans Affairs health care system[60]. Scandinavian patients responded in 96% of cases, an efficacy of 98% was obtained in the Polish EpiTer-2 study and an SVR reached 99% in a German cohort[6,61,62]. The effectiveness of the SPR regimen in cirrhotic individuals in these RWE studies was also promising, reaching 92%, 81%, 91%, and 88%, respectively[6,60-62].

RWE data revealed a failure of previous therapy, with a history of treatment with IFN and RBV shown to be a negative prognostic factor of the response to SPR treatment. This triple regimen was still recommended by the guidelines of the European Association for the Study of the Liver in 2015 for GT3-infected non-cirrhotic patients and those with compensated liver cirrhosis, regardless of treatment history[63]. Irrespective of the high effectiveness of SPR, accompanied by reasonable tolerability due to the short treatment period, any IFN-based therapy was refused by patients[61]. Therefore, further research on the treatment of GT3 infections has focused on highly efficient IFN-free therapeutic options.

DAA HOME ALONE

The first available IFN-free regimen, SOF and RBV, used in GT3-infected patients for 12 wk or 16 wk, did not result in increased efficacy when compared to standard of care therapy, which demonstrated significantly lower response rates in patients with liver cirrhosis, especially in those who had previously failed IFN and RBV therapy (Table 1)[40,58,64].

The extension of treatment duration to 24 wk enabled effectiveness of up to 95%,

Table 1 Efficacy of interferon-free regimens in genotype 3 patients in clinical trials

Ref.	Phase	Number of GT3 participants	Regimen	Treatment duration	SVR			
					Noncirrhotics		Cirrhotics	
					Treatment-naive (%)	Treatment-experienced (%)	Treatment-naive (%)	Treatment-experienced (%)
Lawitz *et al*[40], FISSION	3	173	SOF + RBV	12 wk	61	-	34	-
Jacobson *et al*[64], POSITRON	3	98	SOF + RBV	12 wk	68	-	21	-
Jacobson *et al*[64], FUSION	3	127	SOF + RBV	12 wk	-	37	-	19
				16 wk	-	63	-	61
Foster *et al*[58], BOSON	3	363	SOF + RBV	16 wk	83	76	57	47
				24 wk	90	82	82	77
Zeuzem *et al*[65], VALENCE	3	250	SOF + RBV	24 wk	95	87	92	62
Nelson *et al*[69], ALLY-3	3	152	SOF + DCV	12 wk	97	94	58	69
Leroy *et al*[70], ALLY-3+	3	50	SOF + DCV + RBV	12 wk	100	100	50 (1/2)	93
				16 wk	100	100	100	86
Poordad *et al*[71], ALLY-3C	3	78	SOF + DCV + RBV	24 wk	-	-	93	79
Esteban *et al*[81], NCT02781558	2	204	SOF/VEL	12 wk	-	-	91	
			SOF/VE + RBV	12 wk	-	-	96	
Foster *et al*[80], ASTRAL-3	3	552	SOF + RBV	24 wk	90	73	71	58
			SOF/VEL	12 wk	98	93	91	89
Bourlière *et al*[102], POLARIS-1	3	78	SOF/VEL+ VOX	12 wk	-	100[1]	-	93[1]
Jacobson *et al*[103], POLARIS-2	3	181	SOF/VEL	12 wk	97		-	-
			SOF/VEL+ VOX	8 wk	99		-	-
Jacobson *et al*[103], POLARIS-3	3	219	SOF/VEL	12 wk	-	-	99	91
			SOF/VEL+ VOX	8 wk	-	-	96	97
Bourlière *et al*[102], POLARIS-4	3	106	SOF/VEL	12 wk	-	85[2]	-	85%[2]
			SOF/VEL+ VOX	12 wk	-	96[2]	-	96[2]
Gane *et al*[114], LEPTON	2	41	SOF/VEL+ VOX	6 wk	-	-	83	-
				8 wk	-		100	100
Gane *et al*[115], NCT02378961	2	74	SOF/VEL+ VOX	6 wk	100	-	-	-
				8 wk	-	-	94	-
				12 wk	-	100	-	94
Zeuzem *et al*[92], ENDURANCE-3	3	505	GLE/PIB	8 wk	95	-	-	-
				12 wk	95	-	-	-
			SOF + DCV	12 wk	97	-	-	-
Kwo *et al*[93], SURVEYOR-II (part 2)	3	53	GLE/PIB	8 wk	97	-	-	-
				12 wk	-	92	-	-

Wyles *et al*[94], SURVEYOR-II (part 3)	3	131	GLE/PIB	12 wk	-	91	98	-
				16 wk	-	95	-	96
Brown *et al*[90], EXPEDITION-8	3	124	GLE/PIB	8 wk	-	-	98	-
Wyles *et al*[104], MAGELLAN-3	3	14	GEL/PIB+ SOF + RBV	16 wk	-	100	-	100
Gane *et al*[105], ELECTRON-2	2	101	SOF/LDV	12 wk	64	-	-	-
			SOF/LDV + RBV	12 wk	100	89	-	73
Pawlotsky *et al*[106], VITAL-1	2	181	ALV	24 wk	76	-	-	-
			ALV + RBV	24 wk	93	-	-	-
Lawitz *et al*[109], NAVIGATOR	2	21	OBV/PTV/r	12 wk	40	-	-	-
			OBV/PTV/r + RBV	12 wk	9	-	-	-
Shafran *et al*[110], QUARTZ II-III	2	51	OBV/PTV/r + SOF	12 wk	98	-	-	-
			OBV/PTV/r + SOF + RBV	12 wk	91	-	100	-
Gane *et al*[107], C-WORTHY (part D)	2	41	GZR/EBR + RBV	12 wk	45	-	-	-
				18 wk	57	-	-	-
Lawitz *et al*[111], C-SWIFT	2	41	GZR/EBR + SOF	8 wk	93	-	-	-
				12 wk	100	-	91	-
Foster *et al*[112], C-ISLE	2	100	GZR/EBR + SOF	8 wk	-	-	91	-
				12 wk	-	-	96	100[3]
				16 wk	-	-	-	94
Lawitz *et al*[113], C-CREST-1 and -2	2	337	GZR + EBR + UPR ± RBV	8 wk	95			
				12 wk	97			
				16 wk	96			
Lawitz *et al*[108], C-BREEZE-2	2	61	RZR + UPR	12 wk	80		68	

[1]NS5A-inhibitor-experienced.

[2]No detailed information on the response rate in patients with and without liver cirrhosis.

[3]Recommended by American Association for the Study of Liver Diseases/Infectious Diseases Society of America as an alternative option for pegylated interferon + ribavirin-experienced patients with compensated liver cirrhosis. SVR: Sustained virologic response; GT: Genotype; SOF: Sofosbuvir; RVB: Ribavirin; DCV: Daclatasvir; VEL: Velpatasvir; VOX: Voxilaprevir; GLE: Glecaprevir; PIB: Pibrentasvir; LDV: Ledipasvir; ALV: Alisporivir; OBV: Ombitasvir; PTV/r: Paritaprevir boosted by ritonavir; GZR: Grazoprevir; EBR: Elbasvir; UPR: Uprifosbuvir; RBV: Ribavirin; RZR: Ruzasvir.

however, the difference in response rates between patients without and with liver cirrhosis was significant[58,65]. The results of phase 3 clinical trials confirmed by RWE data became a basis for treatment guidelines, according to which IFN-ineligible GT3-infected patients should receive SOF/RBV for 24 wk. However, this regimen was recognized as suboptimal, due to unsatisfactory effectiveness for those with liver cirrhosis who had previously failed IFN and RBV therapy[66-68].

Searching for the optimal antiviral regimen, the combinations of SOF and another DAA with a different mechanism of action were studied. Promising results were obtained with a SOF and DCV combination administered for 12 wk and 24 wk, with or without RBV[69-71]. Although the difference in response rates with SOF + DCV ± RBV between non-cirrhotic and cirrhotic patients was still noticeable, this regimen was recommended for the treatment for GT3-infected patients regardless of liver fibrosis and history of previous therapy and has been widely used in RWE settings[66,72-74]. High efficacy and good tolerability demonstrated in both clinical trials and real-world cohorts have made this regimen a reasonable choice for therapy for GT3 infection as long as highly potent pangenotypic options became broadly available[75-77].

BETTER IS THE ENEMY OF GOOD ENOUGH

The final stage of the revolution in antiviral treatment which improved the outcome and simplified the management of GT3-infected patients was the implementation of potent pangenotypic regimens (Table 1).

According to the most recent guidelines, two basic options of DAAs are currently approved for the treatment of GT3 infection; a fixed-dose combination of SOF and velpatasvir (VEL), and dual treatment with glecaprevir (GLE), an NS3/4A protease inhibitor, and pibrentasvir (PIB), an NS5A inhibitor (Table 2)[78,79]. A single-tablet regimen containing SOF and VEL was registered based on the results of the ASTRAL-3 study, which confirmed effectiveness exceeding 93% in non-cirrhotic and 89% in cirrhotic patients[80]. The 12-wk regimen is recommended for non-cirrhotics and patients with compensated cirrhosis, regardless of the previous treatment history. The addition of RBV may be considered in compensated individuals.

It is noteworthy that SOF/VEL combined with RBV is the only option recommended for patients with decompensated liver cirrhosis[81,82]. Data on the high efficacy and favorable safety profile of SOF/VEL achieved in clinical trials were supported by RWE studies reporting comparable SVR rates. Prior treatment-experience, as well as advanced liver fibrosis, were significant predictors of reduced effectiveness[83-87]. As resistance-associated substitutions at the NS5A position can be responsible for a reduction in the efficacy of the NS5A inhibitors, the resistance-associated substitutions testing at baseline should be considered for treatment-experienced patients and cirrhotic individuals, irrespective of treatment history, for whom SOF/VEL is being considered. The identification of the Y93H substitution indicates the need for RBV addition or an alternative regimen administration[78,79].

The second potent pangenotypic option is a combination of GLE and PIB, which was approved for the treatment of patients without or with liver cirrhosis irrespective of previous therapy. As protease inhibitors containing regimens carry a risk of decompensation during antiviral treatment, GLE/PIB is not recommended for decompensated cirrhotic patients[88]. This regimen provides the opportunity for shortening therapy to 8 wk in the majority of patients[89]. Based on findings from the ENDURANCE-3, SURVEYOR-II, and EXPEDITION-8 clinical trials, an 8-wk regimen has been registered for all previously untreated patients, including those with compensated liver cirrhosis, whereas treatment-experienced GT3-infected individuals should be treated for 16 wk regardless of liver fibrosis[90-94]. RWE studies reported effectiveness for an 8-wk GLE/PIB regimen, which exceeded 96% in treatment-naïve patients without liver cirrhosis[95-99]. Since the shortening of therapy in previously untreated cirrhotic patients infected with GT3 has been approved very recently, the available RWE data are very limited and only include a small number of patients[100,101]. Therefore, further studies are needed to determine the treatment outcome in this subpopulation. Although the implementation of SOF/VEL and GLE/PIB regimens has resulted in a high efficacy rate among GT3-infected individuals, there is still room for improvement, especially in those who did not achieve SVR, particularly following NS5A containing regimens. For such patients, a 12-wk salvage therapy with a single-tablet combination of SOF/VEL and next-generation NS3/4A protease inhibitor voxilaprevir (VOX) is recommended[102]. Safety and efficacy of SOF/VEL/VOX in GT3-infected patients without and with liver cirrhosis, both treatment-naïve and treatment-experienced were demonstrated in the POLARIS studies (Table 1)[102,103].

The other option to address failed DAA treatment in GT3-infected patients is a combination of GLE/PIB plus SOF and RBV, as investigated in the MAGELLAN-3 study. This demonstrated a 100% SVR rate, however, the small number of patients enrolled may have limited the broad applicability of these findings[104].

SOMETHING WENT WRONG

On the way to developing highly effective pangenotypic regimens against GT3, there were multiple paths that appeared to be dead ends. Some of them were not investigated despite showing encouraging initial results, due to disappointing treatment outcomes in selected subpopulations of GT3 patients. One good example is an open-label study of 12-wk treatment with an NS5A inhibitor—ledipasvir and SOF, plus RBV, which demonstrated a 100% SVR rate among treatment-naïve GT3 infected individuals. Unfortunately, due to limited efficacy in treatment-experienced patients, especially those with liver cirrhosis, as well as low antiviral potency without RBV against GT3, that direction of search has proved a blind alley[105]. Alisporivir, a

Table 2 European Association for the Study of the Liver and American Association for the Study of Liver Diseases/Infectious Diseases Society of America current recommendations on the treatment of genotype 3-infected patients

Recommendations	Genotype/subtype	Cirrhosis status	Prior treatment experience	SOF/VEL	GLE/PIB	SOF/VEL/VOX	GZR/EBR + SOF
European Association for the Study of the Liver[78]	GT3	No cirrhosis	Treatment-naïve	12 wk	8 wk	-	-
			Treatment-experienced		12 wk	-	-
		Compensated cirrhosis	Treatment-naïve	12 wk with RBV[1]	8-12 wk[2]	12 wk	-
			Treatment-experienced		16 wk		-
		Decompensated cirrhosis	Treatment-naïve and experienced	12 wk with RBV or 24 wk	-	-	-
	GT3, subtype b, g or any other subtype naturally harbouring one or several NS5A RASs[3]	No cirrhosis	Treatment-naïve	Unknown	Unknown	12 wk	-
			Treatment-experienced				-
		Compensated cirrhosis	Treatment-naïve				-
			Treatment-experienced				-
AASLD/IDSA (Ghany *et al*[79])	GT3	No cirrhosis	Treatment-naïve	12 wk	8 wk	-	-
			Treatment-experienced	12 wk	16 wk[4]	12 wk[4]	-
			SOF + RBV ± PEGIFN-experienced	-	16 wk	12 wk	-
			DAA-experienced[5]	-	-	12 wk, + RBV for NS5A failures	-
		Compensated cirrhosis	Treatment-naïve	12 wk, + RBV for 12 wk[4]	8 wk	12 wk[4]	-
			PEGIFN + RBV-experienced	+ RBV for 12 wk[4]	16 wk	12 wk	12 wk[4]
			SOF + RBV ± PEGIFN-experienced	-	16 wk	12 wk	-
			DAA-experienced[5]	-	-	12 wk, + RBV for NS5A failures	-
		Decompensated cirrhosis	Treatment-naïve and experienced	12 wk with RBV or 24 wk	-	-	-

[1]If resistance testing is performed, only patients with the nonstructural protein 5A Y93H resistance-associated substitutions at baseline should be treated with sofosbuvir/velpatasvir plus ribavirin or with sofosbuvir/velpatasvir/voxilaprevir, whereas patients without the Y93H resistance-associated substitutions should be treated with sofosbuvir/velpatasvir alone.

[2]In treatment-naïve patients infected with genotype 3 with compensated (Child-Pugh A) cirrhosis, treatment with glecaprevir/pibrentasvir can be shortened to 8 wk, but more data are needed to consolidate this recommendation.

[3]As determined by sequence analysis of the nonstructural protein 5A region by means of population sequencing or deep sequencing (cutoff 15%).

[4]Alternative regimen.

[5]Including nonstructural protein 5A inhibitors except glecaprevir/pibrentasvir failures. NS5A: Nonstructural protein 5A; SOF: Sofosbuvir; VEL: Velpatasvir; GLE: Glecaprevir; PIB: Pibrentasvir; VOX: Voxilaprevir; GZR: Grazoprevir; EBR: Elbasvir; EASL: European Association for the Study of the Liver; GT: Genotype; RBV: Ribavirin; PEGIFN: Pegylated interferon; DAA: Direct-acting antivirals; AASLD/IDSA: American Association for the Study of Liver Diseases/Infectious Diseases Society of America.

cyclophilin inhibitor, applied alone or with RBV in treatment-naïve non-cirrhotic patients has resulted in SVR rates of 76% and 93%, respectively, however, research involving other subgroups of patients was suspended due to a safety issue[106]. Efficacy was observed to be below expectations with the combination of the NS3/4A inhibitor grazoprevir (GZR) and the NS5A inhibitor elbasvir (EBR) with RBV, as well as with a regimen consisting of the NS5A inhibitor ruzasvir and the NS5B inhibitor uprifosbuvir[107,108]. The unsatisfactory outcome of the treatment with NS35A inhibitor ombitasvir, and NS3/4A inhibitor paritaprevir boosted by ritonavir with or without RBV, was subsequently improved by the addition of SOF, but ultimately these regimens were not further evaluated, because there were new potent pangenotypic options on the horizon[109,110]. For this same reason, investigations into a regimen of GZR/EBR combined with uprifosbuvir or SOF were discontinued, despite the high effectiveness demonstrated in phase 2 clinical trials. However, GZR/EBR + SOF is currently recommended by AASLD/IDSA as an alternative option for the specific subpopulation of pegIFNα+RBV-experienced patients with compensated liver cirrhosis[79,111-113].

CONCLUSION

Despite the high efficacy and safety of pangenotypic therapies, that may sooner or later cure all or at least almost all identified HCV infections, including GT3, there will still be many infections that go unrecognized and are therefore impossible to cure with even the best drug. The major problem that remains to be solved worldwide is screening people who are unaware of the risk of liver disease progression from a virus in their body. It is a shame for national governments that, despite having access to the perfect tool to eliminate a dangerous virus and rule out one of the most difficult-to-treat cancers, are not doing enough.

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REVIEW

How to manage inflammatory bowel disease during the COVID-19 pandemic: A guide for the practicing clinician

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Abstract

Managing inflammatory bowel disease (IBD) during the coronavirus disease 2019 (COVID-19) pandemic has been a challenge faced by clinicians and their patients, especially concerning whether to proceed with biologics and immunosuppressive agents in the background of a global outbreak of a highly contagious new coronavirus (severe acute respiratory syndrome coronavirus 2, SARS-CoV-2). The knowledge about the impact of this virus on patients with IBD, although it is still scarce, is rapidly evolving. In particular, concerns surrounding medications' impact for IBD on the risk of acquiring SARS-CoV-2 infection or developing COVID-19, and potentially exacerbate viral replication and the COVID-19 course, are a current thinking of both practicing clinicians and providers caring for patients with IBD. Managing patients with IBD infected with SARS-CoV-2 depends on both the clinical activity of the IBD and the occasional development and severity of COVID-19. In this review, we summarize the current data regarding gastrointestinal involvement by SARS-CoV-2 and pharmacologic and surgical management for IBD concerning this infection, and the COVID-19 impact on both the patient's psychological functioning and endoscopy services, and we concisely summarize the telemedicine roles during the COVID-19 pandemic.

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Core Tip: The knowledge on coronavirus disease 2019 (COVID-19) is rapidly evolving. Although patients with inflammatory bowel disease (IBD) do not appear to be at increased risk for COVID-19, the potential impact of immunosuppressive therapies on patients with IBD infected with severe acute respiratory syndrome coronavirus 2 calls for concern for clinicians and patients. Several recommendations and guidelines have recently been published, including the necessary reorganization of gastroenterology and endoscopy services to attendance of these patients, the growing role played by telemedicine, and the importance of addressing aspects of mental health in this context. We provide an overview and practical guidance for managing patients with IBD medically and surgically in the COVID-19 era.

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INTRODUCTION

Over the past year, the coronavirus disease 2019 (COVID-19) pandemic has evolved as a public health emergency of international concern. The epidemiological panorama is constantly evolving, and the data updated to January 12, 2021 have 191 countries involved, with more than 90947243 confirmed cases and 1947243 confirmed deaths globally[1]. The first opportunity to eradicate the virus over the long term and to protect specific patients from COVID-19 is by introducing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines[2]. Although efforts to implement mass vaccination programs are currently in place globally, high rates of COVID-19 infections and fatalities are still expected in the months ahead.

Inflammatory bowel disease (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory conditions of the gastrointestinal (GI) tract affecting millions of people worldwide[3]. Patients with IBD and other immune-mediated diseases often require treatment with corticosteroids, immunomodulators (thiopurines, methotrexate), biologics, and Janus kinase inhibitors, which can increase the risk of infections[4-6]. However, until now, the incidence of SARS-CoV-2 infection in patients with IBD and immunosuppressive therapy did not appear to differ from the general population[7]. Also, based on data from an international registry developed to collect information from patients with IBD from all over the world with confirmed COVID-19 and its outcomes, and the Surveillance Epidemiology of Coronavirus Under Research Exclusion for Inflammatory Bowel Disease (SECURE-IBD), the evolution of COVID-19 does not seem to be worse in patients with IBD[8].

Notwithstanding, most elective clinical activities involving IBD care were drastically decreased during the pandemic[9-11]. Moreover, as suggested by the most qualified international societies and organizations, outpatient visits, colonoscopies, and non-urgent surgery have been postponed to prevent patient contact with the hospital and to enable patients with IBD to maintain social isolation[12,13].

As the world is gradually attempting to normalize, IBD physicians must face new challenges in terms of both future uncertainty, given the lack of supply of COVID-19 vaccines worldwide, and the ability to reorganize clinical activities for patients with IBD to provide optimal care while avoiding new outbreaks. Facing this scenario, this review aims to critically analyze the evidence on the effect of medications commonly used to treat IBD, as well as the management of the disease in its different degrees of activity, including the setting of SARS-CoV-2 infection.

WHAT IS THE RISK OF SARS-CoV-2 INFECTION IN THE IBD POPULATION?

Data on the incidence of SARS-CoV-2 infection among patients with IBD have been conflicting. Initial evidence suggested that patients with IBD had a lower risk of COVID-19 compared to the general population, as subsequent studies reported that no case of COVID-19 was diagnosed among patients with IBD followed in referral centers in China and Italy[14,15]. However, other studies assessing the risk of COVID-19 among patients with IBD reported incidence rates of 4.9 cases per 1000 patients with IBD in a Spanish cohort[16] and 2.5 cases per 1000 patients with IBD in France and Italy cohorts[17]. In a recent systematic review and meta-analysis by Aziz *et al*[7] comprising six studies that incorporated data from 9177 patients with IBD, the pooled incidence of COVID-19 in the IBD population was approximately 0.3%, which is greatly reassuring, as the incidence is on the lower side compared with the general population (0.2%-4.0%)[7]. Although there is limited evidence available, it seems that patients with IBD are not at greater risk of acquiring COVID-19, and SARS-CoV-2 infection does not seem to be more prevalent in patients with IBD than in the general population. However, this data must be interpreted with caution given that IBD patients might have better adherence to protection, social distancing and hygiene measures, which could explain the lower incidence in this population.

MANAGING IBD MEDICATIONS DURING THE COVID-19 PANDEMIC

Managing IBD medications during SARS-CoV-2 infection and COVID-19

Managing IBD during the COVID-19 pandemic has been a challenge faced by clinicians and their patients, especially concerning whether to carry on with biologics and immunosuppressive agents in the background of a global outbreak of a highly SARS-CoV-2. The knowledge about the impact of this virus on patients with IBD, although it is still scarce, is rapidly evolving[18].

In particular, concerns surrounding medications' impact for IBD on the risk of acquiring SARS-CoV-2 infection or developing COVID-19, and potentially exacerbate viral replication and the COVID-19 course, are a current thinking of both practicing clinicians and providers caring for patients with IBD. Confounding a systematic management strategy reveals that evidence-based data are scarce[19].

Providers caring for patients with IBD during the COVID-19 outbreak are opportune to reduce the burden of COVID-19 by assuming or sharing responsibility for multidisciplinary management of patients with IBD in this common clinical difficulty. The main goal that must be kept in mind is to treat active disease and maintain remission[20].

Fortunately, the worldwide management of patients with IBD during the COVID-19 pandemic presents considerable agreement. Indeed, gastroenterologists, both adult and pediatric, and colorectal surgeons were attending the practical recommendations/guidance and consensus statements from several societies of gastroenterology, endoscopy, surgery, and from the International Organization for the Study of Inflammatory Bowel Disease (IOIBD) with adaptations based on local regional characteristics[18]. For instance, an international survey on this issue showed that most gastroenterologists reduced clinic visits, restricted steroid use, and postponed elective endoscopic procedures and surgery. Also, if a patient was diagnosed with COVID-19, biologics and immunomodulators were mostly held[21].

Understanding the short- and long-term safety of drugs used in patients with IBD remains an important area of research, especially now in the middle of the COVID-19 pandemic. Preliminary evidence specified that except high-dose systemic steroids, using aminosalicylates, budesonide, antibiotics, rectal therapies, nutritional therapy, immunomodulators, and biologics, including anti-tumor necrosis factor (TNF) agents, anti-integrin, or anti-interleukin (IL)-12/23, were well tolerated without an increased risk of unfavorable evolution or duration of viral disease in patients with IBD that develop infection by SARS-CoV-2 and COVID-19[8]. However, recent accumulating evidence from SECURE-IBD registry based on data on over 1400 patients with IBD suggests that compared with anti-TNF monotherapy, thiopurine monotherapy, combotherapy of thiopurines with anti-TNF agent, and, surprisingly, aminosalicylates were associated with significantly increased risk of severe COVID-19, although the association with latter will require further replication in other IBD populations[22]. The authors of this study hypothesized that the influence of combotherapy on increased

COVID-19 severity appears to be guided by thiopurines, as the estimated impact for thiopurines monotherapy and combotherapy compared with anti-TNF monotherapy were similar[22]. Furthermore, this hypothesis followed previous observations that found a higher risk of viral infections in patients during treatment with thiopurines alone or in combotherapy with anti-TNF agents[4]. In line with the British Society of Gastroenterology (BSG) statements[13], these researchers proposed the withdrawal of thiopurine while the ongoing COVID-19 pandemic in high-risk patients with IBD, for instance, those with older age or multiple comorbidities that are in stable remission on combotherapy with anti-TNF agent[22]. Also, in this international registry in progress, there are no significant differences between biological classes (anti-TNF, anti-IL12/23, and integrin antagonists) on the risk of developing severe COVID-19.

Interestingly, some anecdotal reports have shown improvement in pulmonary symptoms and multisystem inflammatory syndrome related to COVID-19 in patients with active IBD treated with infliximab[23,24]. However, whether anti-TNF alpha therapy or other anti-inflammatory agents may protect against cytokine release syndrome in patients with COVID-19 will require further investigation[25]. Moreover, as advances in IBD therapy broaden the therapeutic arsenal, it will be necessary to maintain investigations using collaborative multi-center registries for evaluating the possible impact of novel agents, such as Janus kinase inhibitors, IL-23 antagonists, and others, on SARS-CoV-2 or COVID-19. It should be highlighted that as the knowledge regarding SARS-CoV-2 progresses, it is likely that IBD-specific recommendations in the COVID-19 setting also undergo substantial changes[26].

Managing patients with IBD infected with SARS-CoV-2 depends on both the clinical activity of the IBD and the occasional development and severity of COVID-19[12,27]. For practical purposes, we will present current recommendations for managing drugs for IBD concerning the SARS-CoV-2 infectious status into distinct clinical scenarios: (1) The patient attending outpatient clinic with IBD in remission in the setting of the asymptomatic SARS-CoV-2 infection or confirmed or suspected COVID-19; (2) The patient with active IBD undergoing outpatient follow-up in the setting of the asymptomatic SARS-CoV-2 infection or confirmed or suspected COVID-19; and (3) The patient with IBD hospitalized with asymptomatic SARS-CoV-2 infection or COVID-19.

Management of patient attending outpatient clinic with IBD in remission in the setting of asymptomatic SARS-CoV-2 infection or confirmed or suspected COVID-19 without systemic hyperinflammation syndrome

As the COVID-19 pandemic expands, an increasing number of tests for SARS-CoV-2 are being conducted, including asymptomatic contacts of COVID-19 index cases. Thus, the situation in which an individual tests positive for the virus but remains asymptomatic will be more and more frequent.

The IOIBD recommends for quiescent patients with IBD with asymptomatic SARS-CoV-2 infection — quick withdrawal of prednisone or de-escalating to < 20 mg/d or switch to budesonide or budesonide MMX (Multi Matrix System) when appropriate[12]. Immunomodulators such as thiopurines, methotrexate, and tofacitinib (or other Janus kinase inhibitors) should be temporarily held for 2 wk while monitoring for the appearance of COVID-19 symptoms[12]. Similarly, biologic administration, including anti-TNF drugs, vedolizumab, and ustekinumab, should be postponed for 2 wk if the dose is due, even recognizing that the half-lives of these biologics are relatively long, so that immunosuppressive effects of these drugs will persist for a few additional weeks despite the withdrawal of these agents[26]. Conversely, nonimmune-based anti-inflammatory therapies such as aminosalicylates, antibiotics, budesonide, or rectal therapy may be continued[22,26]. However, for patients with IBD who have had the closest contact with an individual with proven or suspected COVID-19, it is suggested that they isolate themselves and follow local recommendations from health managers. In this situation, European Crohn and Colitis Organization (ECCO) experts recommend that it is unnecessary to hold biologics or immunomodulators based on exposure only[20].

For patient attending outpatient clinic with quiescent IBD but with confirmed or suspected COVID-19, the approach to drug management is like that adopted for patients with asymptomatic infection by SARS-CoV-2. The American Gastroenterology Association (AGA) and IOIBD experts suggest that budesonide, aminosalycilates, antibiotics, and topical therapy may be maintained while systemic corticosteroids (prednisone) should be avoided and withdraw speedily, if possible. Likewise, it is recommended to hold immunomodulators, Janus kinase inhibitors, and biologics until after disappearance of symptoms, usually for 2 wk during the acute disease[12,26].

Management of patient with active IBD undergoing outpatient follow-up in the setting of asymptomatic SARS-CoV-2 infection or confirmed or suspected COVID-19 without systemic hyperinflammation syndrome

Currently, in the COVID-19 era, if a patient with IBD presents an apparent flare, it is important to always question the presence of concomitant symptoms suggestive of COVID-19, such as fever, cough, anosmia, or dyspnea, because GI symptoms including diarrhea, nausea, vomiting, and abdominal pain have been reported in 2%-33% of patients on initial presentation of COVID-19[28]. Moreover, in a few cases, these digestive symptoms may be the only clinical features of COVID-19[29]. This context is a clinical challenge, compounded by the frequent finding of remarkably elevated serum inflammatory biomarkers in patients with COVID-19.

When a patient with IBD presents diarrhea, it is doubtful whether this is secondary to a disease flare or COVID-19, a wait-and-see approach for the next 5-7 d is a reasonable strategy[27], once the diarrhea due to COVID-19 is mostly mild and self-limited, usually with an average duration of 5 d (range, 1 d to 14 d) and a mean frequency of four bowel movements per day[30]. Also, follow-up using interval assessment of fecal calprotectin (FC) may be useful, as FC levels are typically both transiently raised and mildly elevated in patients with diarrhea caused by COVID-19[31]. In contrast, in active IBD, sustained and substantial elevation of FC is commonly seen. In any case, in the current era of COVID-19, the joint expert consensus from ECCO recommends that all patients with a suspected IBD flare be tested to exclude COVID-19 preferably with oropharyngeal and nasopharyngeal swabs reverse transcription polymerase chain reaction (RT-PCR) assays when the first symptoms emerge[20].

In cases where, after initial assessment, diagnostic doubts remain, computed tomography (CT) scans of the chest, abdominal cross-sectional imaging methods, and, more restrictively, ileocolonoscopy assessment can allow the real cause of diarrhea to be established[20]. Another question that remains unknown is whether SARS-CoV-2 can cause a flare of or *de novo* IBD[19]. Moreover, in patients with apparently active IBD (especially colonic IBD), it is important to be aware to exclude enteric superinfections, mainly due to *Clostridioides difficile* (*C. difficile*) and cytomegalovirus (CMV), assess adherence to therapy, and perform therapeutic drug monitoring of biologics[27].

If GI symptoms (including diarrhea) are not caused by COVID-19 and other causes for IBD flare are excluded, such as enteric superinfection, nonsteroidal anti-inflammatory use, and non-adherence to therapy, the drug management for IBD will depend on the equilibrium between the severities of the IBD flare and those of the COVID-19[18-20]. For a flare of mild severity in outpatients with asymptomatic infection by SARS-CoV-2 or with mild to moderate COVID-19 without systemic hyperinflammation syndrome (SHS), it is recommended tapering off prednisone or its equivalent for < 20 mg/d with complete weaning where possible, balancing with the potential for possible adrenal insufficiency in the setting of chronic corticosteroid therapy[12,26,27]. Another option that can be considered in patients using systemic steroids is converting to oral budesonide or budesonide MMX on adequate dosing, provided the patient is in the appropriate clinical setting (*e.g.*, mildly to moderately active ileocecal CD or UC, respectively). Further, it is suggested stopping or avoiding commencing immunomodulators, tofacitinib (or other Janus kinase inhibitors), and biologics for at least 2 wk during viral illness, while budesonide, aminosalycilates, antibiotics, and topical therapy may be initiated or maintained if needed[26,27].

The approach for a IBD flare-up moderate to severe in patients attending outpatient clinic with COVID-19 without SHS may include continuation of current biological therapy for IBD with optimization to rescue a state of remission or starting a new biological agent if needed, preferably in monotherapy and with subcutaneous biologics to reduce the risk of exposure to SARS-CoV-2 in infusion units[20,27].

In this clinical context, if glucocorticosteroids are considered essential, the dose of prednisone (or its equivalent) should be ≤ 40 mg/d limiting the duration of use, if practicable[26]. Also, it is advised to stop if in use or avoid commencing immunomodulators or tofacitinib. If COVID-19 is progressive with significant pulmonary involvement and hospitalization, consultation with infectious diseases experts for possible COVID-19 treatment with antiviral or experimental anticytokine therapy may be interesting[19,20,27]. In Tables 1-3, we present an approach for managing IBD medications in patients attending outpatient clinic who are infected with SARS-CoV-2 with or without COVID-19.

Table 1 Management of patients attending outpatient clinic with quiescent inflammatory bowel disease in the scenario of asymptomatic severe acute respiratory syndrome coronavirus 2 infection or confirmed or suspected coronavirus disease 2019[12,20,26,27]

	Management
Asymptomatic infection with SARS-CoV-2	(1) Budesonide, aminosalycilates, antibiotics, and topical therapy may be maintained; (2) Hold immunomodulators, tofacitinib, and biologics for 2 wk; (3) Taper or withdraw systemic corticosteroids (prednisone); and (4) Monitoring for 2 wk for COVID-19 symptoms
Mild COVID-19	(1) Budesonide, aminosalycilates, antibiotics, and topical therapy may be maintained; (2) Hold immunomodulators, tofacitinib, and biologics for 2 wk; and (3) Taper or withdraw systemic corticosteroids (prednisone)
COVID-19 with pulmonary involvement without SHS	(1) Budesonide, aminosalycilates, antibiotics, and topical therapy may be maintained; (2) Hold immunomodulators, tofacitinib, and biologics for 2 wk; and (3) Taper or discontinue systemic corticosteroids

Immunomodulators refer to thiopurines and methotrexate. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; SHS: Systemic hyperinflammation syndrome.

Table 2 Management of patients attending outpatient clinic with mildly active inflammatory bowel disease in the scenario of the asymptomatic severe acute respiratory syndrome coronavirus 2 infection or confirmed or suspected coronavirus disease 2019[12,20,26,27]

	Management
Asymptomatic infection with SARS-CoV-2	(1) Budesonide, aminosalycilates, antibiotics, and topical therapy may be used if needed; (2) Hold immunomodulators, tofacitinib, and biologics for 2 wk; (3) Taper or withdraw corticosteroids (prednisone < 20 mg/d); and (4) Monitoring for 2 wk for COVID-19 to present
Mild COVID-19	(1) Budesonide, aminosalycilates, antibiotics, and topical therapy may be used if needed; (2) Hold immunomodulators, tofacitinib, and biologics for 2 wk; (3) Taper or withdraw systemic corticosteroids); and (4) Monitoring for 2 wk for COVID-19 symptoms to disappear
COVID-19 with pulmonary involvement without SHS	(1) Budesonide, aminosalycilates, antibiotics, and topical therapy may be used if necessary; (2) Hold immunomodulators, tofacitinib, and biologics for at least 2 wk or until COVID-19 resolves; and (3) Taper or withdraw systemic corticosteroids

Immunomodulators refer to thiopurines and methotrexate. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; SHS: Systemic hyperinflammation syndrome.

Table 3 Management of patients attending outpatient clinic with moderately to severely active inflammatory bowel disease in the scenario of asymptomatic severe acute respiratory syndrome coronavirus 2 infection or confirmed or suspected coronavirus disease 2019[12,20,26,27]

	Management
Asymptomatic infection with SARS-CoV-2	(1) Restrict the use of prednisone ≤ 40 mg/d if necessary; (2) Avoid immunomodulators and tofacitinib; (3) Escalate to biologics as necessary (preferably in monotherapy); and (4) Thromboprophylaxis
Mild COVID-19	(1) Restrict the use of prednisone ≤ 40 mg/d if necessary; (2) Avoid starting or stopping, if in use, immunomodulators, and tofacitinib; (3) Escalate to biologics and dose optimization as necessary (preferably in monotherapy); and (4) Thromboprophylaxis
COVID-19 with pulmonary involvement without SHS	(1) Restrict the use of prednisone ≤ 40 mg/d if necessary; (2) Avoid starting or stopping immunomodulators, and tofacitinib; (3) Escalate to biologics and dose optimization as necessary (preferably) in monotherapy based on balance of benefits and risks; consultation with infectious diseases expert for possible COVID-19 treatment with antiviral or experimental anticitokine therapy; and (4) Thromboprophylaxis

Immunomodulators refer to thiopurines and methotrexate. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; SHS: Systemic hyperinflammation syndrome.

Management of patients with IBD hospitalized with asymptomatic SARS-CoV-2 infection or COVID-19

Although the ongoing COVID-19 pandemic has substantially impacted the management of IBD, with unprecedented restriction of hospitalizations, many patients with IBD will still be hospitalized in the COVID-19 era either because of severely active or complicated IBD, or because they were infected with SARS-CoV-2 and developed progressive or severe COVID-19 with SHS requiring supplemental oxygen or ventilator support, use of vasopressors, or present evidence of end organ damage[19,26]. In fact, hospitalization should be restricted to life-threatening circumstances or complications[20].

For patients hospitalized with severe COVID-19, the priority is life support, while IBD therapy is likely to have less priority. Nonetheless, when possible, therapy for COVID-19 should consider the underlying IBD[12,27]. Ideally, treatment decisions should be personalized and decision-making should include a multidisciplinary approach, involving teams of experts on both conditions. This way, consultation with pulmonary medicine and infectious disease experts is crucial, with discussion involving the possibilities of COVID-19 treatment with experimental antiviral drugs or anticytokine therapy trials, and staying aware of potential interaction with IBD medications[32]. If IBD is mildly active, budesonide, aminosalycilates, and rectal therapies may be initiated while reducing or holding prednisone. Also, stopping biologics, immunomodulators, and tofacitinib throughout the duration of the COVID-19 is appropriate[18,19,26]. For moderately-to-severely active IBD, the limited use of intravenous corticosteroids for IBD is acceptable, if necessary. Topical therapy may be ordered if deemed adequate, while immunomodulators, tofacitinib, or biologics that failed for IBD should be stopped[18,19,27]. Other therapies for IBD should only be used if definitely required. Intravenous cyclosporine may be a reasonable option for severe UC, based on limited evidence of its benefit against coronavirus[26,27].

Interestingly, in a recent trial involving patients without IBD hospitalized with COVID-19, patients were randomized to therapy with oral or intravenous dexamethasone at a dose of 6 mg once daily for up to 10 d or until discharge, whichever was sooner, or to receive usual care only[33]. The group treated with dexamethasone presented significantly lower 28-d mortality in patients receiving either oxygen alone or with invasive mechanical ventilation (23.3% and 29.3% *vs* 26.2% and 41.4% with usual care, respectively). Importantly, the same benefit was not found in subjects receiving no respiratory support[33]. Whether other glucocorticoids like methylprednisolone or hydrocortisone on equivalent doses might also be effective in this setting is unknown and will require future studies. From the perspective of pharmacological effects and on the immune system, there is nothing specific about dexamethasone that other steroids do not offer as well, and so other glucocorticosteroids could in theory be used if dexamethasone is unavailable[34]. If this hypothesis is confirmed in prospective studies, this important therapeutic strategy can help us clarify our approach for suitably managing the challenging and life-threatening scenario of a patient hospitalized that concomitantly presents with a severe flare of IBD and moderate-to-severe COVID-19, where intravenous methylprednisolone or hydrocortisone could be one of the first-line therapies for both conditions.

However, for patients hospitalized due to severe flare of UC and who also have asymptomatic infection with SARS-CoV-2 or mild-moderate COVID-19, priority must be given to address issues pertinent to severe exacerbation of IBD, and usually a standard approach directed to the care of hospitalized patients with IBD should be followed[26,35]. In this clinical setting, expert opinions of the AGA recommend limiting intravenous steroids to three days and then transitioning to infliximab or cyclosporine[20]. Evaluating enteric superinfections, especially those caused by *C. difficile* or CMV, using fecal toxin A/B enzyme immunoassay and/or PCR for detecting toxins A and B genes or for detecting CMV DNA by quantitative PCR should be a routine practice for these patients[27]. In contrast, when CMV superinfection remains suspected despite the results of non-invasive tests, urgent colonoscopy should be reserved for patients in whom the procedure may change or target a specific therapy[12,19,20]; also, colorectal surgery expert consultation in the first days of hospitalization of the patient should also be a standard practice in this context[35]. Foremost, a recent 'RAND appropriateness panel' adapted from the BSG guidelines for managing acute severe UC recommended that regarding the COVID-19 pandemic, prophylactic anticoagulation post-discharge is ordered for patients hospitalized with acute severe colitis that had a positive SARS-CoV-2 testing due to the predisposition to develop thromboembolic complications in both conditions[36]. Indeed, the British Thoracic Society proposes that it is a reasonable approach to consider extended venous thromboembolism prophylaxis on discharge with low molecular weight heparin or direct oral anticoagulant during four weeks in high-risk patients with COVID-19[37]. Table 4 depicts the suggested approach for managing patients with IBD hospitalized with severe COVID-19.

When to restart biological and other immunosuppressive agents in patients with IBD infected with SARS-CoV-2 with or without COVID-19

Considering that patient safety should be a priority in the context of still limited but rapidly expanding knowledge regarding the management of IBD during the COVID-

Table 4 Management of patient with inflammatory bowel disease hospitalized with severe coronavirus disease 2019[12,19,20,26,27]

	Management
Quiescent IBD	(1) Budesonide, aminosalycilates, and rectal therapy may be kept; (2) Taper or withdraw prednisone; (3) Stop immunomodulators, tofacitinib, and biologics; and (4) Prioritize life support; consultation with infectious diseases expert for possible COVID-19 treatment with antiviral or experimental anticitokine therapy; thromboprophylaxis
Mildly active IBD	(1) Budesonide, aminosalycilates, and rectal therapy may be initiated; (2) Taper or withdraw prednisone; (3) Non starting or stopping if in use biologics, immunomodulators, and tofacitinib; and (4) Prioritize life support; consultation with infectious diseases expert for possible COVID-19 treatment with antiviral or experimental anticitokine therapy; thromboprophylaxis
Moderately to severely active IBD	(1) Limited use of intravenous steroids for IBD if necessary; (2) Topical therapy may be initiated if needed; (3) Quit immunomodulators, tofacitinib, or biologics that failed for the IBD; and (4) Consider other therapies for IBD only if absolutely necessary; intravenous cyclosporine may be a reasonable option for ulcerative colitis, based on limited evidence of its benefit against coronavirus. Prioritize life support; consultation with infectious diseases expert for possible COVID-19 treatment with antiviral or experimental anticitokine therapy; thromboprophylaxis

Immunomodulators refer to thiopurines and methotrexate. IBD: Inflammatory bowel disease; COVID-19: Coronavirus disease 2019; Severe COVID-19: Patient with systemic hyperinflammation syndrome needing mechanical ventilation ± vasopressors or evidence of end organ damage.

19 pandemic, most consensus statements and expert opinions recommend temporarily holding biologics and other immunosuppressant drugs in patients with IBD with asymptomatic SARS-CoV-2 infection or in the presence of symptoms suggestive of COVID-19 until a patient recovers[38]. However, if used prolongedly, this practice can lead some patients to both lose the effectiveness of their therapy and present an IBD flare[39]. Therefore, guidance on when to restart IBD medications in this setting is very welcome.

Although the timing for treatment restart is nonconsensual, currently, for decision making, the IOIBD expert panel recommends preferably for most patients a symptoms-based strategy due to the lack of accuracy of current molecular tests available for diagnosing SARS-CoV-2 infection, and also because of the clinical significance still unclear of the prolonged persistence of viral RNA detected by these tests in individuals that had COVID-19[38].

In asymptomatic patients with SARS-CoV-2 infection, some experts advise waiting at least 10 d from the first positive COVID-19 test for restarting immunosuppressive drugs as long as there is no development of symptoms suggestive of COVID-19 in this time interval[12,38]. In patients with COVID-19, the timing of resumed biologics or other immunosuppressants should be guided by the balance between the severity of both viral disease and IBD[38]. Thus, based on updated guidelines from the IOIBD, using a symptom-based strategy, it is recommended to wait at least 10 d since the appearance of the first symptoms of COVID and at least 3 d since recovery, defined as resolution of fever and significant improvement in respiratory symptoms, to re-start these medications[38]. Having two consecutive negative PCR tests in swab specimens, collected at least 24 h apart, is no longer required when this strategy is embraced[38,40]. In severe COVID-19, a longer time frame for re-initiating immunosuppressant may be necessary according to the personalized clinical strategy, if possible awaiting full patient recovery[41]. However, when doctors require a test-based strategy to decide about restarting IBD medications, in addition to the patient having clinically recovered based on the described parameters of the symptom-based strategy, he must have two consecutive negative nasopharyngeal swabs COVID-19 molecular assays collected at least 24 h apart[38].

MONITORING IBD TREATMENT DURING THE PANDEMIC

The outbreak of the COVID-19 infection forced government authorities to impose several restrictions, including lockdown[42]. Hospitals were then forced to rapidly restructure their activities to accommodate this critical and emergent situation. Institutional rearrangements have challenged IBD units worldwide, forcing them to adapt and generate specific approaches to maintain appropriate IBD care[10]. Referral IBD centers and Gastroenterology/IBD Societies published their guidance, helping clinicians tackle this troublesome situation[10,12,13,19,26,42-47]. In common, they advised remote monitoring, drug home delivery whenever possible, infusion unit restrictions, and patient education concerning protective measures (Table 5). Although necessary, all these measures and restrictions may negatively impact patients with IBD. A recent survey among 225 patients with IBD from a referral center showed depressive mood

Table 5 Approach to diminish the spread of coronavirus disease 2019 for patients with inflammatory bowel disease[10]

	Approach to diminish the spread of COVID-19 for patients with IBD
Inpatient clinic	(1) Hospitalized patients with IBD relocated to an isolated area/building, if possible, minimizing exposure to the virus; and (2) Test for coronavirus 2019 with nasopharyngeal swabs (PCR) before hospitalization
Outpatient clinic	(1) Visits rescheduled if possible; (2) Medical staff monitor patients *via* telemedicine (*e.g.*, remote video and telephone call); (3) Laboratory tests strictly limited; use fecal calprotectin (home modality, stool collection kit picked up by express mail services, if possible); (4) Endoscopy and image procedures only for urgent cases; (5) Patients should be advised to keep hygienic measures, avoid nonessential travels, and stay at home or work on a home-office basis; (6) Recommendations to maintain adequate hydration and nutrition status; and (7) Advise patients to continue their therapies, especially if in remission
Infusion center	(1) No accompanying person permitted; (2) Rearrangement of seats allowing a distance of at least 1.5 m in between; (3) Surgical masks for both patients and healthcare professionals; (4) Pre-admission protocol to assess for acute respiratory tract symptoms among patients with IBD and their contacts; (5) Selection of patients that could have their infusion postponed for 1-2 wk to let more space available for rearrangements of seats (those with clinical and endoscopic remission); and (6) Preference, if possible, for those biologics that can be offered subcutaneously, at home, instead of intravenously, to avoid overcrowding in the infusion center

IBD: Inflammatory bowel disease; COVID-19: Coronavirus disease 2019; PCR: Polymerase chain reaction.

as the most prevalent social impact (80.2%), followed by anxiety/fear of death (58.2%), insomnia (51.4%), daily activity impairment (48%), sexual dysfunction (46.2%), and productivity impairment (40%)[11]. These health repercussions in patients with IBD are essential, and healthcare professionals should be aware of them when talking remotely with the patients.

A common feature in the various IBD units is that telemedicine has replaced follow-up visits[48]. Although not the ideal way to follow these patients, that was the best way to do it now. Some authors prefer to term these remote visits video office or telephone office visits[44]. They avoid the term "virtual," which could mistakenly connote that the visits were not "real"[44]. In general, patients found those remote visits worthwhile, but no doubt, this was exhausting for the healthcare professionals that ended up with the well-described "zoom-fatigue"[44]; consequently, the personal visits were reduced by 30%-40%[10,42-44]; for comparison, endoscopic procedures were reduced by 90%-95%[10,42-44] and were indicated in selected cases[42,44,49,50]. Image procedures were also largely deferred and only indicated in cases of intestinal obstruction or suspected abscess[44]. There has been a lack of information on the impact of remote monitoring of patients with IBD during the SARS-CoV-2 pandemic. In a single-center cross-sectional Italian study with 1083 patients with IBD, telemonitoring of patients by phone or videoconference was compared with clinical evaluation in person (control group)[48]. Despite fewer relapses in the control group, there were no statistically significant differences between the groups regarding the quality of life measured by the IBDQ-32 questionnaire[48]. Thus, the possibility of contacting the IBD staff through remote monitoring, although not ideal, partially contributes to maintaining the quality-of-life parameter.

FC has been a valuable tool during this pandemic[27]. Many centers have relied on commercial labs to send an overnight home stool collection kit for FC that can be picked up by express mail services, avoiding the patient from leaving home[44]. A home FC test (IBDoc) was compared with a laboratory test (Quantum Blue® calprotectin test, BÜHLMANN, Schönenbuch, Switzerland) and the correlation between both tests was good ($r = 0.776$, $P < 0.0001$)[51]. Using 250 μg/g as the cutoff, the agreement between the home and laboratory tests was 80%[51]. Diarrhea can be one of the manifestations of COVID-19 or an IBD flare[27]. FC can be particularly useful to differentiate these situations since FC is usually only mildly elevated in patients with COVID-19 with diarrhea, whereas in patients with IBD and active disease, significant and sustained elevation may occur[27].

ENDOSCOPY IN PATIENTS WITH IBD IN THE COVID-19 ERA

Endoscopy is not only one fundamental pillar for diagnosing IBD but also plays an important role in its management, treatment, and colorectal cancer (CRC)/dysplasia surveillance. Differential diagnosis concerning other etiologies and between UC and CD as well as full evaluation of disease extension, activity, response to treatment, and even some therapeutic approaches are some endoscopic indications in a patient with IBD. Ileocolonoscopy, flexible proctosigmoidoscopy, and esophagogastroduodenoscopy are the most commonly used endoscopic methods, but enteroscopy and

videocapsule endoscopy also play a role in IBD management[52,53]. The overcrowded healthcare system worldwide by the COVID-19 pandemic and the need to control the spread of infection required restructuring primary care and hospital activities, including endoscopy units[43,54].

Risk of infection during endoscopy

Regardless of being more contagious than the other coronavirus (SARS-CoV and Middle East respiratory syndrome coronavirus), SARS-CoV-2 has similar ways of infection and complications[55], with aerosolized droplets produced by coughing, sneezing, or breathing as the main route of infection. SARS-CoV-2 infects the GI epithelium, and its RNA can be detected in stool samples, sometimes in high concentrations, even in patients without GI symptoms, lightening the possibility of fecal–oral transmission[55]. Although this route of transmission is controversial, some papers proved the presence of live virus in fecal specimens and even a positive PCR in material collected from the surface of the toilet and sink used by infected patients, thereby corroborating this route of transmission[56].

Both upper and lower GI endoscopies should be considered as aerosol-generating procedures. Upper GI endoscopy can generate respiratory droplets by coughing or gagging induced mainly during endoscopic insertion and lower GI endoscopy by passing flatus or pathogen-containing stools[57]. Therefore, endoscopy can be considered a high-risk procedure for SARS-COV-2 transmission for both patients and healthcare professionals, being reasonable to ration the endoscopic resources.

Indications of endoscopy during COVID-19 pandemic

To minimize the exposure and risk of infection, different Societies of Gastroenterology and Endoscopy worldwide (American Gastroenterological Association[58], BSG[13], European Society of Gastrointestinal Endoscopy and European Society of Gastroenterology and Endoscopy Nurses and Associates[59], Asian Pacific Society for Digestive Endoscopy[60], and Brazilian Society of Digestive Endoscopy[61]) agreed to postpone all the endoscopic procedures during the pandemic period, except for the emergency ones and on a case-by-case basis, some urgent ones. The emergency and urgent endoscopic procedures can be resumed and divided into the following three categories: Emergent, urgent, and elective (Table 6)[62]. The restriction in the indications for GI endoscopy aimed not only to reduce the risk of patients and healthcare professional infection but also to save personal protective equipment (PPE) and other medical supplies[57].

Endoscopy, especially ileocolonoscopy, plays a fundamental role in the diagnosis and management of IBD, ruling out some other diagnoses and providing important information about the extension and activity of the disease, response to treatment, and even a therapeutic approach to stenosis and other complications. During the COVID-19 pandemic, these indications for patients with IBD were reviewed and based on expert opinions, the IOIBD[63] and French Society of Digestive Endoscopy (SFED) recommendations[64], the advice was again to postpone all endoscopic procedures that were not urgent and extremely necessary. However, sometimes, this emergent/urgent indication for endoscopy in patients with IBD is slightly unclear. Thus, next we are going to discuss some possible scenarios[57]:

Confirm IBD diagnosis: In highly suspected cases with moderate-to-severe symptoms and grounded by positive biomarkers and cross-sectional imaging findings (bowel ultrasonography or magnetic resonance enterography) of IBD, lower GI endoscopy (proctosigmoidoscopy or colonoscopy) with biopsies is still indicated[57]. Depending on the clinical situation and the presence of rectal symptoms, proctosigmoidoscopy might be preferable to full colonoscopy, as the former is faster and can be done without sedation (no need for companion) or oral bowel preparation[63]. Cases with mild symptoms can have their endoscopy postponed[57,63,64]. If an isolated small bowel disease is the hypothesis, after negative cross-sectional imaging and in the absence of obstructive symptoms, videocapsule endoscopy should be preferred to enteroscopy for its lower risk[64].

Monitoring IBD treatment: Disease activity monitoring, if possible as mentioned before, should be made by checking patient report outcomes (PROs) and noninvasive tests such as FC, C-reactive protein (CRP), or even cross-sectional imaging[64,65].

Acute severe UC: This is an emergency condition with a high morbimortality. A patient who fulfills the criteria of a stool frequency (≥ 6 per day), with bloody stools, heart rate above 90 bpm, temperature exceeding 37.8 °C, hemoglobin levels below 105

Table 6 Category of endoscopic procedures[13,62]

Emergent endoscopy	Urgent endoscopy	Elective endoscopy
Ascending/acute cholangitis; Foreign body retrieval; GI obstruction; Life-threatening GI bleeding	Cancer staging; Stable GI bleeding; Tumor biopsy; Palliative procedures (stenting, neurolysis); Planned EMR/ESD for complex/high-risk lesions	Biliary stent removal; Clinical trials; Colorectal cancer screening; Percutaneous endoscopic gastrostomy; Post-polypectomy surveillance; Surveillance/follow-up endoscopy (excluding high-risk neoplasia)

GI: Gastrointestinal; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

g/L, and high CRP levels (> 30 mg/L) needs to be hospitalized and undergo investigation to rule out infections other than IBD activity, especially COVID-19, *C. difficile*, and CMV[66,67]. Symptoms such as fever, cough, dysosmia, dysgeusia, and fatigue need to be questioned, and a PCR for SARS-COV-2 asked. If negative, analysis of stool samples should be performed looking for *C. difficile* and parasitological infection and, in this setting, it is advised to perform flexible proctosigmoidoscopy with biopsies to exclude CMV infection[57,63,64].

Postoperative recurrence assessment: Endoscopy plays an important role in the postoperative management of both CD and UC patients. In CD, mainly in cases of ileocecal resection, since endoscopic inflammation precedes biological and clinical activity, ileocolonoscopic findings allow us to tailor therapy. In UC patients, postoperative endoscopy is mainly indicated for pouchitis and surveillance of this segment for dysplasia. However, when we consider the risks and benefits of the procedure during this pandemic period, endoscopy to check postoperative recurrence and dysplasia could be delayed, and for inflammation, patients should be followed noninvasively[63,64,68].

Surveillance colonoscopies: It is known that patients with IBD have twice more risk of CRC than the general population, justifying the importance of its screening and surveillance. Based on ECCO guidelines, surveillance colonoscopy is indicated for all patients with UC, except if the disease is restricted to the rectum eight years after the beginning of the symptoms. Patients with colonic CD with more than 1/3 of the colon affected should follow the same protocol. If primary sclerosing cholangitis (PSC) is present, surveillance is recommended to start immediately when the diagnosis is confirmed and continues yearly. The interval between colonoscopies will depend on the risk factors and the results obtained in the index exam[66]. However, for all the risks listed above, the recommendation is to postpone the colonoscopy in patients without alarming signs. With the extension of the pandemic period, this recommendation needs to be reviewed, and probably the patients will be stratified by risk factors for CRC, as with PSC or previous dysplastic lesions, and will be rescheduled as priorities for endoscopic examination[57,64].

Partial GI obstruction: Patients with IBD are at higher risk of some complications, such as benign or malignant strictures. In cases of a new colonic stricture, a colonoscopy is indicated to exclude malignancy, and if this hypothesis is confirmed, a stent or balloon dilatation might be needed if surgery could not be performed. In CD patients with a known short stricture (< 4 cm) with recurrent obstructive symptoms (nausea, vomiting, weight loss, and abdominal pain), endoscopic dilatation is a therapeutic option to avoid future admission to the emergency room, overloaded during COVID-19 pandemic[57,68].

Upper GI endoscopy: The indications for upper GI endoscopy in patients with IBD during this pandemic period are restricted to acute GI bleeding or dilatation of upper GI strictures[64].

Worsening cholangitis and jaundice in patients with IBD and PSC with a dominant bile duct stricture: Acute cholangitis in a patient with PSC is an emergency, and if there is a dominant bile duct stricture detected on a magnetic resonance cholangiography, an urgent endoscopic retrograde cholangiopancreatography (ERCP) will access the stricture to exclude cholangiocarcinoma and may decompress the bile duct[57].

Endoscopy in industry-sponsored clinical trials: Most of the recruitment of new patients and screening visits for clinical trials have been discontinued during this

pandemic period, but the status of monitoring colonoscopies for participants already recruited should be discussed with trial sponsors and research ethics committees. A case-by-case discussion might be necessary, as for some patients, the investigational product is the only therapeutic option to avoid surgery and/or corticosteroids. Trial visits should occur virtually whenever possible[57,64].

In summary, endoscopy in patients with IBD during the COVID-19 pandemic should be restricted for the indications listed in Table 7.

Safety measures for protection against COVID-19 infection

Aiming to mitigate the risk of the spread of infection from possible COVID-19 patients, reduce the risk of cross-infection, and preserve resources and PPE, some measures are recommended by different GI endoscopy societies[62,69]. The safety recommendations can be targeted to the unit structure, patient safety, healthcare professional safety, and equipment/endoscopy room care.

Unit structure[57,70]**:** Limit the number of patients scheduled; Consider at least a one-hour interval between exams; Select the indications of endoscopy and postpone the others; Remote pre-exam evaluation with a questionnaire about COVID-19 symptoms and contact; Inform the need to wear a mask; Apply social distancing rules in the waiting room; Avoid physical contact; Limit the waiting room time and number of people; Reassure that just exams with sedation or anesthesia require a companion; Relatives and caregivers are forbidden from entering the hospital or endoscopy unit unless required; Medical and nursing students are restricted in the endoscopy units during pandemic crises; Allow only essential staff with proper PPE inside the endoscopic unit; Keep doors closed; Provide information about hand hygiene; Follow-up call one week after endoscopy: As symptoms of COVID-19 can occur after infection, patients undergoing endoscopy could develop symptoms after the procedure if they have contracted the SARS-CoV-2 infection at the community level just before endoscopy. It is a way to verify whether the protective measures at the unit are working[64].

Patient safety[64]**:** Check again for COVID-19 symptoms (fever > 37.5 °C; cough, dysgeusia, dysosmia, and dyspnea) or contact at admission; Access body temperature; Provide the patient alcoholic solution to clean hands; Before entering the endoscopy room, the patient is asked to dress a cotton gown, a hairnet, and a surgical mask; Nasal swabs are not a routine recommendation, as they are not generally available or validated.

Healthcare professionals' safety[64,70,71]**:** AGA and the SFED recommend the use of filtering face piece (FFP) respiratory class 2 or 3 (FFP2 or FFP3) instead of surgical masks to protect Healthcare professionals (HCPs) during upper and lower GI procedures, regardless of the COVID-19 status of the patient. The suggestion for mask changing depends on regional recommendations; Limit the number of endoscopy personnel and operational endoscopy rooms; Endoscopy work teams should comprise a consultant endoscopist, a highly trained endoscopy nurse, and, if possible, a consultant anesthesiologist; HCPs should be checked for COVID-19 symptoms and having their body temperature accessed; HCPs should wear FFP2 mask during the entire time at endoscopy unit as SARS-CoV-2 remained viable in aerosols for at least 3 h; HCPs should be trained in dressing and undressing the PPE, and hand washing is mandatory before both phases; HCPs must remove contact lenses and dress: Hairnet, a long water-resistant gown with back closure, an FFP2 mask, goggles for eye protection, and over-sleeve gloves over the gown. Over the other layers, a single-use gown and another pair of gloves; HCPs should change the disposable gown and the second gloves in each procedure; Hand washing is mandatory before and after every interaction with patient and other HCPs; Conscious sedation remains the most feasible option and can be provided and managed even if the patient is wearing a mask.

Equipment/endoscopy room[62,64,72]**:** Negative-pressure room to prevent generated aerosols from diffusing outside the room is recommended mainly in COVID-19 confirmed or highly suspected cases; Disinfection and decontamination by neutral detergent and virucidal disinfectant using 0.05% sodium hypochlorite or 70% ethanol on surfaces and devices are effective in clearing the virus; All used endoscopes must undergo standardized reprocessing and disinfection; All used accessories must be disposed of; Beds must be cleaned with specific disinfection products and bed sheets changed for each patient.

Table 7 Indications for gastrointestinal endoscopy in patients with inflammatory bowel disease during the coronavirus disease 2019 pandemic[57,63,64]

Recommended	Considered case-by-case	Postpone
Confirm IBD diagnosis in patients with moderate to severe activity; Acute severe ulcerative colitis; Partial GI obstruction; Life-threatening GI bleeding; Worsening cholangitis and jaundice in patients with IBD and PSC with a dominant bile duct stricture	Surveillance colonoscopies of high-risk patients; Specific clinical trials	Confirm IBD diagnosis in patients with mild symptoms; Monitoring IBD treatment; Postoperative recurrence assessment; Surveillance colonoscopies of low-risk patients; Clinical trials

GI: Gastrointestinal; IBD: Inflammatory bowel disease; PSC: Primary sclerosing cholangitis.

Endoscopy post-pandemic phase

When the COVID-19 pandemic ends, endoscopy units must face a dammed demand list with the impossibility of returning to a regular agenda, as the interval between the exams will need to remain longer to prevent new outbreaks. A stratification of priorities will be necessary[73]. Some algorithms based on available point-of-care tests considering epidemiological regional data and an accurate clinical risk assessment are being proposed to stratify the patients[74]. Whatever method is used to prioritize endoscopy, it is important to maintain close contact with patients with IBD by phone or e-mail to monitor for specific symptoms. Planning carefully the post-pandemic phase is primordial, and a case-by-case analysis with reassessments of patients' conditions by both clinician and endoscopist will be demanded, and telemedicine might be a useful tool to help this conversation[73].

SURGICAL INTERVENTIONS IN PATIENTS WITH IBD AND COVID-19

Despite significant advances in the medical management of IBD in recent years, many patients will require surgery. The most common indications for CD surgery include stenosis, fistulae, and abscesses[75]. In UC, the most common indications are acute severe colitis refractory to medical therapy and chronic refractory UC. Other indications of surgery in UC are dysplasia and CRC[76]. Many of these situations are elective or semi-elective surgeries that can be postponed for a few days or weeks in some patients with confirmed COVID-19. However, emergencies, such as perforation, acute severe colitis, and uncontrolled hemorrhage, may occur, and the surgery in these cases cannot be postponed and must be treated promptly. At the peak of the pandemic, one of the collateral effects was that elective surgeries were canceled or temporarily suspended[77]. Elective surgeries in IBD cannot be delayed too long when they are strictly indicated, mainly due to increased morbidity given the patient's weakened condition (*e.g.*, steroids and malnutrition)[78]. It is not recommended to delay surgical treatment for these patients with IBD, regardless of their COVID-19 status.

Conducting emergency surgeries during a pandemic such as COVID-19 is challenging for IBD surgeons and the entire hospital infrastructure[79,80]. The information available on COVID-19 and the possibility of contamination through aerosols and droplets lead to the need for modifications to perform surgery with success, reducing the risk of contaminating hospital facilities and protecting health teams and patients[79]. It is pivotal to protect all surgical teams (*e.g.*, masks, glasses, face shields, and surgical caps) to avoid contagion with COVID-19 when they perform surgery in patients with IBD with suspected or confirmed COVID-19 and protect the patient to prevent him from contracting COVID-19.

There is a concern among surgeons with minimally invasive techniques (*e.g.*, laparoscopy, and robotic surgery) due to a possible risk of viral transmission of the COVID-19 with the creation of pneumoperitoneum[81]. However, minimally invasive surgery (MIS), including laparoscopic surgery, is feasible and safe in IBD and has many advantages, such as reduced length of hospital stay, less pain, reduced trauma, less impact on respiratory movements, reduction of morbidity, and faster postoperative recovery[82].

There is little evidence of viral transmission through laparoscopic or open approaches. As shown by Somashekhar *et al*[79], the risk of infection by COVID-19 for the healthcare team during MIS (laparoscopic or robotic) is considered low. Therefore, in IBD, we should not postpone surgery, even if considered "elective," due to the COVID-19 pandemic. Thus, the care that must be followed by the surgical, nursing,

and anesthesia teams must be standardized at the referred hospital and replicated to other health services referenced in the surgical treatment of IBD.

Precautions to avoid contamination of the surgical team are described in several studies published during the pandemic and include care with airway management during the anesthetic procedure and specific care during laparoscopy[79,81,83-85]. There seems to be a consensus in the literature that intubation and extubation are high-risk healthcare professionals' procedures and that the maximum amount of PPE is needed. However, there is little evidence of the real risk of contamination by healthcare professionals during laparoscopy itself, nor of operating room (OR) pressure, surgical smoke, tissue extraction, or CO_2 deflation[83]. If surgery is considered necessary, the surgeon must minimize the risk of exposure to the virus, involving a minimum number of health professionals and shortening the occupation of the ORs. As said, there are no absolute contraindications to MIS; however, appropriate PPE for the OR team and smoke evacuation/filtration systems are unanimously recommended[85].

If there is a lack of safety measures to allow safe laparoscopy, open surgery should be considered[84]. Nevertheless, previous studies have shown that bacterial and viral aerosols can be detected in both open and laparoscopic surgical operations[86]; then, a surgical aspirator/smoke evacuation device should also be used in open procedures. Electrical instruments and energy devices should be used at the lowest energy level to preclude excessive aerosol and smoke production[87].

There are some tips and tricks to make MIS safer during the COVID-19 pandemic, such as avoid creating a leak for smoke evacuation, use a closed suction system; use leak-free trocars such as balloon trocars, aspirate the entire pneumoperitoneum before retrieving the surgical specimen at the end of a procedure before removing the trocars, or before conversion to open surgery[84]. Hospitals must prepare specific internal protocols and arrange adequate training of the involved personnel[88].

If possible, postpone elective surgery and consider screening every surgical patient for COVID-19 either by RT-PCR swab or CT scan of the thorax[89]. In emergent (< 24 h) surgeries, such as perianal abscess, bowel perforation, toxic megacolon, the surgery must be done without any delay, and all patients must be treated as if they were COVID-19-positive. All surgical teams must strictly follow all rules related to infection against COVID-19. The same rules apply to patients under urgent (< 72 h) situations, such as bowel obstruction (without ischemia). Elective (up to 4 wk to 3 mo) surgeries must have an individualized approach. Seton replacements, "J" pouch confection, can be postponed up to three months. However, colectomy in patients with chronic refractory UC or dysplasia and CRC in UC should be referred to surgery, preferably before this period, so that there will be no worsening of the primary clinical condition.

It is important to emphasize that all known or suspected COVID-19-positive patients requiring surgical intervention must be treated as positive until proven otherwise to minimize infection spread[88]. Besides, whenever possible, dedicate specific OR to patients with COVID-19[83]. Create negative pressure ORs because they are considered ideal; yet, most ORs work at positive pressure, and their use is therefore permitted. An air exchange rate of ≥ 25 cycles/h is considered sufficient to effectively reduce the OR's viral load. Only essential staff members should be admitted into the OR, limiting in/out traffic, and doors should be kept closed. Use level III PPE during intubation and extubation. Use proper filters and closed systems for CO_2 desufflation and do not perform transanal surgery[83].

There are some critical considerations for transanal surgery during the COVID-19 pandemic[90]. Several lines of evidence have supported the possible fecal-oral transmission of the COVID-19[91,92]. It is important to emphasize that positive pressure transanal surgery, such as transanal MIS and transanal endoscopic microsurgery, are aerosol-generating procedures. Hence, it is appropriate to perform routine preoperative fecal testing for SARS-CoV-2, in addition to nasopharyngeal screening, in patients undergoing transanal surgery under positive pressure. For patients with confirmed SARS-CoV-2 infection, conventional open and robotic approaches may be safer alternatives when surgery cannot be postponed.

MENTAL HEALTH AND EDUCATIONAL INITIATIVES

It has been recognized that the pandemic will greatly increase the incidence of severe psychological problems, such as mood disorders, anxiety disorders, or posttraumatic stress disorder (PTSD), as a consequence of isolation, human losses, and financial hardships[93]. For patients with IBD, psychological distress is already a common feature, with studies suggesting that active disease is strongly related to comorbid

anxiety and depression[94]. A recent cross-sectional survey exploring the emotional state, perception, and concerns of Saudi patients with IBD during the pandemic found a diagnosis of anxiety in 48.4% of surveyed patients. In this context, patients with IBD require greater attention, and clinical or cognitive behavioral treatment should be offered to all patients who exhibit psychological distress.

Provided that patients with IBD are experiencing substantial changes to the routine management of their conditions during the pandemic, it seems highly critical to assess patients' perceptions and viewpoints. In the earlier phase of the pandemic, the Federation of Crohn's and Ulcerative Colitis Associations (EFCCA) conducted an anonymous online survey to investigate the concerns, fears, and behaviors of patients with IBD. Based on responses from 3815 participants from 51 countries worldwide, it was shown that about half of respondents reported receiving COVID-19 information or specific recommendations from doctors to prevent infection. However, most patients (60%) would have preferred to receive more recommendations regarding COVID-19 from their physicians[95]. These results emphasized the urgent need for better communication between physicians and patients and for clear and specific recommendations for people with chronic conditions in these unprecedented times. In this context, educational initiatives involving patient associations might play a crucial role in allowing dissemination of the correct messages regarding patient management. Also, patient compliance with healthcare providers' guidelines could be achievable by enhanced collaboration, and long-term, trusted partnerships could also be established.

VACCINATION

The recent availability of vaccines to prevent SARS-CoV-2 infection has raised concerns regarding the safety and efficacy of immunization in patients with IBD. Until now, there has been international agreement among the main international IBD expert groups that the risks of SARS-CoV-2 vaccination in patients with IBD are anticipated to be very low, and it is strongly recommended that patients with IBD should be given a COVID-19 vaccine once it is widely available[2,96-98]. All coronavirus vaccines, which are licensed or in the final stages of testing, are considered suitable for patients on biologics, steroids, and immunosuppressants, as they are not live vaccines. These include the mRNA (Pfizer, Moderna), the non-replicating adenovirus vector (Oxford), and the inactivated SARS-CoV-2 (Coronavac) vaccines. Analogous to other vaccines used for many years, such as influenza and pneumonia vaccines, there is no indication of worsening IBD symptoms or flares following vaccination, and immunization appears improbable to affect IBD activity[99]. SARS-CoV-2 vaccines have also been tested in tens of thousands of patients with safety profiles analogous to other vaccines commonly used in patients with IBD, such as the flu vaccine.

For patients under immunosuppressive treatments, it is anticipated that the vaccine may be slightly less effective, as other studies have shown that immunosuppressant medications may induce some reduction in antibody formation and lower immune response with other common vaccines. For instance, it has been demonstrated that the serologic conversion rate to influenza vaccine is lower in immunosuppressed patients with IBD[99,100] and that the immune response to pneumococcal polysaccharide vaccination is reduced in patients with CD by combining TNF-blockers and immunomodulators[101]. Conversely, treatment with newer biologics, such as ustekinumab or vedolizumab, does not seem to decrease responses to flu vaccine[102,103]. We still do not know which IBD treatments, if any, will reduce the effectiveness of the coronavirus vaccine; however, it is important to emphasize that even if the COVID-19 vaccine works slightly less well in immunosuppressed patients, it will still offer greater protection than not having the vaccine.

It is not advisable that patients should stop their treatments to get vaccinated, as it can induce an exacerbation, putting patients at a greater risk of serious complications of COVID-19. Also, patients should avoid receiving their vaccine on the same day of an infusion/subcutaneous dose of biologic, just in the exceptional circumstance that the patient develops a reaction or adverse effect. It would be important to identify which one (vaccine or biologic) has caused it.

CONCLUSION

In this review, we presented a guide for the practicing clinician for managing IBD during the COVID-19 pandemic. We also reviewed the risk of infection during

endoscopy, highlighting the restricted conditions where we still should indicate GI endoscopy in patients with IBD and the recommendations of the most important endoscopy societies for a safer procedure. All known or suspected COVID-19-positive patients requiring surgical intervention must be treated as positive until proven otherwise to minimize infection spread. It is not advisable that patients should stop their treatments to get vaccinated, as it can induce an exacerbation, putting patients at a greater risk of serious complications of COVID-19. In this time of an unprecedented pandemic, where knowledge about COVID-19 rapidly expands, we suggest that clinicians caring for patients with IBD should periodically check for updates in the SECURE-IBD registry and Gastroenterology Societies statements and guidelines to update knowledge about SARS-CoV-2 and COVID-19 in patients with IBD for better information and follow the approach to manage medications in IBD in this challenging context.

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ORIGINAL ARTICLE

Retrospective Study

Efficacy and safety of endoscopic submucosal dissection for gastric tube cancer: A multicenter retrospective study

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Abstract

BACKGROUND

Recent improvements in the prognosis of patients with esophageal cancer have led to the increased occurrence of gastric tube cancer (GTC) in the reconstructed gastric tube. However, there are few reports on the treatment results of endoscopic submucosal dissection (ESD) for GTC.

AIM

To evaluate the efficacy and safety of ESD for GTC after esophagectomy in a multicenter trial.

METHODS

We retrospectively investigated 48 GTC lesions in 38 consecutive patients with GTC in the reconstructed gastric tube after esophagectomy who had undergone ESD between January 2005 and December 2019 at 8 institutions participating in the Okayama Gut Study group. The clinical indications of ESD for early gastric cancer were similarly applied for GTC after esophagectomy. ESD specimens were evaluated in 2-mm slices according to the Japanese Classification of Gastric Carcinoma with curability assessments divided into curative and non-curative resection based on the Gastric Cancer Treatment Guidelines. Patient characteristics, treatment results, clinical course, and treatment outcomes were analyzed.

RESULTS

The median age of patients was 71.5 years (range, 57-84years), and there were 34 men and 4 women. The median observation period after ESD was 884 d (range, 8-4040 d). The median procedure time was 81 min (range, 29-334 min), the *en bloc* resection rate was 91.7% (44/48), and the curative resection rate was 79% (38/48). Complications during ESD were seen in 4% (2/48) of case, and those after ESD were seen in 10% (5/48) of case. The survival rate at 5 years was 59.5%. During the observation period after ESD, 10 patients died of other diseases. Although there were differences in the procedure time between institutions, a multivariate analysis showed that tumor size was the only factor associated with prolonged procedure time.

CONCLUSION

ESD for GTC after esophagectomy was shown to be safe and effective.

Key Words: Endoscopic submucosal dissection; Gastric tube; Gastric cancer; Esophagectomy; Multicenter study; Retrospective study

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Core Tip: Despite increasing occurrence of gastric tube cancer (GTC) after esophagectomy, there are few reports on the treatment results of endoscopic submucosal dissection (ESD) for GTC. This multicenter study showed that treatment results and complications of ESD for GTC were similar to those of standard ESD and there were not significantly difference between institutions except for procedure time. ESD for GTC after esophagectomy is a safe and effective treatment.

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INTRODUCTION

Recently, the survival of patients with esophageal cancer after esophagectomy has improved[1-5]. However, the risk of a subsequent occurrence of primary cancer is high in these patients. The most frequent cancer that overlaps with esophageal cancer is head and neck cancer, while the second most common is gastric cancer, including gastric tube cancer (GTC)[6-9]. Therefore, the improved prognosis of esophageal cancer patients has led to an increase in the occurrence of GTC in the reconstructed gastric tube.

For the treatment of GTC after esophagectomy, total gastric tube resection (TGTR) or partial gastric tube resection (PGTR) has been proposed. However, surgical resection for GTC, being a secondary operation following esophagectomy, may lead to high mortality and morbidity[10,11]. On the other hand, in recent years, endoscopic therapy for early gastric cancer (EGC) has developed and become widespread[12]. Endoscopic submucosal dissection (ESD) enables the treatment of large lesions with a higher rate of *en bloc* resection that cannot be achieved by using conventional endoscopic mucosal resection. In addition, ESD is less invasive than surgery. For this reason, ESD has become widely used as a standard treatment for EGC, and ESD is often performed for GTC.

However, ESD for GTC after esophagectomy is a technically difficult procedure compared with that for an unresected stomach because of the limited working space, unusual fluid-pooling area, food residue, bile reflux, fibrosis, and staples under the suture line[13]. Therefore, a high degree of skill is required for ESD of GTC. There are few reports about ESD for GTC after esophagectomy, and most are case reports and case series of a small number of patients[13-17]. A study by Nonaka *et al*[18] reported the effectiveness and safety of ESD for GTC in a high-volume national center, which had largest number of cases but was nonetheless a single-center study. Therefore, the aim of this study was to evaluate the efficacy and safety of ESD for GTC after esophagectomy in a multicenter context.

MATERIALS AND METHODS

Patients

We retrospectively investigated patients with GTC in the reconstructed gastric tube after esophagectomy for esophageal squamous cell carcinoma who had undergone ESD between January 2005 and December 2019 at 8 institutions participating in the Okayama Gut Study group (O-GUTS). All of the participating institutions in O-GUTS, except Okayama University Hospital (OUH), were considered core hospitals in each area. During the study period, 48 GTC lesions in 38 consecutive patients were treated. The clinical indications of ESD for EGC were based on the Gastric Cancer Treatment Guidelines[19]. These indications were similarly applied for GTC after esophagectomy with gastric tube reconstruction.

Study measurements were as follows: patient characteristics, endoscopic findings, treatment results, adverse events, histopathological results, and clinical courses. In addition, we defined OUH as a high-volume center and compared the patients' background and clinical outcomes between OUH and other facilities.

The institutional review board of each hospital approved this study, and informed consent was obtained from all patients.

Endoscopic procedures

All endoscopic procedures were performed by experts in ESD who had experience with more than 500 clinical cases. There were no restrictions on the scopes and devices used by each endoscopist for ESD. The scopes used were GIF-Q260J or GIF-H260 (Olympus, Tokyo, Japan), and the devices were an insulation-tipped diathermic knife (IT Knife), IT Knife 2, IT Knife nano, or Dual Knife J (Olympus, Tokyo, Japan). Other devices, such as an argon plasma coagulation probe (ERBE, Tubingen, Germany) for marking dots or a needle knife (ZEON MEDICAL, Tokyo, Japan) for the initial incision, were occasionally used.

First, marking dots for the incision lines were placed around the lesion. Next, fructose-added glycerol (Glyceol; TAIYO Pharma CO, Tokyo, Japan) with a minute amount of indigo carmine dye was injected into the submucosal layer. In some cases, 0.4% sodium hyaluronate (MucoUp; Boston Scientific, Tokyo, Japan) was used. After submucosal injection, a precut was made with the Dual Knife J or needle knife, followed by a circumferential mucosal incision around the lesion using the dots as a

landmark and submucosal dissection with the IT Knife, IT Knife 2, IT Knife nano, or Dual Knife J. The resected specimens were evaluated pathologically.

Histopathological assessment of curability

ESD specimens were evaluated in 2-mm slices according to the Japanese Classification of Gastric Carcinoma with curability assessments divided into curative and non-curative resection based on the Gastric Cancer Treatment Guidelines[20]. R0 resection indicated that the lesion was resected *en bloc* with both the horizontal and vertical margins tumor-free histopathologically, but did not include findings regarding lymphovascular infiltration, the type of adenocarcinoma, or an assessment of the depth of invasion for curability. A curative resection was divided into eCura A and eCura B. A non-curative resection was defined as not meeting the criteria of curative resection and was further separated into 2 groups, eCura C-1 and eCura C-2, based on histopathological results per the Gastric Cancer Treatment Guidelines[19].

Statistical analysis

Continuous and categorical variables are expressed as median (range) and *n* (%), respectively. Overall survival was calculated according to the Kaplan-Meier method. Differences in the clinical outcomes of ESD for GTC between institutions were evaluated using the Mann-Whitney *U* test for continuous data and the Chi-squared test for categorical variables. The risk factors for long procedure time were evaluated using logistic regression analysis. All statistical analyses were performed using the statistical analysis software JMP Pro, version 15 (SAS Institute Inc., Cary, NC, United States). *P* values < 0.05 were considered statistically significant.

RESULTS

Patients' characteristics and endoscopic findings

A total of 38 consecutive patients with 48 GTC lesions were treated with ESD between January 2005 and December 2019 (Table 1). The median age of these patients was 71.5 years (range, 57-84 years), and they included 34 men and 4 women. The median period from esophagectomy to the treatment of GTC was 2106 d (range, 38-9523 d). This included patients who had a diagnosis of EGC before surgery for esophageal cancer and had undergone ESD after esophagectomy (5 patients). The reconstruction routes were antethoracic, retrosternal, and posterior mediastinal in 7, 11, and 20 patients, respectively. The location of the GTC lesion was upper, middle, and low in 2, 18, and 28 patients, respectively. Regarding the macroscopic type, there were 21 lesions of 0-IIa, 22 lesions of 0-IIc, 2 lesions of 0-IIb, 1 lesion of 0-III, and 2 combined lesions. The median observation period after ESD was 884 d (range, 8-4040 d).

Treatment results of ESD and histopathological findings

Treatment results of ESD for GTC after esophagectomy and pathological findings are shown in Table 2. The median procedure time was 81 min (range, 29-334 min). *En bloc* resection was performed in 44 of 48 lesions (91.7%). The median tumor size of the resected specimen was 15 mm (range, 4-60 mm). Among the 48 lesions, 43 were differentiated (90%) and 5 were undifferentiated (10%). Regarding the tumor depth, 40 lesions were intramucosal carcinoma (M, 84%), 4 were submucosal superficial carcinoma (SM1, 8%), and 4 were submucosal deep invasive carcinoma (SM2 or deeper, 8%). Ulcerative findings were seen in 6 lesions (13%). Lymphatic infiltration was seen in 3 lesions (6%), and vascular infiltration was seen in 1 lesion (2%). According to the Japanese Gastric Cancer Treatment Guidelines, 38 lesions (79%) achieved curative resection (eCura A) and 10 lesions (21%) were classified as non-curative resection. The reasons for non-curative resection were as follows: 3 lesions were horizontal margin positive (HM1) or cutting into the lesion (eCura C-1), 2 were undifferentiated and showed SM invasion, 2 showed lymphatic infiltration, 2 showed SM invasion with ulcerative findings, and 1 was undifferentiated and showed SM invasion and lymphatic and vascular infiltration (eCura C-2).

Adverse events

Complications during ESD were seen in 2 cases (4%), with 1 case of perforation, and 1 case of bleeding. Complications after ESD were seen in 5 cases (10%), with 2 cases of bleeding, 1 case of subcutaneous abscess, 1 case of liver failure, and 1 case of respiratory failure (Table 2).

Table 1 Patients' characteristics and endoscopic findings

Characteristics	
Patients/lesions, *n*	38/48
Median age, yr (range)	71.5 (57-84)
Sex, *n* (%)	
Male	34 (89)
Female	4 (11)
Median period from esophagectomy to ESD for GTC, d (range)	2106 (38-9523)
Reconstruction route of gastric tube, *n* (%)	
Antethoracic	7 (18)
Retrosternal	11 (29)
Posterior mediastinal	20 (53)
Median observation period after ESD, d (range)	884 (8-4040)
Location of lesion, *n* (%)	
U	2 (4)
M	18 (38)
L	28 (58)
Macroscopic type, *n* (%)	
0-IIa	21 (44)
0-IIb	2 (4)
0-IIc	22 (46)
0-III	1 (2)
Combined	2 (4)

ESD: Endoscopic submucosal dissection; GTC: Gastric tube cancer; U: Upper; M: Medium; L: Lower.

It was the same patient who had perforation during ESD and who formed subcutaneous abscess after ESD (Figure 1). In this case, perforation during ESD was sealed immediately with endoclips. Nevertheless, 20 d after ESD, the patient was admitted to the hospital with redness of the skin in the precordial area and excretion of pus from the skin. Computed tomography showed formation of a subcutaneous abscess around the gastric tube of the antethoracic reconstruction route. The patient was treated conservatively with antibiotics and percutaneous drainage and was discharged on the 16th day after the start of re-admission.

Patients' clinical courses

Of the 38 cases, 2 had local recurrence and 3 had metachronous recurrence. In the 2 cases with local recurrence, 1 received additional surgery and the other received additional ESD. In the 3 cases with metachronous recurrence, 1 received additional surgery and the others received additional ESD. The patients' overall survival curve is shown in Figure 2. The survival rate at 5 years was 59.5%. During the observation period after ESD, no patient died of GTC. However, 10 patients died of other diseases, including pneumonia, which was the most common and occurred in 4 patients, heart failure and hepatocellular carcinoma in 1 patient each, and other unknown diseases.

Comparison of clinical outcomes

A comparison of the patients' background and clinical outcomes between OUH and other hospitals is shown in Table 3. In terms of the patients' backgrounds, the posterior mediastinal route was used as a reconstruction route in more cases at other hospitals. Treatment results were generally similar in both groups; however, procedure time was significantly longer at other hospitals.

Since there were differences in procedure time between institutions, we divided

Table 2 Treatment results of endoscopic submucosal dissection for gastric tube cancer and histopathological findings

Lesions, *n*	48
Median procedure time, min (range)	81 (29-334)
En bloc resection, *n* (%)	44 (91.7)
Adverse events during ESD, *n* (%)	
Bleeding	1(2)
Perforation	1(2)
Adverse events post ESD, *n* (%)	
Bleeding	2 (4)
Subcutaneous abscess	1 (2)
Liver failure	1 (2)
Respiratory failure	1 (2)
Median tumor size, mm (range)	15 (4-60)
Histrogical type, *n*	
Differentiated	43
Undifferentiated	5
Tumor depth, *n*	
M	40
SM1	4
SM2	4
Ulcerative findings, *n*	
Absent	41
Present	7
Lymphatic infiltration, *n*	
Negative	3
Positive	45
Lymphatic infiltration, *n*	
Negative	1
Positive	47
Horizontal margin, *n*	
Negative	45
Positive	3
Vertical margin, *n*	
Negative	47
Positive	1
eCura, *n* (%)	
A	38 (79)
C-1	3 (6)
C-2	7 (15)

ESD: Endoscopic submucosal dissection; M: Intramucosal; SM1: Submucosal superficial; SM2: Submucosal deep invasive.

patients into two groups, a short procedure time group (< 90 min) and a long procedure time group (≥ 90 min), and examined the factors affecting the procedure time. In univariate analysis (Table 4), the treatment institution and tumor size showed

Table 3 Comparison of clinical outcomes between Okayama University Hospital and other hospitals

Institution (patients/lesions)	OUH (17/20)	Other hospitals (21/28)	*P* value
Median age, yr (range)	70 (57-83)	73 (58-84)	0.28
Male, *n* (%)	15 (88)	19 (79)	0.72
Reconstruction route of gastric tube, *n*			< 0.01
Antethoracic	7	0	
Retrosternal	7	4	
Posterior mediastinal	3	17	
Median tumor size, mm (range)	18 (8-60)	15 (4-40)	0.21
depth, M/SM, *n*	16/4	24/4	0.6
Ulcerative findings positive, *n* (%)	3 (15)	4 (14)	0.94
Median procedure time, min (range)	50 (20-180)	108 (32-334)	< 0.01
En bloc resection, *n* (%)	19 (95)	25 (89)	0.48
Curative resection (eCura A or B), *n* (%)	17 (85)	21 (75)	0.4
Adverse events during ESD, *n* (%)	1 (5.0)	1 (3.6)	0.8
Adverse events post ESD, *n* (%)	3 (15)	2 (7.1)	0.37

OUH: Okayama University Hospital; ESD: Endoscopic submucosal dissection; M: Intramucosal; SM: Submucosal.

Table 4 Comparison of short (< 90 min) and long (≥ 90 min) procedure time groups

	< 90 min, *n* = 26	≥ 90 min, *n* = 22	*P* value
Okayama University Hospital/other hospitals, *n*	15/11	5/17	0.01
Reconstruction route of gastric tube, *n*			
Antethoracic/retrosternal/posterior mediastinal	6/11/9	3/4/15	0.06
Location of lesion, *n*			
U/L/M	0/8/18	2/10/10	0.08
Median tumor size, mm (range)	13 (4-26)	15 (6-60)	0.06
Tumor depth, *n*			
M/SM	23/3	17/5	0.30
Ulcerative findings positive, *n*	2	5	0.14

U: Upper; M: Medium; L: Lower; M: Intramucosal; SM: Submucosal.

significant differences between the two groups. However, in multivariate analysis (Table 5), tumor size was the only factor associated with a long procedure time.

DISCUSSION

This study was the first multicenter study on ESD for GTC in the reconstructed gastric tube after esophagectomy, and it included the second largest number of patients. According to a systematic review of GTC after esophagectomy, there are two surgical options for the treatment of GTC: PGTR or TGTR plus lymphadenectomy with colon or jejunal reconstruction[21]. However, surgical treatment for GTC is highly invasive and carries a certain degree of risk. Sugiura *et al*[10] reported that 5 of 7 cases of TGTR had surgical complications (leakage) and 2 died. In addition, 1 of 3 cases of PTGR had fatal complications. Akita *et al*[11] reported that 1 of 5 cases of TGTR died of postoperative complications. On the other hand, in previous studies on ESD for GTC,

Table 5 Multivariate analysis about risk factors for a long procedure time of endoscopic submucosal dissection for gastric tube cancer

	Risk ratio (95%CI)	*P* value
Other hospitals	3.18 (0.59-19.6)	NS
Posterior mediastinal route	3.18 (0.61-19.4)	NS
Location of lesion, U/M	2.12 (0.52-8.84)	NS
Median tumor size ≥ 20 mm	4.90 (1.09-29.6)	0.04

U: Upper; M: Medium; CI: Confidence interval.

the proportions of R0 resection and curative resection were 87.5%-92% and 65%-85%, respectively, and complications were seen in 12.5%-18% of patients[13-18]. In the present study, the proportions of R0 resection and curative resection were 91.7% and 79%, respectively, and complications were seen in 10%. Overall, the treatment results of ESD for GTC in this study were similar to those of previous studies. In a previous study on gastric ESD of the unresected stomach, the proportions of R0 resection, curative resections, and complications were 92%-94.9%, 80.4%-94.7%, and 5.9%-6.3%, respectively[22,23]. Furthermore, in gastric ESD of the remnant stomach after gastrectomy, the proportions of R0 resection, curative resection, and complications were 84.7%-85%, 70.9%-78%, and 2.8%-21.1%, respectively[24,25]. ESD for GTC was considered a minimally invasive, effective, and relatively safe treatment.

There are some points of note in GTC. First, detection of early GTC requires long-term regular endoscopic surveillance after esophagectomy. GTC is often found long after esophagectomy; in some cases, GTC is detected after more than 10 years and the risk of metachronous GTC is high[15-18]. Second, GTC may be difficult to diagnose. The reasons are as follows: Food residue and bile reflux are often seen in the gastric tube, and the lumen of the gastric tube is long and narrow and can constrain endoscopic observation[13]. Therefore, it is necessary to pay attention to these points during endoscopy for patients with gastric tube reconstruction after esophagectomy. Third, when performing ESD for GTC, it is necessary to pay attention to complications specific to GTC. For example, in our study, a subcutaneous abscess formed after treatment in a case with perforation during ESD for GTC in the antethoracic reconstruction route. This case was cured by conservative treatment with antibiotics and percutaneous drainage. Moreover, Miyagi *et al*[26] reported that post-treatment precordial skin burns occurred in 5 of 8 patients with GTC in the antethoracic reconstruction route. In this report, all burns were diagnosed as first-degree burns based on the clinical classification of burn depth, developed on postoperative day 1-2, and took 4-7 d to heal.

In this study, since approximately half of the patients were treated at OUH, we defined it as a high-volume center and compared clinical outcomes with those of other institutions. As a result, there were no significant differences in the clinical outcomes of ESD between institutions. In addition, lesion size was the only factor related to long procedure time in multivariate analysis. We believe these results were attributable to the fact that all of the participating institutions specialized in gastrointestinal diseases with more than 500 cases of ESD for EGC. Moreover, ESD for GTC may have been performed by leading specialists given the relative rarity of GTC. For these reasons, ESD for GTC seems safe if performed by specialists with sufficient ESD experience.

Previously, not a few patients had complications or died of other diseases during the course after esophagectomy[27,28]. However, due to the widespread use of minimally invasive esophagectomy, such as thoracoscopic and laparoscopic surgery, the incidence of postoperative complications, including respiratory complications, has decreased and the general condition of patients after esophagectomy has improved in recent years[29-32]. With continued improvements in the prognosis of esophageal cancer, the number of cases of GTC after esophagectomy will likely increase in the future and the demand for ESD for GTC is expected to increase further.

There were several limitations to this study. First, as this was a retrospective study, the ESD indications and devices used for treatment were not standardized. However, treatment was performed according to the typical standards. Second, since data on *Helicobacter pylori* infection status were missing in some patients, the association between GTC and *Helicobacter pylori* could not be evaluated. Third, as some patients were observed for only a short time, the assessment of long-term prognosis after ESD for GTC was insufficient. Further follow-up studies are needed in the future.

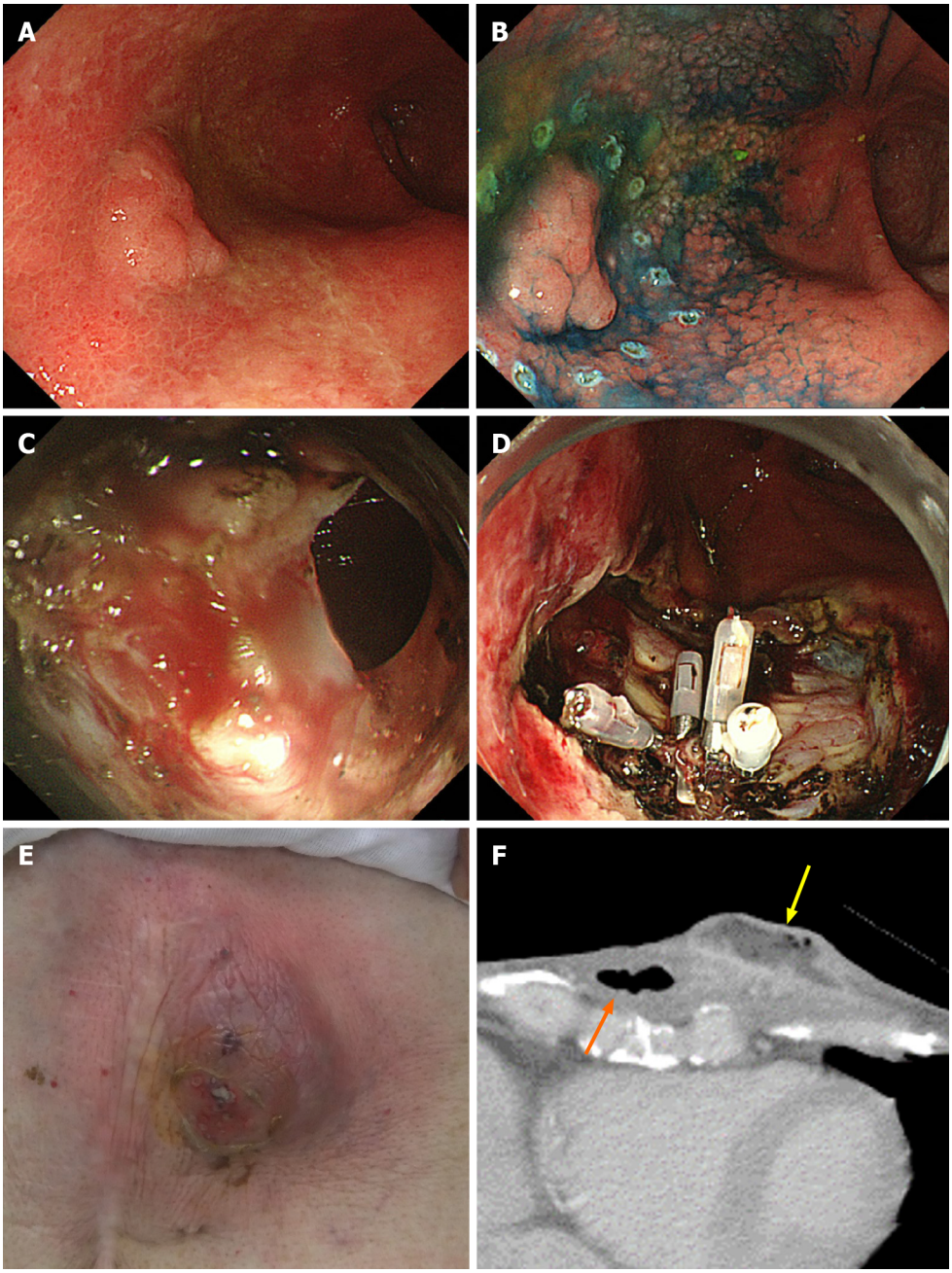


Figure 1 A case of subcutaneous abscess formation after perforation during endoscopic submucosal dissection for gastric tube cancer.
A: Gastric tube cancer located at the anterior wall of gastric body; B: Marking dots were placed around the lesion, and endoscopic submucosal dissection (ESD) was performed as usual; C: Perforation occurred during ESD; D: Perforation was sealed immediately with 4 endoclips; E: Redness of the skin in the precordial area, 20 d after ESD; F: Computed tomography performed 20 d after ESD. A subcutaneous abscess (yellow arrow) had formed around the gastric tube of the antethoracic reconstruction route (orange arrow).

CONCLUSION

In conclusion, ESD for GTC after esophagectomy is a safe and effective treatment that can be performed without significant variability in treatment results at any specialized institution where standard gastric ESD can be performed with sufficient expertise. Further accumulation and follow-up of cases of GTC are necessary in the future.

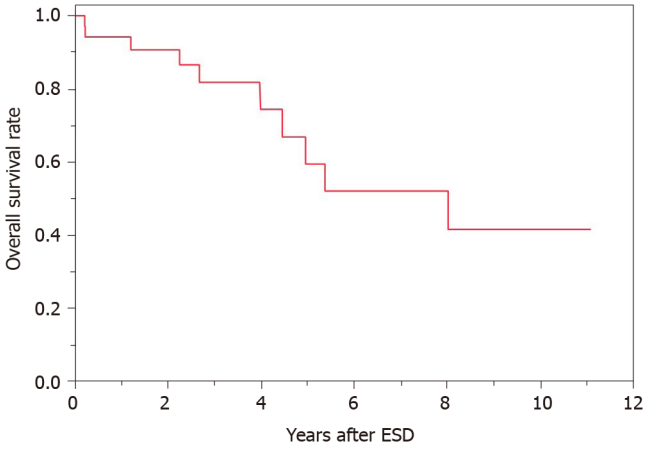


Figure 2 Overall survival curve after endoscopic submucosal dissection for gastric tube cancer. The survival rate at 5 year was 59.5%. ESD: Endoscopic submucosal dissection.

ARTICLE HIGHLIGHTS

Research background

Recent improvements in the prognosis of patients with esophageal cancer have led to the increased occurrence of gastric tube cancer (GTC) in the reconstructed gastric tube.

Research motivation

There are few reports on the treatment results of endoscopic submucosal dissection (ESD) for GTC.

Research objectives

This retrospective study aimed to evaluate the efficacy and safety of ESD for GTC after esophagectomy in a multicenter trial.

Research methods

We retrospectively investigated 48 GTC lesions in 38 consecutive patients with GTC in the reconstructed gastric tube after esophagectomy who had undergone ESD between January 2005 and December 2019 at 8 institutions participating in the Okayama Gut Study group. Patient characteristics, treatment results, clinical course, and treatment outcomes were analyzed.

Research results

The median age of patients was 71.5 years (range, 57-84years), and there were 34 men and 4 women. The median observation period after ESD was 884 d (range, 8-4040 d). The median procedure time was 81 min (range, 29-334 min), the *en bloc* resection rate was 91.7% (44/48), and the curative resection rate was 79% (38/48). Complications during ESD were seen in 4% (2/48) of case, and those after ESD were seen in 10% (5/48) of case. The survival rate at 5 years was 59.5%. During the observation period after ESD, 10 patients died of other diseases. Although there were differences in the procedure time between institutions, a multivariate analysis showed that tumor size was the only factor associated with prolonged procedure time.

Research conclusions

ESD for GTC after esophagectomy was shown to be safe and effective.

Research perspectives

As some patients were observed for only a short time, the assessment of long-term prognosis after ESD for GTC was insufficient. Further accumulation and follow-up of cases of GTC are necessary in the future.

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ORIGINAL ARTICLE

Retrospective Study

Study on the characteristics of intestinal motility of constipation in patients with Parkinson's disease

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Author contributions: Zhang M designed the research and wrote the paper; Yang S and Li XC performed the research; Li BY and Peng D analyzed the data; Jia TX and Tian C collected the data; all authors approved the final version.

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Informed consent statement: The research subjects of this survey have fully understood and agreed to the research content.

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Abstract

BACKGROUND

Constipation is one of the most important nonmotor symptoms in Parkinson's disease (PD) patients, and constipation of different severities is closely related to the pathogenesis of PD. PD with constipation (PDC) is considered a unique type of constipation, but its mechanism of formation and factors affecting its severity have been less reported. Understanding the gastrointestinal motility characteristics and constipation classification of PDC patients is essential to guide the treatment of PDC. In this study, the colonic transit test and high-resolution anorectal manometry were used to identify the intestinal motility of PDC to provide a basis for the treatment of PDC.

AIM

To investigate the clinical classification of PDC, to clarify its characteristics of colonic motility and rectal anal canal pressure, and to provide a basis for further research on the pathogenesis of PDC.

METHODS

Twenty PDC patients and 20 patients with functional constipation (FC) who were treated at Xuanwu Hospital of Capital Medical University from August 6, 2018 to December 2, 2019 were included. A colonic transit test and high-resolution anorectal manometry were performed to compare the differences in colonic transit time, rectal anal canal pressure, and constipation classification between the two groups.

RESULTS

There were no statistically significant differences in sex, age, body mass index, or

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duration of constipation between the two groups. It was found that more patients in the PDC group exhibited difficulty in defecating than in the FC group, and the difference was statistically significant. The rectal resting pressure, anal sphincter resting pressure, intrarectal pressure, and anal relaxation rate in the PDC group were significantly lower than those in the FC group. The proportion of paradoxical contractions in the PDC group was significantly higher than that in the FC group. There was a statistically significant difference in the type composition ratio of defecatory disorders between the two groups ($P < 0.05$). The left colonic transit time, rectosigmoid colonic transit time (RSCTT), and total colonic transit time were prolonged in PDC and FC patients compared to normal values. The patients with FC had a significantly longer right colonic transit time and a significantly shorter RSCTT than patients with PDC ($P < 0.05$). Mixed constipation predominated in PDC patients and FC patients, and no significant difference was observed.

CONCLUSION

Patients with PDC and FC have severe functional dysmotility of the colon and rectum, but there are certain differences in segmental colonic transit time and rectal anal canal pressure between the two groups.

Key Words: Parkinson's disease; Parkinson's disease with constipation; Colonic transit time; High-resolution anorectal manometry

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Core Tip: In this study, we used the colonic transit test and rectal anal manometry to subtype constipation and detect corresponding indicators in patients with Parkinson's disease with constipation (PDC) and functional constipation, with the aim of clarifying the colonic and rectal motility characteristics of PDC and providing a basis for the treatment of PDC. It was found that the segmental colonic transit time and the constituent ratio of types of defecation disorders were statistically different between the two groups.

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INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disease, with a prevalence of 1% in the elderly and about 10% in patients before the age of 50[1]. The pathological signs of PD are abnormal deposition of α-synuclein and progressive degeneration and loss of dopamine neurons in the substantia nigra compacta[2]. The main clinical manifestations are resting tremor, myotonia, bradykinesia, and abnormal postural gait. Over the past 15 years, non-motor symptoms (NMS), mainly autonomic dysfunction, have attracted much attention in patients with PD, including gastrointestinal dysfunction (dysphagia, delayed gastric emptying, and constipation), hypo olfaction, and sleep disorders, which are caused by neuronal loss in other areas of the brain[3]. Among them, constipation is one of the most common NMS in patients with PD. Studies have shown that 20%-89% of PD patients experience constipation, and the incidence gradually increases with the progression of the disease[4]. Compared with non-PD subjects, the frequency of constipation in PD patients increased by 2 to 4 times[5], and the degree of constipation was positively correlated with PD. Autopsy of constipated patients revealed a significant decrease in neuronal density in the substantia nigra, which also provided pathological evidence that constipation increased the risk of PD. Constipation can occur more than 10 years earlier than motor symptoms, making it one of the earliest indicators in the process of PD formation;

recently, constipation has been included in the predecessor diagnostic criteria for PD[6]. PD with constipation (PDC) is considered a unique type of constipation, but its mechanism of formation and factors affecting its severity have been less reported, and the conclusions are not uniform[7]. It is likely that there is a causal relationship between PD and constipation, such that they exacerbate one another and form a vicious cycle. To stop constipation, this cycle may be terminated to prevent or delay the occurrence of PD. However, PDC treatment is very difficult and prone to drug resistance[8,9]. Compared with functional constipation (FC), it is not clear whether the clinical characteristics and influencing factors of PDC are unique. Understanding the gastrointestinal motility characteristics and constipation classification of PDC patients is essential to guide the treatment of PDC.

MATERIALS AND METHODS

Patient recruitment

A total of 20 patients with PDC and 20 patients with FC who visited Xuanwu Hospital of Capital Medical University between August 6, 2018 and December 2, 2019 were recruited as test and control groups, respectively. All patients underwent a colonic transit test (CTT) and high-resolution anorectal manometry (HRAM) to compare the differences in colonic transit time, rectal anal canal pressure, and constipation classification between the two groups. Inclusion criteria were: (1) Age older than 18 years; (2) Fulfilled the diagnostic criteria for PD and Rome IV functional constipation; and (3) Symptom onset of more than 6 mo. Exclusion criteria were: (1) Presence of chronic diseases such as hypertension, diabetes mellitus, and coronary heart disease; (2) Organic diseases of the colon; (3) Diseases that may affect gastrointestinal motility function, such as thyroid disease, renal dysfunction, connective tissue disease, mental disorders, *etc*.; (4) History of abdominal surgery; and (5) Taking drugs that affect gastrointestinal motility in the last 2 wk.

Colon functional assessment

HRAM: The ManoScan™ High Resolution Anorectal Manometry System (given, United States) was used for all patients to measure rectal resting pressure, anal sphincter resting pressure, rectal maximum squeeze pressure, anal canal maximum squeeze pressure, intrarectal pressure, anal relaxation rate, residual anal pressure, and rectoanal pressure difference. According to the results, conditions can be divided into either defecatory impetus deficiency, or pelvic floor muscle synergism disorders.

CTT: Radiopaque markers (Sitzmarks, Konsyl Pharmaceuticals, United States) were used for detection. Each capsule contained 24 radiopaque markers, measuring 1 mm × 4.5 mm, of three different shapes: The O-Ring shape, the Double D shape, and the Tri-chamber shape. On day 1, subjects took one capsule containing the Double D shape under supervision, and swallowed the second capsule containing the O-Ring shape and the third capsule containing the Tri-chamber shape after 24 h and 48 h, respectively. After 72 h, a kidney, ureter, and bladder examination of the abdomen was performed. If the transverse colon could not be fully displayed, an X-ray of the upper abdomen was taken. The transit time of the whole colon and segmented colon was then calculated according to Metcalfe's method[10] (Figure 1).

Statistical analysis

IBM SPSS 22.0 was used to analyze all data. Continuous variables were analyzed using a *t*-test (normally distributed) and expressed as mean ± SD. Data with a non-normal distribution were expressed as percentiles, and the Kruskal-Wallis test was used to compare groups. Categorical data were calculated using the Chi-square test and expressed as a ratio (%). A $P < 0.05$ was considered statistically significant.

RESULTS

Twenty patients with PDC and 20 patients with FC were included in the study, and there were no statistically significant differences in sex, age, body mass index (BMI), or duration of constipation between the two groups. The chief complaints of constipation across the two groups were compared, and it was found that more patients in the PDC group exhibited difficulty in defecating than in the FC group, and the difference was

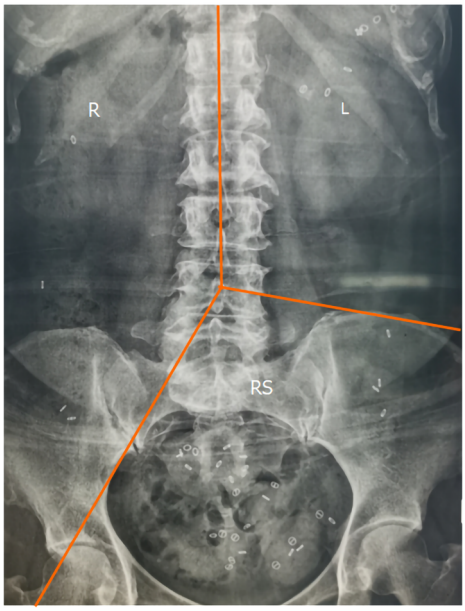


Figure 1 A line is made from the thoracic spinous process to the fifth lumbar spinous process; tangent lines are then made from the fifth lumbar spinous process to both sides of the pelvic outlet. The dashed lines from the fifth lumbar spine to the right pelvic outlet and the left ilium are distinguished as projections into the right colon, left colon, and rectosigmoid colon. R: Right; L: Left; RS: Rectosigmoid colon.

statistically significant (Table 1).

Comparison of HRAM results

The rectal resting pressure and anal sphincter resting pressure in the PDC group were significantly lower than those in the FC group. The intrarectal pressure and anal relaxation rate in the PDC group were significantly lower than those in the FC group. The proportion of paradoxical contractions in the PDC group was significantly higher than that in the FC group. These results are shown in Table 2. The study found that the proportion of defecatory impetus deficiency was 50% (10/20) in the PDC group and 40% (8/20) in the FC group; the proportion of pelvic floor muscle dyssynergia was 35% (7/20) in the PDC group and 20% (4/20) in the FC group. In addition, 10% (2/20) of patients in the PDC group demonstrated inadequate defecatory propulsion combined with pelvic floor muscle dyssynergia. Rectal anal manometry was normal in 40% (8/20) of the FC group and in only 5% (1/20) of the PDC group, with a statistically significant difference in the composition type ratio of defecatory disorders between the two groups ($P < 0.05$) (Table 3).

Comparison of colonic transit time between the two groups

Left colonic transit time (LCTT), rectosigmoid colonic transit time (RSCTT), and total colonic transit time (TCTT) were prolonged in PDC and FC patients when compared with normal values. A comparison of the segmented colonic transit time between the two groups revealed that the patients with FC had a significantly longer right colonic transit time (RCTT) and a significantly shorter RSCTT than the patients with PDC ($P < 0.05$), whereas there were no significant differences in the LCTT and TCTT between the two groups (Table 4).

Comparison of constipation classification between the two groups

Patients with PDC were divided into three subtypes based on CTT and HRAM: (1) Slow transit constipation: Prolonged CTT only (1, 5%); (2) Defecatory disorder: Altered HRAM only (4, 20.0%); and (3) Mixed constipation: Prolonged CTT and altered HRAM (15, 75.0%). Patients with FC were divided into four subtypes: (1) Slow transit constipation: (7, 35.0%); (2) Defecatory disorder: (2, 10.0%); (3) Mixed constipation: (10, 50.0%); and (4) Normal transit constipation: No functional abnormality (1, 5.0%). There were no statistically significant differences in constipation subtypes between the two groups, as seen in Table 5.

Table 1 Comparison of clinical data between the Parkinson's disease with constipation and functional constipation groups

	FC	PDC	*P* value
Male	7 (35.0%)	12 (60.0%)	0.205
Female	13 (65.0%)	8 (40.0%)	
Age, years	70.80 (6.24)	72.32 (4.80)	0.402
BMI	23.32 (2.47)	22.25 (4.32)	0.343
Decreased defecation frequency	10 (50.0%)	16 (80.0%)	0.097
Dry stools	9 (45.0%)	6 (30.0%)	0.514
Defecatory difficulties	13 (65.0%)	20 (100.0%)	0.013
Manual assisted defecation	7 (35.0%)	12 (60.0%)	0.205
Constipation duration, years	10.00 (5.00, 20.00)	5.00 (2.75, 6.50)	0.051

PDC: Parkinson's disease with constipation; FC: Functional constipation; BMI: Body mass index.

Table 2 Comparison of high-resolution anorectal manometry results between the Parkinson's disease with constipation and functional constipation groups

	FC	PDC	*P* value
Rectal resting pressure (mmHg)	82.36 (20.18)	63.98 (30.94)	0.032
Anal sphincter resting pressure (mmHg)	89.55 (16.25)	66.50 (19.35)	< 0.001
Rectal maximum squeeze pressure (mmHg)	189.50 (159.95,250.68)	188.95 (139.60,234.00)	0.516
Anal canal maximum squeeze pressure (mmHg)	222.16 (86.26)	195.25 (63.62)	0.269
Intrarectal pressure (mmHg)	43.08 (20.74)	21.73 (14.61)	0.001
Anal relaxation rate (%)	23.00 (16.00, 42.58)	0.16 (-0.07, 0.22)	< 0.001
Residual anal pressure (mmHg)	75.83 (33.55)	69.70 (30.92)	0.551
Rectoanal pressure difference (mmHg)	-36.77 (36.56)	-47.98 (27.38)	0.279
Paradoxical contraction	1 (5.0%)	8 (40.0%)	0.023

PDC: Parkinson's disease with constipation; FC: Functional constipation.

Table 3 Comparison of the composition type of defecatory disorders between the Parkinson's disease with constipation and functional constipation groups, *n* (%)

	FC	PDC	*P* value
Defecatory impetus deficiency	8 (40.0)	10 (50.0)	0.037
Pelvic floor muscle dyssynergia	4 (20.0)	7 (35.0)	
Inadequate defecatory propulsion combined with pelvic floor muscle dyssynergia	0 (0.0)	2 (10.0)	
Normal	8 (40.0)	1 (5.0)	

PDC: Parkinson's disease with constipation; FC: Functional constipation.

DISCUSSION

Constipation is one of the most important nonmotor symptoms in PD patients, and can occur several years or even decades before the onset of exercise symptoms[11,12]. Taiwanese scholars[5] conducted a follow-up survey of the relationship between the severity of constipation and the risk of PD over 5 years. The study showed that the incidence rate of PD was 1.57/100000 for those without constipation. The incidence

Table 4 Comparison of colonic transit time between the two groups

	FC	PDC	*P* value
RCTT (h)	10.50 (3.12)	8.40 (3.28)	0.045
LCTT (h)	19.50 (6.07)	20.15 (5.07)	0.715
RSCTT (h)	24.45 (4.56)	27.60 (3.68)	0.021
TCTT (h)	55.10 (7.46)	56.00 (8.31)	0.721

Normal reference values: right colon transit time ≤ 17.4 h; left colon transit time ≤ 16.8 h; rectosigmoid transit time ≤ 22.9 h; total colonic transit time ≤ 39 h for men and ≤ 50 h for women. PDC: Parkinson's disease with constipation; FC: Functional constipation; RCTT: Right colonic transit time; LCTT: Left colonic transit time; RSCTT: Rectosigmoid colonic transit time; TCTT: Total colonic transit time.

Table 5 Comparison of constipation classification between the Parkinson's disease with constipation and functional constipation groups, *n* (%)

	FC	PDC	*P* value
Slow transit constipation	7 (35.0)	1 (5.0)	0.067
Defecatory disorder	2 (10.0)	4 (20.0)	
Mixed constipation	10 (50.0)	15 (75.0)	
Normal transit constipation	1 (5.0)	0 (0.0)	

PDC: Parkinson's disease with constipation; FC: Functional constipation.

rate of PD in patients with mild, moderate, and severe constipation increased 5 years later, to 4.04/100000, 5.28/100000, 12.67/100 000, and 12.67/100000, respectively. Therefore, constipation of different severities is closely related to the pathogenesis of PD. Although constipation may be the first symptom of PD, the incidence rate of constipation is high and is influenced by many factors. It is not specific for predicting PD[13]. Thus, this study compared PDC and FC and analyzed the constipation symptoms of the two groups of patients. At the same time, the colonic transit test and HRAM were used to classify constipation and detect the corresponding indicators, in order to find more sensitive and specific indicators to predict which constipation patients will eventually develop PD and to identify the gastrointestinal motility of PDC. It is expected to provide a basis for the treatment of PDC.

The rectal anal manometry classification in this study identified that the incidence of rectoanal dysfunction in the PDC group was 95%, suggesting that anal dysfunction plays an important role in the formation of PDC. In addition, this study compared the rectal and anal dynamics of patients with PDC and FC and found that the resting rectal and anal canal pressures of the PDC group were significantly lower than those of the FC group. This is mainly derived from the internal anal sphincter tension, accounting for about 70%-85% of resting pressure[14], suggesting that patients with PDC have internal sphincter dysfunction. Some previous[14,15] neuropathological studies have found Lewy bodies in the central nervous system, peripheral autonomic system, and enteric nervous system (ENS) of PD patients. The latter can cause neurodegenerative changes in the ENS. It is speculated that neuropathy may involve the autonomic nerves innervating the internal anal sphincter, causing low resting pressure in the rectum and anal canal. In simulated defecation, the rectal defecation pressure and anal relaxation rate of the PDC group were significantly lower than those of the FC group. Rectal defecation pressure is mainly produced by abdominal pressure. Fontana *et al*[16] has found that the increase in abdominal pressure in patients with PD is significantly lower than that in healthy controls. Fontana *et al*[16] believed that the mechanism of impaired abdominal pressure may be due to increased axial muscle tension and decreased contractility. In this study, patients with PDC had an insufficient rectal defecation driving force, such that the anus could not be effectively relaxed resulting in abnormal contraction. This is considered a local dystonia in patients with PD, where rectal contraction is reduced during defecation and the abdominal tension becomes weak. A coordinated movement disorder results due to reduced rectoanal muscle contraction and puborectal and pelvic smooth muscle

dysfunction. The performance of this dystonia is aggravated during the "close" phase of PD and lessened during the "open" phase[17].

Our results showed that the LCTT, RSCTT, and TCTT were significantly prolonged in PDC patients, and the distribution of colonic transmission time in each segment of PDC patients was not uniform, especially in the left semicolon and rectum sigmoid colon, which was consistent with previous studies[18-20]. However, to our knowledge, no study has compared the segmental colon transmission time between PDC and FC patients. Our results suggest that the RSCTT of PDC patients was significantly longer than that of FC patients while the RCTT was significantly shorter than that of FC patients; this revealed the similarities and differences in the mechanism of slow colon transmission between patients with PDC and those with FC. The common mechanism may be explained as follows: (1) Compared with healthy individuals, vasoactive intestinal peptide (VIP) and VIP receptors in the colon mucosa of PDC and FC patients were downregulated[20]. VIP is a non-adrenergic non-cholinergic neuroinhibitory neurotransmitter, and its reduction could increase colon segment peristalsis, weakening the effective promotion of movement. At the same time, through abnormal colon secretion, VIP could reduce the fecal water content and lead to stool sclerosis, thus prolonging colon transmission time; and (2) ENS degeneration, a decrease in the number of intermuscular plexuses of the colonic wall, and the formation of ganglion cell vacuoles may all cause a decrease in the peristaltic function of the colon. The differences between PDC patients and FC patients are as follows: (1) PD leads to the loss of dopaminergic neurons between intestinal muscles, which may lead to decreased colonic transport function[21]. The ENS is rich in cholinergic neurons, including intestinal intermuscular motor neurons, primary transmission neurons, intermediate neurons, and other neurons projecting to the prevertebral sympathetic ganglion. Dopamine is a reactant of tyrosine hydroxylase in 4%-11% of enteric muscle neurons. Therefore, dopamine plays an important role in regulating gastrointestinal motility. Moreover, the innervation of the digestive tract gradually decreases from proximal to distal, and the motor function reduction caused by the loss of dopaminergic neurons is more serious in the distal colon segment[22]; and (2) PDC patients have more abnormal rectal-anal manometry results than FC patients. Some researchers believe that PD patients may have an independent "pelvic floor cooperative motion disorder" which is an element of extrapyramidal disease and a sequelae of neurodegeneration. These are also the reasons why the RSCTT of those with PDC was significantly longer than that of FC patients in this study.

In this study, mixed constipation was dominant in both PDC patients and FC patients, suggesting that both groups of patients had severe functional dyspraxia in the colon and rectum. There was no statistical difference between the two groups. According to the results of HRAM, the rectal sensorimotor dysfunction in patients with PDC was mainly caused by insufficient bowel movement impetus, which may in turn be caused by autonomic neuropathy innervating the rectum. A small number of patients with pelvic floor muscle synergy disorder may also have pelvic floor muscle dystonia caused by PD.

This study has several limitations. The overall sample size of this study was small. In addition, a sample of healthy individuals were not included in the study. Increasing the sample size of patients with PD and including healthy subjects will help us to better understand the pathophysiological mechanism of PDC.

In conclusion, cases of PDC and FC were associated with a prolonged CTT and abnormal HRAM. However, the rectal resting pressure, anal sphincter resting pressure, intrarectal pressure, and anal relaxation rate in the PDC group were significantly lower than those in the FC group. The proportion of paradoxical contractions in the PDC group was significantly higher than that in the FC group. The different segments of the CTT were also significantly different. The RSCTT of PDC patients was significantly longer than that of FC patients, and the RCTT was significantly shorter. The above indexes can be helpful for further studies of the mechanism of PDC and FC and for early diagnosis and treatment of patients with PD.

CONCLUSION

Patients with PDC and FC have severe functional dysmotility of the colon and rectum, but there are certain differences in segmental colonic transit time and rectal anal canal pressure between the two groups.

ARTICLE HIGHLIGHTS

Research background

Parkinson's disease (PD) is a common neurodegenerative disease characterized clinically by typical motor symptoms such as tremor, bradykinesia and myotonia and non motor symptoms such as constipation, depression and dysmetria. Constipation is one of the most common clinical manifestations of PD patients. Investigations have shown that the incidence of constipation among PD patients is up to 88%, and constipation is regarded as one of the independent risk factors for PD. PD constipation (PDC) is considered a unique type of constipation that is clinically inadequately treated, severely affecting patient quality of life. Current studies on the characteristics of intestinal motility in patients with Parkinson's disease with constipation are less frequently reported, and the control groups are mostly healthy subjects, and the conclusions are not uniform.

Research motivation

It is likely that there is a causal relationship between PD and constipation, such that they exacerbate one another and form a vicious cycle. However, PDC treatment is very difficult and prone to drug resistance. Compared with functional constipation, it is not clear whether the clinical characteristics and influencing factors of PDC are unique. Understanding the gastrointestinal motility characteristics and constipation classification of PDC patients is essential to guide the treatment of PDC.

Research objectives

To identify the gastrointestinal motility of PDC and provide a basis for its treatment. Moreover, to find more sensitive and specific indicators to predict which constipation patients will eventually develop PD.

Research methods

A colonic transit test and high-resolution anorectal manometry were performed to compare the differences in colonic transit time, rectal anal canal pressure, and constipation classification between Patients with PDC and functional constipation (FC).

Research results

The study found that the rectal resting pressure, anal sphincter resting pressure, intrarectal pressure, and anal relaxation rate in the PDC group were significantly lower than those in the FC group. The proportion of paradoxical contractions in the PDC group was significantly higher than that in the FC group. The different segments of the colonic transit test (CTT) were also significantly different. The rectosigmoid colonic transit time of PDC patients was significantly longer than that of FC patients, and the right colonic transit time was significantly shorter.

Research conclusions

Cases of PDC and FC were associated with a prolonged CTT and abnormal high-resolution anorectal manometry. There are certain differences in segmental colonic transit time, rectal anal canal pressure and composition type ratio of defecatory disorders between the two groups.

Research perspectives

This study can be helpful for further studies of the mechanism of PDC and FC and for early diagnosis and treatment of patients with PD.

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ORIGINAL ARTICLE

Observational Study

Apolipoprotein E polymorphism influences orthotopic liver transplantation outcomes in patients with hepatitis C virus-induced liver cirrhosis

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Abstract

BACKGROUND

Hepatitis C virus (HCV) infection is responsible for a chronic liver inflammation, which may cause end-stage liver disease and hepatocellular carcinoma.

of the manuscript for important intellectual content; and Oriá RB supervised the study.

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Institutional review board statement: All study procedures were in accordance and with the 1964 Helsinki declaration and the study protocol was approved by the Research Ethics Committee of the Federal University of Ceará, protocol No. 2.018.768.

Informed consent statement: All patients were approached by the research team, who explained the study protocol and clarified that failure to participate in the study would not cause discontinuation of care or medical treatment. After that, patients read and signed the informed consent form.

Conflict-of-interest statement: The authors have no conflicts of interest.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE statement, and the manuscript was prepared and revised accordingly.

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Apolipoprotein E (protein: ApoE, gene: *APOE*), a key player in cholesterol metabolism, is mainly synthesized in the liver and *APOE* polymorphisms may influence HCV-induced liver damage.

AIM

To determine whether *APOE* alleles affect outcomes in HCV-infected patients with liver cirrhosis following orthotopic liver transplantation (OLT).

METHODS

This was a cohort study in which 179 patients, both genders and aged 34-70 years, were included before or after (up to 10 years follow-up) OLT. Liver injury severity was assessed using different criteria, including METAVIR and models for end-stage liver disease. *APOE* polymorphisms were analyzed by quantitative real-time polymerase chain reaction.

RESULTS

The *APOE3* allele was the most common (67.3%). In inflammation severity of biopsies from 89 OLT explants and 2 patients in pre-transplant, the degree of severe inflammation (A3F4, 0.0%) was significantly less frequent than in patients with minimal and moderate degree of inflammation (≤ A2F4, 16.2%) $P = 0.048$, in patients carrying the *APOE4* allele when compared to non-*APOE4*. In addition, a significant difference was also found (≤ A2F4, 64.4% *vs* A3F4, 0.0%; $P = 0.043$) and (A1F4, 57.4% *vs* A3F4, 0.0%; $P = 0.024$) in *APOE4* patients when compared to *APOE3* carriers. The fibrosis degree of the liver graft in 8 of 91 patients and the lack of the E4 allele was associated with more moderate fibrosis (F2) ($P = 0.006$).

CONCLUSION

Our results suggest that the E4 allele protects against progression of liver fibrosis and degree of inflammation in HCV-infected patients.

Key Words: Apolipoprotein E; Polymorphism; Liver cirrhosis; Hepatitis C virus; Hepatocellular carcinoma; Liver transplantation

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Core Tip: Hepatitis C virus (HCV) infection is responsible for a chronic liver inflammation, which may cause end-stage liver disease. Apolipoprotein E (protein: ApoE, gene: *APOE*) is key for lipid metabolism. In this study, the *APOE4* allele, which has been associated with increased risk of acquiring late-onset Alzheimer's disease, was found to have a protective effect against the progression of inflammation and/or fibrosis in liver damage induced by HCV pre- and post-liver transplantation. Further studies are needed to unravel the possible contribution of ApoE from the donor liver to this protection.

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INTRODUCTION

The World Health Organization reported in 2015, that around 71 million people had chronic hepatitis C virus (HCV) infection worldwide, with a global prevalence of 1% and that 399,000 died of hepatocellular carcinoma (HCC) or cirrhosis[1].

HCV infection causes a chronic liver inflammatory condition leading to chronic hepatitis[2,3]. The evolution from acute to chronic hepatitis C occurs in up to 80% of cases when HCV infection lasts for more than six months. Without characteristic

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symptoms, HCV evolves slowly, for years, with approximately 20% of those chronically infected progressing to cirrhosis, and between 1% to 5% to HCC [4,5].

Human apolipoprotein E (protein: ApoE, gene: *APOE*) is a 34-KDa glycoprotein of 299 amino acids. ApoE is an important protein constituent of very-low-density lipoproteins (LDL), high-density lipoproteins, and chylomicrons in plasma, and a ligand for the LDL receptor[6]. ApoE is synthesized mainly in the liver (90%), but also in spleen, kidney, lungs, gonads, monocyte-macrophages, and in the nervous system[7,8].

The human *APOE* gene is polymorphic with three common *APOE* alleles on chromosome 19 (19q13), named E2, E3 and E4, giving six possible genotypes: E2/E2, E2/E3, E2/E4, E3/E3, E3/E4 and E4/E4[9]. These alleles form different ApoE isoforms due to amino acid substitutions at positions 112 and 158: E2 = Cys-112/Cys-158; E3 = Cys-112/Arg-158; and E4 = Arg-112/Arg-158[10].

Several studies have documented the association between ApoE isoforms and chronic illnesses such as Alzheimer's disease[11], atherosclerosis[12], herpes simplex virus infection[13] and early childhood diarrhea[12]. The ApoE4 allele has been recently associated with a high risk for severe coronavirus disease 2019 infection, independent of preexisting cardiovascular disease, type-2 diabetes and dementia[14]. In addition, the *APOE3*/3 genotype is implicated in fibrosis progression in HCV-infected patients, likely through mechanisms of competition for viral entry and replication[15].

Although some studies report that *APOE4* exerts a protective effect in HCV-induced liver damage, no studies have investigated the role of *APOE* genotypes in modifying the natural history of HCV-induced liver injury in liver transplanted patients in a Brazilian setting. Our aim, therefore, was to establish whether *APOE4* genotype recipients were associated with more benign HCV-related liver injuries compared to patients with other *APOE* genotypes.

MATERIALS AND METHODS

Study design

This is a cohort study conducted at the University Hospital Walter Cantídio and the Fortaleza General Hospital (HGF). A total of 179 consecutive patients were enrolled from May 2017 to July 2019 for the collection of buccal cells and medical record data. Medical records from liver transplant recipients before May 2017 were also collected retrospectively and prospectively each year. Patients in the orthotopic liver transplantation (OLT) queue, or liver transplanted from May 2017 onwards, had data collected prospectively and annually during the study period.

The study included 179 patients of both genders and aged 34-70 years, with HCV-related end-stage liver disease, 105 of them complicated with HCC and 74 without HCC, in pre- and/or post-OLT. At enrolment, 143 known HCV-infected patients were on antiviral treatment, 126 with direct-acting antivirals and 17 treated with the combination of interferon & ribavirin or pegylated interferon & ribavirin; 36 patients had not received any treatment. The HCV status of all patients was confirmed by identification of serum HCV-RNA and HCV genotypes. Exclusion criteria were: patient refusal, coinfection with HBV or HIV, and HCC associated with metastases.

The study protocol was approved by the Research Ethics Committee of the Federal University of Ceará, Protocol No 2.018.768 and the HGF, Protocol No 2.062.278. The research team explained the study protocol to all patients and clarified that failure to participate in the study would not cause discontinuation of care or medical treatment. Patients then read and signed the informed consent form. All protocols of this study were in accordance with the Helsinki Declaration.

APOE polymorphisms

Of 179 patients, oral cells for DNA extraction were obtained at single time points from 56 (31.3%) pre-OLT patients, and from 123 (68.7%) who underwent liver transplantation. Oral cell DNA was extracted using the Gentra Puregene system (Qiagen, MD, United States)[12].

APOE genotyping was detected by quantitative real-time polymerase chain reaction (qRT-PCR), through enhancement of a fluorescent signal (SYBR® Green) interspersed in the double strand of the amplified DNA[16]. The primers were combined in three PCR amplification mixtures according to Calero *et al*[16] in a Light Cycler® Nano (Roche) with a 32-well RT-PCR system.

Data and statistical analysis

Demographic, clinical and laboratory data were collected after thorough reviews of medical records during the preoperative period (179 patients) and after OLT with follow-up of the 144 transplanted patients over 10 years (Supplementary Figure 1). Data included serological markers such as anti-HCV antibodies to define the agent and quantitative HCV-RNA; severity markers of liver cirrhosis [models for end-stage liver disease (MELD)]; liver imaging (computed tomography, nuclear magnetic resonance, and ultrasonography) for identification of liver tumor and classification according to the Milan criteria[17]. Out of 144 patients who underwent OLT based on Milan classification, 24 (16.7%) were subjected to one or more sessions of chemoembolization to reduce tumor size before Milan criteria were reached.

Staging the degree of liver damage and hepatic fibrosis

Liver damage severity was categorized into different criteria. Less severe cases were identified by Milan scores (single nodule < 5 cm, or up to 3 nodules < 3 cm), METAVIR (≤ A2 and ≤ F2), and MELD < 25. Patients with more severe liver injury were scored according to the Milan expanded criteria of the University of San Francisco[18] (1 nodule ≤ 6.5 cm; ≤ 3 nodules, each ≤ 4.5 cm with total diameter ≤ 8 cm), METAVIR score (A3 and ≥ F3), and MELD > 25. METAVIR scoring was categorized to assess liver inflammation/fibrosis severity (Supplementary Table 1).

Liver fibrosis was assessed by using aminotransferase-platelet ratio index (APRI) and fibrosis-4 (FIB4) scores. APRI = [AST (U/L)/35 (ULN, the upper limit of normal AST is estimated at 35)]/platelets count (10^9/L) × 100 and FIB4 = AST (U/L) × age (years)/[platelets count (10^9/L) × ALT$^{1/2}$ (U/L)]. The cut-off points of severity are: for significant liver fibrosis (APRI ≥ 1.5, METAVIR F3-F4, and FIB4 ≥ 3.25) and for low-degree fibrosis (APRI ≤ 0.5, METAVIR F0-F1, and FIB4 ≤ 1.45)[19].

Liver biopsy data were obtained from liver explants patients in pre-OLT and of post-OLT liver grafts. Liver biopsies were performed post-transplant in 91 of the 144 transplant recipients, who presented positive HCV viral load, or high risk of viral recurrence or rejection. All recipients had a minimum 1-year follow-up period post-OLT, and donated at least one liver biopsy more than 1 year after their OLT.

Data were analyzed with the SPSS statistical 22.0 software. For normality, D'Agostino and Kolmogorov–Smirnov tests were performed. The absolute and relative frequencies were calculated for the categorical variables and mean ± SD for the numerical variables. Fisher's exact test or the Mann–Whitney test was used to compare frequencies or means, respectively, when appropriate. Multilinear regression and correlation analyses were performed to avoid other potential confounders. Either one-way or two-way ANOVA test followed by Bonferroni's or Kruskal-Wallis, and Dunn's test were used for multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

A total of 179 patients (145 males, 34 females) were enrolled in this study with a median age of 61 (range = 34-70). All patients were diagnosed with HCV-induced chronic liver cirrhosis; 105 of them (58.6%) complicated with HCC.

Analysis of pre-OLT data in the overall population

Demographic and clinical data were collected from all patients only in pre-OLT. Their *APOE* allele stratification is reported in Table 1; no statistical difference was found in any comparisons.

The *APOE* allele frequency according to group, HCV viral load and liver inflammation by the METAVIR score are depicted in Table 2. The *APOE* allele frequencies were 67.3%, 17.1% and 15.6% for E3, E2 and E4, respectively. The most frequent genotype was E3/E3 (51.4%).

APOE allele frequencies were associated with liver inflammation based on METAVIR score, assessed in biopsies of liver explants from 89 patients and from 2 patients in pre-OLT. The degree of severe inflammation (A3F4, 0.0%) was significantly less frequent than in patients with minimal and moderate degree of inflammation (≤ A2F4, 16.2%) $P = 0.048$, in patients carrying the *APOE4* allele when compared to non-*APOE4*. In addition, a significant difference was also found regarding METAVIR score (≤ A2F4, 64.4% *vs* A3F4, 0.0%; $P = 0.043$) and (A1F4, 57.4% *vs* A3F4, 0.0%; $P = 0.024$) in *APOE4* patients compared to *APOE3* carriers (Table 3). All patients with advanced liver inflammation (A3) were treated with antivirals only in the post-OLT period.

Among patients with less severe liver disease (MELD ≤ 25), the degree of severe

Table 1 Demographic and clinical profile of all candidate patients for liver transplant according to E2, E3 and E4 allele stratification

Variables	*APOE2*	*APOE3*	*APOE4*	*P* value
Age (yr) (mean ± SD)	60.8 ± 7.26	60.3 ± 7.19	60.3 ± 7.44	0.956
BMI (mean ± SD)	27.6 ± 4.36	26.4 ± 5.01	27.0 ± 4.81	0.167
Gender (n^1 = 179; n^2 = 358), n^2 (%)				
Male (n^1 = 145; n^2 = 290)	52 (17.9)	193 (66.6)	45 (15.5)	0.650
Female (n^1 = 34; n^2 = 68)	9 (13.2)	48 (70.6)	11 (16.2)	
BMI (n^1 = 179; n^2 = 358), n^2 (%)				
Non-obese (n^1 = 137; n^2 = 274)	49 (17.9)	185 (67.5)	40 (14.6)	0.519
Obese (n^1 = 42; n^2 = 84)	12 (14.3)	56 (66.7)	16 (19.0)	
Etiology (n^1 = 179; n^2 = 358), n^2 (%)				
HCV (n^1 = 74; n^2 = 148)	24 (16.2)	96 (64.9)	28 (18.9)	0.357
HCV + HCC (n^1 = 105; n^2= 210)	37 (17.6)	145 (69.0)	28 (13.3)	
HCC Milan (n^1 = 105; n^2= 210), n^2 (%)				
Within the criteria (n^1 = 83; n^2 = 166)	29 (17.5)	115 (69.3)	22 (13.2)	0.885
Outside the criteria (n^1 = 22; n^2 = 44)	8 (18.2)	29 (65.9)	7 (15.9)	

Of 105 patients complicated with hepatocellular carcinoma. n^1: Number of subjects; n^2: Number of alleles; *APOE*: Apolipoprotein E gene; BMI: Body mass index; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

Table 2 Genotypic and allele distribution of apolipoprotein E according to group, hepatitis C virus serology/viral load and severity of liver inflammation by the METAVIR score of the total population

APOE genotype		Group		HCV-RNA		METAVIR		METAVIR	
	All	HCV	HCV + HCC	Positive	Negative	≤ A2F4	A3F4	A1F4	A3F4
	n^1 (179)	n^1 (74)	n^1 (105)	n^1 (33)	n^1 (146)	n^1 (80)	n^1 (11)	n^1 (27)	n^1 (11)
E2/E2	15 (8.4)	6 (8.1)	9 (8.6)	7 (21.2)	8 (5.5)	9 (11.2)	1 (9.1)	4 (14.8)	1 (9.1)
E2/E3	25 (14.0)	9 (12.2)	16 (15.2)	3 (9.1)	22 (15.1)	11 (13.8)	2 (18.2)	4 (14.8)	2 (18.2)
E2/E4	6 (3.3)	3 (4.1)	3 (2.9)	0 (0.0)	6 (4.1)	2 (2.5)	0 (0.0)	1 (3.7)	0 (0.0)
E3/E3	92 (51.4)	36 (48.6)	56 (53.3)	16 (48.5)	76 (52.0)	39 (48.8)	8 (72.7)	12 (44.5)	8 (72.7)
E3/E4	32 (17.9)	15 (20.3)	17 (16.2)	5 (15.1)	27 (18.5)	14 (17.5)	0 (0.0)	3 (11.1)	0 (0.0)
E4/E4	9 (5.0)	5 (6.7)	4 (3.8)	2 (6.1)	7 (4.8)	5 (6.2)	0 (0.0)	3 (11.1)	0 (0.0)
***APOE* alleles**	n^2 (%)	n^2 (%)	n^2 (%)	n^2 (%)	n^2 (%)	n^2 (%)	n^2 (%)	n^2 (%)	n^2 (%)
E2	61 (17.1)	24 (16.2)	37 (17.6)	17 (25.8)	44 (15.1)	31 (19.4)	4 (18.2)	13 (24.1)	4 (18.2)
E3	241 (67.3)	96 (64.9)	145 (69.1)	40 (60.6)	201 (68.8)	103 (64.4)	18 (81.8)	31 (57.4)	18 (81.8)
E4	56 (15.6)	28 (18.9)	28 (13.3)	9 (13.6)	47 (16.1)	26 (16.2)	0(0.0)	10 (18.5)	0 (0.0)
All	358	148	210	66	292	160	22	54	22

METAVIR score of biopsies liver explants from 89 patients and from 2 patients in pre-transplant. n^1: Number of subjects; n^2: Number of alleles; *APOE*: Apolipoprotein E gene; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

inflammation (A3F4, 0.0%) was significantly less frequent in *APOE4* carriers when compared to non-*APOE4* patients with minimal and moderate degree of inflammation (≤ A2F4, 15.7%, P = 0.046), and with minimal degree of inflammation (A1F4, 18.2%, P = 0.044). These results were also significant in *APOE4* patients when compared to *APOE3*, as categorized by their METAVIR scores [≤ A2F4, 65.7% *vs* A3F4, 0.0% (P = 0.042) and A1F4, 61.4% *vs* A3F4, 0.0% (P = 0.040)] (Table 4).

Logistic regression model predicting moderate and severe (A2A3) *vs* minimal (A1)

Table 3 METAVIR scores for liver inflammation in pre-orthotopic liver transplantation patients according to E2, E3 and E4 alleles stratification

METAVIR score	*APOE2*			*APOE3*			*APOE4*		
	Yes	No		Yes	No		Yes	No	
	n^2 (%)	n^2 (%)	*P* value	n^2 (%)	n^2 (%)	*P value*	n^2 (%)	n^2 (%)	*P* value
METAVIR (n^1 = 38; n^2 = 76)									
A1F4 (n^1 = 27; n^2 = 54)	13 (24.1)	41 (75.9)	0.764	31 (57.4)[3]	23 (42.6)	0.064	10 (18.5)	44 (81.5)	0.055
A3F4 (n^1 = 11; n^2 = 22)	4 (18.2)	18 (81.8)		18 (81.8)	4 (18.2)		0 (0.0)[1]	22 (100.0)	
METAVIR (n^1 = 91; n^2 = 182)									
≤ A2F4 (n^1 = 80; n^2 = 160)	31 (19.4)	129 (80.6)	1.000	103 (64.4)[4]	57 (35.6)	0.148	26 (16.2)	134 (83.8)	0.048[a]
A3F4 (n^1 = 11; n^2 = 22)	4 (18.2)	18 (81.8)		18 (81.8)	4 (18.2)		0 (0.0)[2]	22 (100.0)	

n^1: Number of subjects; n^2: Number of alleles.

[3]*APOE3 vs APOE4* allele frequency, $P = 0.024$.

[4]*APOE3 vs APOE4* allele frequency, $P = 0.043$.

[a]$P < 0.05$. METAVIR A1: Minimal degree of inflammation; METAVIR A1A2: Minimal and moderate degree of inflammation; METAVIR A3: High degree of inflammation; *APOE*: Apolipoprotein E gene.

degree of liver inflammation included as significant predictors male-gender ($P = 0.032$), and mean-BMI ($P = 0.017$). In the other analyses, there was no significance without adjustment or with adjustment for potential confounders (MELD, age and BMI) (Table 5).

Analysis of the post-OLT data

With respect to the fibrosis degree, using METAVIR scores, of liver grafts in 91 non-*APOE4* patients undergoing OLT, the frequency of patients with a moderate degree of fibrosis (F2) was significantly higher in up to 1 year when compared to those between 1 and 5 years ($P = 0.006$) (Figure 1D). No other significant differences were found (Figure 1). Of note, patients who progressed to moderate (F2) fibrosis in the post-OLT follow-up were treated with antiviral therapy only in the post-OLT period.

In a 10-year follow-up post-OLT, based on non-invasive tests (APRI and FIB4) of the total transplanted population, a significant higher mean of the 1st year APRI score was found when compared to the 4th and 5th years ($P < 0.001$), as well as between the 1st year FIB4 and the 5th year scores ($P < 0.001$) (Figure 2A). APRI and FIB4 scores over the follow-up time did not significantly change regardless of the E2 and E4 alleles (Figure 2).

DISCUSSION

Increasing evidence associates *APOE* polymorphisms with progression of chronic liver disease[20,21]. Here, we evaluate the impact of ApoE genetic background in patients with HCV-induced liver cirrhosis, with or without HCC, transplanted or non-transplanted, and with positive or negative viral loads; in particular, in a Brazilian population and with a focus on the influence of E2, E3, and E4 alleles. We related E2, E3, and E4 carriers with the degree of liver inflammation, fibrosis and severity of the disease assessed by MELD scores, using liver biopsy and/or non-invasive indices, such as APRI and FIB4, and the METAVIR score. In addition, we also associated *APOE* alleles with co-morbidities and lipid blood levels.

As expected, we found E3 the most common *APOE* allele (67.3%), though slightly lower than the expected 70%-80% seen in the general Brazilian population[22] and in other countries[15,23] The E2 allele frequency was 17.1%, higher than in the Brazilian general population[22] and even greater than the E4 allele (15.6%). This E2 frequency in our study population was also higher than that reported by Wozniak *et al*[24] (7.7%).

Our liver biopsies from 89 liver explants and from 2 pre-OLT patients showed that cirrhotic E4 carriers were less likely to present with severe inflammation. These results were also evident in patients with MELD score ≤ 25. All these patients with severe

Table 4 Association of the models for end-stage liver disease score and variables in pre-orthotopic liver transplantation patients according to E2, E3 and E4 allele stratification

Variables	MELD≤ 25				MELD> 25				MELD≤ 25			MELD> 25		
	APOE2	*APOE3*	*APOE4*		*APOE2*	*APOE3*	*APOE4*		*APOE4*			*APOE4*		
									(Yes)	(No)		(Yes)	(No)	
	n^2 (%)	n^2 (%)	n^2 (%)	*P* value	n^2 (%)	n^2 (%)	n^2 (%)	*P* value	n^2 (%)	n^2 (%)	*P* value	n^2 (%)	n^2 (%)	*P* value
METAVIR (n^1 = 38; n^2 = 76)														
A1F4 (n^1 = 27; n^2 = 54)	9 (20.4)	27 (61.4)[3]	8 (18.2)	0.085	4 (40.0)	4 (40.0)	2 (20.0)	NA	8 (18.2)	36 (81.8)	0.044[a]	2 (20.0)	4 (80.0)	NA
A3F4 (n^1 = 11; n^2 = 22)	4 (18.2)	18 (81.8)	0 (0.0)[3]		0 (0.0)	0 (0.0)	0 (0.0)		0 (0.0)	22 (100.0)		0 (0.0)	0 (0.0)	
METAVIR (n^1 = 91; n^2 = 182)														
≤ A2F4 (n^1 = 80; n^2 = 160)	26 (18.6)	92 (65.7)[4]	22 (15.7)	0.123	5 (25.0)	11 (55.0)	4 (20.0)	NA	22 (15.7)	118 (84.3)	0.046[a]	4 (20.0)	16 (80.0)	NA
A3F4 (n^1 = 11; n^2 = 22)	4 (18.2)	18 (81.8)	0 (0.0)[4]		0 (0.0)	0 (0.0)	0 (0.0)		0 (0.0)	22 (100.0)		0 (0.0)	0 (0.0)	

n^1: Number of subjects; n^2: Number of alleles.

[3]*APOE3 vs APOE4* allele frequency, $P = 0.040$.

[4]*APOE3 vs APOE4* allele frequency, $P = 0.042$.

[a]$P < 0.05$. METAVIR F4: Cirrhosis; METAVIR A1: Minimal degree of inflammation; METAVIR A1A2: Minimal and moderate degree of inflammation; METAVIR A3: High degree of inflammation; *APOE*: Apolipoprotein E gene; MELD: Model for end-stage liver disease; NA: Not applicable.

hepatic inflammation were treated with antivirals only post-OLT. In agreement, others identified a protective role of *APOE4* against severe HCV-related liver damage, when comparing to patients with mild liver disease[24], while another study found that *APOE4* allele was under-represented in 996 patients chronically infected with HCV[23]. Other researchers have also noted a higher frequency of the E4 allele among patients with chronic non-cirrhotic hepatitis C, suggesting that the E4 allele is protective against severe HCV infection[25] However, somewhat inconsistent with these findings a 2003 report by Mueller *et al*[26] was unable to associate the E4 allele in chronic HCV-infected patients with a strong antiviral treatment response, although a later study by Price *et al*[27] found an association of the E2 and E4 alleles with reduced likelihood of chronic infection in HCV patients.

In 2012, Ahn *et al*[20] suggested that high ApoE serum levels in patients with liver cirrhosis may be due to liver inflammation. ApoE is known to modulate immune function by inhibiting CD4 and CD8 lymphocyte proliferation, reducing lymphocyte-derived production of IL-2, a key cytokine in regulating lymphocyte differentiation[28]. We speculate that a reduction in ApoE plasma levels, which is recognized for *APOE4* carriers[6], could be protective to support OLT and to reduce over-inflammation and fibrosis caused by chronic HCV infection.

Table 5 Logistic regression model predicting moderate and severe (A2A3) degree of liver inflammation

Variables	Odds ratio	95%CI	*P* value
Alcohol consumption	0.636	0.188-2.148	0.466
Gender–male	7.609	1.195-48.447	0.032[a]
Mean MELD	0.932	0.829-1.049	0.244
Mean age	0.969	0.893-1.051	0.446
Mean BMI	0.874	0.782-0.976	0.017[a]
*APOE*2	1.519	0.059-39.334	0.801
*APOE*3	2.850	0.121-66.917	0.516
*APOE*4	2.868	0.112-73.560	0.524

[a]$P < 0.05$. MELD: Model for end-stage liver disease; BMI: Body mass index; *APOE*: Apolipoprotein E gene; CI: Confidence interval.

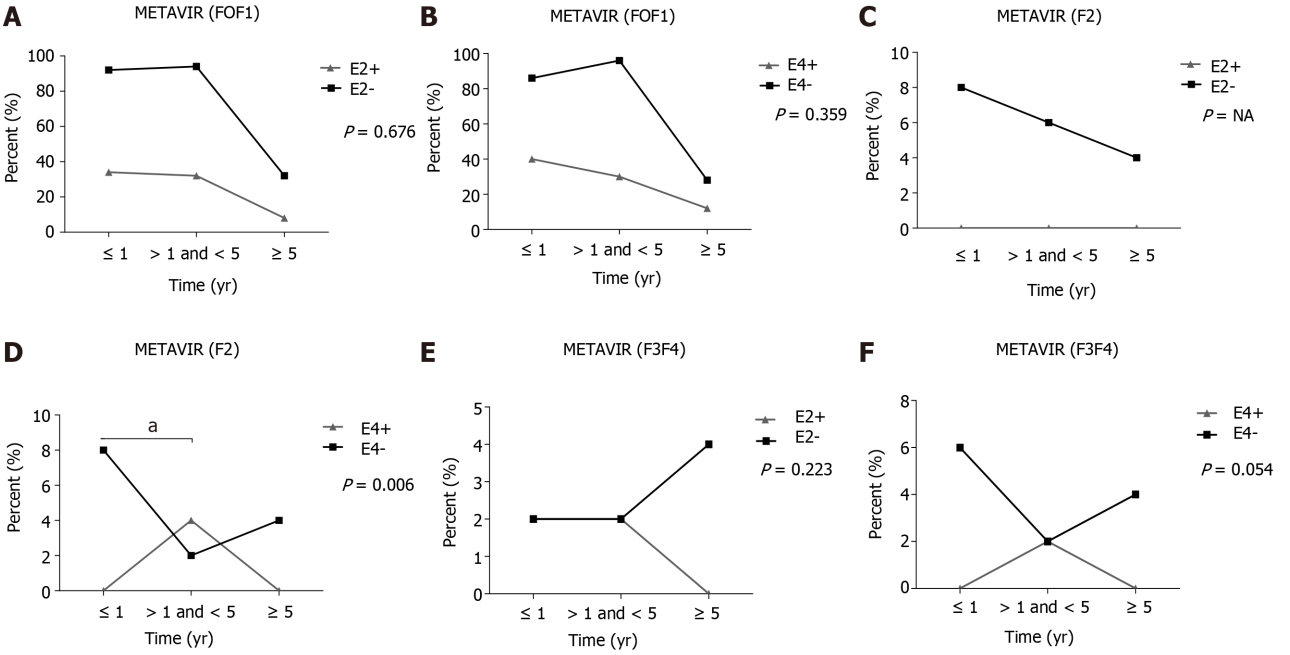


Figure 1 Follow-up of liver fibrosis in liver transplanted patients in the presence or absence of the E2 and E4 allele. A: METAVIR (F0F1) in the presence or absence of the E2 allele; B: METAVIR (F0F1) in the presence or absence of the E4 allele; C: METAVIR (F2) in the presence or absence of the E2 allele; D: METAVIR (F2) in the presence or absence of the E4 allele; E: METAVIR (F3F4) in the presence or absence of the E2 allele; F: METAVIR (F3F4) in the presence or absence of the E4 allele. [a]$P < 0.01$. E2- and E4-: Absence of the respective alleles; E2+ and E4+: Presence of the respective alleles. Fisher chi-square test; F: Degree of fibrosis; NA: Not applicable.

In the follow-up of our 144 liver transplanted patients, we identified the E4 allele as protective against the progression of liver fibrosis in 91 (63.2%) recipients. This protection of *APOE4* against severe HCV-related liver fibrosis agrees with an early report[24]. Other investigators also showed a benefit of the *APOE4* allele against fibrosis progression in liver transplanted patients diagnosed with HCV recurrence. Additionally, it is reported that liver transplanted patients carrying at least one E4 allele may present with reduced graft fibrosis progression during HCV recurrence. Indeed, ApoE polymorphism can be an important tool to monitor fibrosis progression in patients with hepatitis C and normal values of alanine aminotransferase, as there may be competition mechanisms for viral entry and replication in cells[15].

ApoE is a component of several lipoprotein classes and important for lipid transport. ApoE isoforms have several effects on lipoprotein entry into cells, and this mechanism might explain our results, supporting previous investigations in which ApoE4 protects against HCV infection[29,30].

In the follow-up of liver fibrosis progression evaluated between 1 to 10 years post-OLT of our transplanted population, the average score of APRI and FIB4, tended to

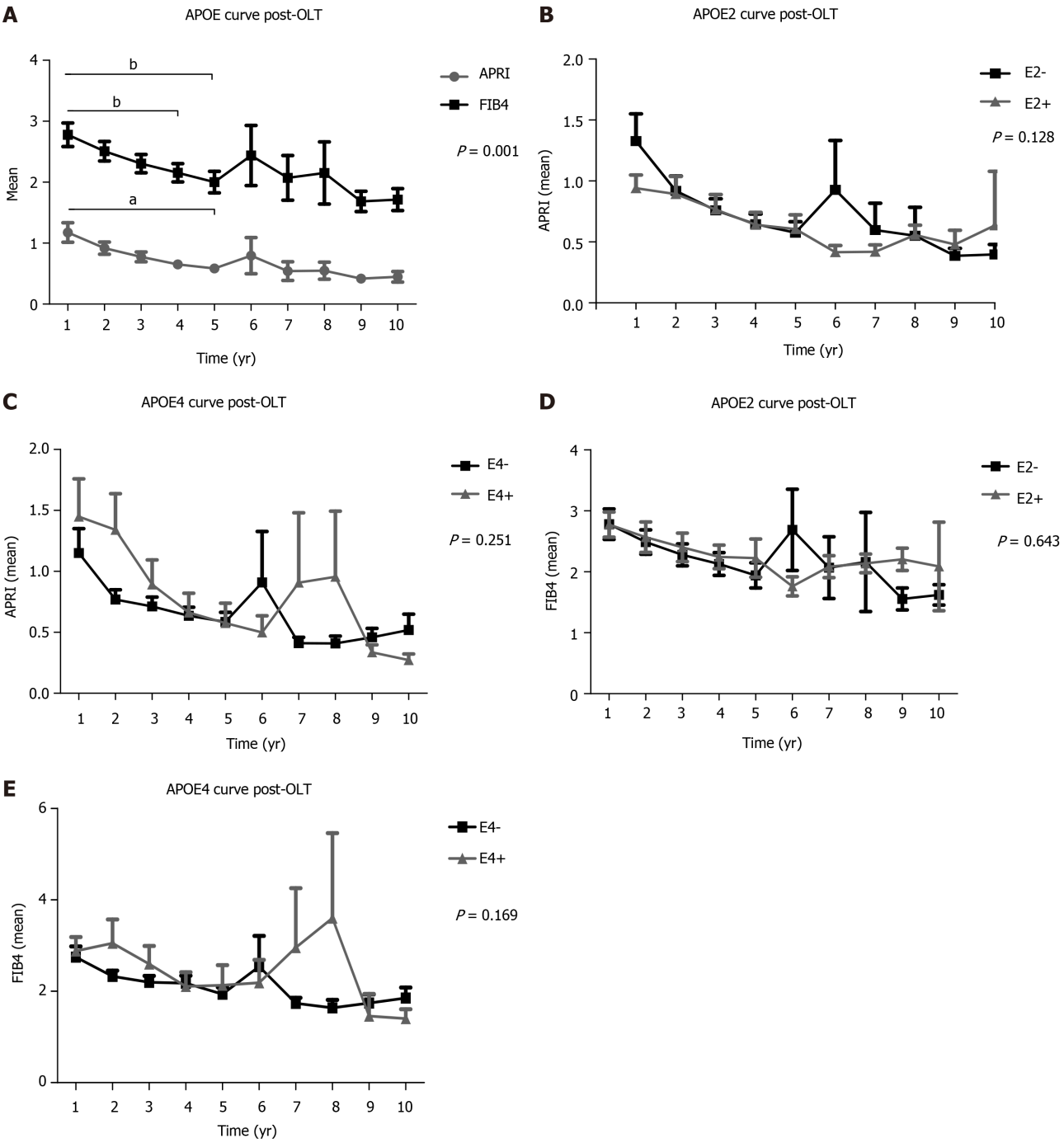


Figure 2 Follow-up using non-invasive methods (aspartate aminotransferase to platelet ratio index and fibrosis 4) of liver transplant patients in the presence or absence of the E2 and E4 alleles. A: Post-orthotopic liver transplantation (OLT) apolipoprotein E gene (*APOE*) curve for mean aspartate aminotransferase to platelet ratio index (APRI) and fibrosis 4 (FIB4); B: Post-OLT curve considering the presence or absence of *APOE2* for mean APRI; C: Post-OLT curve considering the presence or absence of *APOE4* for mean APRI; D: Post-OLT curve considering the presence or absence of *APOE2* for mean FIB4; E: Post-OLT curve considering the presence or absence of *APOE4* for mean FIB4. Two-way ANOVA, results are shown in mean ± SEM. $^{a}P < 0.01$. $^{b}P < 0.01$. E2- and E4-: Absence of the respective alleles; E2+ and E4+: Presence of the respective alleles. *APOE*: Apolipoprotein E gene; OLT: Orthotopic liver transplantation; APRI: Aspartate aminotransferase to platelet ratio index; FIB4: Fibrosis 4.

decrease significantly over the years, implying liver graft survival without progression to fibrosis. Thus, non-invasive methods are now widely used in clinical practice to stage the degree of fibrosis[31,32].

Our findings suggest that *APOE4* can be important tool in the medical management of patients following inflammation and liver fibrosis, since the carriage of *APOE4* may select patients with a more benign clinical course of liver disease. Of note, patients with a degree of severe inflammation and moderate degree of fibrosis (F2) were cured for HCV only in the post-OLT period.

This study has some limitations: Data from liver transplanted patients were

obtained retrospectively from medical records; no data from liver graft donors, including *APOE* genotypes, were collected. The sample size, may have been insufficient to draw strong, robust conclusions. Isoform studies have previously shown that transplanted donor livers supply > 90% of plasma ApoE[10]. The remainder is synthesized by circulating macrophages and immune cells, or by tissues such as kidney, adipose and muscle, and hence retains the phenotype of the recipient. However, to date, there are no reports of how each source, hepatic ApoE or circulating non-liver ApoE, particularly that of macrophages, might affect the inflammation and fibrosis status of the transplanted liver.

CONCLUSION

Our results indicate that *APOE4* genotype may protect against HCV-induced severe hepatic inflammation and fibrosis in pre- and post-OLT patients. Additionally, the *APOE2* allele was over-represented in these patients, suggesting that E2 carriers have increased risk and worse outcomes following HCV infection. Further studies are needed to better understand how ApoE levels *via* liver and extrahepatic derived sources, and biochemical activities, are affected by donor and recipient genetic backgrounds after liver transplantation.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) can cause chronic liver inflammation, end-stage liver disease and hepatocellular carcinoma (HCC). Apolipoprotein E (protein: ApoE, gene: *APOE*) is mainly liver synthesized and *APOE* polymorphisms may affect HCV-induced liver damage after orthotopic liver transplantation (OLT).

Research motivation

Although *APOE4* may protect against HCV-induced liver damage, the role of *APOE* genotypes in modifying HCV-induced liver injury in post-OLT has not been reported.

Research objectives

To establish if *APOE4* genotype OLT recipients have more benign HCV-related liver injuries compared to patients with *APOE2* or *APOE4* genotypes.

Research methods

Patients with HCV-related end-stage liver disease, 105 of 179 complicated with HCC, were assessed pre-OLT (179) and post-OLT (144; with a 1-year follow-up for 132 patients). Liver injury analyses included METAVIR and models for end-stage liver disease scores, while *APOE* genotype was determined by qRT-PCR.

Research results

HCV positive recipients with severe hepatic inflammation had low *APOE4* genotype frequency, compared to those with minimal and moderate inflammation. In addition, liver fibrosis was lower in patients carrying *APOE4* genotype compared to those carrying the most common *APOE3* genotype.

Research conclusions

We found that carriage of *APOE4* genotype protects pre-OLT patients against HCV-induced severe hepatic inflammation, and against fibrosis progression post-OLT.

Research perspectives

We propose that carriage of *APOE*4 genotype protects against progression of inflammation and liver fibrosis in recurrent HCV hepatitis after OLT, but additional studies are needed to assess whether donor-derived ApoE4 protein directly affects these processes.

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ORIGINAL ARTICLE

Observational Study

Fatigue in patients with inflammatory bowel disease in Eastern China

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Abstract

BACKGROUND

Fatigue is a very common but relatively neglected problem in patients with inflammatory bowel disease (IBD). The prevalence rate of IBD in China is the highest in Asia, but there is little research on fatigue in patients with IBD. Neither the relationship between fatigue and quality of life (QoL) nor the relationship between fatigue and work productivity (WP) in Chinese IBD patients has been reported.

AIM

To investigate the prevalence of fatigue related to IBD in Eastern China, to identify the risk factors associated with fatigue, to assess the impact of fatigue on QoL, and to evaluate the relationship between fatigue and WP.

METHODS

A cross-sectional study was conducted in a Regional Tertiary IBD Diagnostic and Treatment Center in Eastern China. Clinical data of patients were collected, and disease activity was evaluated. Blood samples were analyzed to assess anemia, albumin, and inflammation. Fatigue was assessed using the multidimensional fatigue inventory. QoL and WP were measured using the short inflammatory bowel disease questionnaire and the work productivity and activity impairment general health questionnaire, respectively. The patients also completed assessments of depression (Patient Health Questionnaire-9) and anxiety (Generalized Anxiety Disorder 7-item Scale).

RESULTS

A total of 311 IBD patients, comprising 168 Crohn's disease patients and 143

ulcerative colitis patients, were enrolled. The prevalence of fatigue in patients with IBD was 60.77%. In a univariate logistic regression analysis, factors such as disease activity, depression, anxiety, anemia, and IBD-related surgery were individually related to a significantly increased risk of fatigue in IBD patients. Multivariate logistic regression analysis indicated that depression [odds ratio (OR) = 8.078, 95% confidence interval (CI): 4.113-15.865], anxiety (OR = 2.373, 95%CI: 1.100-5.119), anemia (OR = 2.498, 95%CI: 1.290-4.834), and IBD-related surgery (OR = 2.035, 95%CI: 1.084-3.819) were related to fatigue in IBD patients. There was a negative correlation between fatigue and QoL (r = -0.831; P < 0.0001) but a positive correlation between fatigue and WP loss.

CONCLUSION

The prevalence of fatigue in IBD patients in Eastern China is remarkably high even in clinical remission. Factors such as depression, anxiety, anemia, and IBD-related surgery are major risk factors for fatigue in IBD patients. In addition, fatigue has a negative impact on QoL and is positively correlated with WP loss.

Key Words: Inflammatory bowel disease; Fatigue; Quality of life; Work productivity; Risk factors; Eastern China

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Core Tip: Fatigue is a highly prevalent and burdensome symptom in patients with inflammatory bowel disease (IBD), with an important impact on quality of life and (indirect) health expenditures. The prevalence rate of IBD in China is the highest in Asia, but there is little research on fatigue in patients with IBD. In addition, the relationships of fatigue with quality of life and work productivity in Chinese IBD patients have not been reported.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, nonspecific inflammation of the gastrointestinal tract with an unknown etiology that can be classified as ulcerative colitis (UC) and Crohn's disease (CD)[1]. IBD has a high prevalence in young adults and is characterized by a long course, high recurrence rate, and severe complications (such as toxic megacolon, intestinal perforation, intestinal obstruction, intestinal bleeding, and cancer)[2]. Mucus-bloody stools, diarrhea, abdominal pain, weight loss, and anemia are the main clinical manifestations of IBD[3], which seriously impact the quality of life (QoL) of patients and increase the financial burden.

Fatigue is expressed as an overwhelming experience of mental and/or physical exhaustion that affects daily living and is unrelieved by rest or sleep[4,5]. Studies in several countries have shown that fatigue is common in patients with IBD[6-10]. Some studies have found that fatigue is associated with active enteritis[11], especially with mucosal healing in patients with IBD[12]. In addition, fatigue also has a negative psychological impact on patients with IBD, exacerbating clinical symptoms and promoting disease progression[13]. Even as a result of fatigue, IBD patients have to adjust their daily activities and work, and some even choose to resign, which seriously affects their QoL[14] and increases their financial burden[15]. Fatigue is a very common but relatively neglected problem in IBD patients, especially in China. The prevalence rate of IBD in China is the highest in Asia[16], but there is little research on fatigue in patients with IBD. In addition, the relationship between fatigue and QoL and work productivity (WP) in Chinese IBD patients has not been reported.

In this study, we aimed to investigate the prevalence and risk factors for fatigue in patients with IBD in Eastern China through a cross-sectional study. We also

guardian, provided informed written consent prior to study enrollment.

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Data sharing statement: No additional data are available.

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determined the relationships between fatigue and QoL and WP to evaluate the impact of fatigue on IBD patients in Eastern China.

MATERIALS AND METHODS

Study population

This cross-sectional study was conducted at the First Affiliated Hospital of Zhejiang Chinese Medical University, a Regional Tertiary IBD Diagnostic and Treatment Center in Eastern China, from February 2018 to August 2020. The inclusion criteria were a confirmed diagnosis of IBD and signed informed consent. The diagnostic criteria for IBD were based on the Chinese consensus on the diagnosis and treatment of IBD[17]. The exclusion criteria were the inability to understand or complete the questionnaires, refusal to give written informed consent before participation, and concomitant diseases with fatigue as the main symptoms, such as cancer, heart disease, or liver cirrhosis. This study was registered at the Chinese Clinical Trials Registry (ChiCTR1900025890).

Ethics

This study was approved by the ethics committee of the First Affiliated Hospital of Zhejiang Chinese Medical University, and informed consent was obtained from all participants. Patients under the age of 16 were admitted to our study with consent from their parents or guardians.

Clinical and sociodemographic data

The demographic characteristics of the patients were collected, including age, sex, body mass index (BMI), course of the disease, current smoking habits, IBD-related surgery (such as colectomies, other bowel surgery, and perianal surgery), disease activity, type of IBD, location of disease, and current medications. Blood samples were collected (within one week before and after completion of the questionnaires) and analyzed for hemoglobin, albumin, and erythrocyte sedimentation rate.

Definitions

Disease activity and severity were assessed using the following clinical indices: Harvey-Bradshaw activity index[18] was used for CD. Mayo score and Truelove and Witts criteria[19] were used for UC. Anemia was defined as hemoglobin < 130 g/L for males and < 120 g/L for females. Hypoalbuminemia was defined as albumin < 35 g/L. BMI was based on the Chinese criteria of weight for adults[20]. Underweight was defined as BMI < 18.5 kg/m^2; normal weight was defined as 18.5 kg/m^2 ≤ BMI < 24.0 kg/m^2; overweight was defined as 24.0 kg/m^2 ≤ BMI < 28.0 kg/m^2; and obesity was defined as BMI ≥ 28.0 kg/m^2.

The significance level of coefficients is indicated only when they reach the 0.001 criterion. The following cutoffs were used to define the magnitude of the correlation coefficients: < 0.25, low correlation; 0.25 to 0.5, fair correlation; 0.5 to 0.75, moderate-to-good correlation; and > 0.75, good-to-excellent correlation[21].

Questionnaires

Fatigue was analyzed using the multidimensional fatigue inventory (MFI; ranging from 20 to 100, with higher scores indicating more severe fatigue). This questionnaire, previously validated in Chinese and for IBD patients[22], comprises 20 items divided into five subscales: General fatigue, physical fatigue, reduced activity, reduced motivation, and mental fatigue[23]. The definition of fatigue was complicated by a lack of clear cutoff scores. Several studies have found that scales of general fatigue are more psychometrically useful than the use of numerical rating scales, so the MFI of general fatigue can be called "fatigue"[24-26]. Combining the values reported in a domestic study[27] and foreign studies[28-30] on the MFI, fatigue was defined as general fatigue score ≥ 12.

Depression was analyzed using the Patient Health Questionnaire-9 (PHQ-9), which has been validated in Chinese IBD patients[31]. The PHQ-9 scores each of the 9 DSM-IV criteria on a scale ranging from "0" (not at all) to "3" (nearly every day). The total PHQ-9 score that categorizes depression is as follows: Nondepression as 0 ≤ PHQ-9 ≤ 4, mild depression as 5 ≤ PHQ-9 ≤ 9, moderate depression as 10 ≤ PHQ-9 ≤ 14, moderate-severe depression as 15 ≤ PHQ-9 ≤19, and severe depression as 20 ≤ PHQ-9 ≤ 27[32].

The generalized anxiety disorder 7-item scale was completed to measure symptoms

of anxiety and has been validated in Chinese patients with IBD[31]. The generalized anxiety disorder 7-item scale is a 7-item self-report instrument that is scaled from 0–3 (not at all, several days, more than half the days, and nearly every day), with total scores ranging from 0 to 21, and it is interpreted as follows: The absence of anxiety (0-4), mild anxiety (5-9), moderate anxiety (10-14), and severe anxiety (15-21)[33].

The Short Inflammatory Bowel Disease Questionnaire (SIBDQ) was used to assess IBD-specific QoL[34]. The SIBDQ includes 10 items, each with a score from 1 (worst) to 7 (best), with the total score ranging from 10 to 70 (the higher the score, the better the QoL). Furthermore, the SIBDQ has four domains: Bowel symptoms, systemic symptoms, emotional function, and social function.

The work productivity and activity impairment general health questionnaire[35] measures time missed from work and work impairment because of IBD in the past week. The work productivity and activity impairment general health questionnaire includes four items: Work time missed (absenteeism), impaired productivity at work (presenteeism), overall work impairment (OWI; combined absenteeism and presenteeism), and impairment in non-work-related activities due to health problems (activity impairment). Absenteeism was calculated as [hours missed due to health problems/ (hours missed due to health problems + hours worked)] × 100; presenteeism was calculated as (degree health affected productivity while working/10) × 100; OWI was calculated as absenteeism + [(1-absenteeism) × presenteeism]; and (4) daily activity impairment was calculated as (degree of health affected daily activities/10) × 100.

Statistical analysis

Quantitative variables are expressed as the mean ± SD or as medians and interquartile range (IQR), and qualitative variables are expressed as frequencies and percentages. After transforming fatigue from a quantitative to a qualitative variable (with/without fatigue), logistic regression analyses were performed. Variables with $P < 0.05$ in the univariate analysis were included in the multivariate analysis, and the results are expressed as odds ratios (ORs) with their corresponding 95% confidence intervals (CIs). Correlations between fatigue and QoL and WP were measured with Spearman's rank correlation coefficient. Statistical analyses were performed using Statistic Package for Social Science 24 (Statistic Package for Social Science Inc., Chicago, IL, United States), and $P < 0.05$ was considered statistically significant.

RESULTS

IBD patients' demographic and clinical characteristics

A total of 311 IBD patients, including 168 CD and 143 UC patients, were enrolled in this study. The participants had a median age of 42 (IQR: 31-53) years. Most of the participants had health insurance (90.35%, $n = 281$) and were married (74.28%, $n = 231$). There were 212 (68.17%) patients who had a job, of whom 208 (66.88%) were working full-time. Regarding the duration of disease, the participants reported a median of 5 (IQR: 2-12) years. A total of 51.45% of IBD patients were in the active phase of the disease, and 32.80% of participants had IBD-related surgery. The demographic and clinical characteristics of IBD patients are summarized in Table 1.

Prevalence and score of fatigue in patients with IBD

The prevalence of fatigue in patients with IBD was 60.77%, including 71.88% in patients with active IBD and 49.01% in patients in remission. The median fatigue total score was 43 (IQR: 33-59) in IBD patients, and the median general fatigue, physical fatigue, mental fatigue, reduced activity, and reduced motivation scores were 12 (IQR: 9-15), 8 (IQR: 6-12), 9 (IQR: 6-12), 7 (IQR: 4-9), and 7 (IQR: 4-12), respectively (Figure 1).

Factors associated with fatigue

The univariate analysis showed that disease activity ($P < 0.001$, OR = 2.659; 95%CI: 1.663-4.253), depression ($P < 0.001$, OR = 13.722; 95%CI: 7.608-24.749), anxiety ($P < 0.001$, OR = 8.134; 95%CI: 4.351-15.204), anemia ($P < 0.001$, OR = 3.792; 95%CI: 2.232-6.440), and IBD-related surgery ($P < 0.05$, OR = 1.654; 95%CI: 1.004-2.727) were associated with the presence of fatigue (Figure 2A).

Multivariate logistic regression analysis indicated that depression ($P < 0.001$, OR = 8.078, 95%CI: 4.113-15.865), anxiety ($P = 0.028$, OR = 2.373, 95%CI: 1.100-5.119), anemia ($P = 0.007$, OR = 2.498, 95%CI: 1.290-4.834), and IBD-related surgery ($P = 0.027$, OR =

Table 1 Demographic and clinical characteristics of inflammatory bowel disease patients

	CD (*n* = 168)	UC (*n* = 143)
Age, yr, median (IQR)	39 (IQR: 28-52.75)	45 (IQR: 33-54)
Gender, *n* (%)		
Female	71 (42.26)	64 (44.76)
Male	97 (57.74)	79 (55.24)
BMI, *n* (%)		
Normal	94 (55.95)	81 (56.64)
Thinnish	60 (35.71)	37 (25.88)
Overweight	11 (6.55)	25 (17.48)
Obesity	3 (1.79)	0 (0)
Marital status, *n* (%)		
Unmarried	58 (34.52)	14 (9.79)
Married	105 (62.50)	126 (88.11)
Divorced	3 (1.79)	3 (2.10)
Death of a spouse	2 (1.19)	0 (0)
Employment status, *n* (%)		
No work	16 (9.52)	13 (9.09)
Full time	117 (69.64)	91 (63.64)
Retired	15 (8.93)	35 (24.48)
Long-term sick leave	1 (0.60)	3 (2.10)
Students	19 (11.31)	1 (0.69)
Medical insurance, *n* (%)		
Yes	151 (89.88)	130 (90.91)
No	17 (10.12)	13 (9.09)
Education, *n* (%)		
Primary school or below	14 (8.33)	20 (13.99)
Junior high school	34 (20.24)	36 (25.17)
Senior high school	32 (19.05)	41 (28.67)
Junior college or Undergraduate	81 (48.21)	42 (29.37)
Master degree or above	7 (4.17)	4 (2.80)
Montreal classification, *n* (%)		
L1 ileal	38 (22.62)	
L2 colonic	14 (8.33)	
L3 ileocolonic	72 (42.86)	
L4 upper gastrointestinal tract	11 (6.55)	
L1 + L4	13 (7.74)	
L3 + L4	20 (11.90)	
E1 proctitis		37 (25.88)
E2 left-sided UC		40 (27.97)
E3 extensive UC		66 (46.15)
Disease activity: *n* (%)		
Remission	100 (59.52)	51 (35.66)

Mild activity	26 (15.48)	43 (30.07)
Moderate activity	31 (18.45)	37 (25.88)
Severe activity	11 (6.55)	12 (8.39)
Duration of disease, yr, median (IQR)	5.50 (IQR, 2-11)	5 (IQR, 2.3-13)
Current medication, *n* (%)		
5-ASA	19 (11.31)	88 (61.54)
IS	42 (25)	30 (20.98)
5-ASA + IS	22 (13.09)	8 (5.59)
Biological preparation	45 (26.79)	5 (3.50)
Biological preparation + IS	34 (20.24)	8 (5.59)
Other	6 (3.57)	4 (2.80)
IBD related surgery, *n* (%)		
No	80 (47.62)	129 (90.21)
Yes	88 (52.38)	14 (9.79)

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; 5-ASA: 5-aminosalisylic acid; IQR: Interquartile range; IS: Immunosuppressant.

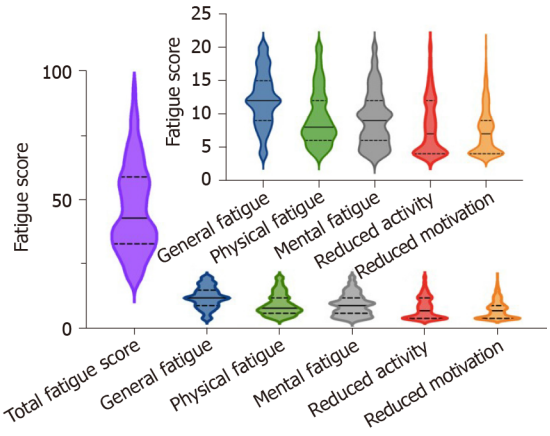


Figure 1 Fatigue score in the inflammatory bowel disease patients. The solid line indicates the median, and the dotted line indicates the interquartile range. The small insert within the graphs in Figure 1 enlarges the scores of the five subscales of multidimensional fatigue inventory (general fatigue, physical fatigue, reduced activity, reduced motivation, and mental fatigue) in the inflammatory bowel disease patients.

2.035, 95%CI: 1.084-3.819) were related to fatigue in IBD patients (Figure 2B).

Fatigue and QoL

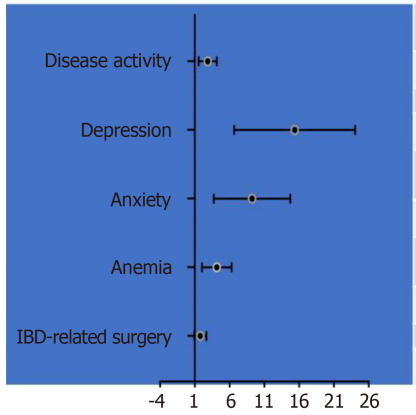
The median QoL total score was 53 (IQR: 44-62), and the median scores of bowel symptoms, social function, emotional function, and systemic symptoms were 17 (IQR: 13-20), 12 (IQR: 9-13), 9 (IQR: 6-12), 15 (IQR: 11-19), and 11 (IQR: 10-13), respectively (Figure 3). Fatigue was negatively correlated with QoL (r = - 0.831; P < 0.0001), particularly with emotional function (r = -0.721; P < 0.0001) (Figure 4A). Further analysis revealed that general fatigue (r = -0.785; P < 0.0001) showed a good-to-excellent correlation with negative QoL, and reduced activity (r = -0.731; P < 0.0001) and psychological fatigue (r = -0.704; P < 0.0001) showed a moderate-to-good correlation with negative QoL (Figure 4B).

Fatigue and WP

There were 208 (66.88%) patients who were working full-time, and their prevalence of fatigue was 58.65%. Further analysis found that their median total fatigue score was 41 (IQR: 32.25-58), with median general fatigue, physical fatigue, mental fatigue, reduced activity, and reduced motivation scores of 12 (IQR: 9-15), 9 (IQR: 7-12), 9 (IQR: 6-11), 6

A

Variables	OR (95%CI)	*P* value
Disease activity	2.659 (1.663-4.253)	< 0.01
Depression	13.722 (7.608-24.749)	< 0.001
Anxiety	8.134 (4.351-15.204)	< 0.001
Anemia	3.792 (2.232-6.440)	< 0.001
IBD-related surgery	1.654 (1.004-2.727)	< 0.05



B

Variables	OR (95%CI)	*P* value
Depression	8.078 (4.113-15.865)	< 0.001
Anxiety	2.373 (1.100-5.119)	0.028
Anemia	2.498 (1.290-4.834)	0.007
IBD-related surgery	2.035 (1.084-3.819)	0.027

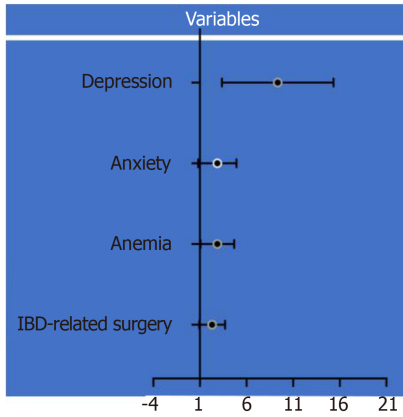


Figure 2 Factors associated with the presence of fatigue. A: Univariate analysis; B: Multivariate analysis. OR: Odds ratio; IBD: Inflammatory bowel disease.

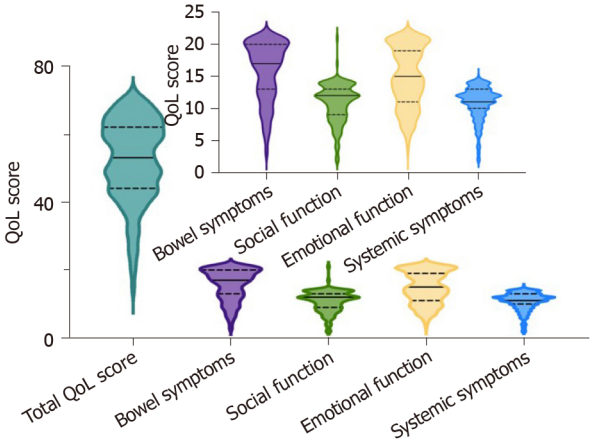


Figure 3 Quality of life score in the inflammatory bowel disease patients. The solid line indicates the median, and the dotted line indicates the interquartile range. The small insert within the graphs in Figure 3 enlarges the scores of the four domains of short inflammatory bowel disease questionnaire (bowel symptoms, systemic symptoms, emotional function, and social function) in the inflammatory bowel disease patients.

(IQR: 4-9), and 6 (IQR: 4-11), respectively (Figure 5). Fatigue had the strongest positive correlation with OWI (r = 0.605; P < 0.0001), followed by activity impairment (r = 0.566; P < 0.0001), presenteeism (r = 0.543; P < 0.0001), and absenteeism (r = 0.480; P < 0.0001) (Figure 6A). Compared with physical fatigue, mental fatigue, reduced activity, and reduced motivation, general fatigue was the most strongly associated with WP loss (OWI: r = 0.552, P < 0.0001; activity impairment: r = 0.549, P < 0.0001; presenteeism: r = 0.519, P < 0.0001; absenteeism: r = 0.442, P < 0.0001) (Figure 6B).

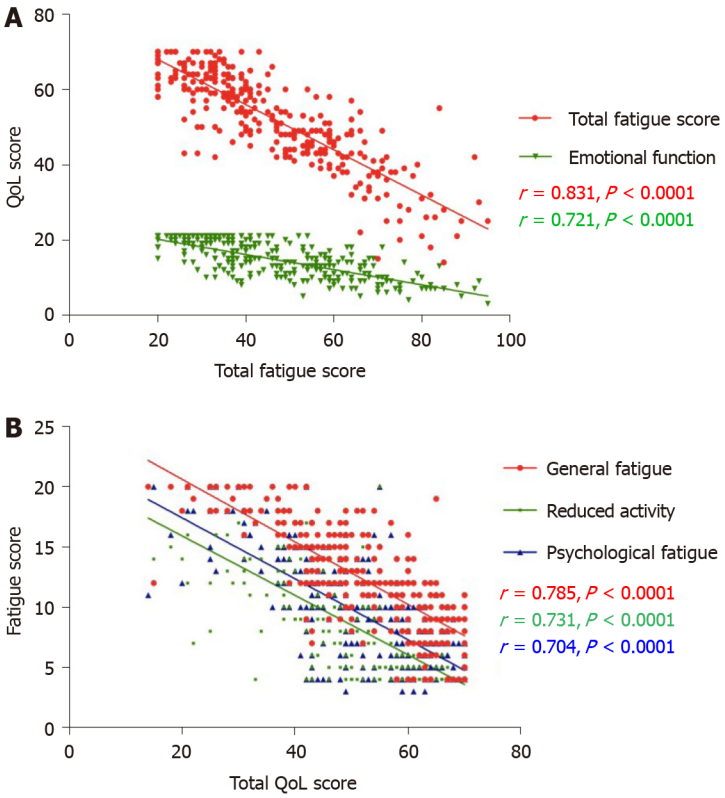


Figure 4 Fatigue and quality of life. A: Correlation between total fatigue scores and quality of life scores (total quality of life scores: Spearman's r = -0.831, P < 0.0001; emotional function: Spearman's r = -0.721, P < 0.0001) in the inflammatory bowel disease patients; B: Correlation between total quality of life scores and fatigue (general fatigue: Spearman's r = -0.785, P < 0.0001; reduced activity: Spearman's r = -0.731, P < 0.0001; psychological fatigue: Spearman's r = -0.704, P < 0.0001) in the inflammatory bowel disease patients.

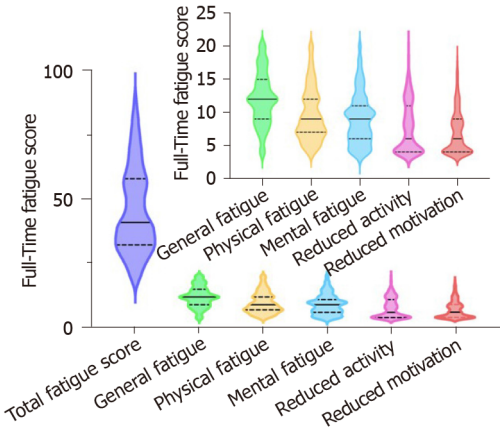


Figure 5 Fatigue score in inflammatory bowel disease patients with full-time jobs. The solid line indicates the median, and the dotted line indicates the interquartile range. The small insert within the graphs in Figure 5 enlarges the scores of the five subscales of multidimensional fatigue inventory (general fatigue, physical fatigue, reduced activity, reduced motivation, and mental fatigue) in the inflammatory bowel disease patients.

DISCUSSION

In the present study, we found that the prevalence of fatigue in patients with IBD in Eastern China was 60.77%, including 71.88% in the active stage and 49.01% in the remission stage. Major factors associated with fatigue were depression, anxiety, anemia, and IBD-related surgery. Female sex, disease activity, and hypoalbuminemia do not increase the risk of fatigue. In addition, fatigue had a negative impact on QoL and was positively correlated with WP loss.

Multifactorial analysis showed that depression and anxiety were risk factors for fatigue, and depression, in particular, is the strongest risk factor for fatigue. Several previous studies are consistent with our findings[36-38]. In chronic diseases, fatigue and

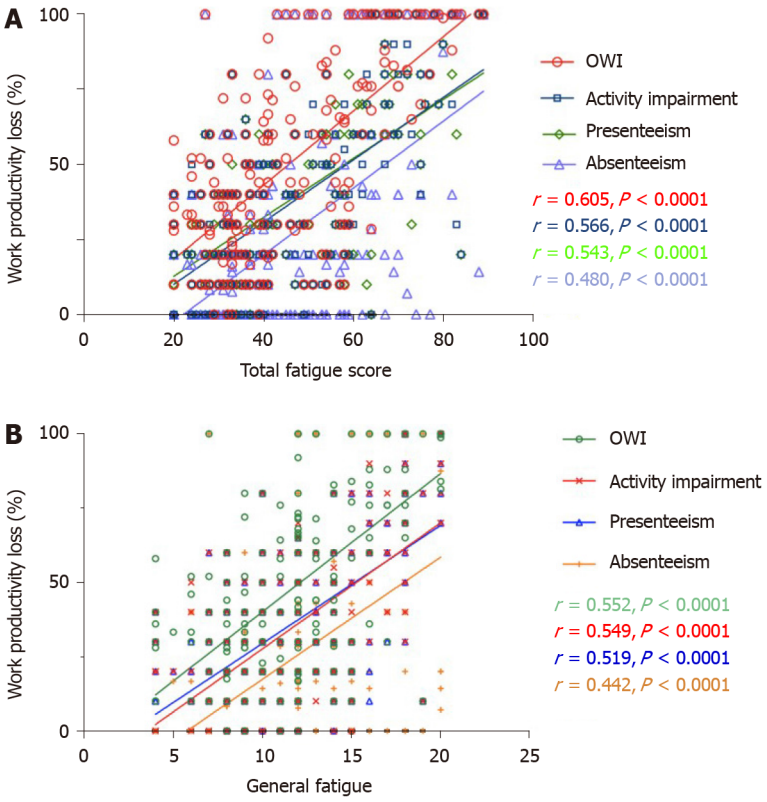


Figure 6 Fatigue and work productivity. A: Correlation between total fatigue scores and work productivity loss [overall work impairment: Spearman's r = 0.605, P < 0.0001; activity impairment: Spearman's r = 0.566, P < 0.0001; presenteeism: Spearman's r = 0.543, P < 0.0001; absenteeism: Spearman's r = 0.480, P < 0.0001] in inflammatory bowel disease patients with full-time jobs; B: Correlation between general fatigue and work productivity loss (overall work impairment: Spearman' s r = 0.552, P < 0.0001; activity impairment: Spearman's r = 0.552, P < 0.0001; presenteeism: Spearman's r = 0.552, P < 0.0001; absenteeism: Spearman's r = 0.552, P < 0.0001) in inflammatory bowel disease patients with full-time jobs. OWI: Overall work impairment.

psychiatric disorders such as depression and anxiety coexist[39,40], including IBD[41]. The immune-inflammatory pathway and gut-brain axis may be possible pathways for the coexistence of fatigue and psychological disorders in IBD[39-42]. One of the reasons for such a high prevalence of fatigue and psychiatric comorbidity in patients with IBD in Eastern China may be due to limited therapeutic drug options. The use of biologics as an effective treatment for IBD in China is very limited. First, the options are limited, with only infliximab entering the Chinese market. When IBD patients fail to respond to infliximab therapy, they are faced with the situation of either having no drugs available or using hormones with more side effects. Second, it is expensive, as only CD is reimbursed by health insurance, which increases the financial burden of patients. The limited availability of medication, the recurrence of disease symptoms, the side effects of hormone therapy, and the heavy financial burden contribute to the development of fatigue and psychiatric disorders in Chinese patients with IBD. Anemia is the most common extraintestinal manifestation of IBD, which occurs in up to 20% of outpatients and up to 68% of inpatients with IBD[43,44]. The major causes of anemia in IBD are iron[45], vitamin B12, and folic acid deficiency[46]. The side effects or complications of some drugs for IBD are anemia. For example, methotrexate can lead to folic acid deficiency and megaloblastic anemia[47]. One of the side effects of azathioprine and 6-mercaptopurine is myelosuppression[48]. Sulfadiazine and 5-aminosalicylate have rare hemolytic complications[49,50]. The relationship between fatigue and IBD-related surgery has rarely been reported. In our study, IBD-related surgery was found to be a risk factor for fatigue in IBD patients, which may be related to postoperative complications, postoperative pain, fear of stoma care, environmental (especially family) reactions, and acceptance of new conditions[29,51,52]. However, a clinical study in Poland that included 60 IBD patients concluded that surgical treatment reduced fatigue symptoms[53], which was contrary to the findings of our study. The difference may be due to different sample sizes, and our study has a larger sample size. In addition, the study in Poland compared the fatigue scores at one day before surgery and three months after surgery. The clinical symptoms of patients at 3 mo after operation were improved, but the postoperative complications were not fully

exposed. Our study included not only patients at 3 mo after the operation but also patients many years after operation and repeated surgery. Postoperative complications, disease activity, the annoyance of anastomotic care, and fear of reoperation were fully exposed. All of these factors will lead to fatigue in IBD patients. Surprisingly, female sex, disease activity, and hypoalbuminemia did not significantly increase fatigue among IBD patients in Eastern China. In previous studies[37,54], female sex was found to be a strong predictor of fatigue, but no good explanation for this association was found. Our study, however, found that female sex was not a risk factor for fatigue. This may have been because of the small sample size in our study. The association between fatigue and disease activity in IBD is controversial. Fatigue scores were higher and more frequent among IBD patients with active disease than in the reference population and among those with quiescent IBD, but contrasts with the findings of others[9,36,55]. In this study, univariate analysis showed that disease activity was a risk factor for fatigue but not in multivariate analyses. Therefore, more research is needed to clarify the relationships between female sex and disease activity and fatigue in Chinese patients with IBD. The common symptom of hypoalbuminemia is fatigue, but our findings suggest that it is not a risk factor for fatigue in patients with IBD in Eastern China. This may have been because of the small number of patients affected (29.3% of all those studied) or because fatigue was strongly associated with other factors, such as depression, anemia, anxiety, or IBD-related surgery.

Risk factors for fatigue, such as depression, anxiety, anemia, and IBD-related surgery, were found to decrease QoL in IBD patients in previous studies[56-59], which explained why fatigue also leads to a decrease in QoL in IBD patients. Our further analysis found that psychological factors are particularly important in the relationship between fatigue and QoL. To improve the QoL of patients with IBD, the risk factors for fatigue should be identified and corrected in time to prevent the occurrence of fatigue. In addition to the impact on the QoL of the patient, fatigue can also lead to WP loss or even unemployment, which has significant economic consequences. Our study found that fatigue had an impact on the OWI, activity impairment, presenteeism, and absenteeism, among which general fatigue had the strongest impact. The appeal conclusion showed that the effect of fatigue on WP loss was also the result of a comprehensive effect, in which physical and psychological factors played an important role.

Although fatigue and its negative consequences are common in patients with IBD, the issue is rarely discussed in China. The underlying cause may be fatigue, especially during remission, which is considered a difficult and frustrating symptom, and the risk factors for fatigue are unclear, so there is little opportunity to help patients. Indeed, in China, there are few studies on the relationship between IBD and fatigue, which cannot provide clinical guidance. It is hoped that our findings will draw the attention of clinicians and patients to the role of fatigue in patients with IBD, improve the QoL of patients with IBD, and reduce the loss of WP by intervening in risk factors that contribute to fatigue. For example, in clinical practice, the joint management of patients' fatigue and psychological disorders is very important. Patients with depression and anxiety should pay close attention to their fatigue through targeted psychological counseling and intervention, such as health lectures, psychological counseling, individual counseling, and other ways to reduce patients' depression and anxiety, improve patients' fatigue, promote patients' health, and improve their QoL. IBD patients with anemia should correct their anemia in time. For IBD patients who have undergone surgery, postoperative complications, postoperative pain, and patients' fear of colostomy nursing should be properly addressed.

There are several limitations to our study. First, the size of the study sample was too small. Further large sample size studies are warranted for a more accurate estimation of the prevalence of fatigue and definitive identification of risk factors for fatigue. Second, our study was a single-center clinical study, which cannot represent the overall situation of IBD patients in China.

CONCLUSION

In conclusion, we have shown that the prevalence of fatigue is considerably high in patients with IBD in Eastern China, even in clinical remission, and the risk factors for fatigue are depression, anxiety, anemia, and IBD-related surgery. Female sex, disease activity, and hypoalbuminemia do not increase the risk of fatigue. In addition, fatigue reduces the QoL of IBD patients in Eastern China and damages WP. The results of our study provide a scientific basis for effectively preventing and improving fatigue in IBD

patients.

ARTICLE HIGHLIGHTS

Research background

Fatigue is frequent and disabling in patients with inflammatory bowel disease (IBD), but the prevalence and risk factors for fatigue in Chinese patients with IBD are unknown. In addition, neither the relationship between fatigue and quality of life (QoL) nor the relationship between fatigue and work productivity (WP) has been reported in Chinese IBD patients.

Research motivation

Fatigue is a very common but relatively neglected problem in patients with IBD. The prevalence rate of IBD in China is the highest in Asia, but there is little research on fatigue in patients with IBD. Neither the relationship between fatigue and QoL nor the relationship between fatigue and WP in Chinese IBD patients has been reported.

Research objectives

Our primary aim was to investigate the prevalence of fatigue related to IBD in Eastern China, and to identify the risk factors associated with fatigue. Our second objective was to assess the impact of fatigue on QoL and to evaluate the relationship between fatigue and WP.

Research methods

A cross-sectional study was conducted in a Regional Tertiary IBD Diagnostic and Treatment Center in Eastern China. Clinical data of patients were collected, and disease activity was evaluated. Blood samples were analyzed to assess anemia, albumin, and inflammation. Fatigue was assessed using the multidimensional fatigue inventory. QoL and WP were measured using the short inflammatory bowel disease questionnaire and the work productivity and activity impairment general health questionnaire, respectively. The patients also completed assessments of depression (Patient Health Questionnaire-9) and anxiety (Generalized Anxiety Disorder 7-item Scale).

Research results

A total of 311 IBD patients were enrolled in this study, 168 of whom were Crohn's disease patients, and 143 of whom were ulcerative colitis patients. The prevalence of fatigue in patients with IBD was 60.77%, including 71.88% in the active stage and 49.01% in the remission stage. The median fatigue total score was 43 (IQR: 33-59) in the full study population. In a univariate logistic regression analysis, factors such as disease activity, depression, anxiety, anemia, and IBD-related surgery were individually related to a significantly increased risk of fatigue in IBD patients. Multivariate logistic regression analysis indicated that depression [odds ratio (OR) = 8.078, 95% confidence interval (CI): 4.113-15.865], anxiety (OR = 2.373, 95%CI: 1.100-5.119), anemia (OR = 2.498, 95%CI: 1.290-4.834), and IBD-related surgery (OR = 2.035, 95%CI: 1.084-3.819) were related to fatigue in IBD patients. There was a negative correlation between fatigue and QoL (r = -0.831; P < 0.0001) but a positive correlation between fatigue and WP loss.

Research conclusions

The prevalence of fatigue in IBD patients in Eastern China is remarkably high even in clinical remission. Factors such as depression, anxiety, anemia, and IBD-related surgery are major risk factors for fatigue in IBD patients. In addition, fatigue has a negative impact on QoL and is positively correlated with WP loss.

Research perspectives

The prevalence of fatigue is considerably high in IBD patients in Eastern China even in clinical remission. In addition, fatigue reduces the QoL of IBD patients in Eastern China and damages WP. Clinicians and patients should be aware of and prevent the incidence of fatigue. The future research direction is to conduct a multicenter study to evaluate the incidence of fatigue in Chinses IBD patients, and more accurately screen out the risk factors leading to the incidence of fatigue in Chinese IBD patients, to effectively prevent the incidence of fatigue.

ACKNOWLEDGEMENTS

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ORIGINAL ARTICLE

Clinical Trials Study

Prospective single-blinded single-center randomized controlled trial of Prep Kit-C and Moviprep: Does underlying inflammatory bowel disease impact tolerability and efficacy?

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Author contributions: Mohsen W and Williams AJ collected data, compiled data, blinded endoscopist (fellow), recruited patients from clinic, organized spreadsheet, developed tables, liaised wrote the manuscript; Wark G collected data and developed the figures; Sechi A inflammatory bowel disease nurse consultant who assisted with education, recruitment of patients and answering patient concerns during the trial process; Koo JH blinded assisted with manuscript review, study design and statistical analysis; Xuan W is statistician from the Ingham institute who assisted with the statistical analysis; Bassan M assisted with the review of the manuscript; Ng W recruited patients, assisted with study design and assisted with review

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Abstract

BACKGROUND

Colonoscopy remains the gold standard for detection of colonic disease. An optimal evaluation depends on adequate bowel cleansing. Patients with inflammatory bowel disease (IBD), require frequent endoscopic assessment for both activity and dysplasia assessment. Two commonly used bowel preparations in Australia are Prep Kit-C (Pc) and Moviprep (Mp). Little is known about tolerability, efficacy and safety of split protocols of Mp and Pc in both IBD and non-IBD patients.

AIM

To primary aim was to compare the tolerability, efficacy and safety of split protocols of Mp and Pc in patients having a colonoscopy. The secondary aim was to compare the efficacy, tolerability and safety of either preparation in patients with or without IBD.

METHODS

Patients were randomized to Pc or Mp bowel preparation. Patients completed a

process and manuscript development; Connor S developed study design and end points for the study, applied for ethics approval, assisted with the development of the manuscript.

Institutional review board statement: Approval was obtained from the Institutional Human Ethics and Research Office (reference HREC/12/LPOOL/108).

Clinical trial registration statement: There was no clinical trial registration for this study.

Informed consent statement: Informed consent form was not required for this study as this was a prospective single blinded single centre study.

Conflict-of-interest statement: All of the authors have no conflict of interest to disclose. We did not receive commercial or government funding for this article.

Data sharing statement: All data included in this study is deidentified.

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questionnaire to assess tolerability. Efficacy was assessed using the Ottawa Bowel Preparation Score. Serum electrolytes and renal function were collected one week prior to colonoscopy and on the day of colonoscopy.

RESULTS

Of 338 patients met the inclusion criteria. Of 168 patients randomized to Mp and 170 to Pc. The efficacy of bowel preparation (mean Ottawa Bowel Preparation Score) was similar between Mp (5.4 ± 2.4) and Pc (5.1 ± 2.1) (P = 0.3). Mean tolerability scores were similar in Mp (11.84 ± 5.4) and Pc (10.99 ± 5.2; P = 0.17). 125 patients had IBD (73 had Crohn's Disease and 52 had Ulcerative colitis). Sixty-four IBD patients were allocated to Mp and 61 to Pc. In non-IBD patients, 104 were allocated to Mp and 109 to Pc. The mean tolerability score in the IBD group was lower than the non-IBD group (mean tolerability scores: IBD: 10.3 ± 5.1 and non-IBD: 12.0 ± 5.3; P = 0.01). IBD patients described more abdominal pain with Mp when compared with Pc; (Mp: 5.7 ± 4.4 *vs* Pc: 3.6 ± 2.6, P = 0.046). Serum magnesium level increased with Pc compared with Mp in all patients (mean increase in mmol/L: Mp: 0.03 ± 0.117 and Pc: 0.11 ± 0.106; P < 0.0001).

CONCLUSION

In this study, the efficacy, tolerability and safety of Mp and Pc were similar in all patients. However, patients with IBD reported lower tolerability with both preparations. Specifically, IBD patients had more abdominal pain with Mp. These results should be considered when recommending bowel preparation especially to IBD patients.

Key Words: Bowel preparation; Inflammatory bowel disease; Tolerability; Efficacy; Moviprep; Prep Kit-C

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Core Tip: When comparing Moviprep (Mp) and Prep-Kit C (Pc) in patients with and without inflammatory bowel disease (IBD): (1) Efficacy, tolerability and safety of Mp and Pc are similar; (2) Participants with IBD reported lower tolerability with both preparations; (3) IBD participants described more abdominal pain with Mp. Consideration of these results are important when discussing bowel preparation with IBD patients.

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INTRODUCTION

Colonoscopy remains the gold standard for detection of colonic disease. An optimal evaluation depends on adequate bowel cleansing. Suboptimal preparation occurs in up to 25% of colonoscopies and results in aborted or incomplete examinations in up to 7% of procedures[1,2]. Suboptimal preparation is associated with longer procedural time, increased need for repeat procedures, lower overall polyp detection rates, including detection of flat (non-polypoid) lesions, small polyps (< 10 mm) and large polyps (> 10 mm)[1,3]. The American Society for Gastrointestinal Endoscopy recommends the rate of inadequate bowel preparation should not exceed 15%[4].

Efficacious bowel preparation is not solely dependent on the type of preparation used. Preparation is enhanced when instructions regarding bowel preparation are explained thoroughly, interpreters are used (when required), a split regime is used and when the type of preparation is individualized to the patient's age and comorbidities[5,6].

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Adequate bowel preparation is particularly important in patients with inflammatory bowel disease (IBD). These patients have an increased risk of developing colonic dysplasia and neoplasia. The increasingly adopted technique of chromoendoscopy is also highly dependent on excellent bowel cleansing[7]. With the increasing annual incidence (24 per 100000) and prevalence (345 per 100000) of IBD in Australia[8], efficacious colonoscopy is crucial. Low tolerability of bowel preparation is reported in IBD patients, although this has not been prospectively validated[9]. The exact mechanism driving such low tolerability is unclear. It may relate to abdominal pain, nausea and vomiting[1,9]. Additional factors that have been reported include previous surgery, intestinal stenosis, altered motility, anxiety, or heightened visceral sensitivity and pre-procedure dietary recommendations[1,9].

In the general population, poor bowel preparation is more commonly seen in males, smokers, the elderly, patients with a history of stroke, dementia, diabetes, previous colonic resection and in patients who take opioids, psychotropic drugs and calcium channel blockers[4,10-12]. Tolerability is one of the most significant factors contributing to efficacy of preparation. Efficacy and tolerability are related, and synergistically both contribute to "effectiveness" of a preparation[13]. If the preparation is not well tolerated, even if otherwise efficacious, it will not be consumed, leading to reduced effectiveness.

In Australia, several bowel cleansing agents are available. Bowel preparations are usually based on solutions of polyethylene glycol (PEG)[14,15]. Prep Kit-C (Pc) is a combination of Picoprep (Sodium picosulphate/magnesium citrate) and glycoprep (PEG). Picoprep is a small volume, hyperosmotic solution, primarily exerting its action through osmotically drawing fluid into the intestinal lumen. Moviprep (Mp) is a combination of low volume PEG solution with ascorbic acid. The ascorbic acid has osmotic laxative effects and a pleasant taste[14,16]. Both Pc and Mp are approved for use under the Australian therapeutic goods administration.

At present, there are no prospective studies which examine tolerability, efficacy and safety of Pc when compared with Mp in both the general and IBD populations. This study's primary aim was to compare tolerability, efficacy and safety of split protocols of Mp with Pc in participants having a colonoscopy. The secondary aim was to compare the efficacy and tolerability of either preparation in participants with or without IBD.

MATERIALS AND METHODS

Methods

A prospective, randomized, single blinded trial was conducted at a single tertiary referral center. Recruitment of patients occurred from March 2013 to December 2016. Approval was obtained from the Institutional Human Ethics and Research Office (reference HREC/12/LPOOL/108).

Inclusion criteria

All patients aged between 18-75 years requiring an outpatient colonoscopy were invited to participate in this study. Patients identified as having IBD required histological evidence of Crohn's disease or ulcerative colitis from a previous colonoscopy.

Exclusion criteria

The following were exclusion criteria: non–English speaking, renal insufficiency (defined as an estimated Glomerular Filtration Ratio of less than 50 mL/min), cardiac failure (New York Heart Association Class greater than two), advanced liver disease (Child-Pugh B or C), poorly controlled diabetes mellitus (uninterrupted Hba1c > 8.0% for greater than one year and/or end organ complications from diabetes mellitus), bowel obstruction, total or limited colonic resection, megacolon, dysphagia and pregnancy or planning to become pregnant during the trial period. Patients with IBD who had a preceding colectomy or ileocolonic resection (that involved or extended beyond the hepatic flexure) were also excluded from this study.

Randomization

All participants were randomly allocated to a bowel preparation regime (Mp or Pc) at time of study recruitment in a 1:1 ratio. The allocation sequence was provided by the coordinating investigator. The investigator drew the patient allocated preparation out of an envelope which had equal numbers of both preparations. Patients were provided

with their assigned bowel cleansing preparation at the time of randomization. The cohort was then stratified according to presence of IBD. Patients were unable to be blinded to their allocated preparation due to associated packaging and the differences in administration. Written information about the bowel preparation including appropriate diet and timing of consumption was provided and explained in detail at a clinic review prior to colonoscopy. These instructions are provided in Supplementary material 1 . All assessing endoscopists were blinded to the assigned bowel preparation.

Outcomes

The primary endpoint was the tolerability and efficacy of each bowel preparation in the entire cohort. The secondary endpoints were comparison of the tolerability and efficacy of the allocated bowel preparation in patients with and without IBD, as well as overall safety of bowel preparation.

Tolerability and side effects

Tolerability was assessed using a Tolerability Questionnaire modified from Lawrance *et al*[17] (Supplementary material 2). Patients received the questionnaire at their pre-assessment visit and completed it after finishing their bowel preparation on the day of their colonoscopy. The questionnaire included a five-point Likert scale to assess tolerability (ranging from 0 to 5) and palatability (ranging from 0 to 5) of the preparation. A lower score indicated poorer tolerance. Common side effects (abdominal discomfort, abdominal pain, nausea, vomiting, abdominal distension, dizziness and shortness of breath) were also measured on a five-point Likert scale (ranging from 0 to 5). A higher score indicated worse reported side effects.

Colonoscopy

Patients were provided with written instructions and the bowel preparation explained in detail by the recruiting investigator at the time of study recruitment (full preparation instructions are available in Supplementary material 2). Apart from the preparation agent, preparation was standardized between the two groups including split dosing and 24 h of clear fluids. Colonoscopies were performed by experienced consultant colonoscopists (n = 4) or advanced gastroenterology trainees under the direct supervision of one of the colonoscopists. All procedures were performed using intravenous sedation administered by an anesthetist.

Efficacy

Efficacy of colon cleansing was assessed using the validated Ottawa Bowel Preparation Score (OBPS)[18]. All colonoscopists attended calibrating sessions prior to study commencement. Two colonoscopists were blinded to the allocated bowel preparation, independently assessed the efficacy of bowel cleansing regime during insertion of the colonoscope, prior to washing. The OBPS grades the quality of bowel preparation (0 to 4, with 0 being no fluid and 4 pertaining to fluid/fecal material unable to be cleared) in three colonic segments (right, left, recto-sigmoid) in addition to an overall fluid score. The total score out of 14 was provided for each patient and an average score calculated from both scores. A score of zero represents excellent preparation and 14 represents solid stool in each segment and excessive fluid. Inadequate bowel preparation is defined as an OBPS score equal to or greater than 8[19,20].

Safety: Electrolyte analysis

Safety of each bowel preparation included determination of electrolyte alteration. Blood was collected from each patient within one week before bowel preparation and on the day of colonoscopy prior to the procedure for serum electrolytes. Changes in serum sodium, chloride, potassium, bicarbonate, urea, creatinine, magnesium, calcium and phosphate were measured.

Statistical analyses

For the primary analysis in the entire cohort, an estimated sample size of 127 patients in each group was calculated to detect a 15% difference in the tolerability of bowel preparation between Mp and Pc, with 95% confidence and 90% power. Preliminary data using the same Tolerability Questionnaire which reported the mean tolerability of Moviprep of 13.3 (standard deviation 4.9) in patients undergoing colonoscopy was used to guide the sample size calculation[21]. The difference in tolerability of 15% between bowel preparation regimes was selected as this was also used in another

study assessing tolerability of different bowel preparations[17]. Assuming a completion rate of 80%, a target of at least 159 participants for recruitment in each group was sought, giving a sample size of at least 318. The student *t*-test was used to compare the differences in mean scores of tolerability and efficacy. Associations between categorical variables and outcomes were assessed using Chi-square test. The IBM Statistical Package for the Social Sciences for Windows version 25.0 (IBM corporation, Armonk, NY, United States) was used to analyze the data.

RESULTS

Participant characteristics

From March 2013 to December 2016, 338 patients were enrolled in the study. 168 patients were randomized to Mp and 170 to Pc (Figure 1). One hundred and twenty-five patients had a pre-existing diagnosis of IBD (58% patients with Crohn's disease and 42% with ulcerative colitis). In the IBD group, 64 patients had Mp and 61 had Pc. In the non-IBD group, 104 patients had Mp and 109 had Pc (Figure 1). Within both the IBD and non-IBD groups, there was no difference in age or gender distribution across the allocated bowel preparation groups (Table 1). Forty percent (n = 86) of the non-IBD cohort were male, compared with 52% (n = 65) in the IBD cohort. The mean ages of the IBD and non-IBD groups were 40.3 ± 14.7 and 50.3 ± 13.4 years respectively (P = 0.65).

Tolerability and side effects

Of the 338 patients, 288 (85%) completed the questionnaire assessing tolerability (Figure 1), this proportion was similar in both the Mp and Pc groups. There were no significant differences in the mean scores for tolerability between Mp (11.84 ± 5.4) and Pc groups (10.99 ± 5.2; P = 0.17). Thirty and 20 patients from the IBD and non-IBD groups respectively did not complete the tolerability questionnaire. The tolerability score in the IBD (n = 95) group was significantly lower than the non-IBD group (n = 193) (10.3 ± 5.1 *vs* 12.0 ± 5.3, P = 0.01) (Figure 2), indicating poorer tolerability in this group of patients.

The IBD group reported higher score (indicating worse) for abdominal pain (mean 4.78 *vs* 3.39; P = 0.031) and lower mean score for dizziness (0.37 *vs* 0.78; P = 0.03), and shortness of breath (mean 0.09 *vs* 0.39; P = 0.042) compared with the non-IBD group. The mean scores for nausea/vomiting were similar in both groups (mean 1.15 *vs* 1.65; P = 0.14) (Figure 3). Within the IBD group, patients who had Mp reported more abdominal pain when compared with Pc (mean 5.7 *vs* 3.62; P = 0.046). There were no other significant differences in the mean scores for other symptoms within the non-IBD or IBD group.

When comparing the overall tolerability of Pc (n = 145) with Mp (n = 143) in both the IBD and non-IBD groups, there was no statistically significant difference in mean tolerability scores between the two bowel preparations, although the study may not have been powered to detect a significant difference (Table 2).

Efficacy

Data on efficacy of the bowel preparation was available in 320 patients (95%). There was no difference in the efficacy within the entire group when comparing Mp to Pc [mean OBPS: Mp (n = 158; 5.4 ± 2.4) and Pc (n = 162; 5.1 ± 2.1; P = 0.73)], nor within both the IBD [mean OBPS: Mp (n = 58; 4.8 ± 2.9) and Pc (n = 56; 5.2 ± 3.3; P = 0.53)] and non-IBD [mean OBPS: Mp (n = 100; 5.5 ± 2.4) and Pc (n = 106; 5.4 ± 2.1; P = 0.84)] groups.

Efficacy of bowel preparation when comparing the IBD (n = 114) to the non-IBD (n = 206) group was not significantly different (P = 0.26). Inadequate bowel preparation (defined as an OBPS of greater than or equal to 8)[17,19] was present in 8.9% (n = 29) of all patients: 10.5% (n = 12) of the IBD group and 8% (n = 17) of the non-IBD group.

Safety: Electrolyte analysis

Electrolyte data was available for 256 patients (78%). There was a statistically significant increase in magnesium in patients who received Pc compared with Mp (mean increase in mmol/L: Mp 0.03 ± 0.117 and Pc 0.11 ± 0.106; P < 0.0001) (Figure 4). There were no additional differences detected in the remaining electrolytes. There were no reported clinical concerns attributed to electrolyte abnormalities during the peri-procedural period, such as arrhythmias, exacerbation of congestive cardiac failure or acute pulmonary edema.

Table 1 Baseline characteristics

	IBD cohort (*n* = 125)			Non-IBD cohort (*n* = 213)			*P* value
mean age +/- SD (yr)	40.3 ± 14.7			50.3 ± 13.4			0.65
	Prep Kit -C	Moviprep	*P* value	Prep Kit -C	Moviprep	*P* value	
mean ± SD, age (yr)	39.7 ± 14.27	40.9 ± 15.1	0.65	52.98 ± 12.97	53.65 ± 13.98	0.72	
Male (*n*)	35	30	0.13	39	47	0.93	

SD: Standard deviation; IBD: Inflammatory bowel disease.

Table 2 Tolerability Scores in the inflammatory bowel disease and non-inflammatory bowel disease cohorts

	Prep Kit-C (*n* = 145)	Moviprep (*n* = 143)	*P* value
IBD, *n* = 95	9.67 ± 4.87; *n* = 47	10.89 ± 5.21; *n* = 48	0.25
Non-IBD, *n* = 193	11.61 ± 5.32; *n* = 98	12.32 ± 5.35; *n* = 95	0.36

IBD: Inflammatory bowel disease.

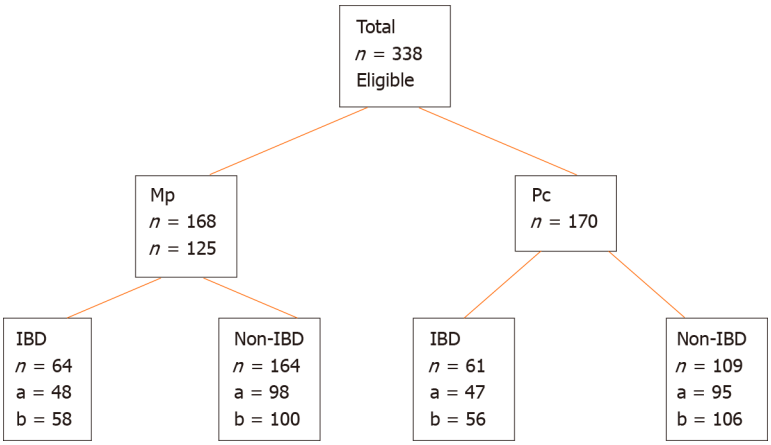


Figure 1 Randomization of bowel preparation. a: Number of patients who completed tolerability questionnaire; b: Number of patients with validated Ottawa Bowel Preparation Scores; Mp: Moviprep; Pc: Prep Kit-C; IBD: Inflammatory bowel disease.

DISCUSSION

This study has demonstrated no significant differences in the tolerability and efficacy of bowel preparation when comparing Mp with Pc. However, subgroup analysis revealed IBD patients were less tolerant of bowel preparation when compared with patients without IBD. IBD patients reported more abdomen pain with both preparations when compared with the non-IBD group. Within the IBD group, Mp produced more abdomen pain compared with Pc. Safety was comparable for IBD and non-IBD patients, although Pc resulted in a higher magnesium level than Mp.

The influence of effective bowel preparation on the quality of colonoscopy is substantial, as recently highlighted by the inclusion of bowel preparation adequacy and safety in the Australian Colonoscopy Care Standards formulated by the Commission on Safety and Quality in Health Care[22]. Systematic reviews have not demonstrated superiority of any specific bowel preparation regimes when assessing efficacy in both the non-IBD population as well as in those with IBD[14,23,24]. At our center, as well as many in Australia, Mp and Pc are commonly recommended bowel preparations. Prior to this study, there have been no prospective studies which compare the efficacy of Pc with Mp in non-IBD or IBD populations. Consistent with systematic reviews for other bowel preparations, our study demonstrated no significant difference in bowel preparation efficacy between Mp and Pc in both IBD and non-IBD populations. Our findings supported both Pc and Mp as suitable choices

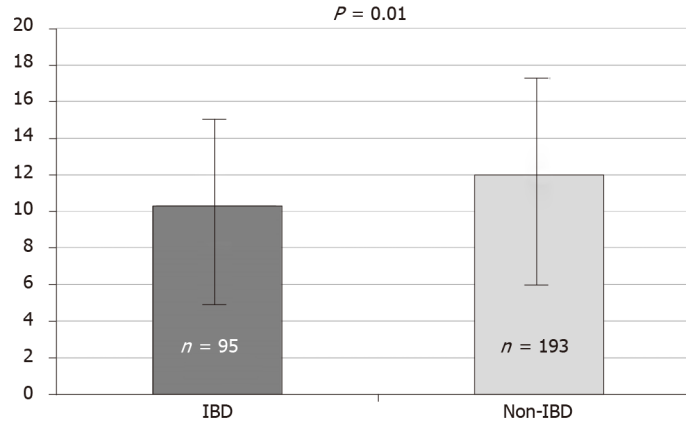


Figure 2 Total tolerability scores when comparing inflammatory bowel disease and non-inflammatory bowel disease cohorts. Of 95 inflammatory bowel disease and 193 non- inflammatory bowel disease participants included. Higher score indicates better tolerability where 0 = poorly tolerated and 5 = well tolerated**.** Total score is out of 20 (0-5 for taste; 0-5 ease of ingestion; 0-5 for palatability; 0-5 for amount). IBD: Inflammatory bowel disease.

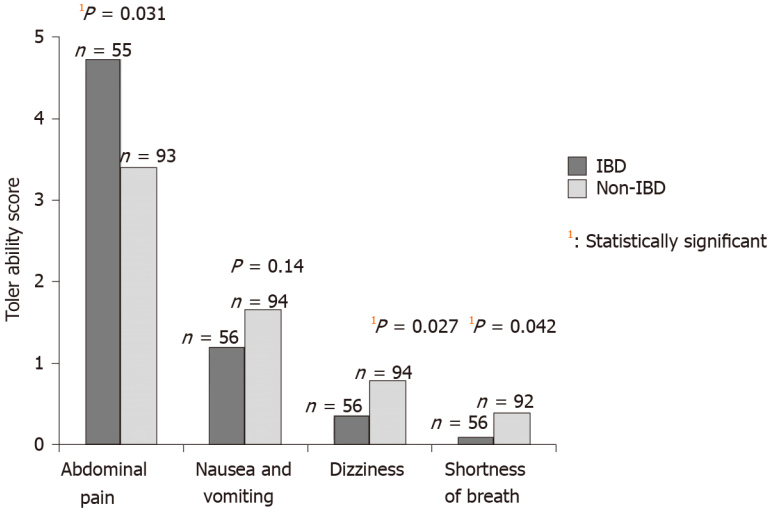


Figure 3 Tolerability scores according to specified symptom. Of 56 inflammatory bowel disease and 93 non-inflammatory bowel disease participants compared. 0 = well tolerated and 5 = poorly tolerated. Maximum score for abdominal pain is 15 (0-5 points abdominal discomfort; 0-5 points for abdominal pain; 0-5 points for abdominal distension). Maximum score for nausea and vomiting is ten (0-5 points for nausea; 0-5 points for vomiting). The maximum points for dizziness or shortness of breath are 5 points. IBD: Inflammatory bowel disease.

when considering efficacy of bowel preparation regimes in patients with and without IBD[1,19,20]. Nine percent of our overall study population had inadequate bowel preparation, which falls within the American Society for Gastrointestinal Endoscopy guidelines for adequate bowel preparation in at least 85% of patients[4].

Our study was unique in that both our IBD and non-IBD patients prospectively completed tolerability questionnaires at the time of bowel preparation ingestion. It was observed that IBD patients were less tolerant of bowel preparation when compared with patients without IBD, though the type of bowel preparation did not affect the total tolerability score when comparing IBD with the non-IBD groups. IBD patients also reported more abdominal pain when compared to non-IBD patients.

Poorer tolerability of bowel preparation within IBD cohorts is consistent with previously published literature. Denters *et al*[25] reported significantly more psychological and physical burden from bowel preparation in patients with IBD when compared with other patient groups. In another study, IBD patients most commonly cited difficulty with bowel preparation as the most important reason for failed compliance with scheduled colonoscopies for colorectal cancer surveillance[9]. Tolerability of bowel preparation in IBD patients may not be entirely related to luminal pathology. In another study, tolerance of bowel preparation was similar when comparing IBD and non-IBD cohorts, however co-morbid anxiety played a role in symptom development during bowel preparation in IBD patients[26].

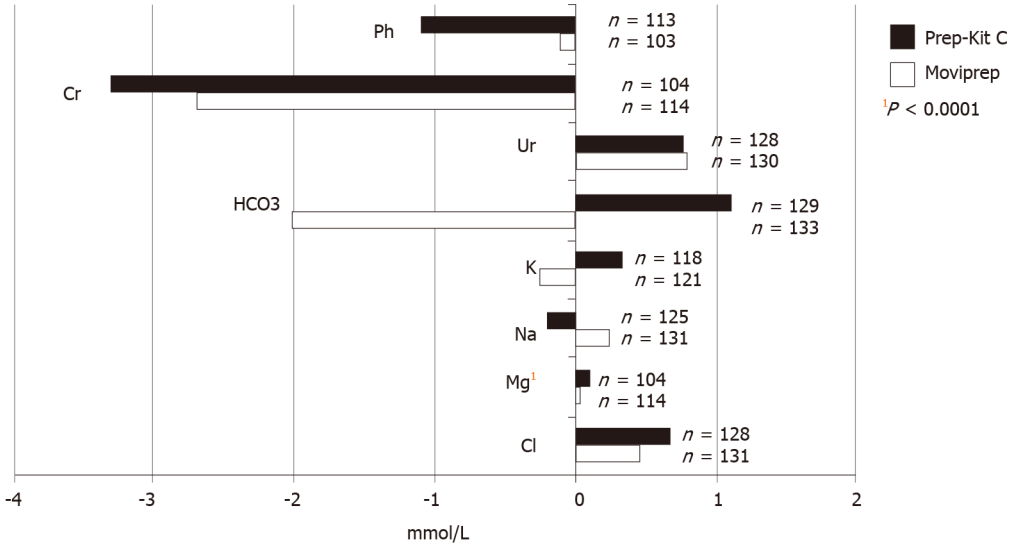


Figure 4 Changes in electrolyte levels (*n* = 256) measured in mmol/L. Levels compared between one week prior to procedure and day of the procedure.

Our study provides further impetus to reinforce the importance of educating IBD patients about bowel preparation, including the possibility for reduced tolerance and more abdomen pain. IBD patient awareness about potentially poor tolerance prior to ingestion may positively impact on the bowel preparation quality and compliance with surveillance protocols. Dietary liberalization, specifically using the white or low residue diet has been shown to be better tolerated and as efficacious as a clear fluid diet[27]. Tolerability of the white diet in comparison with the clear fluid diet, prior to colonoscopy, within the IBD population is a future research area.

Our study supports the safety of both Mp and Pc. There were no reported adverse clinical outcomes. A statistically significant increase in serum magnesium level with the use of Pc when compared with Mp was identified but it was of a small magnitude and unlikely to be clinically significant. Whilst there have been no prospective studies comparing electrolyte changes or adverse outcomes in patients taking Mp compared with Pc, our study is in line with other studies which have shown that Pc can cause electrolyte derangement[24]. Thus, Pc should be avoided in the elderly and patients with renal impairment[24].

Our study has several limitations. In relation to assessment of bowel preparation tolerability, our study utilized a modified, un-validated questionnaire developed by our study team based on an existing questionnaire[17]. Whilst we acknowledge this limitation, the same questionnaire was used in all study arms (Mp and Pc; IBD and non-IBD), and the questionnaire completion rate was equivalent amongst all study arms. Furthermore, tolerability of bowel preparation may have been influenced by the volume of fluid (*e.g.*, water) replacement consumed by each participant in addition to the actual bowel preparation. This was not standardized between groups (Supplementary material 1). The tolerability questionnaire was completed just prior to the colonoscopy. As a result, delayed tolerability side effects from the allocated preparation may have been missed. Lastly, we did not collect data about variables which may influence bowel preparation efficacy. These variables include smoking history, medication history, history of Diabetes Mellitus or disease activity in IBD.

CONCLUSION

Our prospective, randomized controlled study has compared the tolerability, efficacy and safety of Mp and Pc in non-IBD and IBD patients. We demonstrated that both Mp and Pc had similar efficacy of bowel preparation in either the non-IBD or IBD cohorts. However, IBD patients were less tolerant of bowel preparation and reported more abdomen pain compared with patients without IBD. Furthermore, IBD patients reported more abdominal pain with Mp compared with Pc. Future research opportunities in this field include assessing factors contributing to poor bowel preparation tolerability in IBD patients is required.

ARTICLE HIGHLIGHTS

Research background

Colonoscopy remains the gold standard for detection of colonic disease. An optimal evaluation depends on adequate bowel cleansing. Patients with inflammatory bowel disease (IBD), require frequent endoscopic assessment for both activity and dysplasia assessment. Two commonly used bowel preparations in Australia are Prep Kit-C (Pc) and Moviprep (Mp). Little is known about tolerability, efficacy and safety of split protocols of Mp and Pc in both IBD and non-IBD patients.

Research motivation

To determine which bowel preparation is tolerable and effective in both IBD and non-IBD patients. Efficacy and tolerability are related, and both contribute to effectiveness. By maximizing effectiveness we minimise the chances of inadequate bowel cleansing and incomplete colonoscopy. This ensures that hospital and patient resources are not wasted.

Research objectives

This study's primary aim was to compare tolerability, efficacy and safety of split protocols of Mp with Pc in participants having a colonoscopy. The secondary aim was to compare the efficacy and tolerability of either preparation in participants with or without IBD.

Research methods

Patients were randomized to Pc or Mp bowel preparation. Patients completed a questionnaire to assess tolerability. Efficacy was assessed using the Ottawa Bowel Preparation Score. Serum electrolytes and renal function were collected one week prior to colonoscopy and on the day of colonoscopy.

Research results

Of 338 patients met the inclusion criteria. Of 168 patients randomized to Mp and 170 to Pc. The efficacy of bowel preparation (mean Ottawa Bowel Preparation Score) was similar between Mp and Pc. Mean tolerability scores were similar in Mp and Pc. The mean tolerability score in the IBD group was lower than the non-IBD group. IBD patients described more abdominal pain with Mp when compared with Pc. Serum magnesium level increased with Pc compared with Mp in all patients.

Research conclusions

In this study, the efficacy, tolerability and safety of Mp and Pc were similar in all patients. However, patients with IBD reported lower tolerability with both preparations. Specifically, IBD patients had more abdominal pain with Mp.

Research perspectives

These results should be considered when recommending bowel preparation especially to IBD patients. More prospective studies are required in this field.

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ORIGINAL ARTICLE

Clinical Trials Study

Long-term follow-up of cumulative incidence of hepatocellular carcinoma in hepatitis B virus patients without antiviral therapy

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Author contributions: Tong GD conceived, supervised and guided implementation of the experiments; Jiang XY, Huang B, Huang DP and Wei CS conducted the experiments, collected and organized the data, and analyzed the results; Zhong WC, Peng DT and Huang FR wrote the original draft; all the authors revised the manuscript and approved the final version.

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Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of

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Abstract

BACKGROUND

China has a high prevalence of hepatitis B virus (HBV), but most chronic hepatitis B (CHB) patients do not receive standardized antiviral therapy. There are few relevant reports addressing the outcomes of the large number of CHB patients who do not receive antiviral therapy.

AIM

To observe the outcomes of long-term follow-up of patients with CHB without antiviral treatment.

METHODS

This study included 362 patients with CHB and 96 with hepatitis B cirrhosis without antiviral treatment and with only liver protection and anti-inflammatory treatment from 1993 to 1998. The median follow-up times were 10 and 7 years, respectively. A total of 203 CHB and 129 hepatitis B cirrhosis patients receiving antiviral therapy were selected as the control groups. The median follow-up times were 8 and 7 years, respectively. Kaplan-Meier curves were used to analyze the cumulative incidence of hepatocellular carcinoma (HCC), and the Cox regression model was used to analyze the risk factors for HCC.

RESULTS

Among the patients in the non-antiviral group, 16.9% had spontaneous decreases

Shenzhen Traditional Chinese Medicine Hospital, The Fourth Clinical Medical College of Guangzhou University of Chinese Medicine.

Clinical trial registration statement: This study was registered at Chinese Clinical Trial Registry (http://www.chictr.org.cn/index.aspx). The registration identification number is ChiCTR2000029281 (1/20/2020).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All authors and study participants declare no potential conflicting interests related to this paper.

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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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: China

Peer-review report's scientific quality classification

in HBV DNA to undetectable levels, and 32.8% showed hepatitis B e antigen (HBeAg) seroconversion. In the antiviral group, 87.2% of patients had undetectable HBV DNA, and 52% showed HBeAg seroconversion. Among CHB and hepatitis B cirrhosis patients, the cumulative incidence rates of HCC were 14.9% and 53.1%, respectively, in the non-antiviral group and were 10.7% and 31.9%, respectively, in the antiviral group. There was no difference between the two groups regarding the CHB patients (P = 0.842), but there was a difference between the groups regarding the hepatitis B cirrhosis patients (P = 0.026). The cumulative incidence rates of HCC were 1.6% and 22.3% (P = 0.022) in the groups with and without spontaneous HBeAg seroconversion, respectively. The incidence rates of HCC among patients with and without spontaneous declines in HBV DNA to undetectable levels were 1.6% and 19.1%, respectively (P = 0.051). There was no difference in the cumulative incidence of HCC between the two groups regarding the patients with drug-resistant CHB (P = 0.119), but there was a significant difference between the two groups regarding the patients with cirrhosis (P = 0.004). The Cox regression model was used for regression of the corrected REACH-B score, which showed that alanine aminotransferase > 400 U/L, history of diabetes, and family history of liver cancer were risk factors for HCC among men aged > 40 years (P < 0.05). Multifactorial analysis showed that a family history of HCC among men was a risk factor for HCC.

CONCLUSION

Antiviral therapy and non-antiviral therapy with liver protection and anti-inflammatory therapy can reduce the risk of HCC. Antiviral therapy may mask the spontaneous serological response of some patients during CHB. Therefore, the effect of early antiviral therapy on reducing the incidence of HCC cannot be overestimated.

Key Words: Chronic hepatitis B; Anti-inflammatory therapy; Hepatoprotective therapy; Cumulative incidence; Hepatocellular carcinoma; Antiviral therapy

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Core Tip: According to the status quo of antiviral therapy for chronic hepatitis B (CHB) in China, we conducted long-term follow-up of patients with CHB who were recommended to receive nucleoside antiviral therapy in accordance with the guidelines, but did not receive antiviral therapy. We found that early antiviral therapy in patients with CHB did not yield greater benefits in the incidence of hepatocellular carcinoma than hepatoprotective anti-inflammatory therapy. It is suggested that early antiviral therapy with nucleosides may mask spontaneous viral clearance and hepatitis B e antigen clearance in patients with CHB.

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INTRODUCTION

Approximately 45% of hepatocellular carcinomas (HCCs) in patients worldwide and 80% of HCCs in patients in China are caused by hepatitis B virus (HBV) infection[1]. According to the World Cancer Report published by the World Health Organization in 2014, the number of new cases of and deaths from HCC in China accounted for more than half of the total global number in 2012[2]. The high prevalence of HCC in China is mainly due to HBV infection[3,4].

Early studies suggest that effective antiviral therapy can reduce the incidence of HCC in patients with hepatitis B cirrhosis[5-7]. A clinical study in Hong Kong included

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Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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1446 patients with chronic hepatitis B (CHB) (including 482 patients with cirrhosis) who received entecavir treatment. The control group included 424 untreated patients (including 69 with cirrhosis). The cumulative incidence rates of HCC in patients with cirrhosis at 3 and 5 years were reduced in the treatment group[8]. Two studies in Japan showed similar results[9,10].

However, there is no consistent conclusion regarding the effect of antiviral therapy on reducing the incidence of HCC among patients with CHB without cirrhosis who have a low risk of HCC[11,12]. Many studies have found no significant reduction in the incidence of HCC in patients with CHB who benefit from antiviral therapy[13]. A Greek study followed up 818 patients with CHB. The results showed that 49 patients developed HCC and that the cumulative incidence of HCC at 5 years was 3.2%. The incidence rates of HCC among patients aged < 50 years, 50-60 years and > 60 years were 0.7%, 6.7% and 11.7%, respectively. Antiviral therapy did not reduce the incidence of HCC associated with age. Multivariate analysis showed that age, sex and cirrhosis were independent risk factors for HCC, regardless of antiviral therapy[14]. A recent Caucasian study found that among 1666 patients with CHB who received entecavir or tenofovir antiviral therapy, the incidence rates of HCC at 1, 3 and 5 years were 1.3%, 3.4% and 8.7%, respectively[15]. The cumulative incidence of HCC has been increasing even with HBV suppression. With the prolongation of follow-up, the incidence of HCC is predicted to increase.

The occurrence of HCC is related to a high viral load and to a long-term and continuous increase in alanine aminotransferase (ALT). The REVEAL study suggested that HCC is associated with a sustained increase in serum ALT levels[16]. Elevated ALT is an indicator of hepatocyte injury or inflammation. Liver fibrosis and cirrhosis caused by chronic liver inflammation are the pathophysiological and histological bases for HCC progression in patients with hepatitis B[17]. Patients with CHB and persistent or repeated elevations in ALT have significantly higher risks of cirrhosis, hepatic decompensation, and HCC than those with persistently normal ALT levels or with fluctuations that return to normal[18,19].

Anti-inflammatory and hepatoprotective therapy is an important approach for CHB in China[20] and effectively inhibits the inflammatory response of the liver and promotes repair of damaged hepatocytes. Studies have shown that anti-inflammatory and hepatoprotective therapy can delay or even prevent the development of CHB into cirrhosis, indicating its high clinical value[21,22]. Antiviral therapy is also effective in controlling liver inflammation, but the ALT levels of 20% of patients still fail to return to normal afterwards[23]. Abnormal ALT levels during the first year of treatment in patients with CHB are associated with an increased risk of HCC[23].

In China, there are approximately 30 million patients with CHB, but only 11% of these patients receive standardized antiviral therapy[24]. Currently, there are few relevant reports addressing the outcomes of the large number of CHB patients who do not receive antiviral therapy. In our observation group, we included 362 patients with CHB and 96 with hepatitis B cirrhosis who were not treated with antiviral therapy but had been treated with anti-inflammatory and hepatoprotective drugs for a long time. The median follow-up times were 10 and 7 years, respectively. A total of 203 patients with CHB and 129 patients with hepatitis B cirrhosis receiving antiviral therapy were included as the control group. The median follow-up times were 8 and 7 years, respectively.

MATERIALS AND METHODS

Observation group

This study comprised 3500 patients with CHB who were hospitalized for the first time in the Department of Hepatology, Shenzhen Hospital of Traditional Chinese Medicine between January 1993 and December 1998 due to abnormal liver function (ALT ≥ 40 U/L). According to the inclusion and exclusion criteria, we enrolled 362 patients with CHB and 96 patients with cirrhosis who were treated with anti-inflammatory and hepatoprotective drugs without antiviral therapy. The median HBV-DNA (log) load was 7.14, and the median ALT level was 188.62 U/L. These patients should have been treated with antiviral therapy, but for various reasons, they did not receive antiviral therapy.

Control group

We collected data for 3897 patients with CHB who received antiviral therapy when they were admitted to the Department of Shenzhen Hospital of Traditional Chinese

Medicine between January 1999 and December 2007 and who received antiviral therapy at the initial visit. According to the inclusion and exclusion criteria, we enrolled 203 patients with CHB and 129 patients with hepatitis B cirrhosis.

Inclusion criteria

CHB without cirrhosis: (1) Patients were positive for hepatitis B surface antigen (HBsAg) for at least 6 mo; (2) Aged 18-75 years; (3) No treatment with interferon; (4) Patients with anti-inflammatory and hepatoprotective drug treatment had ALT ≥ 2 upper limit of normal, HBV-DNA positivity and follow-up times ≥ 2 years; and (5) Patients with antiviral treatment had voluntary acceptance of nucleoside antiviral therapy, follow-up time of ≥ 2 years, and treatment with anti-inflammatory and hepatoprotective drugs for ≤ 6 mo. Hepatitis B cirrhosis patients: (1) Cirrhosis diagnosed by imaging or histology at enrollment; and (2) Child-Turcotte-Pugh score ≥ 7 points defined as decompensated.

Exclusion criteria

The exclusion criteria were as follows: (1) CHB complicated by drug-induced liver damage, alcoholic liver disease, autoimmune liver disease or other liver diseases; (2) HCC; (3) Liver cancer diagnosed within 1 year after treatment; (4) Patients with anti-inflammatory and hepatoprotective therapy who were followed up for < 2 years after treatment; and (5) Patients with antiviral treatment who were followed up for < 2 years or who received anti-inflammatory and hepatoprotective treatment for > 6 mo.

Study design

This was an ambispective cohort study, with retrospective analyses before December 31, 2007, and prospective cohort analyses thereafter. The study was conducted and reported according to the study protocol, conforming to the ethical guidelines of the 1975 Declaration of Helsinki, which was approved by the Ethics Committee of Shenzhen Hospital of Traditional Chinese Medicine. All of the included patients were required to give signed informed consent.

Treatment

Observation group: Treatment consisted of glycyrrhizin preparation (oral or intravenous injection), glutathione (oral or intravenous injection), schisandra preparation (oral bicyclol, wuzhi capsule or tablet), and Silymarin. Control group: monotherapy consisted of lamivudine (LAM) 100 mg/d (Galans history Ke Pharmaceutical Company), adefovir (ADV) 10 mg/d (GlaxoSmithKline Pharmaceuticals), telbivudine (LDT) 600 mg/d (Beijing Novartis Pharmaceutical Co., Ltd.), or entecavir (ETV) 0.5 mg/d (China-US Shanghai Squibb Pharmaceutical Co., Ltd.), and combination therapy consisted of an initial combination or salvage treatment, namely, LAM + ADV, LDT + ADV, or ETV + ADV.

Follow-up procedure

The starting point was the time when each patient was enrolled for the first time, and the endpoint of follow-up was the time of study discontinuation or last follow-up visit before the patient was lost to follow-up. All patients were followed up at least every 6 mo. The follow-up times of the patients with anti-inflammatory and hepatoprotective treatment were ≥ 2 years. The addition of or switching between antiviral drugs was considered to be the endpoint of follow-up. Patients with antiviral therapy alone were not treated with anti-inflammatory or hepatoprotective therapy for ≥ 6 mo. The anti-inflammatory and hepatoprotective therapy patients were followed up for 2-23 years (1993-2016), and antiviral patients were followed up for 2-17 years (1999-2016) (Figure 1). Follow-up observation indicators were: (1) Liver function: ALT, aspartate aminotransferase (AST), and total bilirubin (TB); (2) HBV-DNA quantification; (3) HBV markers such as HBsAg and hepatitis B e antigen (HBeAg); (4) Routine blood tests; (5) B-mode Doppler imaging, computed tomography (CT) or magnetic resonance imaging (MRI); and (6) a-fetoprotein (AFP) detection.

Laboratory tests

(1) Liver function was tested with an Olympus 2700 automatic biochemical analyzer, and routine analysis of blood was performed with an XS-500i automatic analyzer; (2) HBV marker detection was performed using an ELISA method, with reagents provided by Shanghai Kehua Bioengineering Co., Ltd; (3) HBV-DNA quantitative analysis was performed using real-time fluorescent quantitative polymerase chain reaction (PCR) and COBASTaqMan HBV diagnostic reagents, and the reagents were

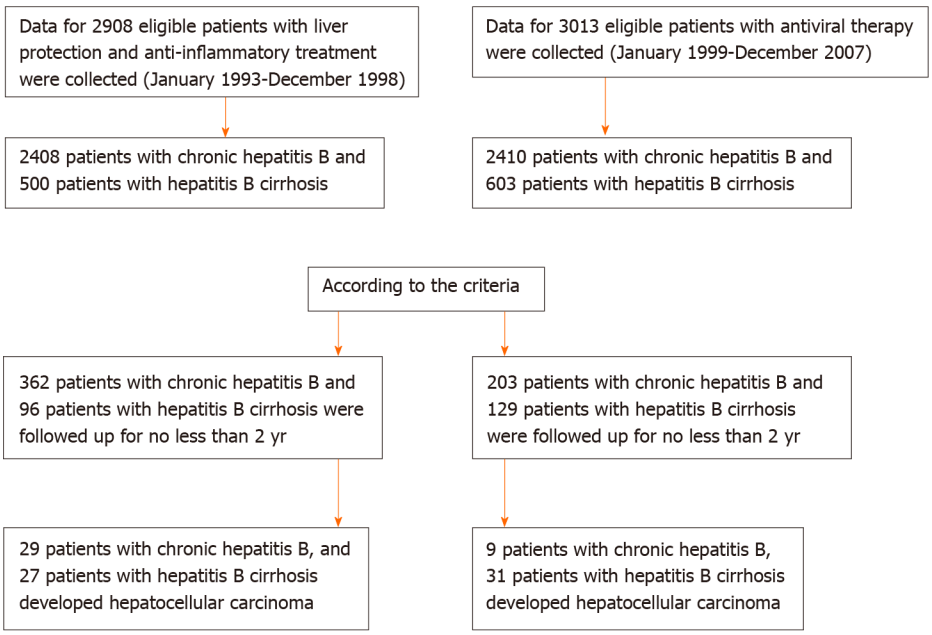


Figure 1 Flow chart of the control group.

provided by Shenzhen Piji Bioengineering Co., Ltd. and Roche Diagnostics Co., Ltd. The instruments used were the ABI PRISM 7000 fluorescence quantitative PCR instrument and COBAS Taqman48analyzer real-time quantitative PCR analyzer; (4) AFP measurements were performed using enzyme immunoassays, with a normal detection value of 20 ng/L; (5) B-mode Doppler imaging was performed using the Fynergy-type color dual-function Doppler produced by the Tyson Corporation. The B-ultrasound diagnostic criteria for cirrhosis were as follows: according to the integral classification standard of liver ultrasound parameters, the score was ≥ 10 points[25]; (6) Lesions in the liver were observed by B-ultrasound, CT and MRI. The CT spiral scanner was the Siemens Picker UltraZ super, and the MRI diagnostic instrument was the Philips intera2.0T, 3.0T high magnetic field superconducting magnetic resonance machine; and (7) For the liver biopsy specimens, the lengths were ≥ 1.5 cm, conventional paraffin sections were prepared for hematoxylin and eosin staining and Masson and reticulum fiber staining, and each specimen contained at least six junction areas.

Statistical analysis

This study used HCC as the endpoint of observation. The study deadline was December 31, 2016. The analysis of all patients with follow-up data and of those who were lost to follow-up was ended with the last clinical datapoints. For statistical analysis of differences between groups, qualitative data were analyzed using the c^2 test or Fisher's exact probability method, and continuous variables were analyzed using the Mann-Whitney test. The cumulative incidence of liver cancer was analyzed by Kaplan-Meier curves, and statistical significance was determined using the log-rank test. The Cox risk regression model was used to analyze the factors influencing liver cancer. All data were analyzed by SPSS version 22.0. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical baseline data of enrolled patients

There were 291 men and 71 women among the 362 patients with CHB in the observation group. There were 175 men and 28 women in the control group. According to the statistical analysis, there were significant differences in sex between the two groups ($P < 0.05$). There were 198 HBeAg-positive patients in the observation group and 123 in the control group. The difference in the proportions of HBeAg-positive patients in the two groups was significant ($P < 0.05$). In the observation group,

the median age was 33 years, and the median follow-up time was 10 years; in the control group, the median age was 39 years, and the median follow-up time was 8 years. There were significant differences in age and follow-up between the two groups ($P < 0.05$). However, there were no significant differences in the remaining indicators (Table 1).

In the observation group, there were 74 men and 22 women among the 96 patients with hepatitis B cirrhosis. In the control group, there were 119 men and 10 women among 129 patients with hepatitis B cirrhosis, and there were significant differences in sex between the two groups ($P < 0.05$). In the observation group, the median TB level was 39.10 mmol/L, and the median platelet count was 99.50 × 10^9/L. In the control group, the median TB level was 37.0 mmol/L, and the median platelet count was 107 × 10^9/L. There were significant differences in the TB and platelet levels between the two groups ($P < 0.05$). There were no significant differences in the other indicators (Table 1).

HBV-DNA, HBeAg, HBsAg and ALT testing at the end of follow-up

At the end of follow-up of the 362 CHB patients, HBV DNA was undetectable in 61 patients (16.6%) and decreased by no less than 2 Log in 216 patients (59.7%). Sixty-five patients (32.8%) were negative for HBeAg, three (0.8%) were negative for HBsAg, and 275 (76.0%) had normal ALT levels. However, among the 203 patients in the control group, 179 were HBV-DNA negative (87.2%), 194 (95.6%) had decreased HBV-DNA levels by no less than 2 Log, 64 (52.0%) were negative for HBeAg, two (0.6%) were negative for HBsAg, and 191 (94.1%) had normal ALT levels (Table 2).

At the end of the follow-up of the 96 patients with hepatitis B cirrhosis, 19 (19.8%) were negative for HBV DNA. Fifty-seven patients (59.37%) showed decreases in HBV-DNA of no less than 2 Log, 12 (40.0%) had HBeAg negative conversion, one had HBsAg negative conversion (1.0%), and 68 patients (70.8%) had normal ALT levels. In the control group of 129 patients, 116 (89.9%) were HBV-DNA negative, 124 (96.1%) had decreases in HBV DNA of no less than 2 Log, 19 (59.4%) had HBeAg negative conversion, one (1.6%) had HBsAg negative conversion, and 110 patients (85.3%) had normal ALT levels (Table 2).

Comparison of cumulative incidence of HCC in CHB patients

Among 362 patients with CHB, the cumulative incidence rates of HCC ($n = 29$) in years 2, 4, 6, 8, 10, 12, 14, 16 and 18 were 0, 0.008, 0.027, 0.045, 0.067, 0.096, 0.111, 0.135 and 0.149, respectively. The cumulative incidence rates of HCC ($n = 9$) among the 203 patients in the control group in years 2, 4, 6, 8, 10, 12, 14 and 16 were 0, 0.005, 0.022, 0.029, 0.066, 0.107, 0.107 and 0.107, respectively. After the Kaplan-Meier log-rank analysis, there was no significant difference in the cumulative incidence of HCC between the two groups ($P = 0.842$) (Figure 2A).

Comparison of cumulative incidence of HCC in patients with hepatitis B cirrhosis

Among the 96 patients with hepatitis B cirrhosis, the cumulative incidence rates of HCC ($n = 27$) in years 2, 4, 6, 8 and 10 in the observation group were 0, 0.065, 0.189, 0.446 and 0.531, respectively. The cumulative incidence rates of HCC ($n = 31$) in years 2, 4, 6, 8, 10, 12 and 14 among the 129 patients with cirrhosis were 0, 0.071, 0.138, 0.264, 0.319, 0.319 and 0.319, respectively. The incidence of HCC accumulation in the control group was lower than that in the observation group, and the results of the Kaplan–Meier log-rank analysis showed that there was a significant difference ($P = 0.026$) (Figure 2B).

Cumulative incidence of HCC after HBeAg negative conversion in HBeAg-positive CHB patients

Among 362 patients with CHB, 198 were HBeAg positive, 65 had HBeAg negative conversion, and one developed HCC after HBeAg negative conversion. The cumulative incidence rates of HCC ($n = 1$) among the 65 patients in years 2, 4 and 6-20 were 0, 0.016 and 0.016-0.016, respectively. Among the 133 patients without HBeAg negative conversion, 12 developed HCC. The cumulative incidence rates of HCC ($n = 12$) in years 2, 4, 6, 8, 10, 12, 14 and 16-20 were 0, 0.050, 0.062, 0.088, 0.104, 0.132, 0.167 and 0.223, respectively. The cumulative incidence rate of HCC in patients with CHB who did not have HBeAg negative conversion was higher than that in patients with HBeAg negative conversion. The cumulative incidence rates of HCC in the two groups were significantly different ($P = 0.022$) (Figure 3A).

Table 1 Data regarding the patients' baseline characteristics

	Chronic hepatitis B patients		Hepatitis B cirrhosis patients	
	Observation group (*n* = 362)	Control group (*n* = 203)	Observation group (*n* = 96)	Control group (*n* = 129)
Sex: Males (%)	291 (80.38)	175 (86.20)[a]	74 (77.08)	119 (92.24)[a]
E antigen-positive patients (%)	198 (54.69)	123 (60.59)[a]	30 (31.25)	32 (24.80)
Age (yr)	33 (25-40)	39 (32-46)[a]	59.50 (48-67)	48 (41-58)
Median follow-up time	10 (7-14)	8 (6-9)[a]	7 (5-8)	7 (6-8)
HBV-DNA (log)	7.14 (5.92-7.70)	6.76 (5.63-7.29)	4.77 (4.37-5.53)	4.92 (4.06-5.57)
ALT	188.62 (141.70-296.55)	185.50 (135.01-260.25)	98.73 (75.91-121.73)	110.00 (78.10-202.00)
AST	132.14 (100.00-173.57)	135.00 (110.00-215.00)	53.15 (40.77-122.58)	76.00 (49.00-105.00)
TB	27.00 (21.30-36.50)	28.50 (21.00-41.25)	39.10 (33.00-45.00)	37.00 (18.00-51.00)[a]
ALB			32.00 (26.00-37.70)	36.00 (33.50-37.00)
PLT			99.50 (58.00-120.00)	107.00 (102.00-119.00)[a]
Diabetes (%)	39 (10.77)	21 (10.34)	21 (21.87)	26 (20.15)
Hypertension (%)	29 (8.01)	18 (8.86)	23 (23.95)	25 (19.37)
Family history of hepatocellular carcinoma (%)	21 (5.80)	10 (4.97)	19 (19.76)	27 (20.93)
REACH-B score	10 (8-12)	10 (8-12)		

[a]$P < 0.05$ compared with the observation group. HBV: Hepatitis B virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; ALB: Albumin; PLT: Platelet.

Table 2 Changes in hepatitis B virus-DNA, hepatitis B e antigen, hepatitis B surface antigen and alanine aminotransferase after anti-inflammatory and hepatoprotective treatment and antiviral therapy in patients with chronic hepatitis B and cirrhosis, *n* (%)

Variable	Chronic hepatitis B		Hepatitis B cirrhosis	
	Observation group (*n* = 362)	Control group (*n* = 203)	Observation group (*n* = 96)	Control group (*n* = 129)
HBV-DNA undetectable	61 (16.85)	179 (87.19)	19 (19.79)	116 (89.92)
HBV-DNA drops no less than 2 log	216 (59.66)	194 (95.56)	57 (59.37)	124 (96.12)
HBeAg negative conversion	65 (32.82)	64 (52.03)	12 (40)	19 (59.37)
HBsAg negative conversion	3 (0.82)	2 (0.55)	1 (1.04)	2 (1.55)
ALT returns to normal	275 (75.96)	191 (94.08)	68 (70.83)	110 (85.27)

Undetectable hepatitis B virus-DNA was defined as hepatitis B virus-DNA < 500 copies/mL; a return of alanine aminotransferase to normal was defined as alanine aminotransferase < 40 mmol/L after treatment. HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen.

Cumulative incidence of HCC after spontaneous decreases in HBV DNA to undetectable levels in patients with CHB

Among the 362 patients with CHB, 61 had undetectable HBV DNA, and one developed HCC after undetectable HBV DNA. The cumulative incidence rates of HCC ($n = 1$) in years 2 and 4-20 were 0.016 and 0.016, respectively. A total of 301 patients did not have undetectable HBV DNA, and 28 of them developed HCC. The cumulative incidence rates of liver cancer in years 2, 4, 6, 8, 10, 12, 14 and 16-20 were 0.013, 0.034, 0.047, 0.068, 0.089, 0.135, 0.157 and 0.191, respectively. The incidence of HCC in patients without undetectable HBV DNA was higher than that of HCC in those with HBV-DNA negative conversion. There was no significant difference in the cumulative incidence of liver cancer between the two groups ($P = 0.051$) (Figure 3B).

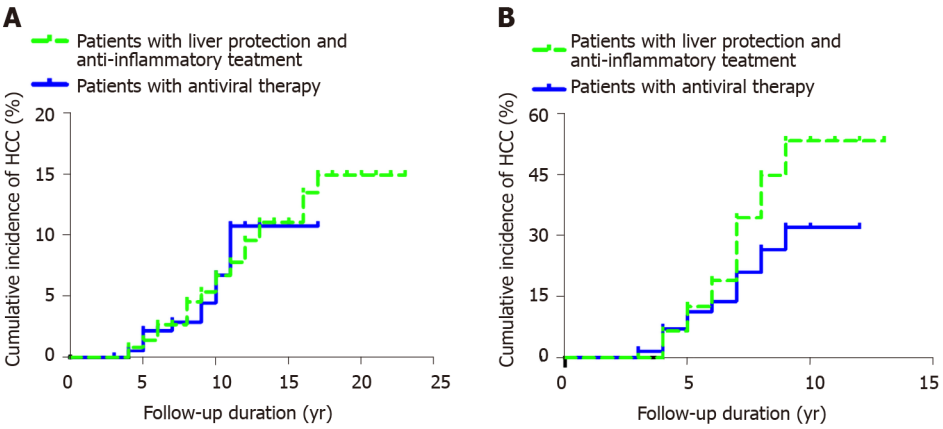


Figure 2 Cumulative incidence of hepatocellular carcinoma. A: Comparison of the incidence of hepatocellular carcinoma over time in two groups of chronic hepatitis B patients (patients with liver protection and anti-inflammatory treatment and patients with antiviral therapy); B: Comparison of the incidence of hepatocellular carcinoma over time in patients with hepatitis B cirrhosis. HCC: Hepatocellular carcinoma.

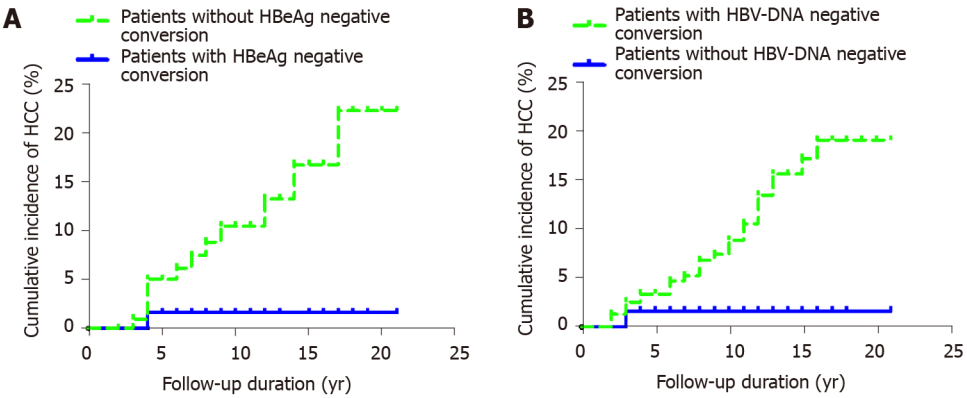


Figure 3 Cumulative incidence of hepatocellular carcinoma. A: Incidence of hepatocellular carcinoma after hepatitis B e antigen negative conversion in hepatitis B e antigen-positive chronic hepatitis B patients; B: Cumulative incidence of hepatocellular carcinoma after hepatitis B virus-DNA negative conversion in patients with chronic hepatitis B. HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

Cumulative incidence of HCC among patients with antiviral resistance in CHB

Among the 203 patients with CHB who received direct antiviral therapy, 79 developed antiviral resistance; of whom, 47 received LAM, 22 ADV, and 10 LDT. Seven of 79 patients developed HCC. The cumulative incidence rates of HCC among the 79 patients with drug resistance at years 2, 4, 6, 8, 10 and 12 were 0.000, 0.000, 0.027, 0.043, 0.130 and 0.185, respectively. The cumulative incidence rates of HCC (n = 2) among the 124 nonresistant patients at years 2, 4 and 6-12 were 0.000, 0.008 and 0.018, respectively. There was no significant difference in the cumulative incidence of HCC between the two groups according to the Kaplan-Meier log-rank test (P = 0.119) (Figure 4A).

Cumulative incidence of HCC in patients with antiviral resistance in hepatitis B cirrhosis

Of the 129 patients with direct antiviral cirrhosis, 30 developed antiviral resistance (HCC = 14); 17 of whom received LAM, eight ADV, and five LDT. The cumulative incidence rates of HCC among the 30 patients with drug resistance at years 2, 4, 6 and 8 were 0, 0.033, 0.240 and 0.506, respectively. The cumulative incidence rates of HCC (n = 16) among the 99 patients who did not develop antiviral resistance at years 2, 4, 6, 8 and 10 were 0, 0.083, 0.105, 0.167 and 0.255, respectively. The cumulative incidence of HCC among patients with antiviral-resistant hepatitis B cirrhosis was higher than that among nonresistant patients. The difference was significantly different according to the Kaplan-Meier log-rank test (P = 0.004) (Figure 4B).

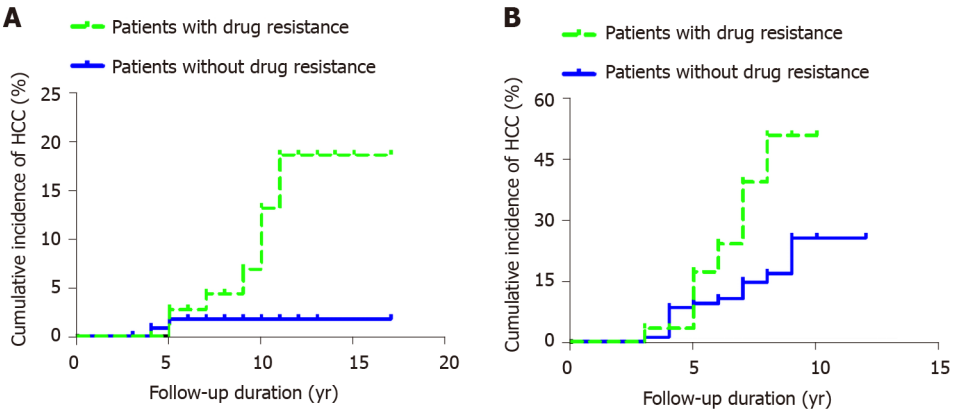


Figure 4 Cumulative incidence of hepatocellular carcinoma. A: Cumulative incidence of hepatocellular carcinoma in patients with antiviral resistance in chronic hepatitis B; B: Cumulative incidence of hepatocellular carcinoma in patients with antiviral resistance in hepatitis B cirrhosis. HCC: Hepatocellular carcinoma.

Cox regression analysis of risk factors for HCC in patients with CHB who were calibrated with REACH-B

We used the Cox regression model of the corrected REACH-B score to determine whether HCC occurred as the endpoint of observation, after adjusting for sex, age, HBeAg, ALT, AST, DNA, and other related parameters. The results showed that men aged > 40 years, ALT > 400 U/L, history of diabetes, and family history of HCC were risk factors for HCC ($P < 0.05$). Multivariate analysis showed that male sex and HCC family history were risk factors for HCC (Table 3).

DISCUSSION

In China, HCC is mainly HBV-associated, and this form of HCC has a worse prognosis than hepatitis-C-virus-associated HCC. Therefore, the effect of antiviral therapy should be discussed based on the incidence of HBV-related HCC rather than on the disappearance of HBV viral markers or serological conversion as the main target of treatment.

The antiviral mechanism of nucleoside analogs (NAs) is propagated mainly through their inhibition of the polymerase of HBV replication, thereby controlling the HBV load in the serum and the circulating pool, thus reducing the pathogenic factors of HBV-related HCC[26]. However, NAs cannot completely eradicate covalently closed circular DNAs, and they cannot block the occurrence of HBV-related HCC. This is mainly related to the carcinogenic mechanism of HBV. It is generally believed that there are three factors contributing to HBV carcinogenesis: the integration of HBV and host genes; accumulation of HBX protein in cells; and the persistence of inflammation. HBV destroys the genes of host cells, and the *trans*-binding carcinogenesis of HBX proteins leads to a series of carcinogenic factors that cannot be countered by NA drugs. It is important to note that persistent liver inflammation is also an important factor in the development of HCC. The causes of HBV inflammation include: (1) Induction of the host immune response by HBV infection; and (2) Uncontrollable inflammatory factors. Specifically, under uncertain conditions, inflammation cannot change from an anti-infection/tissue damage mode to a balanced and stable state[26], leading to continuous progression of inflammation. Proinflammatory cytokines and reactive oxygen species produced in the process of inflammation lead to gene mutations or phenotypic modifications that promote canceration[27].

Antiviral therapy reduces HCC mainly by decreasing the HBV-DNA load, thereby reducing immune-related injury to the body[28-30] and the levels of carcinogenic factors associated with inflammation. NA antiviral therapy can effectively decrease the HBV-DNA load, but it cannot achieve effective immune control. Immunoregulation is generally divided into positive and negative regulation. NA antiviral therapy mainly acts as a negative regulatory factor, but it does not affect positive regulatory factors; thus, it is difficult to achieve true immune functional recovery[31]. Therefore, 50%-70% of patients relapse after stopping drug treatment[32], which confirms the lack of immune recovery.

Current, relevant, long-term follow-up studies that have been published adopted a

Table 3 Cox regression analysis of risk factors for hepatocellular carcinoma in patients with chronic hepatitis B who were calibrated with REACH-B

Variable	Rate ratio (95%CI)	
	Single factor	Multiple factors
Sex		
Female	1.0 (referent)	1.0 (referent)
Male	2.859 (1.835-6.112)[a]	3.076 (1.975-8.437)[a]
Age (yr)		
≤ 40	1.0 (referent)	
> 40	2.677 (1.089-6.579)[a]	
HBeAg		
-	1.0 (referent)	
+	0.614 (0.288-1.310)	
DNA level, IU/L (log)		
≤ 3 (1×10^3 IU/L)	1.0 (referent)	
3-6.30 (2×10^6 IU/L)	1.130 (0.543-1.602)	
> 6.30	2.604 (0.749-3.854)	
ALT level, U/L		
≤ 50	1.0 (referent)	
50-200	1.140 (0.728-6.676)	
200-400	3.310 (0.173-11.112)	
> 400	4.036 (1.678-7.234)[a]	
AST level, U/L		
≤ 40	1.0 (referent)	
40-200	0.592 (0.184-1.904)	
200-400	1.565 (0.124-2.581)	
> 400	8.059 (0.689-11.968)	
TB level, U/L		
≤ 23	1.0 (referent)	
23-46	0.525 (0.211-1.308)	
46-115	1.349 (0.078-1.572)	
> 115	1.605 (0.125-2.936)	
Treatment		
Anti-inflammatory	1.0 (referent)	
Antiviral	0.701 (0.207-1.313)	
Antiviral after anti-inflammatory treatment	0.874 (0.283-1.467)	
Diabetes		
No	1.0 (referent)	
Yes	2.469 (1.079-5.649)[a]	
Hypertension		
No	1.0 (referent)	
Yes	1.932 (0.650-5.748)	
Family history of liver cancer		

No	1.0 (referent)	1.0 (referent)
Yes	30.924 (12.709-75.561)[a]	23.463 (9.372-47.564)[a]

[a]$P < 0.05$ *vs* the observation group. HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin.

retrospective or database observation comparative design, but these studies all had many shortcomings regarding intergroup confounding factors[33]. The present study is a real-world clinical study, lasting 2-23 years, of CHB patients in China. The aim of the study was to evaluate the real clinical outcomes, particularly the occurrence of HCC, in patients who did not receive antiviral therapy but received only anti-inflammatory and hepatoprotective therapy. Notably, a large number of data reported that it generally took 6-12 mo for HCC to be detected by B-ultrasound screening. In order to ensure the reliability and the objectivity of the results, we excluded patients with HCC occurring within 1 year of follow-up. We restricted the follow-up period to at least 2 years, and patients who developed detectable liver cancer within 1 year after enrollment were excluded. Considering that the time to find HCC takes 1-2 years, the follow-up period was determined to be ≥ 2 years. Our results showed that no patient in the CHB group and six patients in the cirrhosis group developed HCC within 1 year and we subsequently excluded these patients in the following observation.

Our results showed that among 362 patients with CHB who were not treated with antiviral therapy but treated only with anti-inflammatory and hepatoprotective therapy, after an average follow-up of 10 years, 16.9% had undetectable HBV DNA, 32.8% had HBeAg seroconversion, and 76.0% had ALT levels that returned to normal. Our results are similar to those of a previous study in Taiwan[34]. In addition, in the antiviral treatment group, 87.2% of patients were HBV-DNA negative, 52.0% had HBeAg seroconversion, and 94.1% had ALT levels that returned to normal. After antiviral treatment, the virological response of patients was significantly higher than that of patients without antiviral treatment; however, neither group showed significant differences.

At present, in China, anti-inflammatory and hepatoprotective drugs, such as glycyrrhizin, glutathione, polyethylene phosphatidylcholine, silymarin, and dicyclol, are classified into multiple categories, including anti-inflammatory, antioxidative and antifibrotic drugs[35,36]. When patients first present with elevated ALT, over the following 5-10 years, approximately 17% of patients may have spontaneous decreases in HBV DNA to undetectable levels, and approximately 33% may have spontaneous HBeAg seroconversion. The results of our study showed that the cumulative incidence of HCC was significantly different between patients with and those without HBeAg seroconversion. As long as the liver inflammatory response is effectively controlled in such patients, once spontaneous HBeAg serological transformation occurs, immune control can be achieved, thereby leading to entry into the inactive HBsAg carrier period, stabilization of the disease for a long time, and a significant reduction of HCC[37]. Although antiviral therapy can significantly inhibit the replication of HBV DNA, 50%-70% of patients relapse after drug withdrawal; even when the serological conversion of HBeAg occurs, it is temporary and unstable, and the cumulative recurrence rate is 44% after a 4-year follow-up period following drug withdrawal[38]. Thus, although these relapsed patients achieve HBV-DNA negative conversion, they do not achieve true immune control, and only 30%-50% of patients have true immune control. There were no significant differences between patients with antiviral therapy who achieved true immune control and spontaneous seroconversion. Therefore, we suggest that antiviral therapy masks the spontaneous relief process of CHB. Several studies have confirmed that the incidence of HCC after interferon therapy is significantly lower than that in patients who benefit from NA antiviral therapy[39-42], which also demonstrates why patients with CHB without cirrhosis who benefit from NA antiviral therapy do not have the advantage of better prevention of HCC. The key is that anti-inflammatory and hepatoprotective treatment can effectively improve the inflammatory response of the liver, slow down the progression of liver fibrosis during spontaneous seroconversion, and thus effectively reduce the incidence of HCC[43].

For patients with CHB complicated by cirrhosis, our results show that effective antiviral therapy can significantly reduce the cumulative incidence of HCC in patients with HBV-related cirrhosis. For hepatitis B cirrhosis patients who are positive for HBV DNA, taking antiviral therapy in a timely manner is important for controlling the persistent inflammatory response in the liver and eliminating the virus[44]. However, the cumulative incidence of HCC in patients with cirrhosis is still increasing with the

prolongation of follow-up, and there is no plateau phase. This shows that antiviral therapy can only delay but not eliminate the occurrence of HCC. Notably, even if patients with cirrhosis are treated with antiviral drugs in a timely manner, the cumulative incidence of HCC is still higher than that of patients with CHB without liver cirrhosis. This indicates that cirrhosis remains the most important factor in the development of HCC[45].

Drug resistance is common in CHB patients receiving antiviral therapy, especially in those treated with LAM and ADV in the early stage. However, will the incidence of HCC be further increased in patients with antiviral resistance? At present, there are still few reports suggesting that drug resistance may offset the benefit of antiviral therapy in patients with cirrhosis[46]. The results of our study showed that there was no significant difference in the cumulative incidence of drug-resistant and nonresistant HCC after antiviral therapy in patients with CHB without cirrhosis. This finding may be related to effective control of the HBV-DNA load in these patients with a low risk of HCC through timely rescue treatment, even when drug resistance occurred. However, in patients with cirrhosis, the incidence of HCC in drug-resistant patients was significantly higher than that in nonresistant patients, and the difference was significantly different. For patients with cirrhosis, the reserve function of the liver decreases, and the effective liver tissue decreases; drug resistance can lead to virological breakthroughs or rebounds, accelerate the progression of the disease, and further aggravate liver injury, thus increasing the risk of cirrhosis and HCC[47]. HBV mutation tends to increase gradually with infection time and disease progression[48], and the selection of antiviral drugs with high resistance barriers is an important factor in preventing viral mutation and reducing the occurrence of HCC in patients with liver cirrhosis.

Taiwanese scholars used data from the Reveal-HBV cohort to quantify HCC risk factors, and they established and preliminarily verified the first HBV-related HCC prediction model, REACH-B. The HCC scoring system includes host factors such as sex, age, family history of HCC, serum ALT levels, and virological indicators such as HBeAg levels, HBV-DNA levels, HBsAg quantification, and HBV genotypes[49]. The optimal cutoff point is 8 points, which is more suitable for the Asian population. Many guidelines recommend this model. The higher the score of this model, the higher the incidence of HCC. In this study, the REACH-B score did not indicate that non-antiviral therapy was an independent factor in the occurrence of HCC, while the occurrence of HCC was closely related to age, sex and family history of HCC.

This study was a single-center, pre-retrospective study, and further prospective cohort studies will be conducted when patients are identified as research subjects. Because antiviral therapy patients were enrolled after 2001 and the enrollment time of each group was different, the results of the study were biased to some extent. In this study, LAM, ADV and other high-resistance and low-potency drugs were used in the early stage of antiviral therapy, which affected the effectiveness of antiviral therapy. The evaluation criteria of the patients with liver cirrhosis were mainly based on B-mode ultrasound, while only 10% of patients were assessed by histopathology, which may have led to an underestimation in diagnosing the degree of liver fibrosis and early cirrhosis.

This study shows that in addition to viruses being the main carcinogenic factor in patients with CHB, inflammation or uncontrollable inflammation of the liver are important carcinogenic factors. Whether it is antiviral therapy or anti-inflammatory and hepatoprotective therapy alone, controlling liver inflammation is one of the mechanisms for improving liver histology. Therefore, once ALT elevation occurs in patients with CHB without cirrhosis, as long as liver inflammation is effectively controlled and immune control is achieved, the incidence of long-term HCC can be reduced to a certain extent. Our results showed that patients with liver cirrhosis had a higher cumulative incidence of HCC, so it was important to prevent patients developing cirrhosis. Patients with cirrhosis must receive antiviral therapy. Antiviral therapy can be implemented at the stage of progressive liver fibrosis to prevent the rapid occurrence of cirrhosis, which will be beneficial to the long-term prevention of HCC. Early NA antiviral therapy for low-HCC-risk patients with CHB without cirrhosis may mask the spontaneous serological response of some patients; therefore, the role of early antiviral therapy in reducing the occurrence of HCC cannot be overestimated.

CONCLUSION

In conclusion, antiviral therapy and non-antiviral therapy with liver protection and anti-inflammatory therapy can reduce the risk of HCC. Antiviral therapy may mask the spontaneous serological response of some patients during CHB. Therefore, the effect of early antiviral therapy on reducing the incidence of HCC cannot be overestimated.

ARTICLE HIGHLIGHTS

Research background

China is one of the leading countries for hepatitis B virus (HBV) prevalence, but most chronic hepatitis B (CHB) patients do not receive standardized antiviral therapy. There are few relevant reports addressing the outcomes of the large number of CHB patients who do not receive antiviral therapy.

Research motivation

The purpose of this study was to provide clinical evidence on the outcomes of CHB patients without antiviral treatment and evaluate the efficacy of antiviral therapy in the development and progression of CHB.

Research objectives

To observe the outcomes of long-term follow-up of patients with CHB without antiviral treatment.

Research methods

This study included 362 patients with CHB and 96 with hepatitis B cirrhosis, without antiviral treatment and with only hepatoprotective and anti-inflammatory treatment in 1993-1998. The median follow-up period was 10 and 7 years, respectively. A total of 203 CHB and 129 hepatitis B cirrhosis patients receiving antiviral therapy were selected as the control groups. The median follow-up period was 8 and 7 years, respectively. Kaplan–Meier curves were used to analyze the cumulative incidence of hepatocellular carcinoma (HCC), and the Cox regression model was used to analyze the risk factors of HCC.

Research results

Among the patients in the non-antiviral group, 16.9% showed spontaneous decreases in HBV DNA to undetectable levels, and 32.8% showed hepatitis B e antigen (HBeAg) seroconversion. In the antiviral group, 87.2% of patients had undetectable HBV DNA, and 52% showed HBeAg seroconversion. Among CHB and hepatitis B cirrhosis patients, the cumulative incidence rates of HCC were 14.9% and 53.1%, respectively, in the non-antiviral group, and were 10.7% and 31.9%, respectively, in the antiviral group. There was no difference between the two groups CHB, but there was a difference between the groups with hepatitis B cirrhosis. The cumulative incidence rates of HCC were 1.6% and 22.3% in the groups with and without spontaneous HBeAg seroconversion, respectively. The incidence rates of HCC among patients with and without spontaneous declines in HBV DNA to undetectable levels were 1.6% and 19.1%, respectively. There was no difference in the cumulative incidence of HCC between the two groups with drug-resistant CHB, but there was a significant difference between the two groups with cirrhosis. The Cox regression model was used for regression of the corrected REACH-B score, and alanine aminotransferase > 400 U/L, history of diabetes, and family history of liver cancer were risk factors for HCC in men aged > 40 years. Multifactor analysis showed that a family history of HCC among men was a risk factor for HCC.

Research conclusions

Antiviral therapy and non-antiviral therapy with hepatoprotective and anti-inflammatory therapy both reduced the risk of HCC. Antiviral therapy may mask the spontaneous serological response of some patients during CHB.

Research perspectives

Our study initially verified the outcomes of patients with CHB without antiviral

treatment. The effect of early antiviral therapy on reducing the incidence of HCC cannot be overestimated. More evidence-based studies are needed to validate the relationship between HCC incidence and antiviral therapy.

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REVIEW

Cascade of care for children and adolescents with chronic hepatitis C

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Abstract

Chronic hepatitis C virus (HCV) infection presents a significant global public health burden. In 2015, over 400000 deaths worldwide were attributed to HCV infection. This led the World Health Organization (WHO) in 2016 to set the ambitious goal of eliminating HCV by 2030. Adult-centered guidelines have been established in order to provide direction for healthcare professionals, allowing integration of the newest screening policies and therapeutic strategies into their practices. However, for children and adolescents, HCV is a significant, unrecognized public health problem. HCV infection rates in the United States in women of childbearing age and those who are pregnant have increased in parallel with the rising opioid epidemic. An estimated 29000 women with HCV infection gave birth each year from 2011 to 2014 in the United States, with approximately 1700 of their infants being infected with HCV. Newer HCV-specific therapeutics, namely direct acting antivirals (DAA), has brought a new and highly successful approach to treatment of hepatitis C. Recent studies have confirmed similar levels of effectiveness and safety of DAA therapies in the pediatric population. Thus, an enhanced cascade of care, which should include the population under 18 years of age, can help achieve the WHO goal by focusing on elimination in the youngest populations. This review will present an overview of the natural history, clinical features, and management of HCV in children and adolescents.

Key Words: Hepatitis C virus; Hepatitis C education; Hepatitis C elimination

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Core Tip: In 2020, the landmark series of accomplishments which started with the discovery of the hepatitis C virus (HCV) and led to the development of pharmaceutical

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agents capable of curing HCV infection was underscored by the awarding of the Nobel Prize in Medicine. These innovative cures are now being applied to the pediatric population. Furthermore, programs such as The Kentucky Hepatitis Academic Mentorship Program have been developed to train general pediatricians on HCV epidemiology, diagnosis, management, treatment and prevention. Thus this cascade of care will hopefully help achieve the World Health Organization goal of eliminating HCV by 2030.

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INTRODUCTION

The arc of discovery–from identification of the hepatitis C virus (HCV) as the causative agent of what was termed "Non-, Non-B Hepatitis" in 1989 to the development of pharmaceutical agent capable of efficiently curing HCV infection was remarkably short. In October 2020, this landmark series of accomplishments was underscored by the awarding of the Nobel Prize in Medicine to Drs. Alter, Houghton, and Rice. The Nobel committee recognized that their work transformed molecular virology/immunology and revolutionized the management of infected patients worldwide[1,2].

HCV infection presents a significant global public health burden. It is currently estimated that over 70 million individuals are chronically infected with HCV and that many are unaware of their infectious status[3]. In 2015, over 400000 deaths worldwide were attributed to HCV infection. This led the World Health Organization (WHO) in 2016 to set the ambitious goal of eliminating HCV by 2030. Despite the advances in HCV therapeutics, significant cost and access to care are the major barriers to the achievement of this goal[4].

Adult-centered guidelines have been established in order to provide direction for healthcare professionals, allowing integration of the newest screening policies and therapeutic strategies into their practices. For children and adolescents, HCV is a significant, unrecognized public health problem. Perinatal transmission accounts for the majority of recognized HCV infections in the pediatric population. HCV infection rates in the United States in women of childbearing age and those who are pregnant have increased in parallel with the rising opioid epidemic. An estimated 29000 women with HCV infection gave birth each year from 2011 to 2014 in the United States, with approximately 1700 of their infants being infected with HCV[5].

Newer HCV-specific therapeutics, namely direct acting antivirals (DAA), have brought a new and highly successful approach to treatment of hepatitis C. Recent studies have confirmed similar levels of effectiveness and safety of DAA therapies in the pediatric population. Thus, an enhanced cascade of care, which should include the population under 18 years of age, can help achieve the WHO goal by focusing on elimination in the youngest populations.

This review will present an overview of the natural history, clinical features, and management of HCV in children and adolescents.

EPIDEMIOLOGY OF HEPATITIS C

All 6 HCV genotypes have been diagnosed in the pediatric population; based on limited reporting, the genotypic distribution appears to mimic what is seen in the adult population, with genotype 1 predominating[6].

HCV infection is most often asymptomatic in the pediatric population; therefore, it is difficult to estimate the true global prevalence. Schmelzer *et al*[7] combined past modelling and epidemiological work in 104 countries and territories to estimate the prevalence in children in 2018. They reported the global estimated viremic prevalence in the population under 18 years of age to be 0.13%, corresponding to 3.26 million children with HCV in 2018, with wide variability. The prevalence increased with age in all countries and territories. The strongest predictor of HCV prevalence in children

aged 0-4 years was the HCV prevalence in women of childbearing age. The proportion of HCV infections in adults who inject drugs was significantly associated with HCV prevalence in children aged 15-19 years[8]. In view of the wide heterogeneity, reliable country- or territory-specific and age-specific HCV prevalence estimates will be required in order to allow countries and territories to improve national HCV elimination and treatment strategies.

The true prevalence of pediatric HCV infection in the United States is also unknown due to a lack of uniform screening strategies. In 2020, the United States Preventive Services Task Force (USPSTF) issued revised recommendations that encourages clinicians to screen all adults aged 18 to 79 years for HCV infection[9]. Previously, they had expressed a concern that HCV screening might be associated with negative psychological and social consequences. However, treatment with DAA therapy has been associated with improved quality of life in addition to high rates of curing HCV[10]. Thus, as screening tests for HCV are highly accurate, they now conclude that the combination of screening with DAA therapy indicates improved long-term outcomes.

The USPSTF recommendations specifically suggest HCV screening for all pregnant women during each pregnancy. This is important since the rate of HCV infection in pregnant women has continued to increase, with an associated increase in the number of infants exposed to HCV.

The risk of perinatal transmission is confined to HCV infected women who have detectable HCV RNA. The risk of transmission is increased with higher levels of HCV viremia, as well as co-infection with HIV[11-14]. The mode of delivery (vaginal *vs* cesarean-section) does not typically affect risk of transmission[13]. However, if the mother is co-infected with HIV, then there may be a protective affect by undergoing a cesarean-section for delivery[11]. HCV RNA may be detected in breast milk and colostrum; however, breast feeding does not appear to increase the rate of HCV transmission (with the exception of HIV co-infected mothers)[15].

While prenatal care settings are potential venues for expanding HCV testing, implementation is sporadic. Epstein *et al*[8] characterized the HCV diagnostic cascade for women attending an obstetric clinic serving individuals with substance use disorders. They reported successfully screening for HCV among pregnant women with opioid use. In retrospective cohort study of infants exposed to HCV who were enrolled in the Tennessee Medicaid program, testing was conducted in only 23% of infants and less frequently among African American infants[16]. These two observations indicate that infant HCV screening is currently imperfect, emphasizing the need for programmatic changes to improve both mother and infant follow-up to bridge gaps in the cascade to cure. Because current testing recommendations may not properly address the barriers to HCV testing among high-risk infants, contributing to missed HCV infections, new policies (such as universal pediatric testing) may address the gaps[6,17,18].

Screening in adolescents may also be improved. Epstein *et al*[19] reported that only 30% of adolescents with identified opioid, amphetamine, or cocaine use were tested for HCV; 7% were found to be positive. Barritt *et al*[20] reported that in the United States from 2006-2012, the hospitalization rates of children with HCV increased by 37%; the majority of these patients were adolescents. This further reflects that our attempts at identifying and treating HCV in early childhood and adolescents are inadequate.

NATURAL HISTORY

Children with chronic HCV infection are typically asymptomatic[6,17]. An estimated 20%-40% will undergo spontaneous clearance within the first 5 years of life[21,22]. A combination of perinatal transmission and genotype 1a is associated with decreased rates of clearance, persistent viremia, and higher likelihood of development of end-stage liver disease in children who are treatment naïve[23]. Albeit uncommon, progression to cirrhosis has been described and hepatocellular carcinoma (HCC) secondary to HCV and cirrhosis in a child has also been reported[24].

Younossi *et al*[25] reported that HCV infection in adolescents was associated with poor social functioning and health-related quality of life (HRQoL). Children chronically infected with HCV had a significant reduction in a wide range of intelligence and memory testing. Vocabulary, reading comprehension, abstract visual reasoning, and short-term memory were all statistically inferior in HCV infected children compared to healthy controls[26]. Treatment of HCV led to improved quality of life, using multiple validated patient reported outcome instruments[25]. Therefore,

while the liver disease in HCV infected children is often absent or mild, treatment may lead to improved HRQoL in addition to prevention of cirrhosis and end-stage liver disease.

CLINICAL FEATURES AND OUTCOMES

Jaundice, fatigue, dyspepsia, and abdominal pain are the most common signs and symptoms reported in adults[27]. Unfortunately, there is less robust prospective data regarding clinical symptoms in children and adolescents. When reported, minimal nonspecific and brief symptoms are found in approximately 15% of children. These symptoms can be in the form of fatigue, anorexia, nausea, vomiting, and abdominal colic[28].

Extrahepatic manifestations of HCV infection are well documented in the adult population. These include glomerulonephritis, polyarteritis nodosa and cryoglobulinemia. Other non-specific extrahepatic symptoms reported in adult studies include fatigue, renal impairment, lymphadenopathy, fever, and thyroid dysfunction[29-31]. Although there appears to be a low incidence of extrahepatic manifestations in children, careful monitoring is still recommended. Indolfi *et al*[32] noted that subclinical thyroiditis (not autoimmune thyroid disease) has been reported in children with HCV. Other extrahepatic manifestations such as myopathy and opsoclonus-myoclonus syndrome have also been reported.

It is rare for HCV-associated liver disease to advance to the point of requiring liver transplant in children or adolescents. Based on retrospective analysis of the United Network of Organ Sharing, Gupta *et al*[33] found that children transplanted for HCV had a one-year survival of 97% and a three-year survival of 89% in the post-pediatric end-stage liver disease era. These findings are consistent with best practice liver transplant outcomes in children.

DIAGNOSIS AND SCREENING CHILDREN AND ADOLESCENTS

In children older than 18 mo of age, diagnostic criteria are the same as those established for adults. An enzyme immunoassay is used to detect antibody (anti-HCV); however, the presence of anti-HCV alone is unable to distinguish if the patient has an active or resolved infection. Thus, in children with detectable anti-HCV antibodies, the next step is to verify viral infection by detecting HCV RNA. This is accomplished *via* polymerase chain reaction (PCR) testing. The diagnosis of chronic HCV infection is made based on presence of detectable HCV RNA for more than 6 mo[34,35].

The diagnosis of perinatal transmission in infants under 18 mo of age is confounded by the passive transfer of maternal antibodies, which can last for one year or more postnatally. Thus, anti-HCV testing is of limited value during the infantile period. Diagnosis in this age group can be reliably established by HCV RNA positivity on two or more occasions after two months of age[36-38]. Criteria for spontaneous clearance requires two negative HCV RNA tests spread at least 6 mo apart, followed by negative anti-HCV testing after 18 mo of age[39,40].

For the population < 18 years of age, the screening guidelines are unclear. Assoumou *et al*[41] completed a cost-effective analysis which revealed improved quality of life years (QALY) gained if universal screening for HCV was expanded to include adolescents (15 years and older). However, as the diagnosis of infants is more difficult to interpret, studies on the cost effectiveness of screening younger patients are needed. Recent efforts in the United States have focused on infants born to HCV infected mothers[42,43].

Jhaveri *et al*[44] endorsed a national strategy for HCV screening that integrates follow-up of infants with HCV exposure by using a model similar to HIV mother-to-child transmission prevention programs. This, and related calls to action to primary care providers, will lead to enhanced recognition and screening for children with HCV exposure, similar to the efforts to combat the HIV epidemic[45].

TREATMENT

The arrival of DAA therapies has led to a paradigm shift in the treatment and

eradication of HCV in all populations[3,4]. The spate of DAAs available have been shown to be as safe and effective in children and adolescents as in the adult populations. Pegylated-interferon (PEG-IFN) and ribavirin (RBV), the initial recommended combination for treatment of HCV in children and adolescents, ae no longer recommended[46]. DAA therapies are specific and more effective at achieving sustained virologic response (SVR) in the pediatric population with few side effects. DAA therapies can also achieve SVR in no more than 12 wk of treatment, as compared to the RBV and PEG-IFN combination which required 48 wk of treatment, close monitoring, and significant side effect profiles including pancytopenia. Furthermore, regimens of PEG-IFN have sustained efficacy of only just above 50%, whereas DAA regimens have been shown to be persistently more effective (SVR > 95%) in children[3,4,17,46,47].

DAAs target three HCV proteins: (1) The nonstructural protein 3/4A (NS3/4A) protease inhibitors (PIs) which work by inhibiting HCV polyprotein processing; (2) NS5A inhibitors, which inhibit viral replication and assembly; and (3) NS5B polymerase inhibitors that block HCV RNA replication[48,49]. By combining two or more of these classes of drugs with different mechanisms attacking the Hepatitis C virus, DAAs are able achieve high SVR rates.

Over the past few years, several phase 2 clinical trials have been completed revealing the safety and efficacy of DAA therapy in children as young as 3 years of age (Table 1)[50-77]. For example, the first pediatric trial showed the safety and efficacy of Harvoni, the combination of Ledipasvir (90 mg) and sofosbuvir (400 mg), for treatment of HCV genotype 1 over a 12 wk period in children ages 12-17 years[50]. Subsequent clinical trials have been completed which show the efficacy and safety of newer combinations of DAA therapy for a wider range of HCV genotypes and pediatric age groups. For example, Jonas *et al*[74] reported the utility of the pangenotypic combination of glecaprevir (300 mg) and pibrentasvir (120 mg) in children ages 12-17 years. They found 100% SVR at 12 wk post therapy (SVR12) in as few as 8 wk of treatment. The safety profile was also consistent with that in adults. Wirth *et al*[75], reported that the fixed-dose combination of elbasvir/grazoprevir in children ages 3-17 years for HCV genotypes 1 and 4 was safe and efficacious in all study participants. Furthermore, SVR12 was achieved by all 57 participants. Sokal *et al*[76] also recently completed a study on the safety and tolerability of sofosbuvir/velpatasvir in pediatric patients aged 3–17 years with chronic HCV infection through 24-wk post-treatment. They found a 92% SVR12 rate regardless of HCV genotype, prior treatment experience, or presence of compensated cirrhosis.

Rosenthal *et al*[72] revealed that sofosbuvir plus ribavirin (RBV) was well-tolerated and highly effective in children aged 3 to < 12 years with chronic HCV genotype 2 or 3 infection. However, over one-third of the participants experienced gastrointestinal symptoms (vomiting, diarrhea), common side effects to RBV treatment. This combination is an option for young children until we have more published evidence for RBV-free DAA regimens. The hope is that in 2021, we will have approval by the United States Food and Drug Administration (FDA) for the use of a wide variety of DAA combination therapies.

Higher risk groups, such as children who are survivors of cancer, have also had high success rates with DAA therapy. El-Shabrawi *et al*[66] prospectively followed 20 childhood cancer survivors ages 8-17 years with HCV genotype 4 in Egypt. They all received Sofosbuvir plus Daclatasvir over a 12 wk period. They achieved 100% SVR12 in their study group without any treatment related adverse events. Furthermore, no relapses were detected during treatment and throughout the follow up period (36 wk) for either the original malignant disease or the HCV infection.

Studies are also assessing the efficacy of smaller doses and shorter duration[60,67,77]. For example, Behairy *et al*[77] reported the effect of a shortened 8-wk regimen of ledipasvir/sofosbuvir at smaller dosing of 45 mg and 200 mg respectively. They found that this regimen is safe and effective with 100% SVR12 in treatment-naïve children aged 4-10 years with chronic HCV infection genotype 4.

WHERE DO WE STAND?

The American Association for the Study of Liver Diseases (AASLD) published updated guidelines for the evaluation and management of HCV infection to reflect the DAA era[78]. The AASLD supports the use of ribavirin-free DAA regimens as early as possible (all children >3 years of age) to avoid future complications. A policy paper from the North American Society of Pediatric Gastroenterology Hepatology and

Table 1 Completed studies of direct acting antivirals regimens in children and adolescents

Ref.	Year	Participant age in years (*n*)	HCV genotype	Therapy (duration)	SVR12 (%)
Balistreri *et al*[50]	2016	12-17 (100)	1	Ledipasvir 90 mg + sofosbuvir 400 mg (12 wk)	98
Wirth *et al*[51]	2017	12-17 (52)	2 or 3	Sofosbuvir 400 mg + ribavirin (variable)	98
Hashmi *et al*[52]	2017	5-18 (35)	1 or 3	Sofosbuvir 400 mg + ribavirin (variable)	97
El-Khayat *et al*[53]	2018	12-17 (144)	1, 4-6	Ledipasvir 45 mg + sofosbuvir 200 mg (12 wk)	99
Murray *et al*[54]	2018	6-11 (90)	1	Ledipasvir 45 mg + sofosbuvir 200 mg (12 wk)	98
El-Karaksy *et al*[55]	2018	12-18 (40)	4	Ledipasvir 90 mg + sofosbuvir 400 mg (12 wk)	100
Leung *et al*[56]	2018	12-17 (38)	1 or 4	Ombitasvir/paritaprevir/ritonavir + dasabuvir +/- ribavirin (variable)	100
Alkaaby *et al*[57]	2018	7-18 (22)		Ledipasvir + sofosbuvir +/- ribavirin (variable)	91
Tucci *et al*[58]	2018	0.5 (1)	4	Ledipasvir 22.5 mg + sofosbuvir 100 mg (12 wk)	100
El-Shabrawi *et al*[59]	2018	6-12 (20)	4	Ledipasvir 45 mg + sofosbuvir 200 mg (12 wk)	95
El-Shabrawi *et al*[60]	2018	12-17 (10)	1-6	Sofosbuvir 400 mg + daclatasvir 60 mg (8 wk)	100
Yakoot *et al*[61]	2018	12-17 (30)	4	Sofosbuvir + daclatasvir (12 wk)	97
Quintero *et al*[62]	2019	6-18 (9)	1 or 4	Ledipasvir + sofosbuvir (variable)	100
Ghaffar *et al*[63]	2019	8-18 (40)	4	Sofosbuvir + daclatasvir (variable)	97.5
Fouad *et al*[64]	2019	11-17.5 (51)	4	Ledipasvir 90 mg + sofosbuvir 400 mg (12 wk)	100
Ohya *et al*[65]	2019	10-13 (3)	1b	Ombitasvir + paritaprevir + ritonavir (12 wk)Or glecaprevir + pibrenastavir (8 wk)	100
El-Shabrawi *et al*[66]	2019	8-17 (20)	4	Sofosbuvir + Daclatasvir (12 wk)	100
Serranti *et al*[67]	2019	12-17 (14)	1	Ledipasvir 90 mg + sofosbuvir 400 mg (8 wk)	100
Marascio *et al*[68]	2019	13, 16 (2)	4	Ledipasvir 90 mg + sofosbuvir 400 mg (12 wk)	100
Fouad *et al*[69]	2020	12-18 (46)	Not performed	Ledipasvir 180 mg + sofosbuvir 400 mg (12 wk)	98
Kamal *et al*[70]	2020	3-6 (22)	4	Ledipasvir 45 mg + sofosbuvir 200 mg (8 or 12 wk)	100
El-Araby *et al*[71]	2020	9-12 (100)	4	Ledipasvir 90 mg + sofosbuvir 400 mg (12 wk)	100
Rosenthal *et al*[72]	2020	3-11 (54)	1 or 4	Sofosbuvir 400 mg + ribavirin (variable)	98
Schwarz *et al*[73]	2020	3-< 6 (34)	1 or 4	Ledipasvir + sofosbuvir (variable)	97
Jonas *et al*[74]	2020	12-17 (47)	1-4	Glecaprevir 300 mg + pibrentasvir 120 mg (8-16 wk)	100
Wirth *et al*[75]	2020	3-17 (57)	1 or 4	Elbasvir + grazoprevir (12 wk)	100
Sokal *et al*[76]	2020	3-17 (216)	1-4, 6	Sofosbuvir + velpatasvir (12 wk)	92
Behairy *et al*[77]	2020	4-10 (30)	4	Ledipasvir 45 mg + sofosbuvir 200 mg (8 wk)	100

Table adapted from Squires *et al*[17]. HCV: Hepatitis C virus.

Nutrition (NASPGHAN), included pediatric guidelines for treating children with DAA therapy[79]. They agreed with starting applicable DAA therapy as early as 3 years of age.

Outside of North America, guidelines are being updated to reflect the advent of DAA therapy. The European Association for the Study of the Liver (EASL) recently published a new guidance for management of HCV[80]. They recommend treating HCV positive children (with or without cirrhosis) as young as 3 years of age with DAA regimens of either combined sofosbuvir and velpatasvir, or glecaprevir and pibrentasvir. Indolfi *et al*[46] as part of the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) also updated their position to

recommend initiation of DAA therapy for children as young as 3 years of age with HCV, regardless of the presence of fibrosis or active inflammation. We agree with AASLD, NASPGHAN, EASL, and ESPGHAN recommendations for an aggressive approach to treating children 3 years of age and older with a RBV-free DAA combination. Furthermore, we agree with these worldwide guidelines that if there is any signs or evidence of fibrosis, then patients should continue to be monitored even after completing DAA therapy and achieving SVR (please refer to monitoring below).

Anecdotally, one of the main hurdles is determining an age when a young child is capable of daily compliance with the medications for the recommended 8-12 wk period. The advent of DAA therapies in the form of granules/pellets is a promising strategy for younger children who cannot swallow whole tablets. For example, Schwarz *et al*[73] allowed for the granules to be sprinkled on a spoonful of nonacidic soft food, such as pudding or ice cream. SVR12 was achieved in 97% of patients, with only one patient discontinuing the trial after 5 d due to "abnormal drug taste".

Cost-effective analyses for treating children with DAA therapy are limited. Nguyen *et al*[81] found that early DAA treatment in adolescent patients with chronic HCV infection was cost-effective compared with deferred treatment, with approximately $27000 per QALY gained after 30 years. Greenaway *et al*[82] published data comparing treatment at age 6 years *vs* delaying treatment until age 18 years. In their model covering 20 years and treating 10000 children early, 330 cases of cirrhosis, 18 cases of hepatocellular carcinoma, and 48 liver-related deaths would be avoided. The incremental cost-effectiveness ratio of early treatment compared to delayed treatment was approximately $12690 per QALY gained and considered cost-effective. Thus delaying treatment until age 18 years results in an increased lifetime risk of late stage liver complications that can otherwise be avoided. Early treatment is associated with saving money and lives, as well as improving quality of life.

IMPROVING THE CASCADE OF CARE

In addition to inadequate screening, a major barrier to treatment and elimination is access to care and treatment. However, several programs have been conceived in order to provide DAA therapy to more individuals, with a focus in the primary care setting. In Australia, DAA treatments are available through the national Pharmaceutical Benefits Scheme (PBS) as of 2016. The PBS is a publicly funded scheme which provides highly subsidized prescription drugs *via* Australia's universal healthcare system[83]. Australia was also one of the first countries to allow DAA treatment to be initiated by general practitioners. Since the advent of these practices, they have seen marked improvements in the cascade of hepatitis C care among patients attending primary care clinics[84].

In the United States, certain regions have much higher rates of HCV infection. For example, the Appalachian region leads the nation in reported new cases. Thus, new developmental strategies have been created focusing on these communities. The Kentucky Hepatitis Academic Mentorship Program (KHAMP) was created with the goal to build a hepatitis C elimination model which would then be easily modified and used to improve the health of rural and underserved communities throughout the Appalachian region. KHAMP has trained primary care providers on HCV epidemiology, diagnosis, management, treatment and prevention. General practitioners in this region are thus equipped with the skills needed to increase the number of individuals treated, ensuring that they will no longer be required to travel and consult with a specialist in order to prescribe DAA therapy[17,85,86].

This blueprint is being applied to the rest of the United States, continuing the focus on the Appalachian region. For example, West Virginia has recently implemented the West Virginia Hepatitis Academic Mentoring Partnership which will use the same strategies as KHAMP to provide education for primary care providers on HCV[86,87]. Virginia and Ohio are also participating to improve their education and access at the primary care level.

The advent of telemedicine has also had a positive impact towards treating HCV. Arora *et al*[88] developed the Extension for Community Healthcare Outcomes (ECHO) model. In a prospective cohort study, the ECHO model through use of video-conferencing technology, trained primary care providers to care for underserved populations with HCV infection who live in New Mexico. Results showed that ECHO was an effective approach to treating HCV infection in underserved communities. Piao *et al*[89] have implemented ECHO to California with improvements in SVR, advocating for such programs to be an essential part of HCV care moving forward.

In Australia, hepatitis C treatment (DAA therapy) using a decentralized, nurse-led telemedicine model of care has been highly effective at reaching a treating large numbers of prisoners, many of which are IV drug abusers[90]. Canada has also implemented a telemedicine program in order to effectively increase the use of DAA therapy with a high success rate of SVR (approximately 95%)[91]. As more programs are being initiated, the possibility of reaching the WHO goal of eradication by 2030 is still possible.

IMPACT OF TREATMENT ON PROGRESSION

Progression of HCV from an inflammatory hepatitis, to fibrosis, and eventually cirrhosis can occur starting in early childhood. In the past, most HCV-infected children would develop chronic HCV with a lifetime risk of liver disease. Modin *et al*[92] quantified the development of long-term liver disease and the effect of treatment in patients infected with HCV in childhood. They reported that liver disease developed in 32% of patients, a median of 33 years after infection; patients with perinatal exposure developed cirrhosis at an earlier age than the rest of the risk groups. The incidence of HCC was 5%, liver transplant 4% and death occurred in 3%. Among those treated there was a higher mortality rate among patients that did not achieve an SVR, and treatment was more effective in patients without cirrhosis. Disease progression was less frequent than in patients with cirrhosis at the time of therapy. The authors make a strong case for early treatment, before development of cirrhosis.

MONITORING

Finding non-invasive methods to assess for progression to fibrosis is an important aspect of monitoring children with chronic HCV. Transient elastography (TE) *via* ultrasound (US) evaluation of the liver is gaining traction in the pediatric population[93]. TE as a measurement of liver fibrosis has been validated in a variety of chronic liver diseases, including HCV[94-97].

Pokorska-Śpiewak *et al*[98] reported their prospective analysis on the prevalence of fibrosis in adolescents (12-17 years) with chronic HCV. Using TE, they found that over 10% of their patients had evidence of significant fibrosis (fibrosis score > 2), and that 9% had evidence of cirrhosis (Fibrosis score of 4). Other markers of liver fibrosis, such as the aspartate transaminase-to-platelet ratio index score, correlated positively with liver stiffness from TE. Otherwise, serial monitoring with in-clinic visits, as well as laboratory testing of aminotransferases and gamma-glutamyl transferase are recommended to occur at least twice yearly. Monitoring for signs of HCC with serum alpha-fetoprotein and US imaging is also warranted[29,99,100].

Based on the previously completed studies on DAA therapy in pediatrics, our current practice involves obtaining HCV PCR at baseline (prior to initiation of DAA therapy), at 4 wk, at 12 wk, and at 24 wk post initiation of therapy[50,54,70]. As long as there was no evidence of long-term damage (fibrosis, cirrhosis, *etc*.), then patients can have a repeat HCV PCR one year after completion of therapy to affirm SVR[50,54,72-76,81]. No pediatric studies with the children completing the DAA therapy has revealed evidence of children being unresponsive to DAA therapies.

Children with evidence of liver fibrosis should continue to be closely monitored even after eradication of their underlying HCV. However, adult studies are emerging which reveal that fibrosis may be to-some-extent reversed by DAA treatment[101-104]. One study revealed a 32% reduction in liver stiffness measurements after DAA completion among 392 adults with chronic HCV and fibrosis[105]. However, for patients with evidence of high-grade fibrosis or cirrhosis, they are still at high risk of developing HCC even after achieving SVR[106]. More histological data is needed to further support the hypothesis of improved liver scarring post DAA treatment. At this time, children with evidence of fibrosis must be closely followed given the continued risk of complications such as HCC and portal hypertension.

IMPACT OF DAA THERAPY ON LIVER TRANSPLANT

The number of patients requiring HCV-related liver transplant has decreased, increasing organ availability for other liver disorders, such as NASH. In addition,

given the safety and effectiveness of DAA therapy, the idea of placing HCV-infected livers into uninfected recipients is gaining traction. With the rising demand of liver transplant, treating recipients with an appropriate course of DAA therapy immediately after transplant appears safe and efficacious[107,108]. In one small study, 8 veterans received HCV-infected livers and all 8 became viremic with HCV. However, after a 12 wk course of DAA treatment, all 8 patients achieved SVR12[109]. Bohorquez *et al*[110] increased this sample size and, after completing an appropriate DAA regimen, had 100% SVR12 in all 51 HCV-naïve patients who received HCV positive livers. Therefore, solid organ transplant from HCV infected recipients appears to be safe, is associated with excellent outcomes, and should be considered for recipients who would benefit from receiving an organ earlier than they would if they waited for an organ from an uninfected donor. Thus, reducing wait-list associated mortality. The same concept applies to other solid organ transplants. While no studies has been performed, based on the efficacy of DAA therapy in children, using HCV-infected donors should be an option.

CORONAVIRUS DISEASE AND HCV

The coronavirus disease 2019 (COVID-19) pandemic has significantly impacted access and healthcare practices for many patients and providers, including the pediatric population. As children will now have access to DAA therapies and potential HCV cure, it is imperative that diagnosis and treatment of this population is not overlooked. A related issue is for patients with chronic liver disease to avoid COVID-19 exposure and infection, by educating patients/parents on the risk and the recommended precautions[111]. This is especially true in rare cases of children with cirrhosis or end-stage liver disease secondary to HCV, as there appears to be a higher risk of a severe course of COVID-19[112].

The advent of telemedicine is playing an important role in the care of children with HCV. This allows patients to undergo lab testing locally, for DAA prescriptions to be sent to their home, and allow providers to safely communicate, educate, and closely monitor their patients during treatment[113]. Thus this pandemic should not be a hindrance to continuing the goal of eradication of HCV in the pediatric population.

CONCLUSION

The discovery of the HCV and the related advances in biomedical research-the establishment and implementation of diagnostic tests to ensure the safety of blood products, and antiviral drug development has, and will continue to have, a major impact on health care outcomes for patients of all ages…including the smallest victims. Enhanced screening and awareness efforts and continued education of healthcare providers will improve the outcomes of HCV infection in the pediatric population.

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MINIREVIEWS

Primary localized gastric amyloidosis: A scoping review of the literature from clinical presentations to prognosis

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Abstract

Localized gastric amyloidosis (LGA) is a rare disease characterized by abnormal extracellular deposition of amyloid protein restricted to the stomach and it is confirmed by positive results of Congo red staining. Over decades, only a few cases have been reported and studies or research focusing on it are few. Although LGA has a low incidence, patients may suffer a lot from it and require proper diagnosis and management. However, the pathology of LGA remains unknown and no overall review of LGA from its presentations to its prognosis has been published. Patients with LGA are often asymptomatic or manifest atypical symptoms, making it difficult to differentiate from other gastrointestinal diseases. Here, we report the case of a 70-year-old woman with LGA and provide an overview of case reports of LGA available to us. Based on that, we conclude current concepts of clinical manifestations, diagnosis, treatment, and prognosis of LGA, aiming at providing a detailed diagnostic procedure for clinicians and promoting the guidelines of LGA. In addition, a few advanced technologies applied in amyloidosis are also discussed in this review, aiming at providing clinicians with a reference of diagnostic process. With this review, we hope to raise awareness of LGA among the public and clinicians.

Key Words: Gastroscopy; Changes of gastric mucosa; Primary localized gastric amyloidosis; Clinical presentations; Prognosis

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Core Tip: Localized gastric amyloidosis (LGA) is rare. Few case reports are available to the public. It is often misdiagnosed as other gastrointestinal diseases due to its atypical manifestation. However, no systemic reviews or guidelines of LGA is published now. Therefore, we present a detailed overview from its clinical manifestations to prognosis for the first time. Based on that, a clinical diagnostic procedure is provided and may benefit clinicians who manage LGA.

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INTRODUCTION

Amyloidosis is a group of conformational diseases characterized by the accumulative extracellular deposition of insoluble fibrils in various tissues and organs as a result of protein folding disorders[1]. Large deposits may lead to the loss of the normal structure of tissues, subsequent organ dysfunction, and even death. At present, 36 different amyloid fibrils have been identified that are associated with amyloidosis[1]. Amyloid fibrils determine the properties of the amyloid diseases, and the subtypes of amyloidosis are also named after the corresponding fibrils. For example, light chain (AL) indicates that amyloid fibrils are derived from immunoglobulin light chains, and the resulting disease is referred to as AL amyloidosis[2]. According to the amyloid distribution, amyloidosis is divided into systemic and localized amyloidosis. Systemic amyloidosis is universal, while localized amyloidosis is a rare condition that only comprises 12% of newly identified amyloidosis cases[3].

Amyloidosis is a rare disease, among which AL amyloidosis is the most common type. An epidemiological study in Sweden reported an incidence of nonhereditary amyloidosis of 8.29 per million person-years, among which AL amyloidosis accounted for 3.2 per million person-years[4]. A nationwide study in the United States reported an increasing incidence of AL amyloidosis, from 9.7 per million person-years in 2007 to 14.0 per million person-years in 2015[5]. Although rare, amyloidosis can result in a severe disease burden, as reflected by patients' poor scores on assessments of the health status compared to the general population in a recent study of 341 patients[6]. Moreover, without proper intervention, it may ultimately develop into a fatal disease. From 2000 to 2008, 0.58 per thousand deaths were due to amyloidosis in England, and its proportion of deaths has doubled, indicating a tendency to increase[7].

Gastrointestinal involvement manifests as systemic amyloidosis (79%), while it is relatively rare in localized cases (21%), according to a retrospective study of 76 patients of biopsy-proven gastrointestinal amyloidosis evaluated in 1998-2011[8]. Localized gastric amyloidosis (LGA) is an extremely unusual condition. Generally, it refers to amyloidosis confined to the stomach without evidence of potential plasma cell dyscrasia or the involvement of other organs, particularly the heart, liver, kidney, or nerve[3,8]. More specifically, the precursor protein of amyloid is produced and deposited in the stomach without detection in a remote site[9]. According to a retrospective study of gastrointestinal biopsies from 542 patients, the most common amyloid subtype in the stomach is AL (λ), followed by transthyretin (ATTR), AL (κ), and serum amyloid A (AA)[10].

Given the rare reports and unsolved problems associated with LGA, we present a case of LGA (Figures 1 and 2) and collect several recent case reports of LGA available in the literature to present its clinical manifestations, diagnosis and differential diagnosis, and treatment. In addition, we will describe our understanding of its pathogenesis.

PATHOGENESIS

To date, the pathogenesis of amyloidosis is unclear, but current studies and

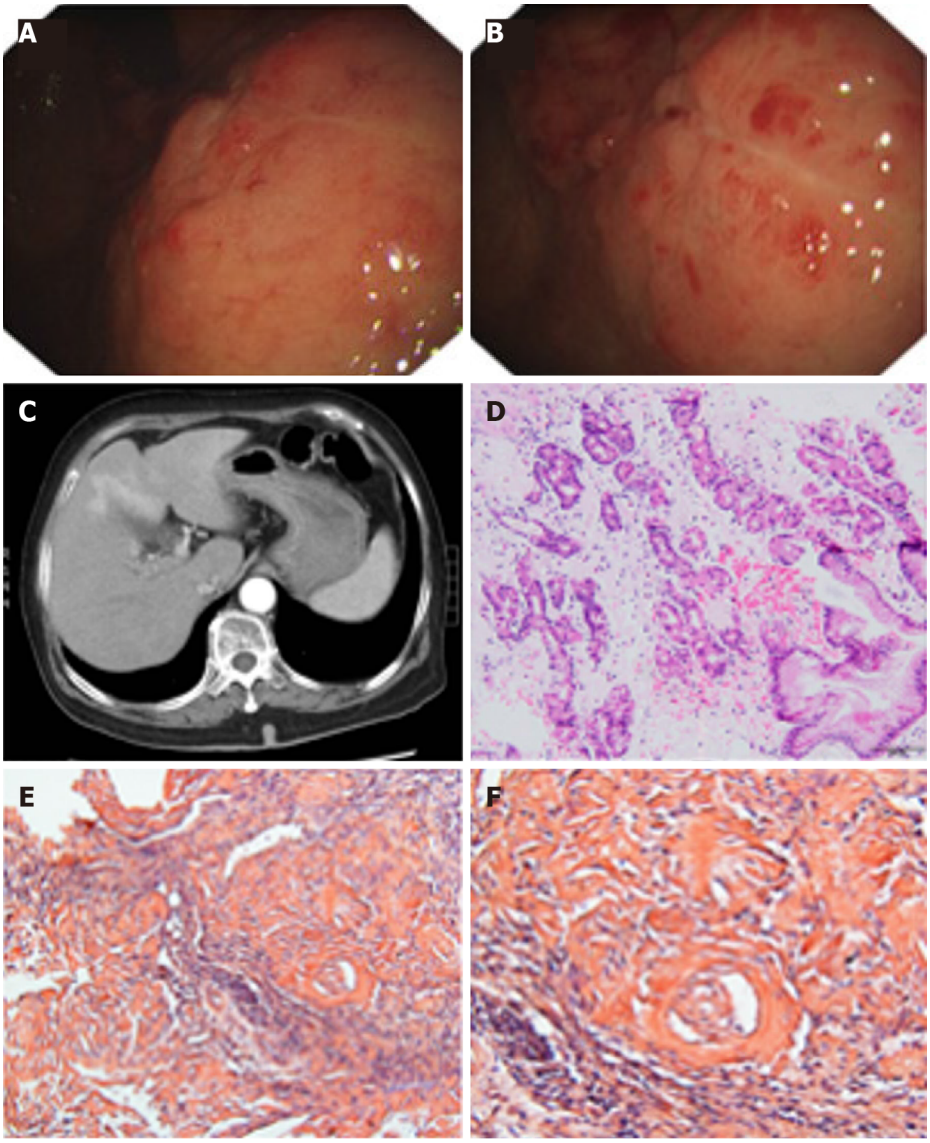


Figure 1 Pathological findings from a 70-year-old woman with localized gastric amyloidosis. The patient came to the hospital with a chief complaint of hematemesis for 2 wk. A and B: Endoscopic findings show multiple congested fragile ulcers scattered in the gastric body and fundus. A 4.0 cm × 4.0 cm area of the mucosa with edema, ulcers, and poorly delineated boundaries was observed in the anterior wall of the gastric body and fundus. The lesion appeared as a rough, congested area with edema, localized superficial fragile ulcers and active bleeding. Spot-like congested erosions exhibited a scattered distribution in the mucus of the sinus; C: CT reflected diffusely thickened gastric walls and shallow folds of the mucosa, while no abnormalities were observed in the enhanced images; D: H&E staining revealed massive amyloid fibrous connective tissues deposited in the interstitium with inflammatory cell infiltration; E and F: Congo red staining confirmed the existence of the amyloid protein (E: Congo red, × 200 magnification; F: Congo red, × 400 magnification).

hypotheses provide a few insights. All amyloid fibrils share the same antiparallel cross-β secondary structure, a structure with a high propensity for self-aggregation, as observed under an electron microscope[11,12]. Tightly bound β-sheets form the protofilament, and several protofilaments twist and eventually form amyloid fibrils[13]. Proteins may have a potential intrinsic propensity of misfolding that is influenced by multiple factors, such as aging and stably high concentrations in serum[11]. For example, wild-type TTR forms amyloid fibrils in older individuals, even at normal concentrations, while serum amyloid A and β_2M only become amyloidogenic at a persistently high concentration[14]. Different amyloid fibrils may be susceptible to different conditions that trigger misfolding. Mutations may induce the formation of amyloidogenic proteins[15,16]. Mutations in genes that encode amyloid fibrils, such as TTR, trigger familial amyloidosis[17]. Somatic mutations have been identified in AL amyloid fibrils. The N-terminal strand of the light chain variable domain prevents protein aggregation, and its mutation destabilizes the protein and accelerates light chain fibrillogenesis[18,19]. However, the exact relationship between mutations and amyloidosis remains unknown. In addition, further thermodynamic investigations reveal that many environmental factors, such as temperature and pH, are related to

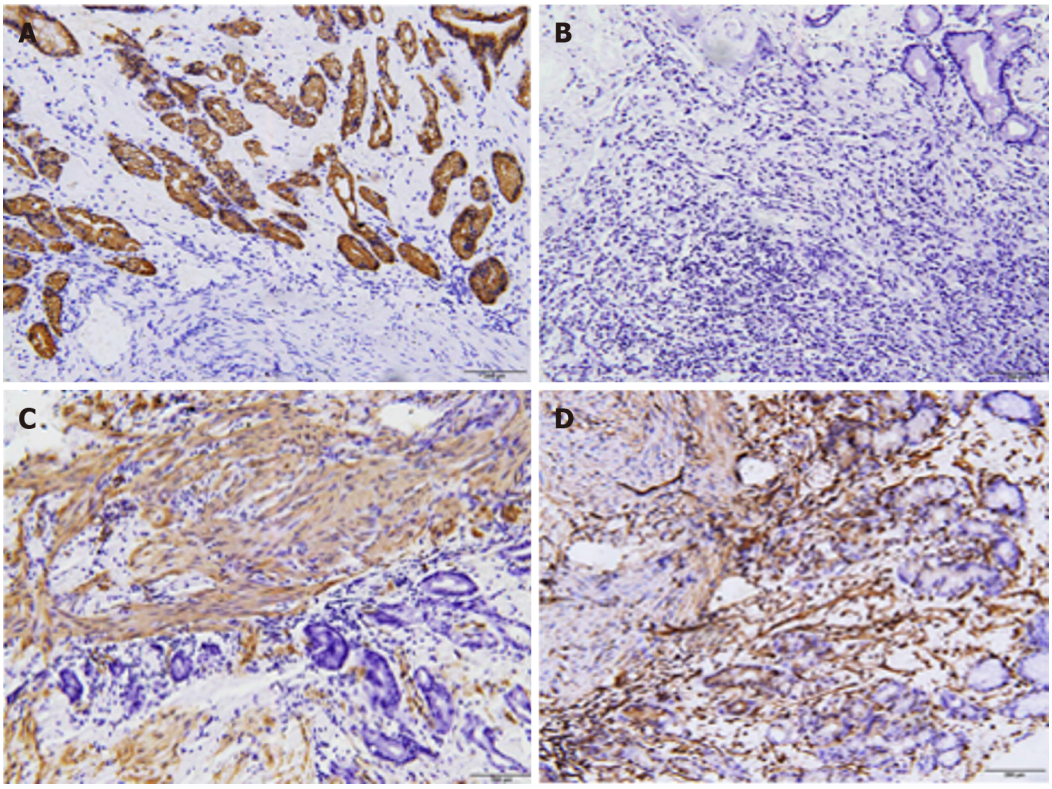


Figure 2 Results of immunochemical staining using several antibodies excluded the diagnosis of gastric cancer. A: CKPAN-positive staining in the glands; B: Periodic Acid-Schiff staining is negative; C: Smooth muscle actin-positive staining in the muscularis mucosa; D: Vimentin-positive staining.

the conformational stability of amyloid fibrils and may be critical factors contributing to the conversion of a normal protein into amyloid fibrils[13,20] (Figure 3).

In addition, several protein cofactors, nonfibrillar components, glycosaminoglycans (GAGs), and serum amyloid P (SAP) are present in all amyloid deposits and are believed to function in amyloidogenesis and persistence. GAGs, a main component of the extracellular matrix, are associated with amyloid fibrils in AL amyloidosis, and their size and charge may play an important role in the acceleration and stability of amyloid formation[19]. SAP is a type of plasma glycoprotein that binds to all types of amyloid fibrils in its calcium-bound state and protects them from proteolysis[21] Amyloid fibrils are digested *in vitro* by proteases and phagocytic cells, while amyloid fibrils likely exhibit relative stability *in vivo* with the assistance of SAP[22]. According to an *in vitro* study, SAP may accelerate and stabilize the formation of $A\beta_{42}$, the amyloid fibrils responsible for Alzheimer's disease[23]. The severity of amyloid deposition in SAP knockout mice is decreased considerably[24]. Therapies targeting SAP have been tested and confirmed to exert stable effects[25].

The mechanism by which amyloid deposits damage the organs and lead to dysfunction is unclear. The site of deposition may be related to multiple factors, such as the pH, protein concentration, proteolytic processing, and fibril seeds. Different amyloid fibrils exhibit a preference for specific organs; for example, β_2-microglobulin prefers joints[11]. In patients with LGA, amyloid is universally present in the walls of small vessels, and most of these amyloid deposits are classified as AL amyloidosis (12/22). In cardiac amyloidosis, amyloid deposits in small vessels lead to symptoms of cardiac ischemia[26]. In gastric amyloidosis, we are able to detect the same distribution patterns, but no connections have been reported to date. Furthermore, the toxicity of amyloidogenic light chain proteins (AL-LC) may be responsible for this condition. An investigation of the potential mechanism revealed that the injection of human AL-LC within a zebrafish model causes cell death. Human AL-LC induces intracellular oxidative stress and alters the cellular redox status, eventually leading to cardiac dysfunction, which is not attributed to the deposition of amyloid protein[27]. As shown in another study, AL-LC mediates cardiomyocyte apoptosis and dysfunction through the activation of p38 mitogen-activated protein kinases[28]. Lysosomal dysfunction has also been reported to provoke the proteotoxicity of AL-LC by contributing to impaired autophagy[29]. Investigations of the precise molecular mechanism of cardiac amyloidosis are ongoing and may explain how the amyloid protein contributes to

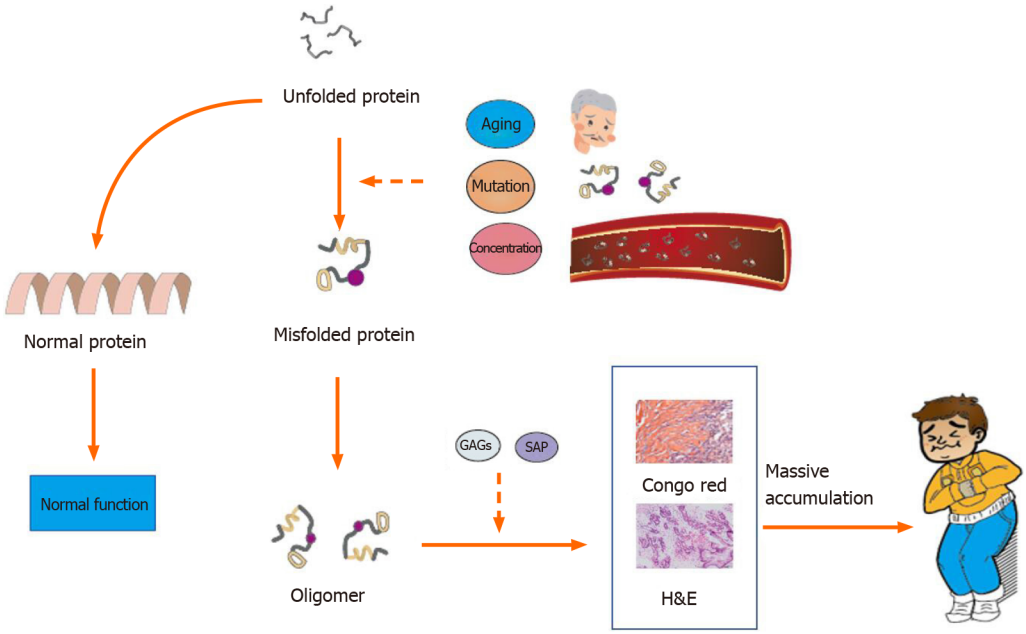


Figure 3 Potential molecular events leading to amyloidosis. Without intervention, the unfolded protein becomes the normal protein. Factors such as aging, mutation, and high blood concentrations may cause protein misfolding. The misfolded protein aggregates into oligomers and forms fibrils with the assistance of glycosaminoglycans and serum amyloid P. Massive deposition of amyloid fibrils leads to amyloidosis. GAGs: Glycosaminoglycans; SAP: Serum amyloid P.

organ damage. These studies may reveal the mechanism and inspire further studies of gastric amyloidosis.

CLINICAL MANIFESTATIONS

LGA mainly targets middle-aged and elderly people aged from 50 to 80 years. Equal numbers of male and female patients are affected, and no sex differences have been detected. The clinical manifestations of LGA often mimic other common gastric diseases and lack specificity, ranging from an asymptomatic disease to epigastric discomfort, pain, weight loss, anemia, heartburn, nausea, hematemesis, tarry stool, fatigue, and other symptoms (Figure 4). Generally, these manifestations depend on the sites and extent of amyloid involvement[30]. Patients in our reviewed case studies often presented without a chief complaint, and LGA was generally diagnosed based on the results of tests for other gastric diseases. Among the 22 cases that we reviewed, 13 cases of AL LGA and 1 case of AA LGA were identified. Most AL LGA cases manifest asymptomatically[31-35] or with epigastric discomfort[36,37], while AA LGA exclusively manifests as epigastric discomfort[38] (Table 1). To date, no association between symptoms and amyloid types has been confirmed[39]. Further discussion of whether a correlation exists between clinical presentations and amyloid fibrils in patients with LGA is worthwhile.

DIAGNOSIS

Imaging findings

Endoscopic results have identified variable features in patients. Amyloid deposits mainly invade the gastric body and antrum (Figure 5). Generally, the deposits manifest as single or multiple lesions in the form of a mass[40-43], ulcer[38,44], fold[36,45], elevation[37,46], or submucosal tumor-like feature[34,47]. The borders may be clear or unclear. These findings are consistent with those of previous studies. A remote study of 37 patients with gastrointestinal amyloidosis revealed that erosions were the most common presentation in the stomach, followed by granules, ulcers, and mucosal friability[48]. However, a recent study provided a different order of a normal appearance, followed by erythema, erosions, and nodularity[39]. The samples included both localized and systemic amyloidosis, and thus, the difficulty in distinguishing localized amyloidosis from systemic involvement simply based on endoscopic features

Table 1 Collection of recent case reports of localized gastric amyloidosis

Ref.	Age/sex	Symptom	Gross	Size	Location	Endoscopic ultrasound	biopsy	Amyloid type	Exclusion test	Suspected diagnosis	Treatment
Ikeda *et al*[59], 1978	68/F	Epigastric pain, nausea	One grayish-white mural elastic soft tumor with an irregular shape and poor margins; thickened and uneven mucosa, partly nodular; swollen mucosal folds	6 cm × 5 cm	Antrum	/	Congo red (+); amyloid deposits in vessel walls; H&E staining: Amyloid deposits with foreign-body reactions; small nodules of amyloid proteins with a scattered distribution	/	Biopsy of the skin rectum, gingiva and liver; urine Bence-Jones protein levels	Gastric carcinoma	Surgery
Dastur *et al*[40], 1980	50/M	Abdominal distension, worse after meals	One mass with central small ulceration and defective mucosa	/	Antrum	/	The mass extended from the mucosa to superficial muscularis and consisted of lymphocytes and germinal centers; normal plasma cells and amyloid proteins; Congo red (+)	/	Urine Bence-Jones protein (-)	/	Surgery
Björnsson *et al*[45], 1987	60/F	Hematemesis	A considerable amount of blood, bleeding and irregular, thickened mucosa folds	/	/	/	H&E staining: Amyloid deposits in the lamina propria and muscularis mucosae, infiltration of plasma cells, mucosa atrophy; Congo red staining: Amyloid deposits in the submucosa, muscularis propria and subserosa, mainly around vessels	AL (κ&λ)	Biopsy of rectum, gingiva, cervix and bone marrow; analysis of renal function; urine analysis	/	Surgery
Yanai *et al*[31], 1991	52/F	None	One irregular erosion	2.5 cm	The lower body of the stomach	Thickened mucosa and submucosa	Amyloid deposits in vessels of the mucosa and submucosa	AL (λ)	Examinations of blood, urine and skin; ultrasound and CT of the abdomen; simple X-ray examination of the chest and abdomen; biopsy of the rectum; ECG; ultrasound of the heart; barium enema; tests of antigens, urine Bence-Jones protein, rheumatoid factors and C-reactive protein levels	Gastric cancer	/
Wu *et al*[38], 2003	50/F	Epigastric discomfort, dull pain in the upper abdomen	One ulcer with heaped-up rough borders and erosive fragile mucosa	3 cm × 1 cm	Lesser curvature of the gastric body	Uneven hypoechoic thickened gastric wall with infiltrated submucosa	Amyloid deposits in the mucosa, submucosa and walls visualized using H&E and Congo red staining (-); atrophy of gastric glands and intestinal metaplasia	AA	Biopsy of the bone marrow and other gastrointestinal tissues (esophagus, duodenum, colon and stomach) (-)	Gastric cancer	Subtotal gastrectomy, clearance of perigastric lymph nodes
Shibukawa *et al*[44], 2004	51/F	Tarry stool	One irregular ulcer with swollen edges and dirty slough-like advanced cancer;	/	/	Structural loss of the first three layers of the gastric wall, a small amount of	H&E staining: Amyloid deposits infiltrated the submucosal connective tissues, lamina propria, and muscularis mucosae	AL or primary type	/	Carcinoma	Partial gastric resection

			bleeding			ascites	and were mainly observed around vascular walls in the submucosa; Congo red (+)				
Deniz *et al*[41], 2006	67/M	Fatigue, weight loss, poor appetite	One mass	5 mm × 5 mm × 5 mm	Paracardiac region	/	H&E staining: Amyloid deposits in the mucosa; Congo red (+)	/	Biopsy of other gastrointestinal tissues (-); urine Bence-Jones protein (-)	/	/
Rotondano *et al*[60], 2007	55/M	Epigastric pain, heartburn, weight loss	Two white-yellow granular-like circular areas	3 cm	Distal portion of the gastric body and angle of the stomach	Mucosal and submucosal layers exhibited slight thickening	H&E staining: Lymphocytes and polyclonal plasma cells infiltrated the lamina propria; Congo red (+)	/	Biopsy and endoscopy of the rectum, duodenum and esophagus (-)	/	None
Ebato *et al*[62], 2012	77/F	Anemia	One flat, depressed area	46 mm × 28 mm	Lower gastric body	/	H&E staining: Amyloid deposits in the mucosa and submucosa; DFS staining (+)	AL	/	/	Endoscopic removal
Sawada *et al*[46], 2012	72/F	/	Flat elevations, tumors; ulcers resemble advanced cancer, intramural hematomas	/	Scattered distribution in the antrum, proximal and middle stomach	Structural loss, thickened hypoechoic mucosa and submucosa	Congo red (+)	AL (κ&λ)	Biopsy and endoscopy of other gastrointestinal tissues (-)	/	/
Rivera *et al*[42], 2012	67/M	Melena, anemia	One round and erosive mass with errhysis	2.5-3 cm	Cardia	/	Confirmed amyloidosis	/	Biopsy of bone marrow (-)	Gastric adenocarcinoma	Surgery, hematology consultations
Kamata *et al*[36], 2012	76/F	Epigastric discomfort	Multiple swollen and reddish folds with a hemorrhagic and erosive mucosa	/	Greater curvature of the gastric body	Thickened submucosal layer	Amyloid deposits in the submucosa and mucosa, Congo red (+)	AL	Biopsy of the rectum and ileum (-); Bence-Jones protein (-); echocardiography (-)	Gastric carcinoma	None
Jin *et al*[61], 2014	33/F	Epigastric pain, dyspepsia, heartburn, acid reflux	One area with irregular borders and a hemorrhagic mucosa; another area with normal borders and smooth surfaces	1.2 cm × 1.2 cm; 10 mm × 20 mm	Lesser curvature of the gastric body; gastric fundus	Hypoechoic thickened stratum mucosum and lamina muscularis protruded in the lesser curvature; nonechoic lesions in the fundus	Amyloid deposits detected from the submucosa to muscularis propria and around small blood vessels using H&E staining; Congo red (+); van Gieson staining (-)	/	/	/	ESD; DMSO
Yamaguchi *et al*[47], 2015	49/M	/	One elevated lesion similar to a submucosal tumor	15 mm	Greater curvature of the lower body	A hypoechoic mass with hyperechoic spots in the submucosa and the muscular layer	Amyloid deposits in the submucosa; Congo red (+)	AL	Biopsy of other tissues in the gastrointestinal tract (-)	/	/
Kobara *et al*[37], 2015	80/M	Epigastric discomfort	One granular, elevated lesion	20 mm	Posterior wall of the prepyloric ring	A hypoechoic mass in the submucosa	Congo red (+)	AL	/	/	/

Kagawa *et al*[32], 2016	73/M	None	One pale, depressed area with clear borders	15 mm	The anterior wall of the lower gastric body	/	H&E staining: Amyloid deposits in the lamina propria and submucosa; Congo red (+)	AL	CT of the chest, abdomen and pelvis (-); urine Bence-Jones protein (-); electrocardiogram, echocardiography	/	None
Ahn *et al*[33], 2018	55/F	None	One pale, round, central-depressed area with irregular and heaped-up edges	20 mm	Lesser curvature of the mid-gastric body	/	Lymphoplasmacytes and Congo red (+) staining in the lamina propria	AL (κ&λ)	Biopsy of colon and duodenum (-); echocardiography (-); CT of chest abdomen and pelvis (-); antineutrophilic antibodies, rheumatoid factors, serum immunoglobulin and components, antinuclear antibodies and urine Bence-Jones protein	cancer	None
Ding *et al*[53], 2018	54/M	None	One well-defined lesion with irregularly distorted vessels	/	/	Thinned superficial mucosa, thickened deep mucosa	Congo red (+); H&E: Amyloid deposits in the mucosa	/	/	Early gastric cancer	Surgery
Kinugasa *et al*[34], 2018	64/M	None	One submucosal tumor with a hard elastic character	40 mm	Middle body of the greater curvature	In the second and third layers of the mucosa	Congo red (+) staining in the mucosal propria	AL (λ)	Bone marrow biopsy; biopsy of other gastrointestinal tissues; ultrasound and CT of the liver, kidney and heart	Myoma, malignant lymphoma, gastrointestinal submucosal tumor	/
Savant *et al*[43], 2018	64/M	/	One mass	3.6 cm	/	One hypoechoic mass in the muscularis propria	Congo red (+); H&E staining: Amyloid deposits with a foreign-body giant cell reaction	AL (λ)	CT, urine analysis and serology (-)	Gastrointestinal stromal tumor	/
Matsueda *et al*[35], 2019	59/M	None	Multiple pale and depressed lesions	/	Through the stomach	/	Congo red (+)	AL	Biopsy of other gastrointestinal tissues; Bence-Jones protein (-); ultrasound and CT	Healing gastric ulcer	/
Present case	70/F	Hematemesis	Multiple congestive erosions; one area of the mucosa with edema and ulcers exhibited unclear boundaries	4.0 cm × 4.0 cm	The anterior wall of the gastric body and fundus	/	Congo red (+); H&E staining: Massive amyloid fibrous connective tissues deposited in the interstitium with inflammatory cell infiltration	/	CT of the liver, colon, kidney; HRCT of the lung; ultrasound of the liver, heart, and kidney	/	None

ESD: Endoscopic submucosal dissection; DMSO: Dimethyl sulfoxide; CT: Computed tomography; ECG: Electrocardiograph; AL: Light chain; AA: Amyloid A; PFS: Progression-free survival.

is noted given the similarities in appearance. Furthermore, some patients appear normal in endoscopic evaluations, and endoscopy should therefore not be used for an independent diagnosis[39].

After reviewing our data, a reasonable hypothesis is that amyloid subtypes and endoscopic findings are correlated, based on the similar appearance and features noted among the patients with the same amyloid subtype. The endoscopic appearance corresponded to amyloid subtypes in the small intestine, which is the most frequent site of gastrointestinal amyloidosis. For instance, the endoscopic presentation of AL is mostly thickening (75%) and multiple polypoid protrusions (63%), whereas a fine

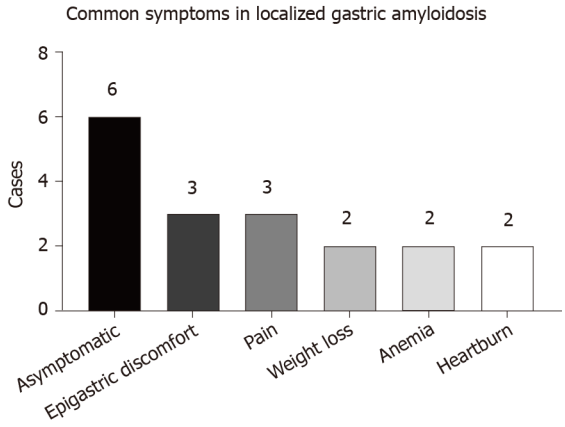


Figure 4 Common symptoms described in case reports of localized gastric amyloidosis and the present times.

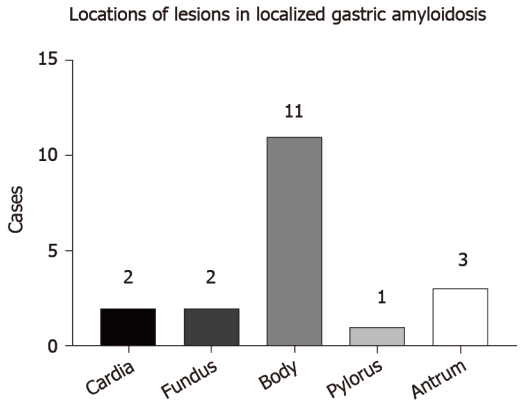


Figure 5 Common lesion locations of amyloid deposition in localized gastric amyloidosis and the present times.

granular appearance is predominant in patients with AA (85%)[49]. However, no association has been confirmed in the gastrointestinal tract according to a study with a small sample size, consistent with the results of the study by Samar M[39,50]. Considering the small sample sizes and limitations of published studies, further studies with large samples are required.

Narrow-band imaging with magnifying endoscopy (NBI-ME), a convenient technique that visualizes the mucosal morphology and microvascular architecture without a dye[51], is also highlighted in the amyloidosis diagnosis. Commonly, it is applied to detect malignancy in the colon and rectum through precise measurements of pit patterns with a promising accuracy (93.4%), sensitivity (100%), and specificity (75%)[52]. To date, the emerging use of NBI-ME has been reported not only in patients with LGA[32,46,47,53] but also in patients with amyloidosis of the rectum, trachea, and pleura[54-56]. Upon enhancement, special pathological patterns are detected in affected lesions, manifesting as abnormal distorted vascular networks on the mucosa with a grayish-green appearance and grooved surface, which may relate to amyloid protein deposits[32,46,56]. The application of NBI-ME provides a better method to reveal the amyloid deposits as a useful tool to assist with the diagnosis.

Endoscopic ultrasound (EUS) is also useful for determining the diagnosis by revealing the loss of the normal structure and thickened hypoechoic gastric walls, which mainly affects the mucosa and submucosa (Table 1). Its use in combination with other methods has certain clinical value, such as EUS-guided fine needle aspiration (EUS-FNA). In a retrospective study of 47 patients with amyloidosis presenting with lymphadenopathy (swollen lymph nodes), the involvement of the gastrointestinal tract was noted in 39% of cases[57]. In addition, EUS-FNA displayed a favorable sensitivity (83%), specificity (94%), and accuracy (86%) for distinguishing malignancy, although the size of the swollen lymph nodes may influence the accuracy[58]. The clearance of peripheral lymph nodes was once suggested as a preferred method to prevent malignancy[38]. However, with the assistance of EUS-FNA, the resection of swollen lymph nodes is not necessary in every case. Further use of EUS is expected.

Biopsy

Biopsy is an essential assessment for diagnosing amyloidosis. Direct biopsy verification in patients with symptoms is the main diagnostic criterion for gastrointestinal amyloidosis, according to the guideline of AL amyloidosis[30]. Specific staining methods, such as H&E staining and Congo red staining, are used to visualize the amyloid deposits. Under a light microscope, amyloid deposits typically manifest as amorphous eosinophilic hyaline materials after H&E staining[38]. Amyloids with foreign-body reactions[43,59] and plasma cell infiltration[40,45,60] are also noted. The gold standard for identifying amyloid protein is Congo red staining. When bound to the dye, amyloid deposits are orange under a light microscope, and exhibit green, orange, or yellow birefringence under a polarized microscope[1,12]. Congo red staining is commonly used to diagnose all types of amyloidosis and has been applied to various tissue samples. Most of the case reports collected in the present study use and present the results of both of H&E and Congo red staining to confirm the diagnosis. Biopsy findings also show that amyloids are mainly deposited in the lamina propria, submucosa, and mucosa. Amyloid involvement of blood vessels is also observed[31,38,59,61]. AA LGA is characterized by deposits in the mucosa, submucosa, and vascular walls[38]. AL LGA deposits are mainly located in the mucosal propria, submucosa, and mucosa[32,34,36,47,62]. In a study of 79 cases of gastric amyloidosis, depositions occurred in the muscularis mucosae, but our data do not reveal this characteristic, likely due to the limited number of cases and exclusion of patients with systemic gastric amyloidosis[39]. Interestingly, the amyloid subtypes may account for the difference in deposit locations, which has been verified in several studies. In patients with gastric amyloidosis, AA amyloid deposits are preferentially located in the lamina propria, while AL is mainly deposited in the muscularis mucosae[63].

Although useful, Congo red staining has limitations. For example, when inadequate amyloid labeling occurs or the procedure is performed by inexperienced examiners in poorly controlled conditions, it presents limitations and improved methods have been constantly introduced over the years[14,64]. The application of some hypersensitive techniques may improve the accuracy of amyloid detection. Luminescent dye-conjugated polymer (LCP) spectroscopy detects every deposit that Congo red does using an easy method and effectively reduces false positives[65]. Based on these findings, several probes have been used in experiments. In a study with a small sample of patients with systemic amyloidosis, heptameric formic thiophene acetic acid (h-FTAA) was reported to be more sensitive than Congo red staining when detecting amyloid in abdominal fat[66]. Furthermore, h-FTTA detects small amyloid-like structures that are negative for Congo red staining due to its high quantum field. Thus, this method might potentially achieve the early diagnosis of amyloidosis and allow considerable progress in early treatment. Moreover, h-FTTA possesses a high sensitivity and relatively low specificity, but its combination with Mayer's hematoxylin staining may compensate for its disadvantage in visual contrast[67]. Although the methods that we mentioned above are still in development, a reasonable expectation is the future development of a hypersensitive approach as a replacement for Congo red staining.

Assessment of amyloid typing

Different forms of amyloid lead to completely different prognoses, and amyloid typing is a routine test performed in patients with amyloidosis. Several assessments have been applied for amyloid typing, including immunohistochemistry (IHC), immunofluorescence (IF) staining, immunoelectron microscopy, and genetic testing[8,16,39]. However, only half (13/22) of the patients receive the test during the diagnosis of LGA, probably due to insufficient tissues and limited techniques[8]. IHC is currently used in the remaining patients and in patients with other forms of amyloidosis, as it is a convenient and rapid method. IHC was demonstrated to be a convincing approach with considerable specificity and sensitivity, according to a systematic study involving 117 patients[68]. However, some of its disadvantages include the presence of unclassifiable and misdiagnosed conditions due to the limited availability, specificity, and sensitivity of antibodies[16,65]. Laser microdissection/mass spectrometry (LMD/MS) requires a small amount of sample and exhibits a high specificity and sensitivity in typing clinical specimens[39,69]. LMD/MS has also been used to analyze samples that do not reach the standards for IF and exhibits a high detection rate of 92% compared to 45% using IHC[70,71]. Moreover, its use in combination with multiple reaction monitoring or liquid chromatography (LMD-LC-MS) results in the detection of amyloid protein that IHC fails to detect and achieves early amyloid detection, even when Congo red results are negative[72,73]. In addition,

the decellularization of an amyloid biopsy can facilitate the diagnosis of AL and ATTR amyloidosis, which are rich in plasma proteins. Here, decellularization provides a solution by removing the unnecessary proteins without altering the amyloid deposits and the basic structures of the biopsy[74,75]. Hopefully, these approaches may be used after further testing and reduce costs. Until then, we still call for the urgent increase in the use of IHC to diagnose LGA.

DIFFERENTIAL DIAGNOSIS

Systemic amyloidosis

The final diagnosis must be confirmed by the exclusion of the systemic involvement of other organs, which is generally performed using ultrasound of the heart and kidney and biopsy of the bone marrow and regions of the gastrointestinal tract, including the esophagus, duodenum, and colon. Occasionally, urine levels of the Bence-Jones protein and other antigens are tested. Under most conditions, the aforementioned tests are selected based on clinicians' experience with a screening procedure. Immunohistochemical examinations also provide a lead for the differentiation of systemic and localized amyloidosis, because the deposited protein exhibits specific distribution patterns. For example, AA and ATTR are often detected in the former type, while AL has been detected in both types[2,11].

Some radiopharmaceuticals present potential abilities to distinguish systemic and localized amyloidosis. SAP is one of the nonfibrillar components present in all types of amyloid deposits, and its abundant accumulation makes it an ideal radiotracer to visualize amyloid deposits in images of the body[2,22]. In a study of 189 patients with confirmed amyloidosis, ^{123}I SAP scintigraphy, a noninvasive qualitative method, presented a high sensitivity and specificity and was applied to diagnose most cases of AA and AL amyloidosis[76]. Through the use of whole-body scintigraphy, the injection of ^{123}I SAP obviously reveals the distribution and extent of amyloid deposits in images that a histopathological examination fails to detect, and the organ involvement identified using this technique exceed the results obtained in the clinic, which may be valuable in excluding systemic involvement. The only limitation is its failure in revealing the heart muscle[76]. It is gradually becoming a universal technique used in relevant studies for scanning systemic involvement in the whole body, except for the heart[3,77]. With the development of proper radiotracers and a considerable increase in use, we postulate that nuclear images will significantly contribute to the diagnosis of LGA.

^{18}F-fluorodeocyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) is also reported as a potential technique to distinguish systemic and localized amyloidosis. Under most conditions, it is introduced as a method for detecting lung malignancy in patients suspected of having the disease, and it is mainly used to scan amyloidosis in the lung[78,79]. Glaudemans *et al*[77] reported that ^{18}F-FDG PET/CT is able to visualize the difference between localized and systemic amyloidosis by imaging the inflammatory reaction of multinuclear giant cells, which are unique in localized amyloidosis, manifesting as positive FDG uptake at sites of amyloid deposits in patients with localized amyloidosis but negative uptake in patients with systemic amyloidosis[77]. However, Mekinian *et al*[80] also reported positive results in patients with systemic amyloidosis, which may be attributed to the limited number of samples or inappropriate designs[80]. To date, ^{18}F-FDG PET/CT is still an immature method to exclude systemic amyloidosis, and patients with gastric amyloidosis were not examined in either study. When used in conjunction with confirmed evidence obtained from other techniques, it may play a supporting role in determining the diagnosis.

Advanced cancer

Due to its rarity and nonspecific presentations, amyloidosis is typically not the first diagnosis suspected by clinicians, and differential diagnosis becomes an important step. Under most circumstances, patients are scheduled for a precise test screening for potential cancers and are confirmed to have amyloidosis. Most lesions were suspected to be advanced gastric cancers or some gastrointestinal tumors (9/22) since they share common appearances, such as ulcers, elevations, and tumor-like lesions. During gastroscopy or esophagogastroduodenoscopy, some of these lesions are easily suspected to be submucosal tumors[34,46]. The most reliable method for excluding the possibility of tumors is a tissue biopsy that does not detect tumor components and the confirmation of the presence of amyloid based on positive Congo red staining results.

Tumor biomarkers are occasionally involved in the examinations based on clinicians' experience. Although NBI-ME is a new technique, it may also facilitate differentiation, given its ability to exclude malignancies. Based on the clear visualization of the microvasculature and microstructure under NBI-ME, vascular and surface pattern classifications are proposed as a reference, and the typical hallmarks of advanced gastric cancer can be observed, thus providing a reliable evaluation of advanced gastric cancer[81]. A subsequent study reported an obviously increased sensitivity and accuracy of NBI-ME compared with routine approaches in scanning for advanced gastric cancer, and the use of NBI-ME was also beneficial to locate the most suspicious lesion for biopsy[82].

Compared to other case reports, we excluded advanced cancer using a more innovative screening method, namely, immunohistochemical staining, given our restricted conditions. Because gastric carcinoma is suspected in some cases[31,36,59], it may be a wise choice. The combination of several antibodies for immunohistochemical staining, including CKPAN, KL067, Periodic Acid-Schiff, spinal muscular atrophy and vimentin, with biopsy results did not reveal strong support for cancers and excluded the possibility of gastric cancer from various origins.

TREATMENT AND PROGNOSIS

Generally, the goal of therapy for amyloidosis is to eliminate harmful extracellular amyloid deposits and restore the normal function of affected organs as much as possible. The main treatments include surgery, observation, and radiotherapy[83]. Chemotherapy is recommended for patients with AL who present with myeloma.

Several novel techniques are also introduced here. As we mentioned above, SAP is a type of plasma protein present in all amyloid deposits, and its interaction with amyloid fibrils may prevent the digestion of amyloid, as evidenced by the results of *in vitro* experiments[21,22]. Because amyloidosis is delayed in SAP knockout mice, SAP clearance is introduced as a potential strategy for treating amyloidosis with the application of relevant antibodies and drugs[24]. Here, (R)-1-{6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl}pyrrolidine-2-carboxylic acid (CPHPC), a small-molecule drug with a high affinity for SAP, depletes most of the circulating SAP[84,85]. Its use in combination with the anti-SAP antibody dezamizumab results in a considerable reduction in the amount of residual amyloid protein visible in ^{123}I SAP scintigraphy, with no obvious side effects[85,86]. After treatment, patients tend to present an improved or stable state, suggesting its potential ability to ameliorate symptoms and reduce damage in the affected organs[84]. A subsequent study further confirmed its effects on the spleen, kidney, and liver[87]. Currently, studies targeting SAP are mainly conducted in mice and small samples of patients with systemic amyloidosis. Although complete removal of all amyloid deposits has not yet been achieved, its usage in patients with LGA as a rapid method for the early clearance of amyloid deposits is worthy of further exploration.

Currently, the first-line therapy for localized AL gastrointestinal amyloidosis is mainly observation/supportive care and the excision of amyloid deposits, and radiotherapy is rarely used, according to the experience of physicians from the Mayo Clinic[83]. Among 13 patients with LGA presented with clear therapeutic strategies, surgery was the main choice (8/13), and a few patients chose observation (4/13). The administration of dimethyl sulfoxide is also recommended to reduce the digestive symptoms and result in a visible improvement on endoscopy[61].

The prognosis of amyloidosis is generally related to the extent of organ damage. Unlike systemic amyloidosis, localized amyloidosis has an excellent prognosis and minimally affects patient survival[3]. After first-line treatment, most patients improved (53%) or achieved a stable state (31%), and only a few progressed (0.2%), according to a study enrolling 413 patients with localized AL amyloidosis. However, the study also mentioned an undeniable recurrence rate, as two or more recurrences occurred in 5% of the 413 patients, and the first 5 years after diagnosis is a crucial period for recurrence[83]. To date, no recurrence of LGA has been mentioned in the case reports published, and all patients presented a healthy state in follow-up visits, but close follow-up and regular examinations are necessary, particularly within the first 5 years (Figure 6).

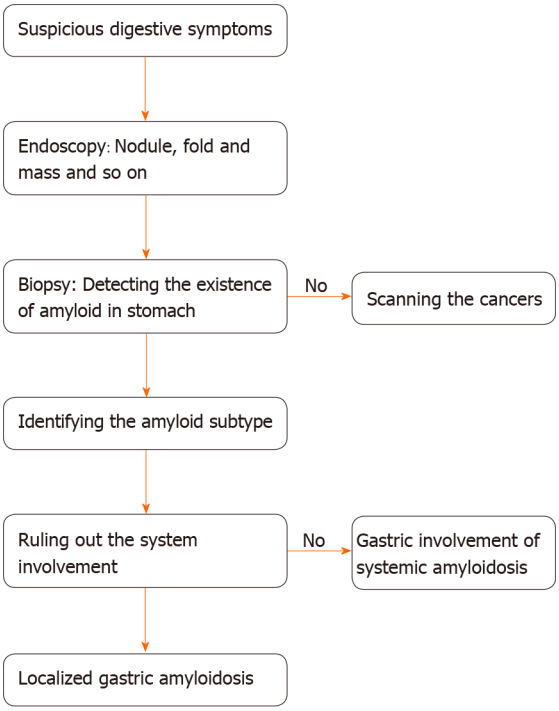


Figure 6 A diagnostic procedure from the perspective of clinicians. When a patient arrives at the hospital with suspected digestive symptoms, clinicians should initially perform endoscopy. Relevant endoscopic manifestations should lead to a biopsy examination to detect the existence of amyloid using Congo red staining. If a negative result is obtained, a screen for cancers is recommended, given the resemblance of their clinical manifestations. For a positive result, clinicians should identify the amyloid protein subtype. Then, a series of tests must be chosen by clinicians according to the patient' conditions to exclude the systemic involvement of amyloidosis. Finally, a diagnosis of localized gastric amyloidosis is determined.

CONCLUSION

Local gastric amyloidosis is such an extremely rare disease that only 22 cases have been reported in the past few decades and the disease is unknown to the public. It is commonly introduced as a human pathological state in which an abnormally misfolded protein accumulates in tissues, causing structure loss, organ dysfunction, and even death. Its pathogenesis remains a mystery, but a few influencing factors that may contribute to the formation of amyloid fibrils are studied and introduced here. Our review may inspire further investigations of the mechanism. Based on the 21 existing cases and our case, we present a detailed description of the main information available on LGA and conclusions regarding its clinical features, diagnostic tools, and treatment, with the goal of establishing future guidelines. Its clinical manifestations are complex and similar to those of other gastric diseases, such as advanced cancer, resulting in minimal awareness among clinicians. The diagnostic tools include biopsy, imaging, and amyloid typing. The final diagnosis mainly depends on the biopsy results, and Congo red staining remains the gold standard. Treatments for LGA mainly include supportive care and surgery. After treatment, most patients receive a good prognosis. Currently, due to its rare incidence, LGA lacks public awareness, and studies that explore its pathogenesis and its clinical features are often unspecific. For clinicians, LGA is a challenge to diagnose using regular tests. Building on that information, we describe the main clinical features and take the lead in proposing a process for diagnosing LGA from the clinicians' perspective, with the aims of promoting awareness of LGA and potentially contributing to the development of LGA guidelines.

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MINIREVIEWS

Emerging wearable technology applications in gastroenterology: A review of the literature

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Abstract

The field of gastroenterology has recently seen a surge in wearable technology to monitor physical activity, sleep quality, pain, and even gut activity. The past decade has seen the emergence of wearable devices including Fitbit, Apple Watch, AbStats, and ingestible sensors. In this review, we discuss current and future devices designed to measure sweat biomarkers, steps taken, sleep efficiency, gastric electrical activity, stomach pH, and intestinal contents. We also summarize several clinical studies to better understand wearable devices so that we may assess their potential benefit in improving healthcare while also weighing the challenges that must be addressed.

Key Words: Wearable technology; Wearables; Ingestibles; Smartphone; Remote patient monitoring; Gastroenterology

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Core Tip: Wearable technology allows continuous health monitoring to provide a novel means of diagnosing and managing patients. Applications of wearable technology such as wrist wearables, abdominal wearables, smartphones and mobile apps, and ingestible sensors, are developing in gastroenterology. The aim of this review is to investigate current data from the literature that studies recent wearable technologies in several gastrointestinal diseases including inflammatory bowel disease, irritable bowel syndrome, and other functional gastrointestinal disorders.

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INTRODUCTION

Wearable devices are revolutionizing medicine and impacting healthcare by enabling continuous health monitoring outside of the clinic[1]. These wearables include devices that can be worn from head to toe and even swallowed. In patients with gastrointestinal diseases such as inflammatory bowel disease and irritable bowel syndrome, these devices collect physical activity, sleep quality, heart rate and rhythm, and more recently, gut activity and gas profiles. Despite the surge of consumer interest in these technologies, there is a lack of sufficient evidence to support their widespread use in clinical practice.

The field of gastroenterology has seen an emergence of wearable technology that has the potential to diagnose, manage, and even prevent disease. As technological advancements continue, classifying devices into categories will become essential. The purpose of this article is to offer focused insights into backgrounds for categorizing devices, the various uses of wearable technology, and future opportunities for clinical applications, with a focus on wrist wearables, abdominal wearables, smartphones, and ingestible sensors (Table 1).

In this review, we performed a PubMed search using the search terms "wearables," "wrist wearables," "abdominal wearables, "smartphones," and "ingestible sensors." We only selected manuscripts, which were original articles, and includes studies in several gastrointestinal diseases including inflammatory bowel disease, irritable bowel syndrome, and other functional gastrointestinal disorders. The objectives of this review were (1) to assess how wearable technology could assist physicians in investigating, diagnosing, or even treating our patients with gastrointestinal diseases; and (2) to recommend how wearable technologies could be applied in the future for several gastrointestinal diseases, including inflammatory bowel disease, irritable bowel syndrome, and other functional gastrointestinal disorders.

WEARABLE DATA TYPES AND USE

Wearable technology may be better understood by categorizing the types of data that can be collected. One type is data collection that requires active patient engagement with the device to obtain data that then can be transmitted in real time or uploaded to a stored source. This allows the user's data to be collected by a device such as a wrist wearable, which then can be uploaded to the electronic health record. For example, active patient engagement may be used to correlate certain symptoms of acute mesenteric ischemia with electrocardiographic assessment to detect the presence of a related arrhythmia. Another type is data collection that does not require active initiation other than the first step of wearing the device. Once the device is worn, it may passively collect data by continuously or intermittently obtaining data to be transmitted or stored and later uploaded. These passive data collections may include continuous measurements of heart rate, respiratory rate, tone of voice, caloric intake, and gastrointestinal activity in a patient with an underlying gastrointestinal condition.

Wearable data may be most useful in its ability to inform individuals and physicians of the effects of the patient's actions, management, or clinical status[2]. Ideally, these devices will provide data to offer decision support and even offer built-in therapies[3]. For example, we know that diet can be modified to modulate the microbiome[4] but to effectively design individualized diets, feedback is needed to close the loop between a prescription and its effects. This feedback can offer automated recommendations for instant modification of a patient's behavior and therapy. Even for devices that are unable to offer built-in therapy, the data collected can be used for diagnosis, prognosis, management, or prevention.

Table 1 Summary of wearable technology along with clinical applications

Ref.	Device name	Device type	Clinical applications
[5,12,13,20,21]	Fitbit; Apple Watch; Amazon Halo	Wrist Wearable	Daily activity monitoring (steps taken, energy expenditure, and sleep hygiene)
[11]	Sweatsenser	Wrist Wearable	Inflammatory bowel disease monitor and management
[27,28]	Electrogastrogram	Abdominal Wearable	Ambulatory monitoring, functional GI disorders screening, diagnosis, and management
[31,32,34]	AbStats; G-Tech Medical	Abdominal Wearable	Bowel sounds and movement monitoring, postoperative ileus and delayed gastric emptying
[27]	N/A	Smartphone App	Meal logs, exercise, bowel movement, and sleep synchronized to electrogastrogram recording
[39]	UCLA eIBD patient app	Smartphone App	Inflammatory bowel disease activity monitor
[40]	HealthPROMISE app	Smartphone App	Tracks symptoms, quality of life, follow up, and intervention integrated with electronic health record
[41,42]	StudentLife app	Smartphone App	Assess stress, sleep, activity, mood, mental well-being, and academic performance
[43]	PoopMD; Pooplog	Smartphone App	Records stool types, records bowel movements
[48]	N/A	Ingestible	Vital sign monitor, motility disorder diagnosis and management
[49]	IMBED	Ingestible	Gastrointestinal bleed diagnosis, management, and monitoring
[50]	N/A	Ingestible	Understand intestinal function, microbiota, and individual response to dietary change
[56]	Colon Capsule Endoscopy	Ingestible	Minimally invasive colonoscopy method
[57]	Digital Pills	Ingestible	Monitor medication adherence

IBD: Inflammatory bowel disease; UCLA: University of California Los Angeles; GI: Gastrointestinal; N/A: Not applicable.

WRIST WEARABLE DEVICES, INFLAMMATORY BOWEL DISEASE AND IRRITABLE BOWEL SYNDROME

Commercially available wrist wearable devices have grown rapidly in popularity during these recent years due to advancements in technology and the public's increased health consciousness. These wrist wearable devices such as Fitbit, Apple Watch, and the new Amazon Halo aim to provide the user with real-time feedback on various aspects of daily activities such as number of steps taken, energy expenditure, sleep hygiene, and time spent in different levels of activity[5]. They also provide personal goal setting options, data summary, and visualizations through synchronization with mobile- and computer-based apps such as health and fitness apps as well as options to connect to social media. Increasing consumer interest and improvement of data collecting capabilities of wearable technology has drawn attention to the devices as a potential avenue to improve the care of patients with inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS).

IBD, which includes Crohn's disease and ulcerative colitis, is characterized by chronic relapsing intestinal inflammation[6]. Although the etiology of IBD remains largely unknown, it is thought that IBD results from an abnormal and continuing immune response to the microbes in the gut, catalyzed by the genetic susceptibility of the individual[6]. Despite advances in therapeutic development, only 40%–60% of IBD patients can achieve remission at 1 year, and symptomatic relapse still occurs in at least 15% of patients per year[7,8]. Prediction of symptomatic relapse would be highly desirable in IBD patients as this would allow for early intervention or prevention. Studies have shown that quality of life for individuals with IBD was poorer than for healthy individuals, for both adults and children[9,10]. Effective and convenient strategies for prediction and prevention of relapse are needed.

IBD represents a chronic disease where the application of wearable technology may be able to improve management and predict or even prevent inflammatory disease flare. In a first study, Jagannath *et al*[11] used EnLiSense's Sweatsenser for noninvasive continuous monitoring of interleukin-1 (IL-1β) and C-reactive protein (CRP), two key biomarkers associated with IBD, in human eccrine sweat. The sensor device demonstrated capability to detect and real-time monitor IL-1β and CRP in sweat. This study signifies a promising non-invasive wearable microsensor device that has the

potential to empower patients to actively engage in monitoring and managing their IBD. This device may also give patients the chance to intervene earlier and help gastroenterologists understand whether treatment is effective.

Wiestler *et al*[12] investigated the association of quality of life with wearable-based physical activity in patients with IBD. A total of 91 patients with IBD were evaluated in terms of disease-specific quality of life, using the Inflammatory Bowel Disease Questionnaire (IBDQ), and physical activity, using an accelerometer. The IBDQ was significantly lower in patients with moderate-severe disease activity as compared to patients in remission, and the physical activity level was higher in remission than in active disease. This study found that parameters of physical activity were significantly correlated with the IBDQ, and steps per day, vigorous activity, and sleep efficiency were significantly associated with the IBDQ. Importantly, the data positively correlate with health-related quality of life and demonstrates the positive effect of physical activity for patients with IBD.

Hirten *et al*[13] surveyed 400 patients with self-reported IBD and found that 89% of them believed that wearable devices can provide important information about their health, and 93.8% reported that they would use a wearable device if it could help their physician manage their IBD. The patients specifically identified wrist wearables as the preferred device type and reported a willingness to wear them at least daily. Because of patients' willingness to participate, wearables allow them to actively engage in their health and further strengthen physician-patient collaboration, which will ultimately improve patient well-being and medicine as whole.

Irritable bowel syndrome, one of the most common disorders of gut-brain interaction worldwide, is a functional disorder of the gastrointestinal tract characterized by chronic abdominal pain or discomfort and bowel habit changes in the form of diarrhea, constipation, or alternating patterns between the two[14,15]. IBS is estimated to affect around 1 in 10 people globally[16] and is associated with reduced quality of life[17].

Many studies have shown that increased physical activity has positive long-term effects on IBS symptoms and psychological symptoms[18,19]. Hamaguchi *et al*[20] investigated the relationship between physical activity and gastrointestinal (GI) symptoms in 101 university students with IBS using the Gastrointestinal Symptoms Rating Scale and a pedometer, which measured gait steps for 1 wk. They found that the probability for daily locomotor activity to discriminate between 5 and 4 points on the Gastrointestinal Symptoms Rating Scale (*i.e.* likely to have reverse symptoms) decreased in accordance with increment of steps per day: 78% probability for 4000 steps, 70% probability for 6000 steps, 59% probability for 8000 steps, and 48% probability for 10000 steps. This study demonstrated that improvement in IBS symptoms increases with number of steps taken per day in IBS patients.

GI symptoms can also be triggered by several lifestyle factors including psychological distress, short sleep duration, and diet. Clevers *et al*[21] investigated the associations between selected lifestyle factors, measures of stress physiology, and GI symptoms. 1002 office employees were asked to report their GI symptoms, psychological distress, sleep times, and intake of caffeine, alcohol, and soft drinks for 5 d. They also recorded skin conductance, heart rate/variability, and acceleration using wearable sensors. Although the physiological variables such as skin conductance and heart rate variability were weakly associated with GI symptoms in this study, they found that short sleep duration was associated with next day GI symptoms and psychological distress mediated the association between short sleep duration and next day GI symptoms (61%).

Stress has been shown to play a major role in the onset and exacerbation of symptoms in IBS patients with stress related disorders such as anxiety and depression either preceding or following the development of IBS[22]. With wearables' capability of monitoring sleep, heart rate, physical activity, and tone of voice, these devices can alert patients of their well-being in real time and potentially recommend therapies to improve their well-being to serve as biofeedback to better control their stress and general health.

ABDOMINAL WEARABLE DEVICES, FUNCTIONAL GASTROINTESTINAL DISORDERS AND POSTOPERATIVE USE

The electrogastrogram (EGG) is a non-invasive device that is used for abdominal surface measurement of the gastric electrical activity of the human stomach[23]. However, it is rarely used due to inconsistent results and signal artifacts that make

interpretation and continuous monitoring difficult. Recent studies have shown the potential of EGG as an effective and non-stationary method to differentiate diabetic gastroparesis and functional dyspepsia patients[23].

Functional GI disorders can affect any part of the GI tract including the esophagus, stomach, and intestines. They are disorders of function, rather than structural or biochemical abnormalities. Examples of functional GI disorders include functional dyspepsia, gastroparesis, and irritable bowel syndrome (IBS). Functional dyspepsia[24], which is characterized by a sensation of pain or burning in the epigastrium, early satiety, fullness during or after a meal, or a combination of these symptoms, has a global prevalence between 5% and 11%[25]. Gastroparesis, which is characterized by delayed gastric emptying in the absence of mechanical obstruction, affects 4% of the United States population[26]. IBS, as stated above, is characterized by chronic abdominal pain and bowel habit changes, which deeply impairs and affects quality of life of many IBS patients. Functional GI disorders are typically diagnosed with subjective symptom-based assessment or objective but invasive procedures such as antroduodenal manometry, a procedure that measures motility with a catheter inserted through the mouth or nose with fluoroscopic or endoscopic guidance[27].

Gharibans *et al*[27] developed an innovative device that overcame the technical issues of the EGG with a wearable multi-channel system and artifact removal signal processing method, making it comparable to antroduodenal manometry, the gold standard diagnostic method. This non-invasive and easily administered approach potentially allows for patient monitoring outside of the clinic, helps better understand functional GI disorders, and leads to more effective screening, diagnosis, and management. Gharibans *et al*[27] also developed a smartphone app to enable the patients to document events or activities such as logging meals, exercise, bowel movement, and sleep, that are time-synchronized to the EGG recording for real-time feedback to the users.

The gut-brain axis consists of bidirectional communication between the central and the enteric nervous system, connecting emotional and cognitive centers of the brain with peripheral intestinal functions[28]. IBS is an example of the disruption of these complex relationships. Vujic *et al*[28] investigated the potential of using GI activity as an index of insula activity, which is the part of the brain associated with cognitive and affective functions. 33 participants with no known GI, neurological, or psychiatric disorders were connected to an EGG and EEG, presented emotionally salient film clips, and answered a self-assessment at the end of each clip. Although positive movie segments did not produce statistically significant changes (P = 0.4706), EGG signal analysis in the frequency domain demonstrated statistically significant changes from negative movie segments (P = 0.0209). Because EGG signals may be a sign of negative emotions, this gut-brain axis should be further studied in IBS patients in hopes of potential use of EGG in diagnosing and managing IBS.

Despite advances in surgical techniques, most patients develop temporary GI paralysis such as postoperative ileus (POI) and delayed gastric emptying (DGE) following abdominal surgery[29]. When prolonged or complicated, POI can worsen patient outcomes, increase resource utilization and cost, and extend hospital length of stay by 30%[30]. Data reveal that continuous audio recordings of bowel sounds strongly correlate with true intestinal motility as measured using antroduodenal manometry[31]. Spiegel *et al*[32] developed an acoustic gastro-intestinal surveillance (AGIS) biosensor – the Gastroinstestinal Logic AbStats system – a disposable plastic device embedded with a microphone that adheres to the abdominal wall and allows continuous and automated analysis of bowel sounds *via* noninvasive vibration and sound sensing. They compared intestinal rates using AGIS in 8 healthy controls, 7 patients tolerating feeding, and 25 with POI. Mean intestinal rates were 0.14, 0.03, and 0.016 events per second, respectively. AGIS separated patients from controls with 100% sensitivity and 97% specificity.

DGE following pancreaticoduodenectomy (PD) is a common complication, which occurs in up to 30% of cases[33]. In primary DGE, which is when not associated with other risk factors or intraabdominal complications, it is difficult to predict early on who will develop DGE after PD[34]. Dua *et al*[34] assessed whether the use of a novel, noninvasive wireless patch system (G-Tech Medical) that acquire gastric myoelectrical signals and transmit data by Bluetooth after PD is reproducible and can serve as an objective tool to identify patients who may be at risk of developing DGE. They found that tolerance of food was noted by 6 *vs* 9 d in the early versus late group by diet tolerance (P < 0.05) with higher cumulative gastric myoelectrical activity. Diminished gastric myoelectrical activity identified delayed tolerance to regular diet. This study introduces an abdominal wearable, wireless patch system capable of accurately monitoring gastric myoelectric activity after surgery, which can not only objectively

identify patients at risk for DGE but also potentially individualize feeding regimens to improve outcomes.

SMARTPHONES AND REMOTE PATIENT MONITORING OF GASTROINTESTINAL DISORDERS

The most common wearable device is the smartphone. The number of smartphone users has increased dramatically with smartphone ownership reported to be 43% globally and 72% in the United States[35]. Digital health refers to the use of digital, mobile, and wireless technologies to support achievement of health objectives, and the term is often interchangeably used with mobile health (mHealth) due to mobile devices' central role[36]. Due to its increasing popularity, smartphones provide one of the most promising platforms for mHealth interventions including activity trackers, telemedicine capabilities, and health-based apps. The integration of smartphones and mobile apps, remote sensor technologies like Fitbit, telemedicine, and electronic health records (EHR) allows for remote patient monitoring (RPM), which refers to digital tools capable of monitoring and reporting real-time data on patients' health activities outside of the usual healthcare settings[37,38].

Chronic GI disorders such as IBD and functional GI disorders are especially appropriate for RPM. Symptom flare risk and interventions required to control disease is heavily influenced by the patient's behaviors, which occurs outside of the healthcare setting and often are not adequately tracked or assessed such as stress levels, depression, smoking, or medication adherence. Because of these factors, patients with chronic GI disorders are ideal candidates for RPM to potentially improve self-management, quality of life, and collaboration.

Van Deen *et al*[39] developed an mHealth index that accurately monitors IBD activity using patient reported outcomes, which is currently implemented in the University of California at Los Angeles eIBD patient app and automated messages are sent to a nurse coordinator when the mHealth index indicates disease activity.

Atreja *et al*[40] created the HealthPROMISE app, a cloud-based patient reported outcome and decision support platform, which helps patients track their symptoms, quality of life, follow up, and interventions in real time and provides point of care intervention from physicians by integrating the app with EHR.

Wang *et al*[41,42] used the StudentLife app, a continuous sensing app that uses the smartphone's GPS, accelerometer, light sensor, and microphone integrated with call history, application usage, and texting patterns, to assess stress, sleep, activity, mood, sociability, mental well-being, and academic performance in college students. They found that the students' depression was significantly negatively correlated with sleep and conversation frequency and duration. These smartphone apps plus the new Amazon Halo, which captures mood using microphone, also have the potential to be integrated with EHR to monitor for depression and anxiety.

Franciscovich *et al*[43] used PoopMD, a mobile app that utilizes a smartphone's camera and color recognition software to analyze an infant's stool, and determined a sensitivity of 100% and specificity of 89%. They found that PoopMD accurately differentiates acholic from normal color stool and may be a valuable tool to help parents identify acholic stool and alert the infants' pediatricians. Apps like PoopMD and Pooplog, which allows patients to record bowel movements using the Bristol Stool Scale, can be further developed to be used in adult patients to identify various stools such as hematochezia and melena and even alert physicians of a possible GI bleed, infection, IBD flare, or constipation.

Studies have also shown that smartphones are widely used for social media and that a majority of social media is accessed through smartphones as compared to computers[44]. These social media such as Facebook, Instagram, and YouTube may have the potential to be used as platforms to broaden health education and outreach to a wider audience especially minority populations with cultural barriers to healthcare[45-47].

INGESTIBLES, GASTROINTESTINAL HEALTH AND BEYOND

Ingestible sensors, which are also known as swallowables, consist of a miniaturized detector and transmitter packed into a capsule that is swallowed and tracked through the intestine. Ingestibles are fast emerging with efforts continuously being made to

optimize these sensors for various clinical applications. These ingestible devices are noninvasive and provide information on pH, manometric pressure, temperature, medication adherence, vital signs, and intestinal lumen contents[48].

Dagdeviren *et al*[48] developed an ingestible sensor that settles on the stomach lining and allows for monitoring of vital signs and mechanical deformation of the gastric cavity. This flexible ingestible pieozoelectric device allows for possibilities in sensing mechanical variations and energy inside the GI tract, which may be applied in diagnosing and treating motility disorders and monitoring ingestion in obesity.

Ingestibles and microbiome

Mimee *et al*[49] created an ingestible micro-bio-electronic device that combines engineered probiotic sensor bacteria with microelectronics that communicates with an external device such as a smartphone. In this study, they engineered heme-sensitive probiotic biosensors and demonstrated accurate diagnosis of GI bleeds in swine (sensitivity and specificity of 83.3% at 60 min and 100% at 120 min). Thus, ingestible micro-bio-electronic device could transform diagnosis, management, and monitoring of GI health and disease.

The human gut is home to diverse microbes that play a fundamental role in the health and well-being of the host. The microbiota, which consists of bacteria, viruses, and eukaryotes, have been shown to interact with an individual's immune system to influence the development of diseases such as obesity, mental health issues, and atopic disease[50]. Gases of the gut, such as hydrogen carbon dioxide, nitrogen, and oxygen, have been significant in understanding the pathogenesis and diagnosis of gut disorders[51]. Gas production from bacterial fermentation is likely to produce symptoms in patients with diseases like IBS[52] and small intestine bacterial overgrowth[53]. Kalantar-Zadeh *et al*[54] developed an ingestible electronic capsule that can sense oxygen, hydrogen, and carbon dioxide. This study showed the potential of this gas-sensing capsule in understanding functional aspects of the intestine, the microbiota, and intestinal response to dietary changes. This allows for a novel diagnostic and monitoring tool that can be used for various clinical indications such as constipation and obesity and can aid in development of individualized diets and lead to more personalized medicine.

Colon cancer screening with ingestibles

Although conventional colonoscopy is currently the gold standard for bowel cancer screening, the colon capsule endoscopy (CCE) continues to be further developed and improved since its introduction in 2007[55]. The currently available second generation CCE has been developed to look at the inside of the gut wall using visible light and two video cameras that cover nearly 360 degrees and transmits images to an external monitor[56]. This is used primarily for incomplete colonoscopy, polyp detection, and IBD, but with further technological advancements and research, CCE has the potential to be a minimally invasive and reliable method for bowel cancer screening.

Ingestibles in medication monitoring

Medication nonadherence is a common issue in healthcare, which may lead to poor outcomes in many patients. Digital pills are an innovative drug-device technology that combines medications with a monitoring system that records in real-time medication adherence[57]. An ingestible event marker is embedded within tablets and activated in the stomach. Once activated, the ingestible event marker communicates to a patch, which is applied to the patient's torso, then the signal transmits *via* Bluetooth to an external device such as a smartphone or computer. These digital pills allow physicians to monitor adherence among patients in hopes of improving rates of adherence and can further remote patient monitoring.

DISCUSSION

Wearable technology could represent a vital method for gastroenterologists to diagnose, manage, and monitor patients with numerous GI conditions and may even prevent disease. Because of the many available technologies such as remote sensor wearables, smartphones and mobile apps, telemedicine, and electronic health records, remote patient monitoring is very promising in the near future. Wearable devices have the ability to connect wirelessly to other devices, allowing the transfer and exchange of information and placing these devices in a category of technology known as the Internet-of-Things[58]. The Internet-of-Things is one framework that will make such a

future possible by providing the framework for exchange and communication of data between sensors and health care providers[58]. This will benefit physicians and patients as wearable sensor systems can help reduce the costs associated with high-quality and continuous health care monitoring by reducing unnecessary hospital admission and length of stay[59], facilitate health behavior in the long run by monitoring and sending alerts to patients to give cues to modify behavior[60], and improve health in vulnerable populations[61].

Although wearable technology is a promising innovation in the field of gastroenterology, their use has also raised a number of concerns such as data accuracy and privacy issues (Table 2). Future studies could continue to investigate data accuracy of these various wearable technology as further developed and improved hardware and software algorithms are necessary before its use in daily clinical practice. Wearable devices store large amounts of information that is accessed by third parties, which creates a potential exposure of personal information to unauthorized users. Technological developments need to be carefully addressed to ensure that patients feel comfortable sharing a significant amount of data regarding their daily lives with health care providers, insurance companies, and data analytic companies[62]. Regulations will also need to evolve continuously to ensure the best interest of the general population. Nonetheless, wearable technology continues to expand and make great impacts in patients' lives from fitness to health and wellness monitoring to possible future diagnostic and management tools.

CONCLUSION

In general, remote patient monitoring in the field of gastroenterology are showing great promise for detection of GI conditions and managing and monitoring patients during their routine daily lives. They also show potential of reducing health care costs by encouraging better self-management and intervention approaches while allowing for a stronger physician patient collaboration and more personalized medicine. With rapidly advancing technological advancements, wearable technology has the potential to revolutionize how physicians provide high quality, reliable, and affordable health care to all.

Table 2 Benefits, challenges, and future advances of wearable technology

Benefits	Challenges	Future research
Method of diagnosis, management, monitoring, and prevention of various gastrointestinal conditions; Remote patient monitoring; Reduce healthcare costs; Encourage better patient self-management and intervention; Improve health in vulnerable populations; Reduce spread of disease and protective tool for healthcare workers; Facilitate physician patient collaboration towards personalized medicine	Data inaccuracy; Privacy issues	Investigate data accuracy with improved hardware and software algorithms; Technological developments to ensure patient privacy; Regulations to ensure patient comfort with sharing data

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ORIGINAL ARTICLE

Retrospective Cohort Study

Perioperative blood transfusion decreases long-term survival in pediatric living donor liver transplantation

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Abstract

BACKGROUND

The impact of perioperative blood transfusion on short- and long-term outcomes in pediatric living donor liver transplantation (PLDLT) must still be ascertained, mainly among young children. Clinical and surgical postoperative complications related to perioperative blood transfusion are well described up to three months after adult liver transplantation.

AIM

To determine whether transfusion is associated with early and late postoperative complications and mortality in small patients undergoing PLDLT.

Joaquim EHG, Seda-Neto J, da Fonseca EA, Pugliese RPS, Vintimilla AM, Carmona MJC, Auler Jr JOC, and D'Alburquerque LAC made critical revisions related to important intelectual content of manuscript; all authors have read and approved the final version of the manuscript.

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METHODS

We evaluated the effects of perioperative transfusion on postoperative complications in recipients up to 20 kg of body weight, submitted to PLDLT. A total of 240 patients were retrospectively allocated into two groups according to postoperative complications: Minor complications (n = 109) and major complications (n = 131). Multiple logistic regression analysis identified the volume of perioperative packed red blood cells (RBC) transfusion as the only independent risk factor for major postoperative complications. The receiver operating characteristic curve was drawn to identify the optimal volume of the perioperative RBC transfusion related to the presence of major postoperative complications, defining a cutoff point of 27.5 mL/kg. Subsequently, patients were reallocated to a low-volume transfusion group (LTr; n = 103, RBC ≤ 27.5 mL/kg) and a high-volume transfusion group (HTr; n = 137, RBC > 27.5 mL/kg) so that the outcome could be analyzed.

RESULTS

High-volume transfusion was associated with an increased number of major complications and mortality during hospitalization up to a 10-year follow-up period. During a short-term period, the HTr showed an increase in major infectious, cardiovascular, respiratory, and bleeding complications, with a decrease in rejection complications compared to the LTr. Over a long-term period, the HTr showed an increase in major infectious, cardiovascular, respiratory, and minor neoplastic complications, with a decrease in rejection complications. Additionally, Cox hazard regression found that high-volume RBC transfusion increased the mortality risk by 3.031-fold compared to low-volume transfusion. The Kaplan-Meier survival curves of the studied groups were compared using log-rank tests and the analysis showed significantly decreased graft survival, but with no impact in patient survival related to major complications. On the other hand, there was a significant decrease in both graft and patient survival, with high-volume RBC transfusion.

CONCLUSION

Transfusion of RBC volume higher than 27.5 mL/kg during the perioperative period is associated with a significant increase in short- and long-term postoperative morbidity and mortality after PLDLT.

Key Words: Liver transplantation; Child; Blood transfusion; Outcome; Liver cirrhosis; Mortality

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Core Tip: This study aimed to assess whether perioperative transfusion is associated with early and late postoperative complications and mortality in small patients undergoing pediatric living donor liver transplantation (PLDLT). The volume of perioperative packed red blood cell (RBC) transfusion was the only independent risk factor for major postoperative complications. The perioperative volume of RBC > 27.5 mL/kg was an independent risk factor for mortality, increasing the risk by 3.031-fold, and was directly related to reduced patient and graft survival. In conclusion, not even massive transfusion, in the perioperative period, was associated with a significant increase in short- and long-term postoperative morbimortality after PLDLT.

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INTRODUCTION

Blood transfusion has been associated with increased morbimortality rates in major surgical procedures such as hepatic resection[1,2], cardiac[3] and non-cardiac major thoracoabdominal surgeries[4], and adult liver transplantation (LT)[5,6]. Regarding adult LT, Boyd *et al*[7] showed that intraoperative red blood cell (RBC) transfusion volume, a positive history of anti-RBC alloantibodies, and the immunosuppressive regimen used are associated with patient mortality. De Boer *et al*[5] reported that the indication for LT and the number of platelets or RBC units transfused during surgery are risk factors for 1-year graft survival; additionally, the number of RBC or platelet units transfused during surgery, cold ischemia time, and surgical team experience are risk factors for 5-year graft survival. Also, RBC transfusion has been associated with an increased rate of cancer recurrence. Indeed, the relative risk of digestive cancer recurrence has been reported to increase by 2.1-fold after the administration of ≥ 3 units[8]. Storage time and the timing of the transfusion related to surgery may also play a role[8]. In patients undergoing adult LT, all blood products (BP) are related to increased postoperative complications[9,10]; consequently, there is a trend towards reducing their use[11-13].

The harmful effects of perioperative transfusion in small pediatric patients are unclear pediatric LT. López Santamaría *et al*[11] suggested decreased graft survival associated with massive intraoperative blood transfusion, which was defined in their study as a loss greater than four volemias. They found four independent risk factors for mortality: Recipient's age < 3 years, retransplantation, severity of the underlying disease, and the transplant team's experience. Blood transfusion was not among them, however. To date, few reports have addressed the risks of transfusion in pediatric LT. In 2012, Nacoti *et al*[14] found that perioperative transfusion of fresh frozen plasma (FFP) RBC are independent risk factors for decreasing 1-year patient and graft survival. Nacoti *et al*[15] reported that intraoperative platelet and RBC transfusion are independent risk factors for developing major complications in the first year after pediatric LT.

Fanna *et al*[16] showed that high intraoperative bleeding is associated with pre-LT abdominal surgeries, factor V level ≤ 30%, and ex-situ parenchymal transection of the liver graft. Jin *et al*[17] identified high white blood cell count, low platelet count, and a deceased donor as independent risk factors for massive transfusion, which was defined as the administration of RBC volume ≥ 100% of the total blood volume (TBV). Although the graft failure incidence was higher in the massive group compared to the non-massive group, they found no difference in survival between the groups. Huang *et al*[18] evaluated pediatric living donor liver transplantation (LDLT) procedures and observed that younger patients with a lower weight, shorter stature, and preoperative prolonged international normalized ratio (INR) required larger blood transfusion volumes. Notwithstanding, preoperative INR was the only risk factor for massive blood transfusion. Kloesel *et al*[19] identified predictors of massive intraoperative bleeding (estimated blood loss of > TBV within a 24 h period): Preoperative hemoglobin (Hb) < 8.5 g/dL, INR > 1.5, platelet count < 100.000/mm^3, and surgery length > 10 h. Except for a longer intensive care unit (ICU) stay, there was no other correlation between massive transfusion and morbimortality.

Pediatric LT includes patients less than 18 years of age with either chronic or acute liver diseases involving deceased and living donors. Underlying pathologies may vary, and even the severity score is different under 12 years old [Pediatric end-stage liver disease (PELD) score]. Importantly, previous studies did not exclusively include young children. In addition, the grafts came from split and whole organs from deceased donors in most samples. Massive transfusion definitions vary across pediatric studies as BP transfusion from one[18] to four[11] volemias. There is still much controversy regarding the type, volume, and timing of BP transfusion and its association with postoperative morbimortality. Thus, this study assessed whether perioperative transfusion is associated with early and late postoperative complications and mortality in small patients undergoing pediatric LDLT (PLDLT).

MATERIALS AND METHODS

The Institutional Research Ethics Committees of ACCamargo Cancer Center and the University of São Paulo School of Medicine approved this observational, retrospective, and analytical cohort study according to the Helsinki Statement. All data were completely anonymized before they were accessed, and both committees waived the requirement for informed consent.

We investigated 254 pediatric patients weighing up to 20 kg with non-acute liver diseases who underwent first LDLT performed at the ACCamargo Cancer Center over 10 years. Fourteen patients were excluded: Five had fulminant hepatitis, three had their first transplant performed in other center, three were lost to follow-up, and three had missing data. All 240 enrolled patients underwent standardized procedures and techniques (total intravenous general anesthesia, piggyback inferior vena cava clamping, and exclusive use of hepatic left lateral segment grafts).

Study groups

Based on the severity of postoperative complications (graded according to the Clavien-Dindo classification during hospital stay), a total of 240 patients were initially allocated into two groups (Figure 1A): The minor complications (MiC) group (n = 109, either with no complications or with grade I-IIIa complications) and the major complications (MaC) group (n = 131, with at least one grade IIIb-V complication). Subsequently, all patients were reallocated into two further groups according to the RBC volume transfused during the perioperative period from 24 h before to 48 h after LT: The low-volume transfusion group (LTr n = 103, RBC ≤ 27.5 mL/kg) and the high-volume transfusion group (HTr n = 137, RBC > 27.5 mL/kg) (Figure 1B).

Classification of postoperative complications and postoperative follow-up

The Clavien-Dindo classification was applied to assess the relationship between blood transfusion and postoperative complications. In this pediatric population, general anesthesia was ostensibly required to ensure stillness and safety for diagnostic and treatment procedures. Thus, the original grade III was modified and subdivided into IIIa and IIIb according to the complexity of the procedures as low and high, respectively (Table 1). Data on mortality and complications were collected over a 10-year period post-LT. Patients who failed to attend outpatient follow-up after hospital discharge were considered lost to follow-up.

Complications were categorized by clinical presentation in 14 types: (1) Bleeding: Epistaxis, gastrointestinal, surgical wound, drains, and systemic hemorrhages due to portal hypertension or coagulopathy with or without need for surgery; (2) Cardiovascular: Systemic arterial hypertension, mismatches of cardiac rhythm, heart failure, hemodynamic instability, and cardiorespiratory arrest; (3) Dermatologic: Skin manifestations of drugs, food, and environmental factors; (4) Gastrointestinal: Malnutrition with weight-for-age z-score, height-for-age z-score[20], weight-for-height z-score, or body-mass-index z-score (BMIZ) ≤ -2 standard deviation, need for enteral or parenteral diet, persistent vomiting or diarrhea ≥ 3 wk, gastroesophageal reflux disease with or without brochoaspiration, visceromegaly, or ascites caused by maintained portal hypertension; (5) Infectious: Positive cultures with clinical or laboratory manifestations except from respiratory infections; (6) Malignancy: Post-transplant lymphoprolipherative disease (PTLD), lymphomas, skin tumors, and relapse of tumors; (7) Metabolic: Hydro-electrolytic serum changes such as hyponatremia (sodium < 133 mEq/L), hypernatremia (sodium > 147 mEq/L), hypokalemia (potassium < 3.0 mEq/L), hyperkalemia (potassium > 5.4 mEq/L), hypocalcemia (ionic calcium < 1.17 mmol/Lol/L), hypomagnesemia (magnesium < 1.8 mg/dL), hypophosphatemia (phosphorus < 2.5 mg/dL), arterial blood gases with pH < 7.2, acidosis and pH > 7.5, alkalosis, hyperlactatemia (lactate > 22 mg/dL), oliguria (diuresis < 0.5 mL/kg/h), adrenal insufficiency, diabetes mellitus, obesity (BMIZ > 2 standard deviations), or dyslipidemia (total cholesterol > 170 mg/dL, LDL fraction > 130 mg/dL, and triglycerides > 130 mg/dL); (8) Miscellaneous: Accidental injuries linked to LT procedure or postoperative follow-up; (9) Neuropsychiatric: Headache, vertigo, seizures, sedation withdrawal syndrome, delayed neuropsychomotor development, school learning difficulties, behavioral changes with psychomotor agitation, attention deficit, mood lability, anxiety, or depression; (10) Primary non-function (PNF) of the graft; (11) Rejection: Clinical and laboratory responsiveness to pulse therapy with methylprednisolone or anatomopathological documentation of acute or chronic rejection; (12) Renal: Renal failure was considered a decay of at least 50% of the estimated glomerular filtration rate (eGFR) applying the simplified revised Schwartz formula[21]; (13) Respiratory: Upper airway infections (rhinitis, sinusitis, otitis, tonsillitis, epiglottitis, pharyngolaryngitis), lower airway infections (tracheitis, bronchopneumonia, pneumonia), prolonged intubations (over 48 h), bronchospasm, atelectasis, effusions, pleural fistulas, hemothorax, pneumothorax, pneumomediastinum, non-cardiogenic edema, or acute respiratory failure; and (14) Surgical: LT specific complications (vascular thrombosis, biliary stenosis and fistulas, reoperation or retransplantation), hernias, dehiscence of anastomoses, or need for exploratory laparotomy except if caused by bleeding.

Table 1 Modified Clavien-Dindo classification for pediatric liver transplantation

Grade	Definition
I	Complication that requires the use of simple analgesics, antipyretics, anti-emetics, diuretics, electrolytes and physiotherapy
II	Complication requiring other drugs, different from grade I, blood transfusion or parenteral nutrition
III	Complication requiring surgical, endoscopic or radiologic intervention under any kind of anesthesia
IIIa	Low complexity procedures: Small and medium surgery, endoscopy and colonoscopy, US, CT, ERCP or PTCD, arteriography and angioplasty of portal vein or suprahepatic veins and hepatic artery, biopsies, simple teeth extractions, drainages, ostomies and central catheter passage for medication, dialysis or chemotherapy
IIIb	High complexity procedures: Large surgery, multiple teeth extractions, vascular and biliodigestive re-anastomosis, laparotomy and thoracotomy
IV	Life-threatening complication (including CNS[1] complications) requiring ICU admission
IVa	Dysfunction of one organ (including dialysis)
IVb	Dysfunction of two or more organs
V	Death of patient

[1]Cerebral hemorrhage, ischemic stroke, subarachnoid bleeding, excluding transient ischemic attack. Suffix "d" (for "disability") indicates that the patient still has complication, at time of discharge, requiring follow-up and must be entered to the degree of complication (*e.g.*, IVa-d degree). US: Ultrasound; CT: Computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography; PTCD: Percutaneous transhepatic cholangiography or drainage; CNS: Central nervous system; ICU: Intensive care unit.

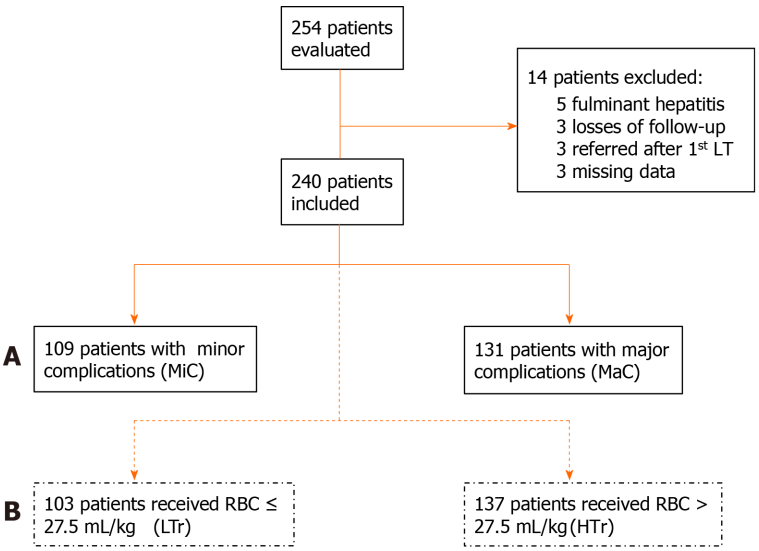


Figure 1 Patient flowchart. A: Minor complication group (grade I-IIIa) and major complication group (grade IIIb-V); B: Low red blood cell (RBC) volume transfusion group (RBC ≤ 27.5 mL/kg) and high RBC volume transfusion group (RBC > 27.5 mL/kg). LT: Liver transplantation; RBC: Red blood cell; MiC: Minor complication; MaC: Major complication; LTr: Low-volume transfusion; HTr: High-volume transfusion.

Statistical analysis

Multiple logistic regression analysis was performed to identify risk factors for major postoperative complications. The stepwise method was used for the selection of the variables. A perioperative RBC transfusion volume was identified as a single risk factor. A receiver operating characteristic curve was constructed using the perioperative RBC transfusion volume, and the occurrence of major complications were input parameters. A cutoff point of 27.5 mL/kg was identified using Youden's index (Figure 2).

Student's *t*-test or the Mann-Whitney test was used for quantitative variables while the chi-square test or Fisher's exact test was used for qualitative variables. Independent risk factors for mortality were identified using simple and multiple Cox regression analyses. Overall patient and graft survival analyses were performed using Kaplan–Meier survival curves, which were compared using the log-rank test. A P value level < 0.05 was used to define statistical significance. Statistical analyses were

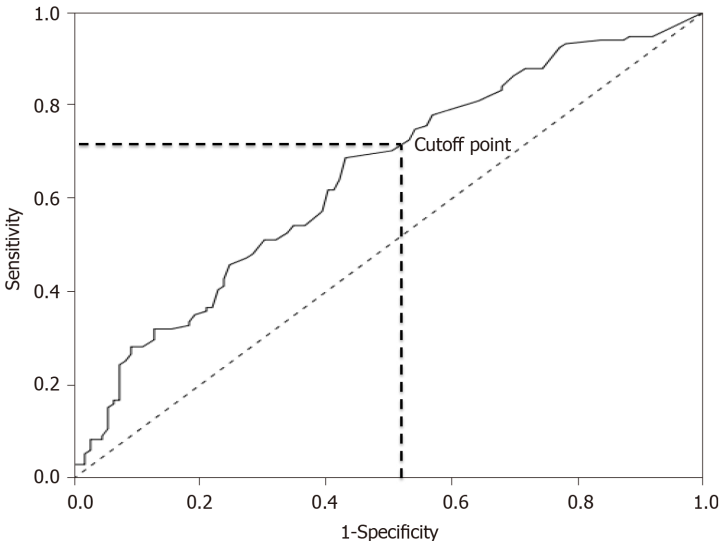


Figure 2 Receiver operating characteristic curve. A Receiver operation characteristic curve determined the optimal volume of perioperative red blood cells transfusion related to the presence of major postoperative complication. (Area under the curve = 0.648, $P < 0.0001$. Sensitivity = 68.7% and specificity = 56.9%. Cutoff point = 27.5 mL/kg; 95%CI: 0.578-0.717).

performed using SPSS version 23 (IBM Corp., Armonk, NY, United States) and R program version 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Patient characteristics

Of the 240 patients included in the study, 136 (56.7%) were females. The median patient age, weight, and stature were 12.4 mo, 8.07 kg, and 70 cm, respectively. Biliary atresia was found in 151 patients (62.9% of the underlying diseases) in our cohort. The Kasai procedure was previously performed in 111 cases (46.3%). The average pre-LT PELD score was 16 (± 7.7).

Perioperative risk factors for major postoperative complications

The overall incidence of major complications was 54.6%. In the MaC, all anthropometric measures, eGFR, Hb, sodium, and albumin levels were significantly lower. The INR, graft-to-body-weight ratio, and transfused BP volume were significantly higher than in the MiC. However, the only independent risk factor for major complications was perioperative RBC transfusion volume (Table 2).

Intraoperative and intensive care unit data

The HTr had lower Hb and sodium levels but significantly higher INR during the intraoperative period than the LTr. Additionally, the HTr had longer anesthetic and surgery time, a higher volume of crystalloids and colloids, higher diuresis rates, a lower incidence of extubation in the operating room, a longer intubation time, and a longer ICU and in-hospital stay than the LTr (Table 3).

Early postoperative complications

During hospitalization, the incidence of major complications *per* patient and the proportion of major complications were significantly higher in the HTr compared to the LTr (Table 4). Metabolic complications accounted for 28.2% of the complications during hospitalization. Additionally, complications such as gastrointestinal, malignancy, miscellany, neuropsychiatric, PNF, renal, and surgical were observed but with no significant difference between transfusion groups. The HTr had significantly more bleeding, respiratory, major cardiovascular, and major infectious complications but less dermatologic complications and rejections than the LTr (Table 5). Early LT-specific complications include PNF (2.1%), biliary fistula (6.2%), hepatic artery thrombosis (HAT) (3.3%), portal venous thrombosis (PVT) (9.2%), and retransplantation (1.2%); these were not related to a higher perioperative transfusion volume (Table 6). In terms of RBC transfusion volume, there was a significantly higher rate of

Table 2 Univariate and multiple logistic regression analyses of perioperative data stratified by the severity of complications

Variables	mean ± SD or No (%)		*P* value	OR	95%CI	*P* value
	MiC, *n* = 109	MaC, *n* = 131				
Male gender	54 (49.5)	52 (39.7)	0.081			
Age (d)	632 ± 440	512 ± 490	< 0.001			
Weight (kg)	9.67 ± 2.91	8.61 ± 3.09	< 0.001			
Height (cm)	76.6 ± 11.2	71.9 ± 11.6	< 0.001			
WAZ	-1.28 ± 1.24	-1.49 ± 1.31	0.105			
HAZ	-0.28 ± 1.15	-0.54 ± 1.24	0.012			
WHZ	-1.60 ± 1.34	-2.43 ± 1.48	< 0.001			
BMIZ	-0.20 ± 1.11	-0.36 ± 1.39	0.001			
Ascites	78 (71.6)	108 (82.4)	0.057			
PELD score	16.2 ± 7.0	17.8 ± 8.2	0.14			
Kasai surgery	50 (45.9)	61 (46.6)	0.698			
Portal hypertension	97 (88.9)	131 (100.0)	0.132			
Hepatopulmonary syndrome	16 (14.7)	15 (11.5)	0.414			
Infections 30 d pre-LT	0.4 ± 0.6	0.6 ± 0.7	0.081			
eGFR (mL/min/1.73 m^2)[1]	228 ± 121	187 ± 104	0.006			
Hemoglobin (g/dL)[2]	10.2 ± 1.5	9.3 ± 1.5	< 0.001			
Platelets (× 10^3/mm^3)[2]	196 ± 112	193 ± 118	0.789			
INR[2]	1.24 ± 0.25	1.49 ± 0.56	< 0.001			
Sodium (mEq/L)[2]	137 ± 2.7	135 ± 4.3	< 0.001			
Potassium (mEq/L)[2]	4.4 ± 0.5	4.3 ± 0.7	0.613			
Glucose (mEq/L)[2]	104 ± 59	92 ± 60	0.313			
Albumin (g/dL)[2]	2.9 ± 0.9	2.5 ± 0.8	0.002			
Total Bilirubin (g/dL)[2]	11.9 ± 7.3	13.4 ± 8.8	0.304			
GBWR (%)	3.3 ± 1.1	3.7 ± 1.2	0.035			
RBC (mL/kg)[3]	31.1 ± 25.4	46.7 ± 39.7	< 0.001	1.018	1.007-1.028	0.001
FFP (mL/kg)[3]	0.3 ± 2.6	3.0 ± 11.5	0.015			
Platelets (mL/kg)[3]	0.1 ± 0.8	1.2 ± 6.0	0.021			
Cryoprecipitate (mL/kg)[3]	0.0 ± 0.0	0.1 ± 1.2	0.196			

[1]Estimated glomerular filtration rate by simplified Schwartz's formula.
[2]Blood samples collected up to 72 h before liver transplantation (LT) anesthetic induction.
[3]Blood samples collected up to 2 h after the onset of LT anesthetic induction.
SD: Standard deviation; MiC: Minor complications group (I-IIIa); MaC: Major complications group (IIIb-V); WAZ: Weight-for-age z-score; HAZ: Height-for-age z-score; WHZ: Weight-for-height z-score; BMIZ: Body-mass-index-for-age z-score; PELD: Pediatric end-stage liver disease; LT: Liver transplantation; INR: International normalization ratio; GBWR: Graft-to-body-weight ratio; RBC: Red blood cells; FFP: Fresh frozen plasma; eGFR: Estimated glomerular filtration rate.

30 d reoperation (26.3% × 8.7%, $P < 0.001$) and 30 d mortality rate (6.6% × 0.0%, $P < 0.001$) in the HTr *vs* LTr, respectively (Table 6).

Late postoperative complications

Major cardiac complications were more frequent at 1, 5, and 10 years post-LT; major respiratory complications were more frequent at 5 and 10 years post-LT in the HTr than in the LTr. Dermatologic complications were less frequent up to 1 year in the HTr than in the LTr. Major infectious complications were more frequent, and rejections were less frequent in the HTr than in the LTr from 1 to 10 years post-LT. Minor

Table 3 Univariate analysis of preoperative, intraoperative and early postoperative data according to perioperative red blood cell volume transfused

Variables	mean ± SD or No. (%)		*P* value
	LTr, *n* = 103	HTr, *n* = 137	
Male gender	48 (46.6)	58 (42.3)	0.565
Age (d)	764 ± 544	419 ± 350	< 0.001
Weight (kg)	10.67 ± 3.20	7.88 ± 2.29	< 0.001
Height (cm)	80.0 ± 12.1	69.4 ± 8.7	< 0.001
WAZ	-0.90 ± 1.20	-1.75 ± 1.21	< 0.001
HAZ	-0.11 ± 1.41	-0.54 ± 1.24	0.012
WHZ	-1.60 ± 1.34	-2.43 ± 1.48	< 0.001
BMIZ	0.20 ± 1.20	-0.36 ± 1.39	0.001
Ascites	72 (69.9)	115 (83.9)	0.036
PELD score	14.1 ± 6.2	19.1 ± 7.9	< 0.001
Extra-hepatic cholestasis	67 (65.0)	105 (76.6)	0.029
Intrahepatic cholestasis	13 (12,6)	5 (3.6)	0.002
Cirrhosis	6 (5.8)	12 (8.8)	0.373
Metabolic diseases	7 (6.8)	10 (7.3)	0.852
Malignant diseases	7 (6.8)	3 (2.2)	0.159
Miscellany	3 (2.9)	2 (1.5)	0.751
Kasai surgery	40 (38.8)	74 (54.0)	0.016
Portal hypertension	96 (93.2)	132 (96.3)	0.132
Hepatopulmonary syndrome	19 (18.4)	12 (8.7)	0.043
Infections ≤ 30 d pre-LT	0 ± 1	1 ± 1	0.01
eGFR (mL/min/1.73 m^2)[1]	229 ± 121	188 ± 105	0.006
Hemoglobin (g/dL)[2]	10.2 ± 1.5	9.3 ± 1.5	< 0.001
Platelets (× 10^3/mm^3)[2]	196 ± 112	193 ± 118	0.789
INR[2]	1.24 ± 0.25	1.49 ± 0.56	< 0.001
Sodium (mEq/L)[2]	137 ± 2.6	135 ± 4.4	< 0.001
Potassium (mEq/L)[2]	4.3 ± 0.5	4.4 ± 0.6	0.613
Glucose (mEq/L)[2]	104 ± 59	92 ± 60	0.313
Albumin (g/dL)[2]	2.9 ± 0.9	2.5 ± 0.8	0.002
Total bilirubin (g/dL)[2]	11.9 ± 7.3	13.4 ± 8.8	0.304
Hemoglobin (g/dL)[3]	8.6 ± 1.5	7.6 ± 1.2	< 0.001
INR[3]	1.23 ± 0.42	1.67 ± 0.75	< 0.001
Sodium (mEq/L)[3]	135 ± 8	134 ± 5	0.002
Warm ischemia time (min)	42.2 ± 12.9	42.9 ± 11.4	0.515
Cold ischemia time (min)	53.3 ± 34.8	67.1 ± 56.3	0.118
Anesthetic time (h)	9.7 ± 1.6	10.8 ± 1.9	< 0.001
Surgical time (h)	7.8 ± 1.5	9.0 ± 1.9	0.001
Crystalloid (mL/kg)[4]	179 ± 67	251 ± 105	0.015
Colloid (mL/kg)[4]	33.6 ± 20.8	43.1 ± 19.7	< 0.001
Diuresis (mL/kg)[4]	47.5 ± 22.9	61.3 ± 36.6	0.013

GBWR (%)	2.9 ± 1.0	3.9 ± 1.1	< 0.001
Extubation in OR	24 (23.1)	16 (11.8)	0.014
Intubation period ≥ 48 h	7 (6.7)	35 (25.7)	< 0.001
ICU length of stay (d)	4 ± 5	8 ± 13	0.001
Hospital length of stay (d)	17 ± 13	23 ± 21	0.010

[1]Estimated glomerular filtration rate, preoperative calculated by Schwartz's formula.
[2]Blood samples collected up to 72 h before liver transplantation (LT) anesthetic induction.
[3]Blood samples collected up to 2 h after the onset of LT anesthetic induction.
[4]Intraoperative volume indexed by body's weight. Extra-hepatic cholestasis, extra-hepatic biliary atresia, coledocus cyst; intrahepatic cholestasis, Alagille's syndrome, non-syndromic biliary hypoplasia, primary sclerosing cholangitis, progressive intrahepatic familial cholestasis; cirrhosis, idiopathic, autoimune and cryptogenic; metabolic diseases, glycogenesis, Cligger-Najar's disease, tyrosinemia, cystic fibrosis, alpha-1 anti trypsin deficiency, urea cycle defects, type 1 oxaluria; malignant diseases, hepatoblastoma and hepatocarcinoma and hepatic miscellany diseases, Budd-Chiari syndrome, Caroli's disease and unclarified fibrosis. SD: Standard deviation; LTr: Low-volume transfusion group; HTr: High-volume transfusion group; WAZ: Weight-for-age z-score; HAZ: Height-for-age z-score; WHZ: Weight-for-height z-score; BMIZ: Body-mass-index-for-age z-score; PELD: Pediatric end-stage liver disease; LT: Liver transplantation; INR: International normalized ratio; GBWR: Graft-to-body-weight ratio; OR: Operating room; ICU: Intensive care unit; eGFR: Estimated glomerular filtration rate.

Table 4 Comparative analysis of postoperative complications during the hospitalization period using the modified Clavien–Dindo classification

Variables	All patients, *n* = 240		
	All complications during hospitalization, *n* = 783		
	mean ± SD or No. (%)	*P* value	
	LTr, *n* = 103	HTr, *n* = 137	
Patients with any complication	95 (92.2)	132 (96.4)	0.264
Patients with major complications (IIIb-V)	41 (39.8)	90 (65.7)	< 0.001
Complications *per* patient	2.5 ± 1.7	3.8 ± 3.6	0.007
Number of major complications (IIIb-V)	54/262 (20.6)	207/521 (39.7)	< 0.001
Median of the highest degrees of complication	IIIa (I-IVb)	IVa (I-V)	< 0.001
Median grade of complications *per* patient	II (I-IIIb)	II (I-V)	< 0.001

SD: Standard deviation; LTr: Low transfusion group; HTr: High transfusion group.

neoplastic complications were more frequent at later points in follow-up in the HTr than in the LTr (Table 5).

Late LT-specific complications were observed after 30 d over 10 years of follow-up, as biliary stenosis (12.1%), HAT (1.2%), PVT (3.7%), reoperation (7.1%) and retransplantation (2.5%), none of these were related to perioperative transfusion (Table 6). Overall, 10-years mortality rate, with respect to RBC transfusion volume, was significantly higher (25.5% *vs* 7.8%, $P < 0.001$), respectively, in the HTr compared to the LTr (Table 6).

Independent risk factors for death

Simple and multiple Cox regression analysis identified perioperative RBC volume > 27.5 mL/kg and preoperative eGFR as independent risk factors for mortality over 10 years of follow-up after LT (Table 7).

Patient and graft survival according to outcome and blood transfusion

The overall patient survival rates were 87.1%, 81.5%, and 80.3%, whereas the overall graft survival rates were 87.1%, 77.7%, and 75.6% at 1, 5, and 10 years post-LT, respectively. The graft survival rates were significantly lower in the MaC than in the MiC at 1, 5, and 10 years post-LT (81.5% *vs* 94.4%, 73.8% *vs* 84.6, and 72.2% *vs* 81%, respectively); however, no significant difference was seen in the patient survival rates between the MaC and the MiC. The patient survival rates were significantly lower in the HTr than in the LTr at 1, 5, and 10 years post-LT: 82.7% *vs* 97.7%, 73.9% *vs* 93.8%,

Table 5 Comparative analysis of type and grade of postoperative complications along the four periods of follow-up

Complication		Hospitalization			1 yr			5 yr			10 yr		
Type	Grade	LTr	HTr	*P* value	LTr	HTr	*P* value	LTr	HTr	*P* value	LTr	HTr	*P* value
Bleeding (%)	Minor	1(0.4)	6(1.1)	0.252	5(0.9)	11(1.3)	0.61	5(0.5)	13(0.9)	0.237	5(0.5)	13(0.9)	0.236
	Major	0(0)	9(1.8)	0.211	1(0.2)	9(1.0)	0.465	1(0.1)	9(0.7)	0.467	1(0.1)	9(0.6)	0.297
	Subtotal	1(0.4)	15(2.9)	0.016	6(1.1)	20(2.3)	0.178	6(0.6)	22(1.6)	0.052	6(0.6)	22(1.4)	0.068
Cardiovascular (%)	Minor	17(6.5)	29(5.6)	0.754	19(3.6)	32(3.6)	0.662	24(2.6)	39(2.8)	0.518	25(2.5)	42(2.7)	0.381
	Major	0(0)	17(3.3)	0.028	0(0)	18(2.0)	0.017	0(0)	18(1.3)	0.005	0(0)	18(1.2)	0.005
	Subtotal	17(6.5)	46(8.9)	0.319	19(3.6)	50(5.8)	0.098	24(2.6)	57(4.1)	0.075	25(2.5)	60(3.9)	0.282
Dermatologic (%)	Minor	9(3.4)	5(1.0)	0.093	25(4.7)	16(1.8)	0.009	52(5.6)	58(4.1)	0.322	59(5.8)	71(4.6)	0.583
	Major	0(0)	1(0.2)	1	1(0.2)	1(0.1)	0.406	7(0.8)	10(0.7)	0.168	8(0.8)	10(0.7)	0.114
	Subtotal	9(3.4)	6(1.2)	0.049	26(4.9)	17(1.9)	0.003	59(6.4)	68(4.8)	0.134	67(6.6)	81(5.3)	0.209
Gastrointestinal (%)	Minor	5(1.9)	13(2.5)	0.336	15(2.8)	30(3.4)	0.353	30(3.3)	44(3.1)	0.721	33(3.2)	49(3.2)	0.65
	Major	0(0)	1(0.2)	1	1(0.2)	4(0.5)	1	3(0.3)	6(0.5)	0.704	3(0.3)	6(0.4)	0.713
	Subtotal	5(1.9)	14(2.7)	0.627	16(3.0)	34(3.9)	0.488	33(3.6)	50(3.6)	1	36(3.5)	55(3.6)	1
Infectious (%)	Minor	21(8)	32(6.1)	1	86(16.2)	116(13.2)	0.577	188(20.3)	235(16.7)	0.434	207(20.3)	257(16.8)	0.394
	Major	32(12.2)	82(15.8)	0.013	43(8.1)	106(12.1)	0.028	56(6.0)	132(9.4)	0.02	60(5.9)	133(8.7)	0.022
	Subtotal	53(20.2)	114(21.9)	0.66	129(24.3)	222(25.3)	0.729	244(26.3)	367(26.1)	0.948	267(26.2)	390(25.5)	0.743
Malignancy (%)	Minor	0(0)	0(0)	1	4(0.8)	9(1.0)	0.576	13(1.4)	32(2.3)	0.067	13(1.3)	35(2.3)	0.027
	Major	0(0)	0(0)	1	4(0.8)	3(0.3)	0.058	9(1.0)	13(0.9)	0.137	11(1.1)	14(0.9)	0.07
	Subtotal	0(0)	0(0)	1	8(1.6)	12(1.4)	1	22(2.4)	45(3.2)	0.296	24(2.4)	49(3.2)	0.253
Metabolic (%)	Minor	86(32.8)	134(25.7)	0.786	89(16.8)	140(16.0)	0.494	96(10.4)	146(10.4)	0.329	102(10.0)	151(9.9)	0.416
	Major	1(0.4)	0(0)	0.207	2(0.4)	0(0)	0.052	2(0.2)	0(0)	0.069	2(0.2)	0(0)	0.077
	Subtotal	87(33.2)	134(25.7)	0.085	91(17.2)	140(16.0)	0.605	98(10.6)	146(10.4)	0.942	104(10.2)	151(9.9)	0.846
Miscellany (%)	Minor	8(3.1)	25(4.8)	0.067	30(5.7)	48(5.5)	0.093	66(7.2)	71(5.1)	0.179	73(7.2)	74(4.8)	0.1
	Major	1(0.4)	5(1.0)	1	3(0.6)	7(0.8)	0.702	4(0.4)	14(1.0)	0.792	5(0.5)	14(0.9)	1
	Subtotal	9(3.5)	30(5.8)	0.217	33(6.3)	55(6.3)	1	70(7.6)	85(6.1)	0.18	78(7.7)	88(5.8)	0.07
Neuropsyquiatric (%)	Minor	3(1.1)	2(0.4)	0.391	7(1.3)	4(0.5)	0.135	17(1.8)	14(1.0)	0.2	24(2.4)	23(1.5)	0.297

	Major	1(0.4)	1(0.2)	0.371	1(0.2)	1(0.1)	0.406	1(0.1)	1(0.1)	0.459	2(0.2)	1(0.1)	0.188
	Subtotal	4(1.5)	3(0.6)	0.169	8(1.5)	5(0.6)	0.134	18(1.9)	15(1.1)	0.116	26(2.6)	24(1.6)	0.109
PNF (%)	Minor	0(0)	0(0)	1	0(0)	0(0)	1	0(0)	0(0)	1	0(0)	0(0)	1
	Major	0(0)	5(1.0)	0.587	0(0)	5(0.6)	0.593	0(0)	5(0.4)	0.332	0(0)	5(0.3)	0.329
	Subtotal	0(0)	5(1.0)	0.175	0(0)	5(0.6)	0.164	0(0)	5(0.4)	0.164	0(0)	5(0.3)	0.164
Rejection (%)	Minor	40(15.3)	32(6.1)	0.004	73(13.8)	66(7.5)	0.003	111(12.0)	102(7.3)	0.106	117(11.5)	125(8.2)	0.03
	Major	0(0)	1(0.2)	1	1(0.2)	4(0.5)	1	4(0.4)	8(0.6)	0.526	5(0.5)	8(0.5)	0.363
	Subtotal	40(15.3)	33(6.3)	<0.001	74(14.0)	70(8.0)	<0.001	115(12.4)	110(7.9)	<0.001	122(12.0)	133(8.7)	<0.001
Renal (%)	Minor	2(0.8)	3(0.6)	1	3(0.6)	3(0.3)	0.693	4(0.4)	3(0.2)	0.469	4(0.4)	4(0.3)	0.731
	Major	2(0.8)	5(1.0)	0.637	2(0.4)	7(0.8)	1	5(0.5)	9(0.7)	0.535	5(0.5)	9(0.6)	0.547
	Subtotal	4(1.6)	8(1.6)	1	5(1.0)	10(1.1)	0.796	9(0.9)	12(0.9)	0.945	9(0.9)	13(0.9)	1
Respiratory (%)	Minor	8(3.1)	24(4.6)	0.093	75(14.2)	110(12.6)	0.934	161(17.4)	247(17.6)	0.141	177(17.4)	261(17.1)	0.106
	Major	7(2.7)	36(6.9)	0.539	9(1.7)	53(6.0)	0.096	14(1.5)	70(5.0)	0.029	15(1.5)	71(4.7)	0.017
	Subtotal	15(5.8)	60(11.5)	0.014	84(15.9)	163(18.6)	0.217	175(18.9)	317(22.6)	0.038	192(18.9)	332(21.8)	0.041
Surgical (%)	Minor	8(3.1)	9(1.8)	0.617	19(3.6)	23(2.6)	0.631	33(3.6)	49(3.5)	0.732	43(4.2)	66(4.3)	0.487
	Major	10(3.8)	44(8.4)	0.711	12(2.3)	51(5.8)	0.509	20(2.2)	56(4.0)	1	20(2.0)	58(3.8)	0.682
	Subtotal	18(6.9)	53(10.2)	0.166	31(5.9)	74(8.4)	0.092	53(5.8)	105(7.5)	0.118	63(6.2)	124(8.1)	0.079
Total minor complications (%)		208(79.4)	314(60.3)	<0.001	450(84.9)	608(69.3)	<0.001	800(86.4)	1053(75.0)	<0.001	882(86.6)	1171(76.7)	<0.001
Total major complications (%)		54(20.6)	207(39.7)		80(15.1)	269(30.7)		126(13.6)	351(25.0)		137(13.4)	356(23.3)	
Total complications per group (%)		262(33,5)	521(66,5)		530(37,7)	877(62,3)		926(39,7)	1404(60,3)		1019(40,0)	1527(60,0)	
Total complications per period (%)		783(30,8)			1407(55,3)			2330(91,5)			2546(100)		

HTr: High-volume transfusion group; LTr: Low-volume transfusion group; PNF: Primary non-function.

and 72.6% *vs* 90.9%, respectively. Likewise, the graft survival rates were significantly lower in the HTr than in the LTr at 1, 5, and 10 years post-LT: 79.5% *vs* 97.7%, 67.2% *vs* 92.3%, and 67.2% *vs* 87%, respectively (Figure 3).

Table 6 Patients with early and late liver transplantation specific complications and 30 d and 10 years mortality rate

Variables	Early complications			Late complications		
	No. (%)	No. (%)	*P* value	No. (%)	No. (%)	*P* value
	LTr, *n* = 103	HTr, *n* = 137		LTr, *n* = 103	HTr, *n* = 137	
PNF	0 (0.0)	5 (3.6)	0.072	0 (0.0)	0	NA
Biliary fistula	4 (3.9)	10 (7.3)	0.267	0 (0.0)	1 (0.7)	NA
Biliary stenosis	0	0	NA	15 (14.6)	14 (10.2)	0.307
Hepatic arthery thrombosis	2 (1.9)	6 (4.4)	0.478	0 (0.0)	3 (2.2)	0.261
Portal vein thrombosis	6 (5.1)	16 (11.7)	0.113	3 (2.9)	6 (4.4)	0.554
Reoperation	9 (8.7)	36 (26.3)	< 0.001	4 (3.9)	13 (9.5)	0.298
Retransplantation	0 (0.0)	3 (2.2)	0.262	2 (1.9)	4 (2.9)	0.306
30 d mortality	0 (0.0)	9 (3.6)	0.008	NA	NA	NA
10 yr mortality	NA	NA	NA	8 (7.8)	26 (19.0)	0.137

LTr: Low-volume transfusion group; HTr: High-volume transfusion group; PNF: Primary non-function.

DISCUSSION

Determining predictive factors for complications of pediatric LT may be hindered by patient heterogeneity and a lack of standardization in the definition of complications. This study is the first retrospective study to assess short- and long-term transfusion-associated postoperative complications in a large number of small pediatric patients with chronic liver diseases who received the same type of graft from living donors.

The Clavien-Dindo classification[22] was first used by Clavien *et al*[23] to assess postoperative complications in adult LT patients and has been increasingly used in most pediatric surgical areas with some adaptations[24]. Beck-Schimmer *et al*[25] classified post-LT complications as minor and major providing the basis for the use of this modified classification primarily because general anesthesia was induced for almost all procedures in this pediatric population (*e.g.*, imaging exams, biopsies, catheter insertions, and other minor invasive procedures).

We defined the interval from 24 h pre- to 48 h post-LT as the perioperative period. Over this period, patients had a greater need for transfusion. Our transfusion goals throughout this period were to keep Hb > 8 and < 10g/dL, INR < 3.5, platelet count > 45 × 10^3/mm^3, and fibrinogen level > 80 mg/dL. Viscoelastic methods were unavailable at our center at this time. Only 16 study participants were not transfused during the perioperative period. Of the 224 transfused patients, 7.1%, 98.8%, and 37.5% received RBC before, during, and after surgery, respectively. Some patients remained within a suitable range during the intraoperative period yet this level dropped during the early postoperative period requiring subsequent transfusion. In this study, the average volume of transfusion of other BP was minimal compared to RBC.

Massive bleeding is usually defined as the loss of 100% or more of circulating TBV within 24 h[26]. Massive transfusion can also be defined as the transfusion of over 10% of TBV *per* minute or 50% in 3 h[27]. Nevertheless, TBV in pediatrics depends on the child's age and weight ranging from 65 to 100 mL/kg. In our study, the child's average age was 567 d (1.5 years) corresponding to a TBV of 75 mL/kg. The mean perioperative RBC volume transfused in the HTr was 57.7 mL/kg. A cutoff point of 27.5 mL/kg was used when the perioperative RBC volume represented less than 37% of TBV transfused within 96 h. Therefore, most of our patients did not meet the definition for massive bleeding or transfusion. Though there was no massive transfusion in the perioperative period in most patients; this lower volume has already been associated with major postoperative complications in PLDLT.

This study identified a perioperative transfusion volume of RBC > 27.5 mL/kg and preoperative eGFR as independent risk factors for mortality in PLDLT patients. A perioperative RBC transfusion volume higher than 27.5 mL/kg was a strong independent risk factor for mortality and increased the risk by 3.031-fold *vs* lower or equal volumes. This volume of RBC is definitely below that reported in other studies, which analyzed risk factors for survival in LT.

Table 7 Independent risk factors for death identified by simple and multiple Cox regression

			All patients *n* = 240			
Variables	**Estimative**	**HR**	**95%CI**	***P* value**	**HR**	***P* value**
		Univariate			**Multivariate**	
Male gender	-0.062	0.94	0.514-1.718	0.841		
Age (d)	-0.024	0.977	0.950-1.004	0.095		
Weight (kg)	-0.17	0.844	0.738-0.964	0.012		
Height (cm)	-0.044	0.957	0.924-0.990	0.013		
WAZ	-0.243	0.784	0.623-0.986	0.037		
HAZ	-0.085	0.918	0.734-1.148	0.455		
WHZ	-0.235	0.791	0.649-0.963	0.019		
BMIZ	-0.084	0.919	0.723-1.168	0.49		
PELD score	0.022	1.022	0.982-1.064	0.282		
Biliary Atresia	-0.426	0.653	0.357-1.195	0.167		
Kasai surgery	-0.214	0.808	0.440-1.483	0.491		
Infections ≤ 30 d pre-LT	0.348	1.416	1.023-1.960	0.036		
eGFR (mL/min/1.73 m^2)[1]	-0.005	0.995	0.991-0.999	0.010	0.995	0.023
Surgical time (hours)	0.012	1.013	0.856-1.197	0.884		
Hemoglobin (g/dL)[2]	-0.206	0.814	0.657-1.008	0.059		
INR[2]	0.259	1.295	0.722-2.322	0.386		
Sodium (mEq/L)[2]	-0.014	0.986	0.916-1.061	0.704		
Albumin (g/dL)[2]	-0.116	0.89	0.615-1.290	0.539		
Lactate (mmoL/L)[3]	-0.021	0.979	0.914-1.048	0.541		
GBWR (%)	0.149	1.16	0.909-1.481	0.232		
RBC > 27.5 mL/kg	1.316	3.73	1.730-8.042	0.001	3.031	0.009
Presence of major complication	0.384	1.469	0.799-2.701	0.261		

[1]Estimated glomerular filtration rate, preoperative calculated by Schwartz's formula.
[2]Blood samples collected up to 72 h before liver transplantation (LT).
[3]Blood samples collected up to 2 h after the onset of LT anesthetic induction. WAZ: Weight-for-age z-score; HAZ: Height-for-age z-score; WHZ: Weight-for-height z-score; BMIZ: Body-mass-index-for-age z-score; PELD: Pediatric end-stage liver disease; LT: Liver transplantation; INR: International normalized ratio; GBWR: Graft-to-body-weight ratio; RBC: Red blood cells; eGFR: Estimated glomerular filtration rate; HR: Hazard ratio.

Matinlauri *et al*[28] identified transfusion of RBC > 20 units as a risk factor for graft survival post-LT in adults. Nacoti *et al*[14] evaluated the effect of transfusion on 1-year patient and graft survival in pediatric LT and estimated a mortality risk of 3.15 for ≥ 3 units of RBC. Preoperative eGFR was a weak risk factor for mortality in our study probably because malnourished children tend to have underestimated serum creatinine values, and the simplified revised formula [eGFR = 0.413 × Height (cm)/serum creatinine (mL/min/1.73 m^2)] increases the error of the results at higher GFR values[29]. In the meantime, serum creatinine is known to be an independent risk factor for mortality in adult LT[30] and in pediatric LT with deceased donor[15].

Though LDLT is currently a standard treatment with good outcomes for young children with end-stage liver diseases, the postoperative complication rate can lead to a high morbimortality[31]. Among the 240 children, early postoperative complications were observed in 94.6% of patients of whom 54.6% had major complications associated with higher rates of graft loss. Although, the 1-year survival rate of 87.1 was slightly lower than in other centers that reported overall survival higher than 90%, the 5 and 10-year survival rates of 81.5% and 80.3%, respectively, are comparable to rates higher than 80%, reported by others[32-34]. This could be attributed to the fact that we studied

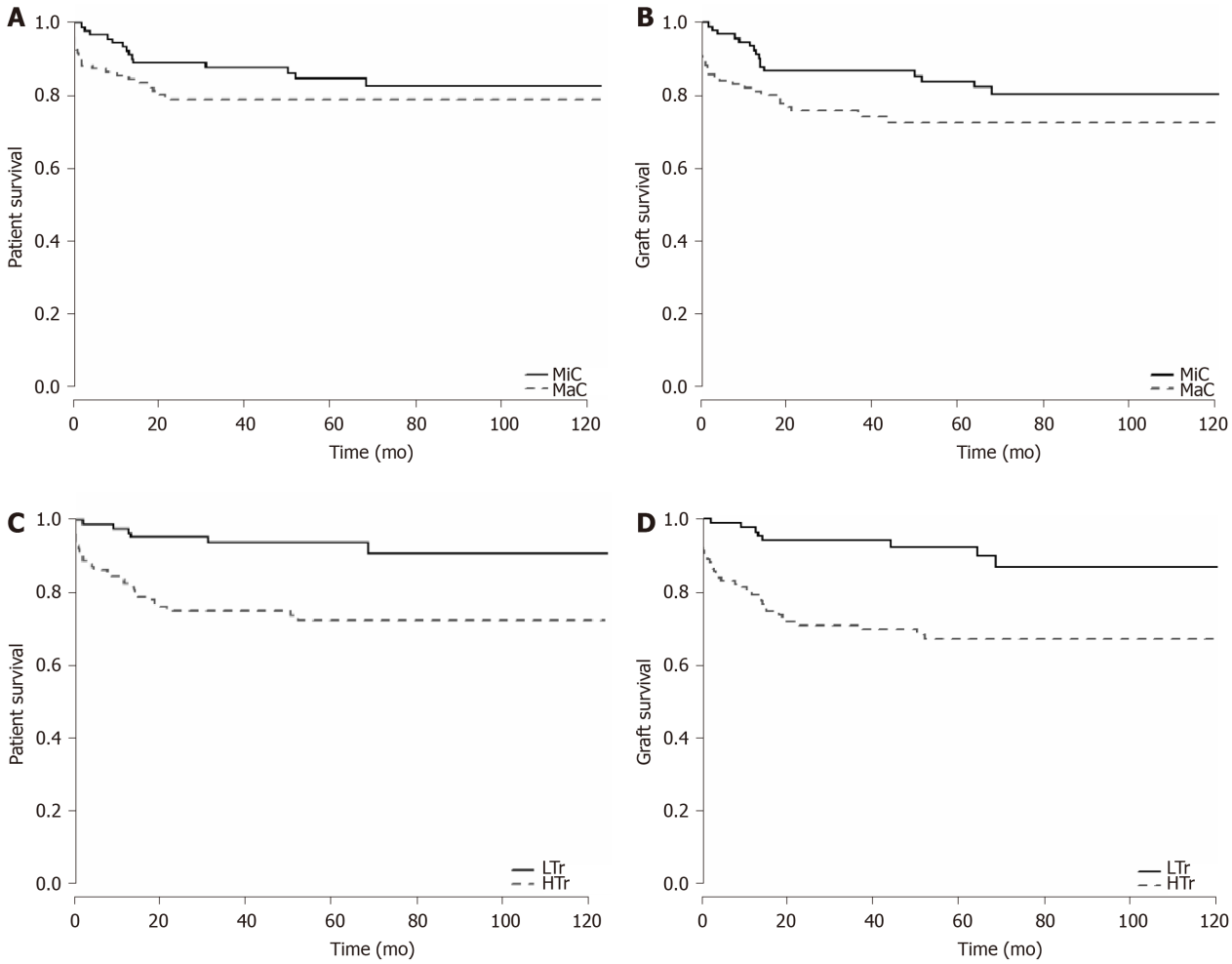


Figure 3 Patient and graft survival curves. A: Patient survival analysis comparing the minor complication (MiC) group (grade I-IIIa, *n* = 109) and the major complication (MaC) group (grade IIIb-V, *n* = 131) with number of patients at risk. Log-rank test: Chi-square = 12.6, degrees of freedom (*df*) = 1, *P* = 0.12; B: Graft survival analysis comparing the MiC and MaC groups. Log-rank test: Chi-square = 14.6, *df* = 1, *P* = 0.03; C: Patient survival analysis comparing the low-volume transfusion (LTr) group (*n* = 103) and the high-volume transfusion (HTr) group (*n* = 137). Log-rank test: Chi-square = 12.6, *df* = 1, *P* < 0.001; D: Graft survival analysis comparing the LTr (*n* = 103) and HTr (*n* = 137) groups. Log-rank test: Chi-square = 14.6, *df* = 1, *P* < 0.001. MiC: Minor complications group (I-IIIa); MaC: Major complications group (IIIb-V); LTr: Low-volume transfusion group; HTr: High-volume transfusion group.

exclusively small children, with inherent risks of early childhood, in comparison to other studies that include patients aged up to 18 years. In this study, the only risk factor for major postoperative complications after PLDLT was the volume of perioperative RBC transfused.

Transfusion was more frequently employed for younger and undernourished patients with extrahepatic cholestasis, previous Kasai procedure, increased rates of ascites, higher preoperative INR, lower Hb and sodium levels, and worse pulmonary and renal function. These findings are consistent with many reports on adult LT[35-37].

BP transfusion has often been reported to be associated with increased rates of both early and late LT complications[38,39]. Our sample consisted of 234 (97.5%) outpatients and 6 (2.5%) patients previously admitted to the ICU. During hospitalization, the total complication rates were similar between patients of the LTr and HTr groups (92.2% *vs* 96.4%). The HTr had more patients with severe complications (65.7% *vs* 39.8%), a higher average of complications *per* patient (3.8 ± 3.6 *vs* 2.5 ± 1.7), and a higher median number of major complications (IIIa *vs* IVa). Although the median grade of complications *per* patient was equal in the groups (II), there was a greater variability (I-V *vs* I-IIIb) in the HTr than in the LTr. Higher transfusion volumes were associated with longer anesthetic and surgery time, prolonged intubation, and longer ICU and in-hospital stays; these conclusions are corroborated by the findings of Massicotte *et al*[39] and Ramos *et al*[40] in adult LT patient studies.

Other specific complications might be related to perioperative BP transfusion in LT patients. Feltracco *et al*[41] reported that intraoperative BP transfusion was a risk factor for early postoperative pulmonary complications. Li *et al*[9] observed an increase in

infectious complications during in-hospital and ICU stays among LDLT adults in the early postoperative period. Furthermore, Pereboom *et al*[12] reported a higher incidence and mortality associated with intraoperative platelet transfusion and acute lung injury related to transfusion. Benson *et al*[42] also reported similar findings with plasma containing BP and a higher incidence of early postoperative infections with dose-dependent RBC transfusion in adult LT.

During hospitalization occurred 30.8% of the total complications observed within the 10 years of follow-up. Minor metabolic complications (*e.g.*, hydro electrolytic disorders, hypo- or hyper-glycemia, acidosis) were the most common. Nevertheless, no association between this type of complication and RBC transfusion volume was observed. Renal complications were not significantly different between groups, and dialysis was performed in seven patients during this period. HTr patients exhibited a higher frequency of major infectious complications (15.7% of complications, *e.g.*, severe sepsis, with hemodynamic instability), general respiratory complications (11.5% of complications, *e.g.*, pulmonary edema, pleural effusion, bronchospasm, pneumonia, and acute respiratory distress syndrome), general bleeding (2.8% of complications, *e.g.*, oozing and draining with a need for reoperation), and major cardiovascular complications (2.1% of complications, *e.g.*, hemodynamic instability requiring vasoactive drugs, severe arrhythmias, and cardiorespiratory arrest) than LTr patients. HTr patients had a lower frequency of rejections (6.3% of complications, *e.g.*, mild acute cellular reaction) and dermatologic complications (1.2% of complications, *e.g.*, oral ulceration and dermatitis.

Kloesel *et al*[19] did not observe significant differences in perioperative complications between the major and minor transfusion groups within the hospitalization period in pediatric LT patients despite an incidence rate of 43% for massive bleeding. In this last study, conflicting results might be attributed to the fact that all patients received an RBC transfusion and 88% of them received FFP transfusion thus creating a bias in the comparison between groups. Aside from increasing the risk of complications, RBC transfusion is a predictive factor for survival in adult[28] and pediatric LT[43]. In our study, patients with MaC presented reduced graft survival but patient survival was not affected relative to patients with no or MiC. Meanwhile, patients who underwent a HTr showed both worse graft and patient survival relative to patients of LTr.

The late period had 69.2% of the total complications with 55.3% up to the first year. Among the late postoperative complications of LT, infections are the leading cause of death[44]. In line with this, the frequency of major complications in HTr patients was higher at 1 up to 10 years post-LT. The most frequent major complications were infectious (15.8%-8.7% of complications, *e.g.*, severe sepsis), pulmonary (5.0%-4.7% of complications, *e.g.*, severe pneumonia requiring mechanical ventilation), and cardiovascular (2.0%-1.2% of complications, *e.g.*, cardiorespiratory arrest). In the HTr patients, the frequency of minor neoplastic complications was significantly higher at 10 years (2.3% of complications, *e.g.*, PTLD) than in LTr patients. The frequency of rejections was lower in the HTr at 1 to 10 years post-LT (8.0%-8.7% of complications, *e.g.*, mild acute cellular reaction) than in the LTr. Renal complications were still not different between groups, and dialysis was necessary in 14 patients up to 10 years. Dermatological complications are common among LT patients and are related to genetics, allergic factors, and the side effects of immunosuppressive drugs. Surprisingly, patients with HTr had fewer dermatological complications than patients with LTr up to 1 year after LT (1.9%). This could be attributed to a lower frequency of rejections and, consequently, less of a need for immunosuppressive drugs. However, this would fail to explain why the rate of dermatological complications does not differ significantly afterward while rejections remained lower in HTr throughout the study. A higher-volume transfusion of RBC might have had some influence. Notwithstanding the fact that such complications are usually related to LT[45], there is no strong evidence of the association of perioperative BP transfusion and long-term outcomes in pediatric LT. We found a significant difference in 30 d and 10-year postoperative mortality rates between the LTr and the HTr (0 *vs* 6.6% and 7.8 *vs* 25.5%), respectively, confirming that HTr patients generally experienced more severe complications.

Concerning LT-specific complications, the incidence of PNF corresponded to 2.1% and was not related to transfusion. This is consistent with others who reported an incidence of 0.9%-8.5%[46]. Hypercoagulability is a risk factor for vascular thrombosis in LT, mainly in children, as determined by rebalanced hemostasis in cirrhosis, technical vascular issues, inflammatory response to trauma, and massive transfusion[47,48]. Incidences of HAT have been reported to be 4%-8% and PVT of 5%-10%[49-51]. In our study, the overall incidence of HAT and PVT was 4.6% and 13.3%, respectively. Nevertheless, no relation between transfusion volume and an increase in arterial or venous thrombosis were observed, in both, short- and long-term periods. The lower

incidence of HAT is likely related to the microsurgical anastomosis technique whereas the higher incidence of PVT may be attributed to previous portal hypoplasia frequently observed in patients with biliary atresia-this was the most prevalent underlying disease in our cohort. Portal vein graft interposition was employed in 37 (15.4%) patients of the total cohort, and only three cases evolved with PVT. Biliary complications accounted for 18.3% of the total cases, and neither fistulas nor stenosis were related to RBC transfusion. The incidence rate of biliary complications in PLDLT has been reported to be 10%-20%[52], depending on the size of the graft and subsequent technical difficulties. Reoperations corresponded to 25.8%, which is consistent with others who reported an incidence of 8%-29%[33] in PLDLT. In the short-term period, reoperation (18.7%) was three-fold higher in the patients who received an RBC volume higher than 27.5 mL/kg (HTr), due mostly to bleeding and intestinal injuries. In the long-term period, reoperations were not related to perioperative transfusion. Retransplantation corresponded to 3.8% and was not related to RBC transfusion. The incidence was below the historical average of 9%-29%[53], probably because of the lower incidence of total hip arthroplasty and the good quality of the grafts. Although the HTr had fewer minor rejection episodes, such as a mild acute cellular reaction over 10 years, this fact did not impact the retransplantation rate in both, short- and long-term periods.

Immunosuppression associated with blood transfusion occurs *via* a decrease in the number and function of natural killer cells, a decrease in cytotoxic T-cell function, an increase in the number of suppressor T-cells, and a reduction in macrophage and monocyte function[54]. Blood transfusion may induce immunomodulatory effects (both proinflammatory and immunosuppressive) that are of variable intensity and long-term duration. These antagonistic effects are associated with a decrease in rejection episodes[55] as well as an increase in the frequency of infection[56], neoplasia[57], and tumor recurrence[8]. We essentially found that HTr patients displayed more major infections and fewer rejections during early and late postoperative periods and more minor neoplastic complications in the late postoperative period than LTr patients.

Detailing the type, severity, and chronology of postoperative complications is of paramount importance for a better understanding of the clinical evolution. It can assist in the implementation of preventive measures that may positively impact the outcome of PLDLT.

Several strategies have been adopted to decrease perioperative transfusion in adult LT patients. They can be classified into three groups of measures: (1) Prophylactic such as the recognition of patients at risk for bleeding and the previous suspension of drugs that interfere with coagulation; (2) Technical such as maintenance of low central venous pressure, controlled hypotension, use of vascular clamping, ultrasonic or argon scalpels, and capture and reuse of blood lost; guided fluid therapy by multiparametric data, reduction of transfusion trigger values, and viscoelastic tests; and (3) Pharmacological such as erythropoietin, desmopressin, vasopressin, antifibrinolytics, prothrombin complex, lyophilized fibrinogen, recombinant factor VIIa, fibrin sealants, and vasoactive drugs[58,59]. Certainly, not all of them apply to this population, that remain to be a challenge in conducting LT. Fluid management in small children undergoing LT cannot be guided by minimally invasive multiparametric monitors. These are of limited use, once their softwares are designed for adult patients. Nonetheless, the analysis of the trend curves can assist in decision-making, there is a lack of accuracy in the assessment of volemia, hemodynamic state and effect of vasoactive drugs during the perioperative period of pediatric LT. Besides, techniques as hemodilution and controlled hypotension are not validated in this group. Hemodilution increases hydrostatic pressure in the portal vein and inferior vena cava system and worsens the coagulopathy, exacerbating surgical bleeding. Controlled hypotension is a debatable issue and might be of potential risk for target organ damage. It is crucial to recognize that small children with chronic liver disease have a tenuous rebalance of the hemostatic system, not entirely understood, which might be easily disrupted by hasted interventions, pushing the patient towards hemorrhage and/or thrombosis. Prophylactic use of FFP is not advised, because it can increase intravascular pressure and increase RBC transfusion. Routine prophilatic use of antifibrinolytic drugs is no longer recommended, tranexamic acid and aminocaproic acid are possibly useful for patients in hyperfibrinolysis, demonstrated by microvascular oozing or viscoelastic tests. Prophylactic use of recombinant factor VIIa, should be avoided in all, except for highest-risk procedures[60]. Although, preoperative blood transfusion has been demonstraded to be independently associated to morbidity up to 30 d of postoperative period and harmful in neonates undergoing general pediatric surgery, neurosurgery, otolaringology, cardiothoracic, plastics and urology surgery[61], no strong evidence is found in pediatric LT in the long-term period.

Concurrent transfusion of "red" and "yellow" BP, in adult liver resection with compromised function, was associated with a significantly higher risk of postoperative morbidity compared to only RBC or only FFP transfusion, what might be attributed to synergistic effects[2]. Though, no similar study was conducted in pediatric LT. If there is an absence of universal definition of massive bleeding or massive transfusion and a scarcity of studies relating survival to specifc BP dosages, ratios, timing and guidance even in adult trauma victims[62], let alone pediatric LT in small children. Specific transfusion trigger thresholds in pediatric LT have not been validated and need to be determined by prospective controlled studies that seek to standardize patient samples, according to age or weight, underlying diseases, type of donor and type of graft.

This study does have some limitations. First, this was a retrospective study performed in a single center. Second, the collection of data was refined on an ongoing basis since the implementation of the LT program; the learning curve may have influenced the results. Third, and most importantly, complications due to increased transfusion volume may be an epiphenomenon related to a sicker patient and of higher technical difficulty, or, indeed, a risk factor for postoperative morbimortality. Nonetheless, this study has several strengths, such as the size and homogeneity of the sample as well as the standardization of the anesthetic/surgical approaches and the immunosuppression regimen. The follow-up was conducted in the same center, which included facilities for patients and their families to remain close during local treatment thus improving patient recruitment and reducing loss to follow-up throughout the study period.

CONCLUSION

In this study, blood transfusion volumes less than one total blood volume, though not considered massive transfusions, were already associated with a higher incidence of more serious complications and mortality as assessed by hospitalization up to 10 years after PLDLT. A perioperative RBC transfusion volume higher than 27.5 mL/kg is associated with not only increased rates of infectious, cardiovascular, respiratory, and neoplastic complications but also decreased frequency of rejection episodes. Furthermore, a perioperative volume of RBC transfusion higher than 27.5 mL/kg is an independent risk factor for mortality and is directly related to reduced patient and graft survival in PLDLT. These results underscore the need for more restrictive criteria to guide the use of blood transfusion in PLDLT patients to prevent potentially related postoperative complications.

Appropriate protocols should be tailored to each center according to the infrastructure, clinical staff experience, and patient's profile. Indeed, some strategies to reduce blood consumption should be implemented. An accurate nutritional assessment with specific dietary support and early supplementation is mandatory. Treatment with iron and vitamins should be considered. Prophylaxis of digestive bleeding and treatment of renal dysfunction and infection can decrease the incidence of preoperative anemia. The use of recombinant human erythropoietin therapy is controversial. Reduce blood tests and perform microsampling. During surgery, a more restrictive fluid management and a reduction of Hb trigger values to less than 8.0 g/dL, could reduce blood transfusion especially when combined with low doses of continuous infusion of norepinephrine. This could mitigate fluid overload, reduce portal hypertension, restore splanchnic and central circulatory imbalances and optimize tissue oxygenation. Assessment of coagulation with viscoelastic tests to improve blood management in pediatric surgery is feasible, but specific algorithms must be developed. The goals are to optimize the erythrocyte mass, minimize blood loss, increase tolerance to anemia and maintain hemostatic balance. As demonstrated, a small reduction in perioperative RBC transfusion volume may determine a better outcome in the short- and long-term postoperative periods. The evaluation of risk, effectiveness, and cost-benefit assessment of these strategies in young children with liver diseases is outside the scope of the present study and should be carried out in future research.

ARTICLE HIGHLIGHTS

Research background

Pediatric living donor liver transplantation (PLDLT) is a multidisciplinary procedure

of high complexity and potential risk of bleeding. The association between transfusion and short- and long-term postoperative complications is poorly established especially in small children. Blood transfusion is frequently indicated in the perioperative period of liver transplant, though there is little robust evidence of associated postoperative complications. Given the good survival results, in the past decade, it is now necessary to identify risk factors for complications in order to improve the long-term evolution.

Research motivation

To study in depth the short- and long-term evolution of this specific group of highly fragile pediatric patients, in order to improve the proficiency acquired in 20 years of working with PLDLT, and to be able to share knowledge.

Research objectives

This study assessed whether perioperative transfusion is associated with early and late postoperative complications and mortality in small patients undergoing PLDLT.

Research methods

Postoperative complications along 10 years of follow up were graduated with Clavien-Dindo modified classification in order to assess relationship between blood transfusion and postoperative complications. Multiple logistic regression analysis identified risk factors for major postoperative complications. Perioperative red blood cells volume was identified as a single risk factor and a receiver operating characteristic curve identified a cutoff point of 27.5 mL/kg. Cox regression analyses identified independent risk factors for mortality. Overall patient and graft survival analyses was performed using Kaplan–Meier survival curves, which were compared using the log-rank test and a $P < 0.05$ was considered statistically significant.

Research results

In terms of red blood cells (RBC) transfusion volume, there was a significantly higher rate of 30 d reoperation (26.3% × 8.7%, $P < 0.001$) and 30 d mortality rate (6.6% × 0.0%, $P < 0.001$) in the high-volume transfusion (HTr) *vs* low-volume transfusion (LTr), respectively. Early liver transplantation (LT)-specific complications include primary non-function, biliary complications, vascular thrombosis, and retransplantation that were not related to a higher perioperative transfusion volume. Over 10 years of follow-up, with respect to RBC transfusion volume, there was a significantly higher rate of reoperation (36.5% × 12.6%, $P < 0.001$) and mortality (25.5% × 7.8%, $P < 0.001$), respectively, in the HTr compared to the LTr. Perioperative RBC volume > 27.5 mL/kg and preoperative estimated glomerular filtration rate were identified as independent risk factors for mortality over 10 years of follow-up after LT. The patient survival rates were significantly lower in the HTr than in the LTr at 1, 5, and 10 years post-LT: 82.7% *vs* 97.7%, 73.9% *vs* 93.8%, and 72.6% *vs* 90.9%, respectively. Likewise, the graft survival rates were significantly lower in the HTr than in the LTr at 1, 5, and 10 years post-LT: 79.5% *vs* 97.7%, 67.2% *vs* 92.3%, and 67.2% *vs* 87%, respectively.

Research conclusions

A perioperative RBC transfusion volume > 27.5 mL/kg is associated with not only increased rates of infectious, cardiovascular, respiratory, and neoplastic complications but also decreased frequency of rejection episodes. Furthermore, a perioperative volume of RBC transfusion higher than 27.5 mL is an independent risk factor for mortality, and is directly related to reduced patient and graft survival in PLDLT.

Research perspectives

The detailed analysis of this study allows the construction of strategy protocols to reduce the need for transfusion of patients undergoing PLDLT improving short- and long-term outcome.

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ORIGINAL ARTICLE

Retrospective Study

R2* value derived from multi-echo Dixon technique can aid discrimination between benign and malignant focal liver lesions

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Abstract

BACKGROUND

R2* estimation reflects the paramagnetism of the tumor tissue, which may be used to differentiate between benign and malignant liver lesions when contrast agents are contraindicated.

AIM

To investigate whether R2* derived from multi-echo Dixon imaging can aid differentiating benign from malignant focal liver lesions (FLLs) and the impact of 2D region of interest (2D-ROI) and volume of interest (VOI) on the outcomes.

METHODS

We retrospectively enrolled 73 patients with 108 benign or malignant FLLs. All patients underwent conventional abdominal magnetic resonance imaging and multi-echo Dixon imaging. Two radiologists independently measured the mean R2* values of lesions using 2D-ROI and VOI approaches. The Bland–Altman plot was used to determine the interobserver agreement between R2* measurements. Intraclass correlation coefficient (ICC) was used to determine the reliability between the two readers. Mean R2* values were compared between benign and malignant FFLs using the nonparametric Mann–Whitney test. Receiver operating characteristic curve analysis was used to determine the diagnostic performance of R2* in differentiation between benign and malignant FFLs. We compared the diagnostic performance of R2* measured by 2D-ROI and VOI approaches.

RESULTS

This study included 30 benign and 78 malignant FLLs. The interobserver reproducibility of R2* measurements was excellent for the 2D-ROI (ICC = 0.994)

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: No additional data are available.

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and VOI (ICC = 0.998) methods. Bland–Altman analysis also demonstrated excellent agreement. Mean R2* was significantly higher for malignant than benign FFLs as measured by 2D-ROI ($P < 0.001$) and VOI ($P < 0.001$). The area under the curve (AUC) of R2* measured by 2D-ROI was 0.884 at a cut-off of 25.2/s, with a sensitivity of 84.6% and specificity of 80.0% for differentiating benign from malignant FFLs. R2* measured by VOI yielded an AUC of 0.875 at a cut-off of 26.7/s in distinguishing benign from malignant FFLs, with a sensitivity of 85.9% and specificity of 76.7%. The AUCs of R2* were not significantly different between the 2D-ROI and VOI methods.

CONCLUSION

R2* derived from multi-echo Dixon imaging whether by 2D-ROI or VOI can aid in differentiation between benign and malignant FLLs.

Key Words: R2*; Multi-echo Dixon imaging; Hypoxia; Malignant lesion; Benign lesion; Focal liver lesion

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Core Tip: Our study showed that mean R2* value of malignant focal liver lesions (FLLs) was significantly higher than that of benign FLLs. R2* derived from multi-echo Dixon imaging is a potential biomarker to differentiate malignant from benign FFLs. The multi-echo Dixon sequence is easy to perform and requires only a single breath-hold of 16 s to image the entire liver, which holds a good potential for clinical application.

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INTRODUCTION

Liver cancer is the sixth most common cancer and the fourth leading cause of cancer deaths worldwide[1]. The liver is also the most frequent site for distant metastases[2]. Clinically, once a focal liver lesion (FLL) is identified, it is essential to distinguish between benign and malignant lesions, as this differentiation determines the individual's prognosis and subsequent treatment strategy[3]. Contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI) are widely used to detect and characterize FLLs[4-7]. However, the use of iodine and gadolinium-based contrast agents is sometimes contraindicated; for example, in patients with severe kidney impairment due to the potential development of contrast-induced nephropathy[8] or nephrogenic systemic fibrosis[9]. Several imaging techniques without the need of contrast agents have been used to diagnose FFLs, including diffusion-weighted image (DWI), intravoxel incoherent motion, diffusion kurtosis imaging, and magnetic resonance elastography, although these techniques have shown mixed success with limited clinical application[10-13].

A hypoxic microenvironment is a hallmark in biology for solid tumors[14,15]. It is known that R2* estimation (R2* = 1/T2*) is inversely related to partial tissue pressure of oxygen, and reflects the paramagnetism of the tumor tissue, such as the presence of deoxygenated hemoglobin[15-17]. Previous studies have demonstrated that R2* can be used to assess oxygenation status in several malignancies[18,19] and offer additive value in identifying metastatic lymph nodes in breast cancer[20]. However, whether R2* can be used to differentiate between benign and malignant FLLs remains to be determined. Besides, 2D region of interest (2D-ROI) and volume of interest (VOI) analyses, which are better for R2* measurement in FFLs, remain elusive.

In this study, the diagnostic performances of R2* derived from multi-echo Dixon imaging in differentiating between benign and malignant FLLs based on 2D-ROI and

VOI analyses were investigated. The purpose of this study was to determine whether R2* derived from multi-echo Dixon imaging can aid in differentiating benign from malignant FLLs, and the impact of 2D-ROI and VOI on the outcomes.

MATERIALS AND METHODS

Patients

This retrospective study was approved by the Institutional Ethics Review Board of our hospital (approval No. SYSEC-KY-KS-2020-147), and the requirement for informed consent from the patients was waived. From January 2019 to December 2019, consecutive patients with FLLs were identified from the hospital database. Patients were included if they had: (1) A solid malignant or benign FLL confirmed by histology, and follow-up contrast-enhanced CT/MRI examination for at least 6 mo, or positron emission tomography (PET)-CT; and (2) Multi-echo Dixon imaging. The exclusion criteria were as follows: (1) Diffuse liver inflammation (n = 5); (2) Maximal lesion diameter < 10 mm (n = 5); (3) Lower signal-to-noise ratio on R2* images; and (4) Obvious breathing artifacts on R2* images (n = 5).

MRI acquisition

MRI was performed on a 3.0 T unit (MAGNETOM Skyra; Siemens Healthcare, Erlangen, Germany). The sequences consisted of conventional sequences and multi-echo Dixon imaging. Conventional MRI included axial BLADE T2-weighted imaging (T2WI) [repetition-time/echo-time (TR/TE) = 9672.9-12331.7/84 ms; flip angle = 130°; averages = 1; matrix = 320 × 320; field of view = 100 mm; slice thickness = 5 mm], axial and coronal T1-weighted imaging (T1WI) volume interpolated breath-hold examination (VIBE) (TR/TE = 3.97/1.29 ms; flip angle = 9°; averages = 1; matrix = 320 × 180; field of view = 75 mm; slice thickness = 3 mm), and axial DWI (TR/TE = 4900/66 ms; flip angle = 90°; averages = 12; matrix = 192 × 113; field of view = 78.125 mm; slice thickness = 5 mm; b values = 0 and 800 s/m^2). The multi-echo Dixon imaging was performed with T2* correction. The acquisition parameters were: TR = 9 ms; six-echo with TE = 1.05/2.46/3.69/4.92/6.15/7.38 ms; averages = 1; matrix = 160 × 136; field of view = 450 mm; slice thickness = 3.5 mm; number of slices = 64; a flip angle = 4° was used to minimize the effects of T1 weighting[21]. This sequence was acquired in a breath-hold of 16 s. After these sequences, multiphase contrast-enhanced imaging was performed after administration of gadolinium contrast medium (Magnevist; Bayer Schering Pharma, Berlin, Germany) using a fat-suppressed dynamic contrast enhancement sequence with the following acquisition parameters: TR/TE = 3.8/1.23 ms; averages = 1; slice thickness = 2.5; field of view = 80.56; matrix = 288 × 186; flip angle = 10°. Then, all patients underwent axial and coronal contrast-enhanced T1WI–VIBE (TR/TE = 3.97/1.26 ms; flip angle = 9°; averages = 1; slice thickness = 2.3 mm; matrix = 320 × 180; field of view = 75 mm).

Image analysis

All the images were assessed by using the ImageJ software (http://rsb.info.nih.gov/ij/). A low flip angle multi-echo Dixon sequence was used to derive R2* to minimize T1-related bias and improve the separation of water and fat. The improved fitting of the signals within fatty tissues allows more accurate R2* mapping and T2* correction of the water-fat separation[22]. Two experienced radiologists (Shi GZ and Gao M, with 6 and 12 years of experience in liver diagnostic imaging, respectively) who were blinded to the diagnosis of patients manually delineated the lesions on R2* maps. For 2D-ROI, a single freehand ROI was drawn to cover the whole tumor area on the section showing the maximal tumor dimension. For VOI, the freehand ROI was placed slice by slice to cover the entire tumor volume. The mean R2* values measured by 2D-ROI and VOI were used for analysis (Figure 1).

Laboratory and anthropometric evaluations

Hepatitis B virus infection, α-fetoprotein (AFP), carbohydrate antigen 19-9 (CA 19-9), and carcinoma embryonic antigen (CEA) were measured using standard reagents. Liver cirrhosis was determined by Masson trichrome staining. The normal ranges are: AFP ≤ 25 ng/mL, CA 19-9 ≤ 34 U/mL, and CEA ≤ 5 ng/mL. Laboratory examination was performed before clinical treatment. The time between laboratory examination and multi-echo MRI examination was within 1 wk.

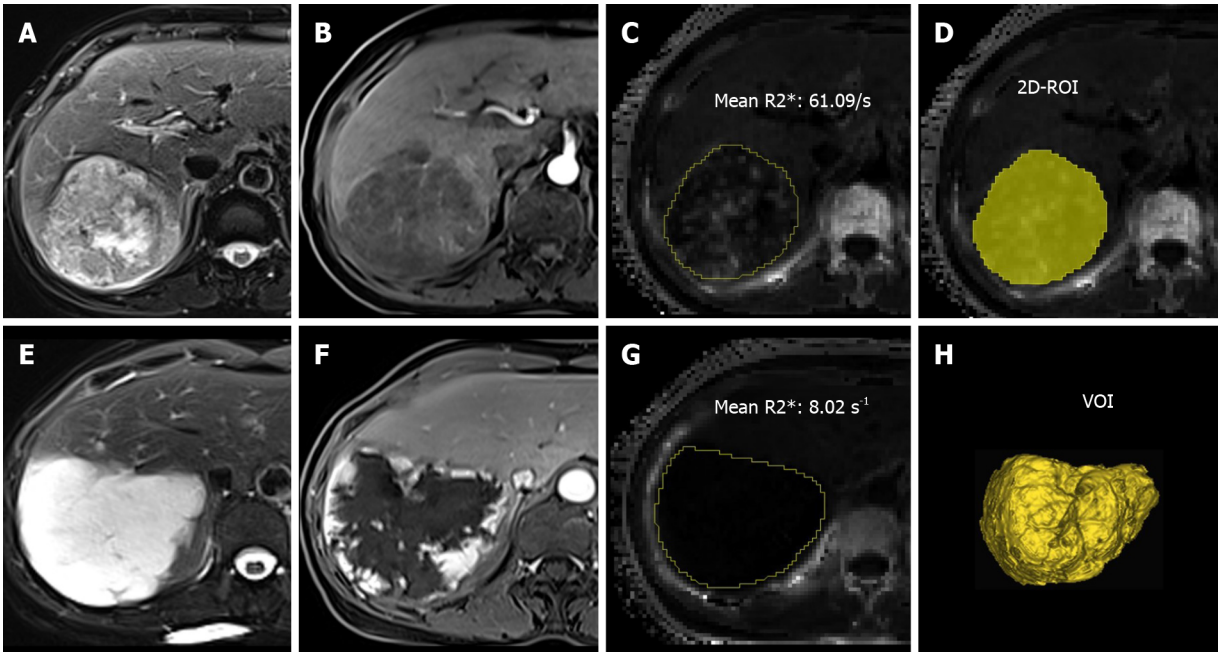


Figure 1 Two-dimensional region of interest and volume of interest. A-C: T2-weighted imaging (T2WI) (A), arterial phase contrast-enhanced T1-weighted imaging (T1WI) (B), and R2* map showed liver metastasis (yellow line) (C) confirmed by histology in a 59-year-old woman with lung cancer; D: Two-dimensional region of interest was drawn on the section showing the maximal tumor dimension; E-G: T2WI (E), arterial phase contrast-enhanced T1WI (F), and R2* map showed a live hemangioma (yellow line) (G) in a 59-year-old woman; H: Volume of interest was placed covering the entire tumor volume on R2* map. 2D-ROI: Two-dimensional region of interest; VOI: Volume of interest.

Diagnosis of FLLs

All analyzed lesions were diagnosed by contrast-enhanced MRI, follow-up contrast-enhanced CT/MRI examination within at least 6 mo, fluorine 18 (^{18}F) fluoro-deoxyglucose (FDG) PET-CT, or histopathological findings (hepatectomy or biopsy)[5,22-25]. Diagnostic reference standard was established based on histopathological confirmation in 29/32 hepatocellular carcinomas (HCCs), 6/9 intrahepatic cholangiocarcinomas (IHCCs), 7/37 metastases, 5/25 hemangiomas, and 2/3 focal nodular hyperplasias (FNHs). In the remaining 69 FLLs without histopathological results, diagnoses were established by well-accepted imaging findings in all acquired MRI sequences (*e.g.*, T1WI, T2WI, T2-SPAIR, DWI, and contrast-enhanced T1WI). Criteria were determined by consensus reading of two experienced radiologists (R1, Shi GZ; and R2, Gao M) by consideration of all acquired images. Further reference standards were required: (1) FFLs were diagnosed as primary malignant FFLs if they showed (a) characteristic imaging appearance during a 6-mo imaging follow-up combined with (b) clinical symptoms and serological results; (2) FFLs were diagnosed as liver metastasis in patients with primary malignancies (pathologically confirmed) when at least one of the following criteria was satisfied: (a) Newly developed lesion or an increase in size with typical imaging appearance during a 6-mo imaging follow-up; and (b) abnormal ^{18}F FDG uptake at PET-CT examination; and (3) FFLs were diagnosed as benign lesions if (a) they were stable at 6-mo imaging follow-up with characteristic imaging appearance in subjects at low risk; and (b) no malignant tumor was found in patients with benign FLLs during imaging examination.

Three HCCs, three IHCCs, and 19 metastases were diagnosed according to 6-mo imaging follow-up. Eleven metastases were confirmed by PET-CT. In liver metastasis patients, the primary tumors were bladder cancer (n = 9), lung cancer (n = 2), colorectal cancer (n = 7), cervical cancer (n = 4), gastric cancer (n = 3), gallbladder cancer (n = 1), breast cancer (n = 1), and HCC (n = 10). For benign FLLs, 20 hemangiomas and one FNH were confirmed by 6-mo imaging follow-up. Two liver abscesses had typical imaging findings in all the MRI sequences and typical imaging findings in a 6-mo follow-up MRI examination after clinical treatment.

Statistical analysis

Numerical data are expressed as the mean ± SD. The Bland–Altman plot was performed to determine the interobserver agreement on R2* measurements. Intraclass correlation coefficient (ICC) was used to determine the reliability between the two

radiologists in R2* measurements using 2D-ROI and VOI methods (0-0.20 poor; 0.21-0.40 fair; 0.41-0.60 moderate; 0.61-0.80 good; and 0.81-1.0 excellent correlation). Mean R2* values from the two readers were used for the final analysis. Nonparametric Mann–Whitney test was used to compare the difference in R2* values between the malignant and benign groups. The receiver operating characteristic (ROC) analysis was used to evaluate the diagnostic performances of R2*. The area under the ROC curve (AUC), optimal cut-off values, sensitivity, and specificity were determined as the maximum Youden index. Differences in the diagnostic performance of the two different ROI positioning methods were analyzed by comparing ROC curves according to the method developed by DeLong *et al*[26]. $P < 0.05$ (two-tail) indicated a statistically significant difference.

RESULTS

Clinicopathological characteristics

A total of 108 FLLs were found in 73 patients, including 78 malignant FLLs (mean maximum diameter, 48.2 ± 37.7 mm; range, 11-163 mm) and 30 benign FLLs (mean maximum diameter, 32.3 ± 22.5 mm; range, 14-94 mm). Forty-nine patients had malignant FFLs (30 men and 19 women; mean age, 56.3 ± 10.3 years; range, 40-81 years), and 24 patients (11 men and 13 women; mean age, 52.1 ± 12.9 years; range, 31-73 years) had benign FLLs. The malignant FFLs included 32 HCCs, nine IHCCs, and 37 liver metastases. Benign FFLs included 25 hemangiomas, three FNHs, and two liver abscesses. The mean maximum diameter of liver metastases, HCCs, and IHCCs was 29.1 ± 24.1 mm (range, 11-122 mm), 66.3 ± 43.0 mm (range, 15–163 mm), and 61.9 ± 25.9 mm (range, 32-111 mm), respectively. In benign FFLs, the mean maximum diameter of hemangiomas, FNHs, and liver abscesses was 29.4 ± 21.8 mm (range, 14-94 mm), 32.0 ± 8.5 mm (range, 23-40 mm), and 69.5 ± 12.0 mm (range, 61-78 mm), respectively. Clinicopathological characteristics and laboratory evaluations of FFLs are shown in Tables 1 and 2.

R2* analysis

Figure 2 shows the Bland–Altman plot measurement of R2* of FLLs for the two readers. For 2D-ROI analysis, the 95% limits of agreement of R2* for the two readers were from -5.68 to 5.04/s, and the mean difference for the two readers was -0.32/s. For VOI analysis, the 95% limits of agreement of R2* for the two readers were from -3.65 to 3.28/s, and the mean difference for the two readers was -0.18/s. The differences between the two readers using two different methods were relatively small. ICC for the 2D-ROI method was 0.994 and ICC for the VOI method was 0.998. The interobserver agreement was excellent.

The mean R2* values measured by 2D-ROI and VOI methods were significantly higher in the malignant group than in the benign group (2D-ROI: 37.99 ± 17.71 *vs* 18.6 ± 8.43/s, $P < 0.001$; VOI: 41.11 ± 19.01 *vs* 20.61 ± 9.01/s, $P < 0.001$). For 2D-ROI measurement, the mean R2* value of liver metastases was 44.17 ± 21.90/s, and the mean R2* values of HCCs and IHCCs were 33.45 ± 10.15 and 28.72 ± 10.21/s, respectively. The mean R2* values of hemangiomas, FNHs, and abscesses were 16.66 ± 8.18, 26.21 ± 5.61, and 23.29 ± 9.31/s, respectively. For VOI measurement, FFLs had a mean R2* value of 48.42 ± 23.61/s for liver metastases, 35.41 ± 10.04/s for HCCs, 31.34 ± 9.65/s for IHCCs, 19.36 ± 8.93/s for hemangiomas, 27.87 ± 7.46/s for FNHs, and 25.29 ± 10.46/s for abscesses. Malignant FFLs had higher R2* values than benign FLLs regardless of ROI placement methods (Table 3).

ROC analysis

The AUC of 2D-ROI was 0.884 (95%CI, 0.819 to 0.950) at a cut-off of 25.2/s, with a sensitivity of 84.6% and specificity of 80.0% for differentiating benign from malignant FFLs. The VOI method yielded an AUC of 0.875 (95%CI: 0.806 to 0.945) at a cut-off of 26.7/s in distinguishing benign from malignant FFLs, with a sensitivity of 85.9% and specificity of 76.7%. There was no significant difference between the AUCs for 2D-ROI and VOI positioning methods for discriminating benign from malignant FFLs ($Z = 1.069$, $P = 0.285$) (Figure 3).

Table 1 Baseline characteristics of malignant and benign focal liver lesions of 73 patients

Characteristic	Malignant	Benign	Total
Per-patient basis			
No. of patients (%)	49 (67.1)	24 (32.9)	73
Age (yr)			
mean ± SD	56.3 ± 10.3	52.1 ± 12.9	55.0 ± 11.2
Range	40-81	31-73	31-81
Sex, *n* (%)			
Male	30 (61.2)	11 (45.8)	41
Female	19 (38.8)	13 (54.2)	32
Per-lesion basis			
No. of lesions	78 (72.3)	30 (27.8)	108
Maximum diameter (mm)			
mean ± SD	48.2 ± 37.7	32.3 ± 22.5	43.8 ± 34.8
Range	11-163	14-94	11-163
Methods of diagnosis (%)			
Pathology	42 (38.9)	7 (6.5)	49 (45.4)
Imaging follow-up	25 (23.1)	23 (21.3)	48 (44.4)
PET-CT	11 (10.2)	–	11 (10.2)

FFL: Focal liver lesion; PET-CT: Positron emission tomography-computed tomography.

DISCUSSION

Our study showed that the mean R2* value of malignant FLLs was significantly higher than that of the benign FLLs. R2* derived from multi-echo Dixon imaging is a potential biomarker to differentiate malignant from benign FFLs.

The combined use of MRI, CT, and ultrasound has a high diagnostic performance for the identification of FLLs, but requires the administration of gadolinium or iodine contrast agents[7]. Gadolinium contrast is contraindicated in patients with severe renal impairment, because it may induce nephrogenic systemic fibrosis, and may even be a greater risk in patients with liver dysfunction[27,28]. Iodinated contrast administration for CT may aggravate renal failure[8]. Currently, no alternative imaging methods have been widely advocated for these patients. Hypoxia is an important factor in cancer progression, affecting the autonomous functions of tumor cells and nonautonomous processes such as angiogenesis, lymphangiogenesis, and inflammation[29]. Hypoxia causes an increase in the concentration of deoxygenated hemoglobin in the tumor. Deoxyhemoglobin can be used as an endogenous hypoxia tracer that may produce local magnetic field inhomogeneities to reduce T2* relaxation time[30]. Furthermore, higher local deoxyhemoglobin may result in a decrease in proton T2* relaxation time and a corresponding increase in R2*, which indicates a link between R2* and the oxygen concentration of local tissues[15]. Recently, susceptibility-weighted imaging, which was originally called blood-oxygen-level-dependent (BOLD) venographic imaging, has demonstrated advantages in the detection of hemorrhagic events due to its sensitivity to paramagnetic substances[31]. Also, BOLD MRI has shown ability in assessing tumor oxygenation and indirectly hypoxia, by detecting signal changes secondary to changes in blood flow and oxygenation[32]. These two sequences were commonly used in the central nervous system[33,34]. Currently, T2* has been used in assessing tissue oxygenation status *in vivo* based on the paramagnetic properties of deoxyhemoglobin[35]. Besides, this technique has been shown to be feasible and accurate in the detection of HCC[27,32]

Previously, R2* values have been used to distinguish cancerous from normal prostatic regions, with higher mean R2* values being related to a higher tumor Gleason score[36]. In addition, higher R2* values were found in high-grade bladder cancer[15] and clear cell renal cell carcinoma[37] than those of low-grade malignancies. In

Table 2 Clinicopathological characteristics of 108 focal liver lesions

Characteristic	Malignant		Benign			
	HCC	IHCC	Hemangioma	FNH	Abscess	
No. of lesions (%)	32 (29.6)	9 (8.3)	37 (34.3)	25 (23.1)	3 (2.8)	2 (1.9)
Maximum diameter (mm)						
mean ± SD	66.3 ± 43.0	61.9 ± 5.9	29.1 ± 24.1	29.4 ± 21.8	32.0 ± 8.5	69.5 ± 12.0
Range	15-163	32-111	11-122	14-94	23-40	61-78
Methods of diagnosis (%)						
Pathology	29 (26.9)	6 (5.6)	7 (6.5)	5 (4.6)	2 (1.9)	0 (0)
Imaging follow-up	3 (2.8)	3 (2.8)	19 (17.6)	20 (18.5)	1 (0.9)	2 (1.9)
PET-CT	–	–	11 (10.2)	–	–	–
Viral infection						
HBV	30	6	34	9	1	2
Non-HBV	2	3	3	9	1	0
NA	0	0	0	7	1	0
Cirrhosis on pathology (%)						
Yes	25	–	–	–	–	–
No	1	–	–	–	–	–
NA	6	–	–	–	–	–
AFP (ng/mL)						
≤ 25	12	9	29	–	–	–
> 25	20	0	7	–	–	–
NA	0	0	1	–	–	–
CA 19-9 (U/mL)						
≤ 34	21	4	15	–	–	–
> 34	9	5	20	–	–	–
NA	2	0	2	–	–	–
CEA (ng/mL)				–	–	–
≤ 5	27	7	15	–	–	–
> 5	5	2	22	–	–	–
NA	0	0	0	–	–	–

APF: -fetoprotein; CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoma embryonic antigen; FFL: Focal liver lesion; FNH: Focal nodular hyperplasia; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; IHCC: Intrahepatic cholangiocarcinoma; NA: Not available; PET-CT: Positron emission tomography-computed tomography. Data are shown as the mean SD.

our study, the mean R2* value of malignant FLLs was significantly higher than that of the benign FLLs. This may be attributed to the rapid growth of liver malignancies, resulting in a relatively hypoxic state and an increase in deoxyhemoglobin[15]. Consequently, the corresponding increase in R2* value may correlate with the degree of malignancy of FFL. R2* may be used as a quantitative imaging biomarker to provide additional information for tumor differential diagnosis.

In our study, mean R2* values, whether derived from 2D-ROI or VOI segmentation positioning methods, were highly reproducible. Moreover, the AUC of R2* measured by 2D-ROI was 0.884 with a sensitivity of 84.6% and specificity of 80.0%, while AUC of R2* measured by VOI yielded an AUC of 0.875 with a sensitivity of 85.9% and specificity of 76.7%, in distinguishing benign from malignant FFLs, respectively. Campo *et al*[38] demonstrated that a large ROI that refers to as large an area of the liver as possible can improve the reproducibility and repeatability of R2* measurements in

Table 3 Mean R2* values for different focal liver lesions

FFL	2D-ROI method	VOI method
Malignant		
Liver metastasis	44.17 ± 21.90	48.42 ± 23.61
HCC	33.45 ± 10.15	35.41 ± 10.04
IHCC	28.72 ± 10.21	31.34 ± 9.65
Benign		
Hemangioma	16.66 ± 8.18	19.36 ± 8.93
FNH	26.21 ± 5.61	27.87 ± 7.46
Abscess	23.29 ± 9.31	25.29 ± 10.46

Numerical data are expressed as the mean ± SD. 2D-ROI: Two-dimensional region of interest; FFL: Focal liver lesion; FNH: Focal nodular hyperplasia; HCC: Hepatocellular carcinoma; IHCC: Intrahepatic cholangiocarcinoma; VOI: Volume of interest.

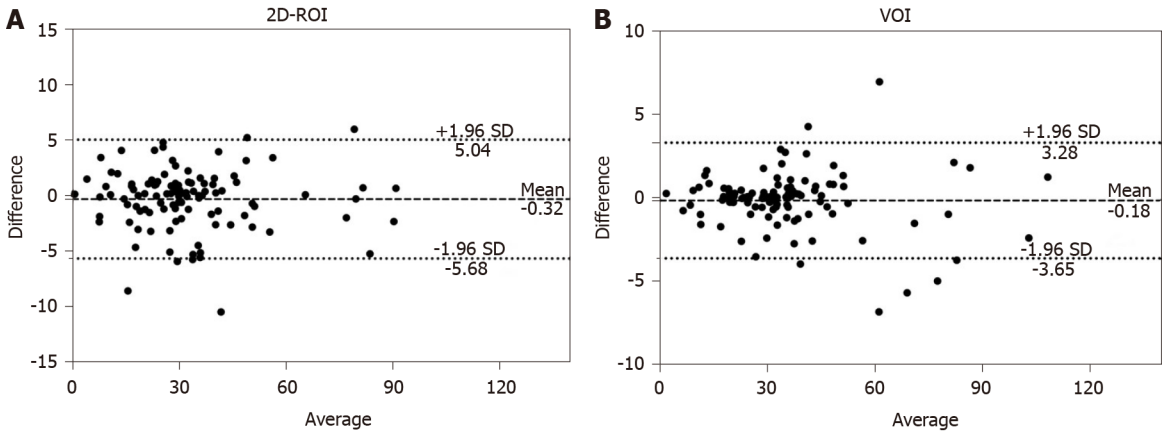


Figure 2 Bland–Altman plots showing interobserver variability in two-dimensional region of interest and volume of interest measurements. A: Two-dimensional region of interest (ROI); B: Volume of interest. The differences between the two readers using the two different ROI positioning methods were small. 2D-ROI: Two-dimensional region of interest; VOI: Volume of interest.

patients with low and high liver iron content. McCarville *et al*[39] reported excellent interobserver agreements in liver R2* for both small (≥ 1 cm diameter) and whole liver ROI methods for iron overloaded patients who underwent biopsy. Sofue *et al*[40] found that R2* measurements of whole liver volume and colocalized ROIs in three different hepatic segments were repeatable between examinations. However, these studies investigated ROI location of R2* measurements in diffusive liver lesions rather than FLLs. To the best of our knowledge, our study was the first to investigate R2* measurements in FFLs.

We found similar results in differentiating between benign and malignant FLLs by using 2D-ROI and VOI methods for R2* measurement. ROC curve analysis demonstrated no significant difference between the AUCs for 2D-ROI and VOI positioning methods for discriminating benign from malignant FFLs. R2* measured by VOI analysis showed an AUC of 0.875, while 2D-ROI analysis showed an AUC of 0.884 in differentiating between benign and malignant FLLs. These results indicate that the impact of the different ROI positioning methods could be ignored for the differential diagnosis of benign and malignant FFLs. Thust *et al*[41] obtained the same results in volumetric and 2D measurements of apparent diffusion coefficient in distinguishing glioma subtypes. Compared with VOI, 2D-ROI is easier to delineate and easily incorporated into clinical practice. The easy implementation of R2* measurements using 2D-ROI will facilitate its clinical application.

There were several limitations to this study. First, this was a single-center study, and the number of patients in the cohort was relatively small. A larger patient cohort in a multicenter setting is needed to validate our findings. Second, R2* is an indirect method for monitoring tumor PO_2[42]. In addition to the oxygenation state, R2* can also

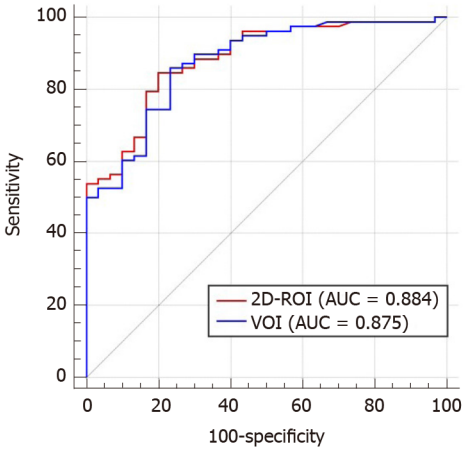


Figure 3 Receiver operating characteristic curve analysis of the two positioning methods in differentiating between malignant group and benign group. Two-dimensional region of interest and volume of interest methods yielded similar results. 2D-ROI: Two-dimensional region of interest; VOI: Volume of interest; AUC: Area under the curve.

be affected by other factors, such as hemoglobin levels, blood volume, and vasculature[15]. Nevertheless, various studies have found that T2WI is a highly sensitive technique for reliably assessing paramagnetic deoxyhemoglobin, methemoglobin, or hemosiderin in lesions and tissues in body imaging[30,35,37]. R2* quantification can yield hypoxia information about malignancies in a noninvasive manner[19,42]. In addition, the sequence used in our study is easy to perform and requires only a single breath-hold of 16 s to image the entire liver, and no image postprocessing is required.

CONCLUSION

In conclusion, R2* values derived from multi-echo Dixon imaging can aid in discrimination between benign and malignant FLLs. 2D-ROI and VOI methods do not affect the diagnostic performance of R2*. R2* measured by 2D-ROI can be adopted to improve diagnostic accuracy of FFLs, particularly in patients with a contraindication to contrast agents.

ARTICLE HIGHLIGHTS

Research background

It is essential to distinguish between benign and malignant focal liver lesions (FLLs), as this differentiation determines the individual's prognosis and subsequent treatment strategy. Since the use of iodine and gadolinium-based contrast agents is contraindicated, imaging techniques without the need of contrast agents have been used to diagnose FFLs, including diffusion-weighted imaging, intravoxel incoherent motion, diffusion kurtosis imaging, and magnetic resonance elastography.

Research motivation

Imaging techniques without the need of contrast agents have shown mixed success with limited clinical application. R2* estimation is inversely related to partial tissue pressure of oxygen, and reflects the paramagnetism of the tumor tissue, which may be helpful to differentiate between benign and malignant FLLs.

Research objectives

To investigate whether R2* derived from multi-echo Dixon imaging can aid differentiating benign from malignant FLLs. The findings obtained can provide information for differential diagnosis of FLLs using R2*.

Research methods

This study retrospectively enrolled 73 patients with 108 benign or malignant FLLs. All patients underwent conventional abdominal magnetic resonance imaging and multi-

echo Dixon imaging. The mean R2* values of lesions were measured using 2D region of interest (2D-ROI) and volume of interest (VOI) approaches. Mean R2* values were compared between benign and malignant FFLs using the nonparametric Mann–Whitney test. Receiver operating characteristic curve analysis was used to determine the diagnostic performance of R2* in differentiation between benign and malignant FFLs. The diagnostic performance of R2* measured by 2D-ROI and VOI approaches was compared.

Research results

The study included 30 benign and 78 malignant FLLs. Mean R2* was significantly higher for malignant than benign FFLs as measured by 2D-ROI ($P < 0.001$) and VOI ($P < 0.001$). The area under the curve (AUC) of R2* measured by 2D-ROI was 0.884 at a cut-off of 25.2/s, with a sensitivity of 84.6% and specificity of 80.0% for differentiating benign from malignant FFLs. R2* measured by VOI yielded a AUC of 0.875 at a cut-off of 26.7/s in distinguishing benign from malignant FFLs, with a sensitivity of 85.9% and specificity of 76.7%. The AUCs of R2* were not significantly different between the 2D-ROI and VOI methods. However, due to the relatively small sample size, a large population from multiple centers is needed for further validation of our findings.

Research conclusions

R2* derived from multi-echo Dixon imaging can aid in differentiation between benign and malignant FLLs. 2D-ROI and VOI methods do not affect the diagnostic performance of R2*.

Research perspectives

This study describes that R2* value derived from multi-echo Dixon imaging can aid in differentiation between benign and malignant FLLs. The multi-echo Dixon sequence is easy to perform and requires only a single breath-hold of 16 s to image the entire liver, which holds a good potential for clinical application.

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ORIGINAL ARTICLE

Retrospective Study

Cytapheresis re-induces high-rate steroid-free remission in patients with steroid-dependent and steroid-refractory ulcerative colitis

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Informed consent statement:

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Abstract

BACKGROUND

It is a crucial issue for patients with refractory ulcerative colitis (UC), including steroid-dependent and steroid-refractory patients, to achieve and maintain steroid-free remission. However, clinical studies focused on the achievement of steroid-free remission in refractory UC patients are insufficient. Cytapheresis (CAP) is a non-pharmacological extracorporeal therapy that is effective for active UC with fewer adverse effects. This study comprised UC patients treated with CAP and suggested the efficacy of CAP for refractory UC patients.

AIM

To clarify the efficacy of CAP in achieving steroid-free remission in refractory UC patients.

METHODS

We retrospectively reviewed the collected data from 55 patients with refractory UC treated with CAP. We analyzed the following points: (1) Efficacy of the first course of CAP; (2) Efficacy of the second, third, and fourth courses of CAP in patients who experienced relapses during the observation period; (3) Efficacy of CAP in colonic mucosa; and (4) Long-term efficacy of CAP. Clinical efficacy was evaluated using Lichtiger's clinical activity index or Sutherland index (disease activity index). Mucosal healing was evaluated using Mayo endoscopic subscore.

Written or oral informed consent was obtained from patients and/or parents of patients aged younger than 20 years.

Conflict-of-interest statement: We declare no conflict-of-interest associated with this manuscript.

Data sharing statement: No additional data are available.

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The primary and secondary endpoints were the rate of achievement of steroid-free remission and the rate of sustained steroid-free remission, respectively. Statistical analysis was performed using the paired t-test and chi-squared test.

RESULTS

The rates of clinical remission, steroid-free remission, and poor effectiveness after CAP were 69.1%, 45.5%, and 30.9%, respectively. There were no significant differences in rate of steroid-free remission between patients with steroid-dependent and steroid-refractory UC. The mean disease activity index and Lichtiger's clinical activity index scores were significantly decreased after CAP ($P < 0.0001$). The rates of steroid-free remission after the second, third, and fourth courses of CAP in patients who achieved steroid-free remission after the first course of CAP were 83.3%, 83.3%, and 60%, respectively. Mucosal healing was observed in all patients who achieved steroid-free remission after the first course of CAP. The rates of sustained steroid-free remission were 68.0%, 60.0%, and 56.0% at 12, 24, and 36 mo after the CAP. Nine patients (36%) had maintained steroid-free remission throughout the observation period.

CONCLUSION

Our results suggest that CAP effectively induces and maintains steroid-free remission in refractory UC and re-induces steroid-free remission in patients achieving steroid-free remission after the first course of CAP.

Key Words: Ulcerative colitis; Cytapheresis; Steroid-dependent; Steroid-refractory; Steroid-free remission; Inflammatory bowel disease

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Core Tip: Management of steroid-dependent and steroid-refractory ulcerative colitis (UC) is a critical issue, and the goal of the therapy for such refractory UC should be steroid-free remission. However, clinical studies focused on the achievement of steroid-free remission in refractory UC patients are insufficient. In this study, we demonstrated that cytapheresis (CAP) was effective in inducing and maintaining steroid-free remission even in both steroid-dependent and steroid-refractory UC patients. Furthermore, it is notable that we also showed that CAP re-induced high-rate steroid-free remission repeatedly in such refractory UC patients who achieved steroid-free remission after the first course of CAP.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) of unknown etiology, which can affect the entire colon. Several treatments for UC are available to induce and maintain the clinical remission of the disease. Among these treatments, corticosteroids (CSs) were first introduced by Truelove and Witts[1] and currently remain the first-line treatment to induce remission in moderate to severe UC patients. Faubion *et al*[2] reported that 34% of UC patients were treated with CSs and that immediate outcomes were complete remission in 54%, partial remission in 30%, and no response in 16% of patients. They also showed that 1-year outcomes were prolonged response in 49%, CS dependence in 22%, and operation in 29% of patients[2]. Despite the effectiveness of CSs in inducing clinical remission in UC patients, it has been reported that 16%-18% of patients had no response to steroids (steroid-refractory), and the rate of steroid dependence was 17%-22% at 1 year following treatment with the initial CS therapy and increased to 38% mostly within 2

years[2-7].

Refractory UC generally includes both steroid-dependent and steroid-refractory UC. Along with the recent advancements of the treatment for UC, several breakthrough treatments, including biologics, have been developed for refractory UC[8-23]. A meta-analysis showed that anti-tumor necrosis factor-α (TNF-α) antibodies had more clinical benefits than placebo control as evidenced by the former's increased frequency of clinical remission, steroid-free remission, endoscopic remission, and decreased frequency of colectomy[24]. It was also reported that the rates of induction of steroid-free remission in refractory UC patients with anti-TNF-α antibodies ranged from 40.0% to 76.5%[6,9,11,15,16,18]. However, studies that analyzed the efficacy of biologics focused on the achievement of steroid-free remission in refractory UC patients are insufficient. On the contrary, despite the efficacy of anti-TNF-α antibody for UC, secondary loss of response (LOR) is a common clinical problem with its incidence rate ranging from 23% to 46% at 12 mo after anti-TNF-α initiation[25]. Moreover, it was reported that the incidence rates of LOR were 58.3% (adalimumab) and 59.1% (infliximab) during maintenance therapy (mean follow-up: 139 wk and 158.8 wk, respectively)[26]. Regarding vedolizumab, it was also reported that the cumulative rate for LOR in UC patients was 39% at 12 mo[27]. Concerning the adverse events of biologics, similar with other biological therapies, anti-TNF-α therapy may lead to serious infection, demyelinating disease, and associated mortality[28]. It was also reported that the use of anti-TNF-α antibody combined with thiopurines was associated with an increased risk of lymphoma in IBD[29].

Thiopurines have been conventionally used for the treatment of steroid-dependent UC[30-35]. Two randomized controlled trials have shown that the rates of the induction of CS-free remission with thiopurines in steroid-dependent UC patients were 44% and 53%, respectively[34,35]. However, Jharap *et al*[32] reported that thiopurine therapy has failed in approximately one-quarter of IBD patients within 3 mo after treatment initiation, which is mostly due to drug intolerance or toxicity. Moreover, thiopurines are associated with potential serious adverse events, such as an increased risk of lymphoma and nonmelanoma skin cancer[33].

Cytapheresis (CAP) is a non-pharmacological extracorporeal therapy and has been developed as a treatment for UC[36-42]. CAP is performed using two methods, namely, granulocyte and monocyte adsorptive apheresis (GMA), which uses cellulose acetate beads (Adacolumn, JIMRO Co., Ltd., Gunma, Japan), and leukocytapheresis (LCAP), which uses polyethylene phthalate fibers (Cellsorba., Asahi Kasei Medical Co., Ltd., Tokyo, Japan)[42,43]. GMA selectively depletes elevated granulocytes and monocytes from the patients' circulation, but spares most of the lymphocytes[42]. LCAP exerts anti-inflammatory effects by removing activated leukocytes or platelets from the peripheral blood through an extracorporeal circulation[43]. It has been shown that CAP is an effective therapeutic strategy for patients with active UC with fewer adverse effects[36-42]. However, to date, the number of studies focused on the efficacy of CAP in both steroid-dependent and steroid-refractory UC has been limited[43-54].

Despite the excellent therapeutic effects of CS for UC patients, prolonged CS therapy can result in multiple serious side effects such as diabetes mellitus, infection, osteonecrosis, and steroid-associated osteoporosis[55]. Furthermore, McCurdy *et al*[56] showed that IBD patients receiving CSs and immunomodulators were more likely to be diagnosed with cytomegalovirus diseases than IBD patients not receiving CSs and immunomodulators. Therefore, management of refractory UC patients is a crucial issue, and the goal of the treatment for such patients should be steroid-free remission. However, as described above, clinical studies focused on the achievement of steroid-free remission in refractory UC patients are insufficient. We had treated many UC patients with CAP and consequently suggested the efficacy of CAP for refractory UC patients. Considering these backgrounds, we retrospectively analyzed the efficacy of CAP specifically focused on the achievement of steroid-free remission in patients with steroid-dependent and steroid-refractory UC.

MATERIALS AND METHODS

Patients

We retrospectively reviewed the collected data from 55 (male 29, female 26) patients aged 16-82 years (mean ± SD, 38.7 ± 16.7 years) with active refractory UC (steroid-dependent type 33, steroid-refractory type 21, refractory but refused steroid therapy 1) treated with CAP (GMA 38, LCAP 17) between September 2002 and December 2019 (Table 1). The detailed clinical profiles of the patients enrolled in this study are shown

Table 1 Patients' characteristics in this study

Characteristics	
Age (yr, mean ± SD)	16-82 (38.7 ± 16.7)
Sex	Male 29, female 26
Disease duration from diagnosis (mo, mean ± SD)	1-384 (59.4 ± 78.8)
Disease extent	
Left-sided colitis	12
Pancolitis	43
Disease refractory type	
Steroid-dependent	33
Steroid-refractory	21
Refusal of steroids	1
Clinical type	
One-attack	4
Relapsing-remitting	50
Chronic continuous	1
Mean CAI (mean ± SE) (pre first course of CAP)	9.0 ± 0.62
Mean DAI (mean ± SE) (pre first course of CAP)	11.3 ± 0.55
Medication (pre first course of CAP)	
PSL (oral)	Yes 54, no 1
5-ASA	Yes 52, no 3
Thiopurines	Yes 12, no 43
TNF-α antibodies	Yes 1 (adalimumab), no 54
Vedolizumab, tofacitinib, tacrolimus, ustekinumab	Yes 0, no 55
Dose of PSL at the start of CAP (mean ± SD)	0-60 mg (33.4 ± 19.2)
Type of CAP	GMA 38, LCAP 17
Observation period after the first course of CAP (mo, mean ± SD)	18-193 (81.5 ± 47.3)

CAI: Lichtiger's clinical activity index; DAI: Sutherland index (disease activity index); PSL: Prednisolone; 5-ASA: 5-Aminosalicylic acid; CAP: Cytapheresis; GMA: Granulocyte and monocyte adsorptive apheresis; LCAP: Leukocytapheresis.

in Table 1. The dosage of prednisolone and the concomitant therapies at apheresis commencement are also shown in Table 1. The rates of concomitant use of prednisolone, 5-aminosalicylic acid, and immunomodulators were 98.2% (54/55), 94.5% (52/55), and 21.8% (12/55), respectively. Anti-TNF-α antibody (adalimumab) was administered to one patient. In most patients, concomitant medications except prednisolone were continued at the same dosage. The dosage of prednisolone was tapered or discontinued according to patients' clinical improvement during the CAP therapy.

This retrospective study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Akita Red Cross Hospital (approval No: 195) and Akita University School of Medicine (approval No: 2419). Written or oral informed consent was obtained from patients and/or parents of patients aged younger than 20 years.

The primary endpoint of this study was the rate of achievement of steroid-free remission in refractory UC patients after the CAP therapy. The achievement of steroid-free remission included the induction of steroid-free remission in the first course of CAP and re-induction of steroid-free remission in the second, third, and fourth courses of CAP. The secondary endpoint was the rate of sustained steroid-free remission in refractory UC patients after the CAP therapy.

Definition of steroid-dependent and steroid-refractory UC

Steroid-dependent UC was defined as the disease that initially responds to steroids but could not maintain control of symptoms without steroids and requires low doses of steroids to remain symptom-free[6, 57]. Steroid-refractory UC was also defined as active UC characterized by the failure to respond to 0.75-1.5 mg/kg per day of prednisolone administered over at least 1 wk[43,57].

CAP

Each patient was treated with 5 to 20 GMA or LCAP sessions (mean ± SD, 8.8 ± 3.8 sessions). A total of 20 patients were treated with 5 sessions of CAP, 29 patients with 10 sessions, 1 patient with 9 sessions, 1 patient with 15 sessions, 1 patient with 18 sessions, and 3 patients with 20 sessions. Under the Japanese health insurance treatment system, the 11th CAP session was performed at 1 mo after the 10th CAP session in patients who received more than 10 CAP sessions. CAP was performed once weekly in principle. However, in some patients with severe UC, CAP was exceptionally performed twice a week for the first 2-3 wk (intensive CAP). CAP was also exceptionally performed once 2 wk for the last several weeks in some patients whose symptoms improved to mild after the treatment with several sessions of CAP.

Exclusion criteria

Patients with serious cardiac, kidney, or liver diseases; malignancy; coagulation disorders; infections; history of hypersensitivity to heparin; severe dehydration, granulocytopenia, anemia, thrombocytopenia; and patients taking angiotensin-converting enzyme inhibitor were excluded.

Evaluation of the efficacy of CAP

Efficacy of the first course of CAP: Clinical efficacy between April 2008 and December 2019 were evaluated using the Lichtiger's clinical activity index (CAI)[58] and that between September 2002 and March 2008 was evaluated using Sutherland index (disease activity index, DAI)[59]. Clinical remission was defined as decreased Lichtiger's CAI in 4 or less or decreased DAI in less than 2.5[60]. In this study, we assessed patients who did not achieve clinical remission after CAP, suggesting the "poor effectiveness of CAP". We evaluated the efficacy of CAP approximately 4 wk after the last apheresis session. We also examined the rate of steroid-free remission. We have defined "steroid-free" as the point when both oral steroids and enemas including steroids were discontinued. However, suppositories including small amounts of steroids were permitted, as an exception.

Laboratory data (C-reactive protein level, serum albumin concentration, neutrophil count, and monocyte count) before and after CAP were also examined in 28 patients treated between April 2008 and December 2019.

Efficacy of the second, third, and fourth courses of CAP: Efficacy of the second course of CAP in patients experiencing a relapse during the observation period was assessed. Furthermore, efficacy of the third and fourth courses of CAP was also assessed specifically in patients who achieved steroid-free remission after the first course of CAP and experienced relapses during the observation period.

Efficacy of CAP in colonic mucosal inflammation: Endoscopic findings after the first course of CAP in patients who achieved steroid-free remission were evaluated using the Mayo endoscopic subscore[61]. A score ≤ 1 suggested mucosal healing.

Long-term efficacy: Long-term efficacy of CAP in patients who achieved steroid-free remission after the first course of CAP was examined by assessing (1) the rate of sustained steroid-free remission at 12, 24, and 36 mo after the first course of CAP and (2) overall rate of maintaining sustained steroid-free remission throughout the observation period.

The surgical operation rate: The surgical operation rates of the patients within 6 mo, 3 years, and throughout the observation period after the first course of CAP were examined.

Statistical analysis: Statistical analysis was performed using the paired *t*-test, and chi-squared test, and a P value < 0.05 was considered statistically significant.

RESULTS

Efficacy of the first course of CAP

The rates of clinical remission, which includes steroid-free remission and clinical remission but not steroid-free remission, steroid-free remission, and poor effectiveness after CAP were 69.1%, 45.5%, and 30.9%, respectively (Figure 1). The rates of clinical remission, steroid-free remission, and poor effectiveness after GMA were 69.2%, 43.6%, and 30.8%, respectively. The rates of clinical remission, steroid-free remission, and poor effectiveness after LCAP were 68.8%, 50.0%, and 31.2%, respectively. There were no significant differences in the rates of both clinical remission and steroid-free remission after CAP between patients who received GMA therapy and patients who received LCAP.

In this study, thiopurines were concomitantly used in 12 patients. The rates of clinical remission, steroid-free remission, and poor effectiveness after CAP in patients who concomitantly received thiopurines were 66.7%, 41.7%, and 33.3% respectively. There were no significant differences in the rates of both clinical remission and steroid-free remission after CAP between patients who concomitantly received thiopurines and patients who did not receive thiopurines.

For patients with steroid-dependent UC, the rates of clinical remission, steroid-free remission, and poor effectiveness after CAP were 69.7%, 42.4%, and 30.3%, respectively (Figure 2). On the contrary, the rates of clinical remission, steroid-free remission, and poor effectiveness after CAP in patients with steroid-refractory UC were 66.7%, 47.6%, and 33.3%, respectively (Figure 3). There were no significant differences in both rates of clinical remission and steroid-free remission between patients with steroid-dependent UC and patients with steroid-refractory UC.

DAI and CAI scores (mean ± SE) before and after the first course of CAP are shown in Figures 4 and 5. The mean DAI score before CAP was 11.4, which decreased significantly to 3.36 after the CAP therapy ($P < 0.0001$) (Figure 4). The mean CAI score before CAP was 9.0, which decreased significantly to 3.63 after the CAP therapy ($P < 0.0001$) (Figure 5).

Laboratory data before and after CAP are shown in Table 2. As shown in Table 2, the inflammatory parameter (C-reactive protein) and the nutritional parameter (serum albumin concentration) significantly improved after CAP. Neutrophil count significantly decreased after CAP therapy. Monocyte count tended to decrease after CAP, but no significant difference was observed.

The rates of steroid-free remission after the second course of CAP

The second course of CAP was performed in 24 patients (12 patients who achieved steroid-free remission after the first course of CAP, 8 patients who achieved clinical remission but not steroid-free remission after the first course of CAP, 4 patients who had poor effectiveness in the first course of CAP) experiencing a relapse or worsening condition during the observation period. The rates of steroid-free remission after the second course of CAP in patients who achieved steroid-free remission after the first course of CAP, patients who achieved clinical remission but not steroid-free remission after the first course of CAP, and patients who had poor effectiveness in the first course of CAP were 83.3% (10/12), 12.5% (1/8), and 0% (0/4), respectively (Figure 6). The rate of steroid-free remission after the second course of CAP was significantly higher in patients who achieved steroid-free remission after the first course of CAP compared with that in patients who achieved clinical remission but not steroid-free remission after the first course of CAP ($P = 0.0018$) and that in patients who had poor effectiveness in the first course of CAP ($P = 0.0029$).

The rates of steroid-free remission after the second, third, and fourth courses of CAP in patients who achieved steroid-free remission after the first course of CAP

As shown above, the rate of steroid-free remission after the second course of CAP in patients who achieved steroid-free remission after the first course of CAP was 83.3%. In these patients, the rate of steroid-free remission after the second course of CAP in patients with steroid-dependent UC (83.3%) was the same as that of patients with steroid-refractory UC (83.3%).

The third and fourth courses of CAP were performed in 6 patients and 5 patients, respectively, who achieved steroid-free remission after the first course of CAP and experienced relapses during the observation period. The rates of steroid-free remission after the third and fourth courses of CAP in these patients were 83.3% (5/6) and 60% (3/5), respectively (Figure 7).

Table 2 Laboratory data obtained (mean ± SE) before and after cytapheresis

	Before CAP	After CAP	*P* value
CRP (mg/dL)	1.795 ± 0.721	0.312 ± 0.130	$P = 0.0396$
Albumin (g/dL)	3.579 ± 0.139	3.911 ± 0.117	$P = 0.0358$
Neutrophil count (μL)	6826 ± 561	5475 ± 456	$P = 0.0124$
Monocyte count (μL)	588 ± 73	425 ± 46	$P = 0.0626$

CRP: C-reactive protein; CAP: Cytapheresis.

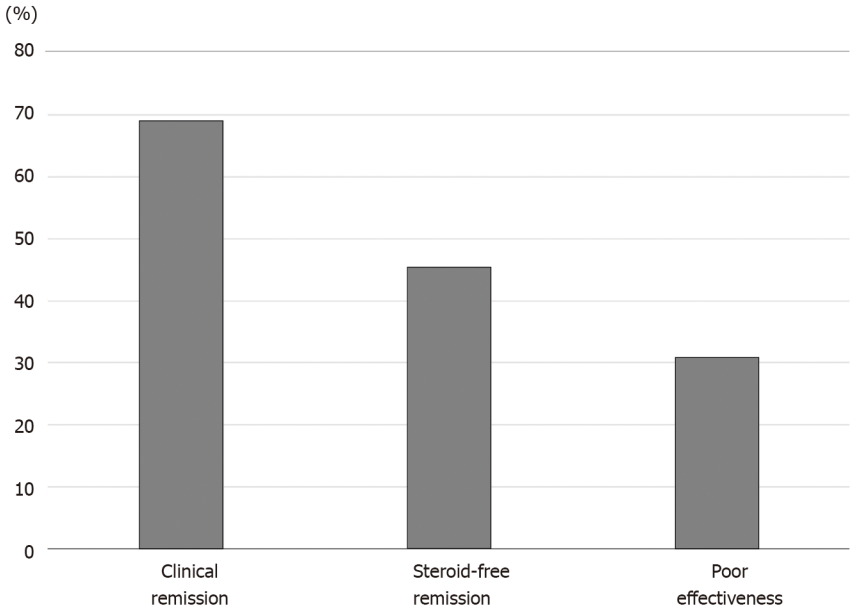


Figure 1 Efficacy of the first course of cytapheresis. The rates of clinical remission, which includes steroid-free remission and clinical remission without steroid-free remission, steroid-free remission, and poor effectiveness after cytapheresis were 69.1%, 45.5%, and 30.9%, respectively.

Endoscopic findings of patients who achieved steroid-free remission after the first course of CAP

Colonoscopic examination was performed in 21 out of the 25 patients (84%) who achieved steroid-free remission after the first course of CAP. Mucosal healing was observed in all 21 patients after the first course of CAP [Mayo endoscopic subscore 0 in 17 patients (81.0%), Mayo endoscopic subscore 1 in 4 patients (19.0%)]. None of the patients showed a Mayo endoscopic subscore ≥ 2 after the CAP. Endoscopic images before and after the CAP therapy of 5 patients are shown in Figure 8.

Long-term efficacy of CAP in patients achieving steroid-free remission in the first course of CAP

We could correctly follow the rate of sustained steroid-free remission for 3 years (36 mo) in all 25 patients who successfully achieved steroid-free remission after the first course of CAP. The rates of sustained steroid-free remission in these patients were 68.0% at 12 mo, 60.0% at 24 mo, and 56.0% at 36 mo after the first course of CAP (Figure 9). The rates of sustained steroid-free remission in patients with steroid-dependent UC were 69.2% at 12 mo, 53.8% at 24 mo, and 46.1% at 36 mo, respectively. On the other hand, the rates of sustained steroid-free remission in patients with steroid-refractory UC were 63.6% at 12 mo, 63.6% at 24 mo, and 63.6% at 36 mo, respectively.

The mean observation period of these 25 patients was 81.5 ± 9.7 mo (mean ± SE). Although the observation periods varied in these 25 patients, 9 patients (36.0%) had maintained sustained steroid-free remission throughout the observation periods. The mean period of maintained steroid-free remission of these 9 patients was 86.6 ± 14.3 mo (mean ± SE). Periods of sustained steroid-free remission and refractory type of the

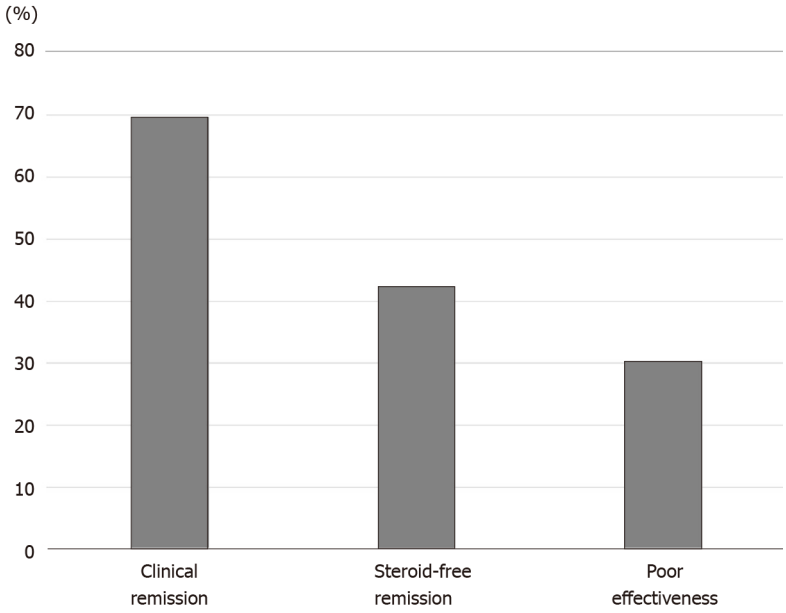


Figure 2 Efficacy of the first course of cytapheresis in the patients with steroid-dependent ulcerative colitis. The rates of clinical remission, steroid-free remission, and poor effectiveness after cytapheresis were 69.7%, 42.4%, and 30.3%, respectively.

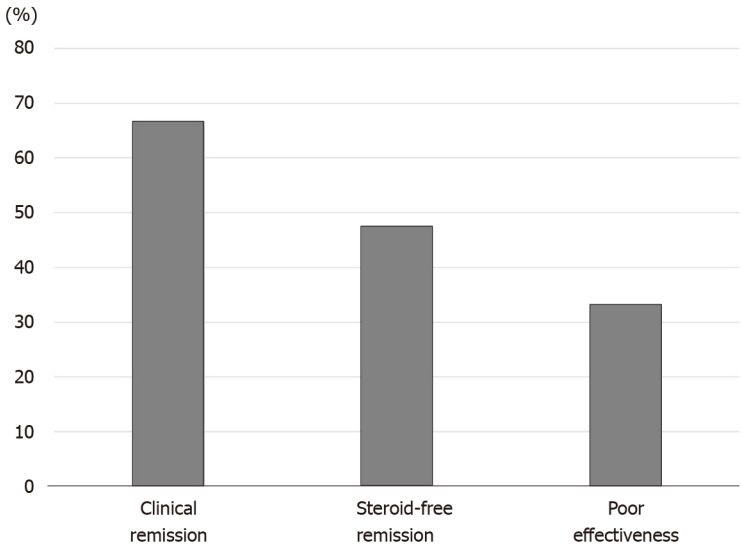


Figure 3 Efficacy of the first course of cytapheresis in the patients with steroid-refractory ulcerative colitis. The rates of clinical remission, steroid-free remission, and poor effectiveness after cytapheresis were 66.7%, 47.6%, and 33.3%, respectively.

9 patients are shown in Figure 10. Two patients had maintained sustained steroid-free remission over 10 years after the first course of CAP. The summary of the results of this study is shown in Figure 11.

The surgical operation rates

The surgical operation rate of the patients within 6 mo after the first course of CAP was 9.1% (5/55). The surgical operation rate within 6 mo after the CAP was significantly lower in patients who achieved steroid-free remission after the first course of CAP (0%) compared with that in patients who had poor effectiveness in the first course of CAP (29.4%) (P = 0.0039). The surgical operation rate within 3 years after the first course of CAP was 12.7% (7/55). The surgical operation rate within 3 years after the CAP was significantly lower in patients who achieved steroid-free remission after the first course of CAP (4%) compared with that in patients who had poor effectiveness (29.4%) (P = 0.0209). The surgical operation rate throughout the observation period [18-193 mo (81.5 ± 47.3 (mean ± SD)] after the first course of CAP was 20 % (11/55). The surgical operation rate throughout the observation period after

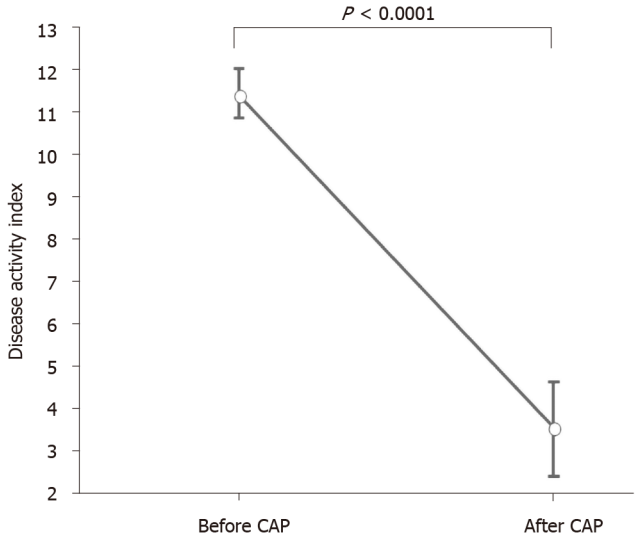


Figure 4 Mean disease activity index score before and after cytapheresis. Disease activity index score (mean ± SE) before and after cytapheresis is shown. The mean disease activity index score before cytapheresis was 11.4, which decreased significantly to 3.36 after treatment ($P < 0.0001$). CAP: Cytapheresis.

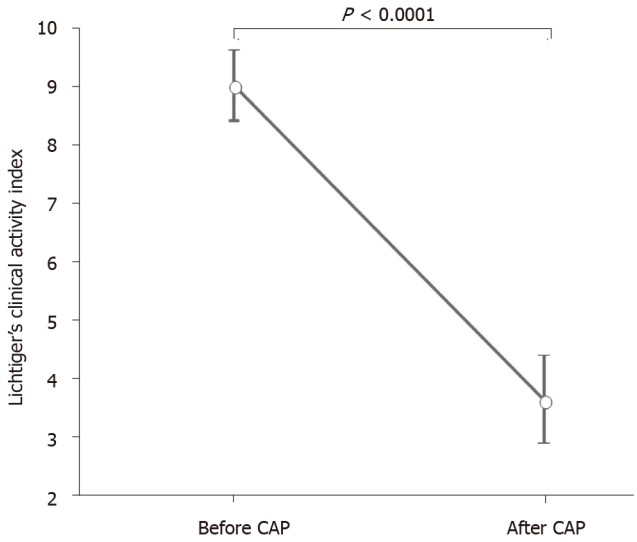


Figure 5 Mean Lichtiger's clinical activity index score before and after cytapheresis. Lichtiger's clinical activity index score (mean ± SE) before and after cytapheresis is shown. The mean Lichtiger's clinical activity index score before cytapheresis was 9.0, which decreased significantly to 3.63 after treatment ($P < 0.0001$). CAP: Cytapheresis.

the CAP was significantly lower in patients who achieved steroid-free remission after the first course of CAP (12%) compared with that in patients who had poor effectiveness in the first course of CAP (41.2%) ($P = 0.0293$).

Adverse events

Headache and slight fever were observed in one patient during the CAP therapy. No serious adverse events were observed in all patients in this study.

DISCUSSION

The primary endpoint of this study was the rate of achievement of steroid-free remission in refractory UC patients after the CAP therapy. In this context, we demonstrated that CAP effectively induced steroid-free remission not only in patients with steroid-dependent (42.4%) but also in patients with steroid-refractory (47.6%) UC. We also showed that mucosal healing was observed in all patients who achieved steroid-free remission after the first course of CAP. Previous studies examining the

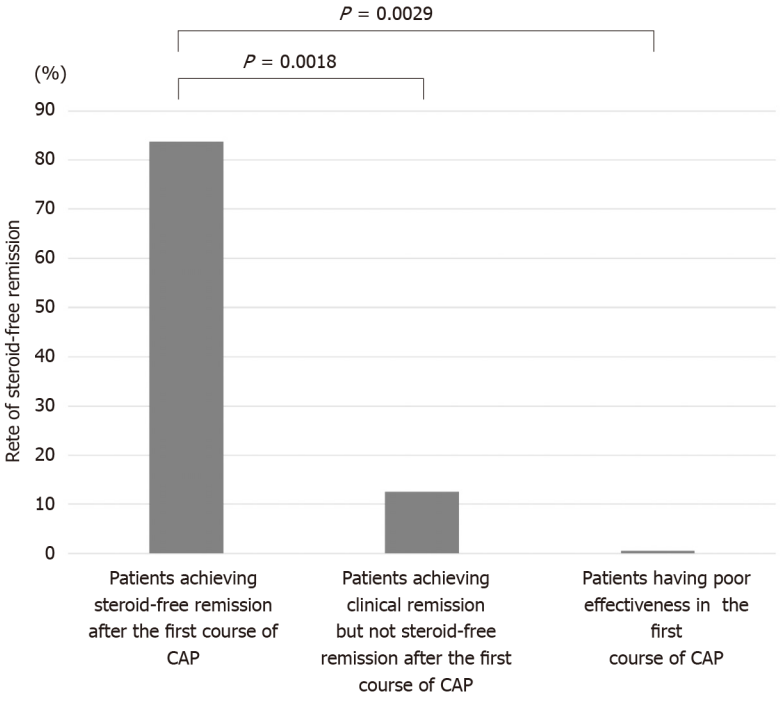


Figure 6 The rates of steroid-free remission after the second course of cytapheresis. The rate of steroid-free remission after the second course of cytapheresis (CAP) was significantly higher in patients who achieved steroid-free remission after the first course of CAP (83.3%) compared with that in patients who achieved clinical remission but not steroid-free remission after the first course of CAP (12.5%, $P = 0.0018$) and that in patients who had poor effectiveness after the first course of CAP (0%, $P = 0.0029$). CAP: Cytapheresis.

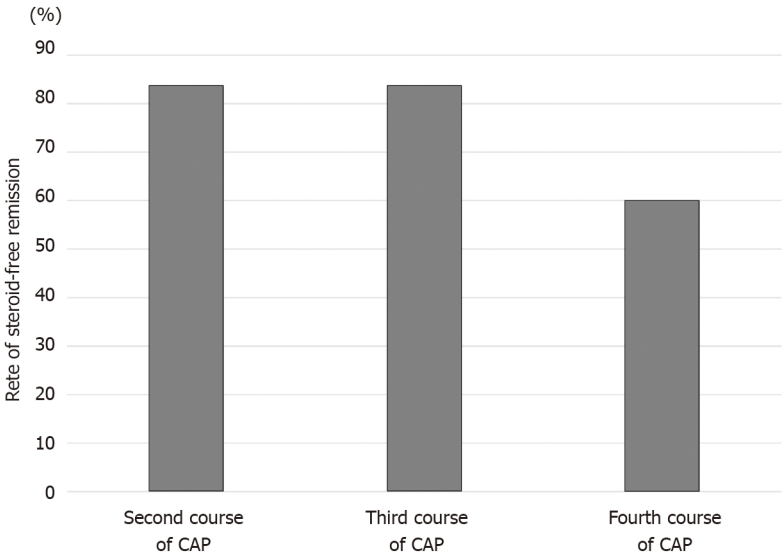


Figure 7 Rates of steroid-free remission after the second, third, and fourth courses of cytapheresis in patients who achieved steroid-free remission after the first course of cytapheresis. The rates of steroid-free remission after the second, third, and fourth courses of cytapheresis in patients who achieved steroid-free remission after the first course of cytapheresis and then experienced relapses were 83.3%, 83.3%, 60%, respectively. CAP: Cytapheresis.

efficacy of CAP in refractory UC and the results of this study are shown in Table 3[43-53]. In these studies, eight studies[45-48,50-53] examined the efficacy of CAP for induction of steroid-free remission and three studies[43,44,49] examined that for induction of only clinical remission. Here, we discuss the eight studies examining the efficacy of CAP for induction of steroid-free remission. In the eight studies, seven studies[46-48,50-53] examined the rate of the induction of steroid-free remission in patients with steroid-dependent UC and one study[45] examined that in patients with steroid-refractory UC. Regarding steroid-refractory UC, it is difficult to evaluate the results of the study because there is only one study, which only comprised eight steroid-refractory patients, that assessed

Table 3 Previous studies examining the efficacy of cytapheresis in refractory ulcerative colitis

Ref.	Refractory type	Number of patients	Evaluation items[1]	Rate of remission (evaluation time)	Rate of steroid- free remission (evaluation time)
Naganuma *et al*[44] (2004)	SR	10	Induction	Severe 20%, moderate 70%	
Giampaolo *et al*[45] (2006)	SR	8	Induction, sustained remission	100%	100%, 12.5% (12 mo)
Ricart *et al*[46] (2007)	SD	20	Induction, sustained remission	42.1% (17 wk)	36.8% (17 wk), 85.7% (12 mo)
Cabriada *et al*[47] (2010)	SD	18	Induction, sustained remission		55% (1 mo), 75% (12 mo)
Cabriada *et al*[48] (2012)	SD	142	Induction, sustained remission		37% (1 mo), 51%[2] (12 mo)
Sacco *et al*[49] (2013)	SD + SR	83 (SD 55, SR 28)	Induction, sustained remission	71%, 48% (12 mo)	
Yokoyama *et al*[43] (2014)	SD + SR	401 (SD 229, SR 172)	Induction	SD: 64.6% (2 wk), SR: 70.9 (2 wk)	
Dignass *et al*[50] (2016)	SD	86	Induction	39.3% (12 wk)	22.6% (12 wk)
Imperiali *et al*[51] (2017)	SD	33	Induction		36% (12 mo)
Dignass *et al*[52] (2018)	SD	95	Induction	34.0% (24 wk), 33.0% (48 wk)	19.2% (24 wk), 19.2% (48 wk)
Domènech *et al*[53] (2018)	SD	63	Induction		13% (24 wk)
Present study	SD + SR	55 (SD 33, SR 21)	Induction, sustained remission	SD: 69.7% (4 wk), SR: 66.7% (4 wk)	SD: 42.4% (4 wk) SR: 47.6% (4 wk); SD: 69.2% (12 mo), SR: 63.6% (12mo)

[1]Evaluation items, which include Induction (evaluation for induction of remission or steroid-free remission) and sustained remission (evaluation for maintenance of sustained remission or steroid-free remission).
[2]Rate of sustained steroid-free remission in the patients with no additional apheresis. SR: Steroid refractory; SD: Steroid dependent.

this type of UC[45]. With regard to steroid-dependent UC, according to the seven previous studies[46-48,50-53], the rates of the induction of steroid-free remission ranged from 13% to 55% (mean 31.4%). Although it is difficult to compare the results of these studies with that of our study because of the diversity of the patients' background enrolled in the studies, the rate of the induction of steroid-free remission of our study is higher than that of the six previous studies[46,48,50-53]. Based on the following reports[43,46,50], we suggest that the differences of the history of previous medication and the differences of the methods of CAP treatment of the studies might influence the rates of steroid-free remission. Dignass *et al*[50] showed that remission was achieved at week 12 after Adacolumn apheresis by 40.3% of patients who failed on immunosuppressants, but only 27.8% of patients who failed on anti-TNF-α treatment. On the other hand, Yokoyama *et al*[43] showed that a multivariate logistic regression analysis comparing the patients' backgrounds, concomitant medications, and therapeutic variables of LCAP between the remission and nonremission groups identified intensive LCAP (≥ 4 LCAP treatments within the first 2 wk) as the only factor that was significantly related to remission after LCAP. On the contrary, Ricart *et al*[46] showed that increasing the number of apheresis sessions affords a significant steroid-sparing effect in steroid-dependent UC. Looking back with reference to these reports, in our study, only one patient who had insufficient response to anti-TNF-α treatment was included, and intensive CAP was performed in some severe cases in contrast to the six previous studies[46,48,50-53] performing weekly apheresis in all patients. Additionally, it appears that patients in our study received more CAP sessions [5-20 sessions (mean 8.8)] compared with the previous studies. We suggest that a selection of an appropriate CAP treatment method for each patient is important to induce steroid-free remission effectively in refractory UC patients.

Regarding the achievement of steroid-free remission, assessing the rate of re-induction of steroid-free remission with CAP in patients who experience relapse after the first course of CAP is also required. In this regard, our study showed that the

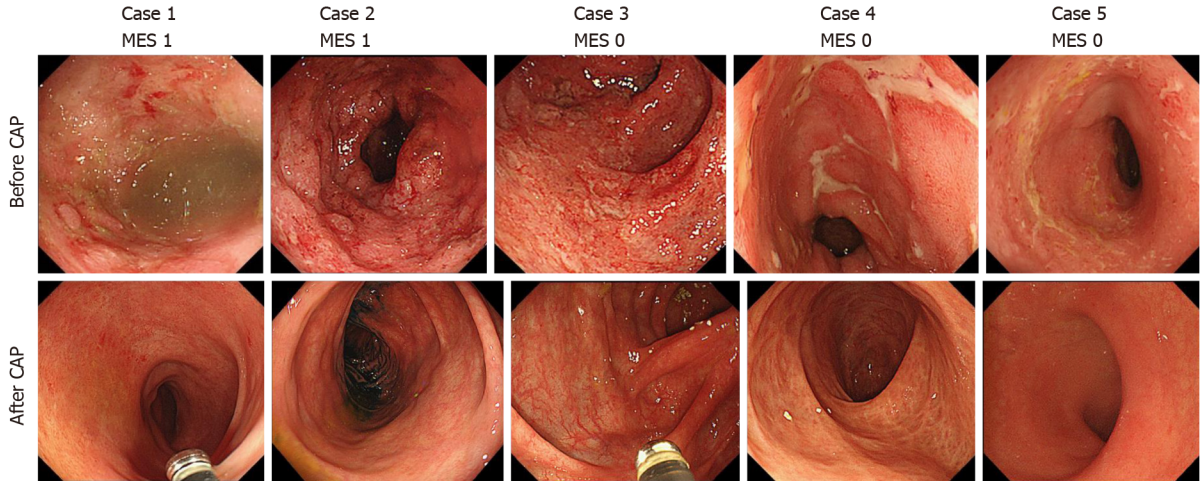


Figure 8 Endoscopic images of 5 patients who achieved steroid-free remission after the first course of cytapheresis. Endoscopic images before and after the cytapheresis (CAP) therapy of 5 patients who achieved steroid-free remission after the first course of CAP are shown. Active inflammation (Mayo endoscopic subscore ≥ 2) was observed in the colonic mucosa in all 5 patients before the CAP therapy. On the contrary, mucosal healing (Mayo endoscopic subscore ≤ 1) was observed in all 5 patients after the CAP therapy. MES: Mayo endoscopic subscore; MES 1/MES 0: Mayo endoscopic subscore after cytapheresis; CAP: Cytapheresis.

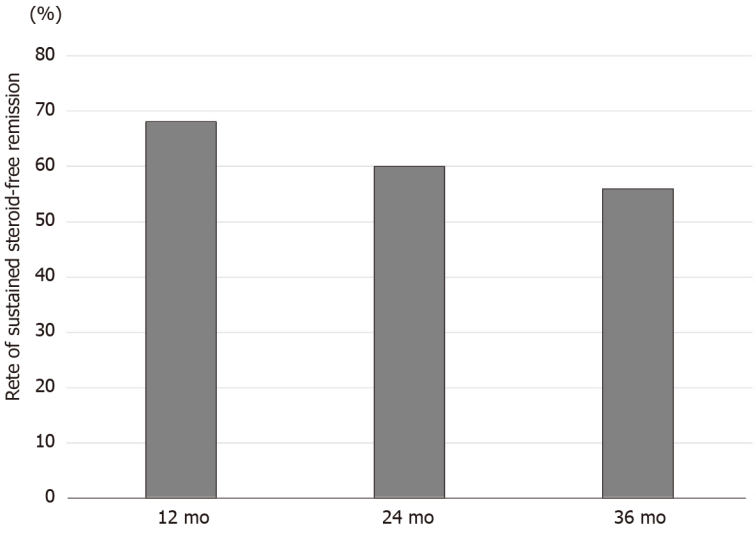


Figure 9 Rates of sustained steroid-free remission at 12, 24, and 36 mo after the first course of cytapheresis in patients who achieved steroid-free remission after the first course of cytapheresis. The rates of sustained steroid-free remission in patients who achieved steroid-free remission after the first course of cytapheresis were 68.0% at 12 mo, 60.0% at 24 mo, and 56.0% at 36 mo after the first course of cytapheresis.

second course of CAP effectively re-induced steroid-free remission (83.3%) in both steroid-dependent and steroid-refractory UC patients who had achieved steroid-free remission in the first course of CAP. The rate of re-induction of steroid-free remission was significantly higher in patients who achieved steroid-free remission in the first course of CAP (83.3%) compared with that of patients who had achieved clinical remission but not steroid-free remission (12.5%) and that of patients who had poor effectiveness in the first course of CAP (0%). Furthermore, our study also showed that the third and the fourth courses of CAP repeatedly induced steroid-free remission at a high rate in patients who achieved steroid-free remission in the first course of CAP. Based on these results, we suggest that patients achieving steroid-free remission in the first course of CAP are significantly likely to have a high sensitivity to CAP, namely, high responders to CAP. There have been no studies assessing the rate of re-induction of steroid-free remission of CAP in patients with steroid-dependent and steroid-refractory UC. However, there have been two studies that examined the re-efficacy of CAP in patients with active UC or Crohn's disease (CD)[37,41]. Takayama *et al*[41] examined the effects of the second course of CAP in UC patients with moderate to severe activity experiencing relapse during the disease course. They showed that the

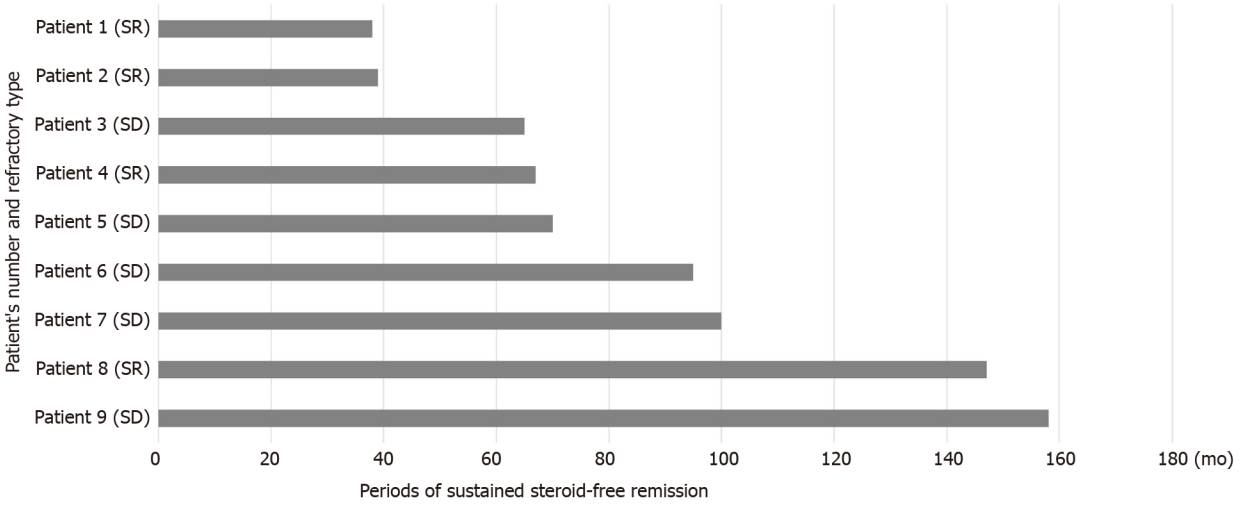


Figure 10 Periods of sustained steroid-free remission and refractory type of the 9 patients who had maintained steroid-free remission throughout the observation periods. Nine patients (36.0%) had maintained sustained steroid-free remission throughout the observation periods. Periods of sustained steroid-free remission of the 9 patients are shown in the figure. The mean period of maintained steroid-free remission of these 9 patients was 86.6 ± 14.3 mo (mean ± SE). Nine patients included 5 steroid-dependent patients and 4 steroid-refractory patients. Periods of sustained steroid-free remission of the 9 patients are shown in the figure. SR: Steroid-refractory patient; SD: Steroid-dependent patient.

percentage of remissive and effective responses of the second course of CAP was 79% in patients who had remissive and effective responses in the first course of CAP, whereas 40% in patients who had noneffective responses in the first course of CAP. Lindberg *et al*[37] presented 14 patients (UC 4, CD 10) who experienced relapse after showing initial remission and were re-treated with GMA. Although the remission rates of the re-treatments of GMA in UC patients were unclear, they showed that 13 of the 14 patients (93%) achieved a second remission. They also showed that following further relapses, all patients were successfully re-treated with GMA for the third, fourth, and fifth time. Thus, the previous two studies also showed that re-treatment of CAP seemed to be effective in UC patients who had remissive responses in the first course of CAP, supporting our results.

The secondary endpoint of this study was the rate of sustained steroid-free remission in refractory UC patients after the CAP therapy. In this regard, we showed that CAP had good long-term efficacy for the maintenance of sustained steroid-free remission (68% at 12 mo, 60% at 24 mo, 56% at 36 mo) in refractory UC patients who achieved steroid-free remission in the first course of CAP. Furthermore, interestingly, 36% of patients had maintained sustained steroid-free remission throughout the observation periods, and two patients had maintained it over 10 years. Previous studies examining the rate of sustained steroid-free remission after the CAP therapy in refractory UC patients are also shown in Table 3. Among them, three studies examined the rate of sustained steroid-free remission in patients with steroid-dependent UC[46-48]. The rates of sustained steroid-free remission at 12 mo after CAP of the three studies ranged from 51% to 85.7% (mean 70.6%). Thus, these studies and our study (69.2% in steroid-dependent patients) showed good long-term efficacy in the rates of sustained steroid-free remission. In this regard, in our study, mucosal healing was observed in all patients who achieved steroid-free remission after the first course of CAP. Ricart *et al*[46] also showed that all patients who experienced clinical remission also experienced endoscopic remission and good long-term efficacy. Cabriada *et al*[48] showed that among those patients in steroid-free remission, 96% also achieved endoscopic remission. They also showed that a tendency for sustained remission at 1 year was observed when initial endoscopic remission was achieved[47]. Based on these findings, we suggest that endoscopic mucosal healing was closely involved in the maintenance of sustained steroid-free remission and good long-term efficacy of CAP.

In this study, no serious adverse events were observed during the CAP therapy. It has been reported that other therapies, such as anti-TNF-α antibody administration, are associated with risk of serious infections, lymphoma, and associated mortality in IBD[28,29,50]. In this context, several studies reporting on the safety of CAP have been considered important[39,42,43,49,50]. Among these studies, Hibi *et al*[39] evaluated the safety and clinical efficacy of Adacolumn in 697 patients with UC in 53 medical institutions. They showed that no serious adverse events were observed, and mild to moderate

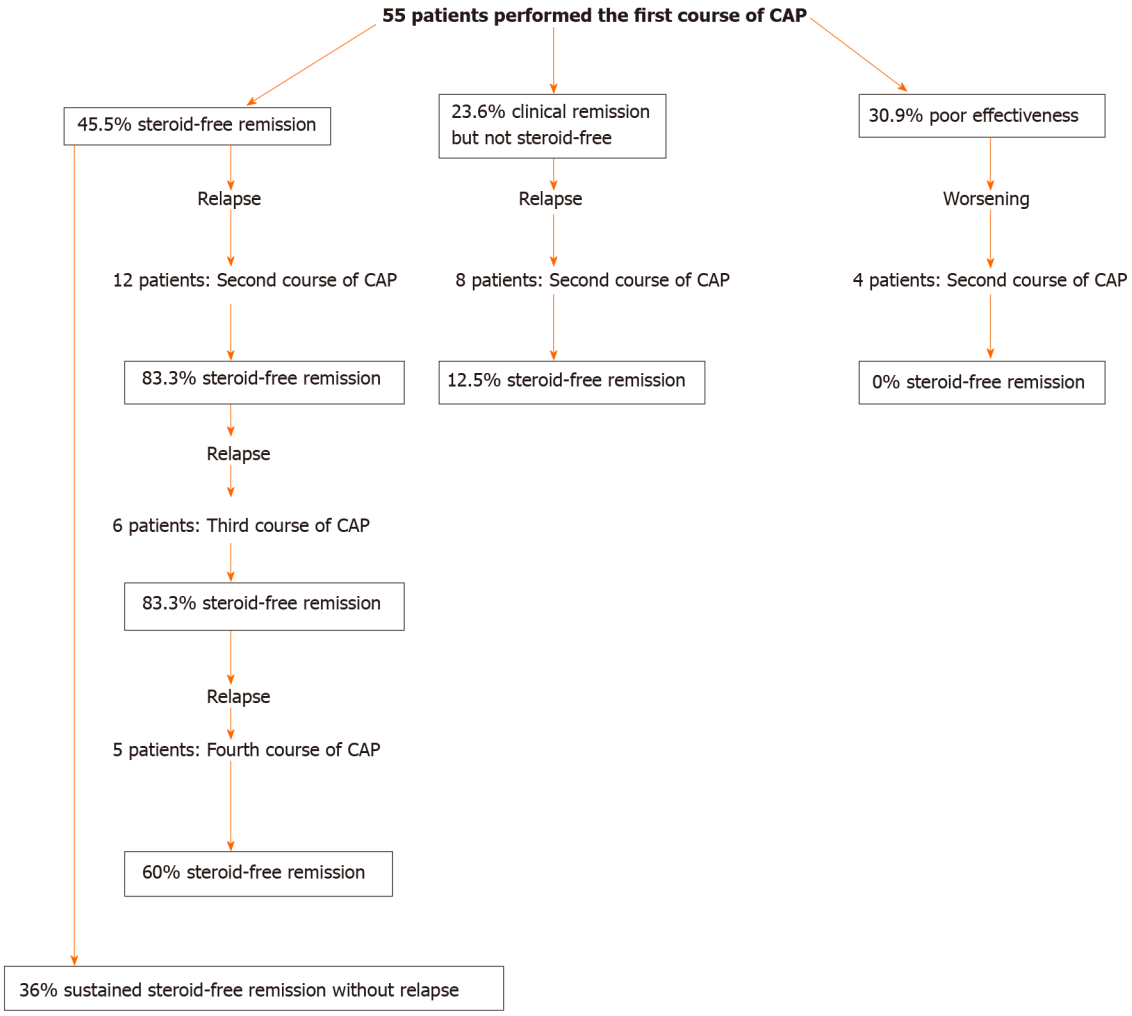


Figure 11 Summary of the results of the study. The results of this study are summarized in the figure. CAP: Cytapheresis.

adverse events were observed in 7.7% of patients. Motoya *et al*[42] conducted a retrospective multicenter cohort study that evaluated the safety and effectiveness of GMA in 437 IBD patients under special situations. They showed that the incidence of adverse events among elderly patients was similar in all patients.

There have been several studies comparing the impact of CAP in the clinical practice with the conventional pharmacotherapy for UC[53,62-64]. A meta-analysis showed that GMA is effective for inducing clinical remission in patients with UC compared with CS [odds ratio (OR), 2.23; 95% confidence interval (CI): 1.38-3.60] and that the rate of adverse events by apheresis was significantly lower than that by CS (OR, 0.24; 95%CI: 0.15-0.37)[62]. Another meta-analysis showed that comparing with conventional pharmacotherapy including CS, LCAP supplementation presented a significant benefit in promoting a response rate (OR, 2.88, 95%CI: 1.60-5.18) and remission rate (OR, 2.04, 95%CI: 1.36-3.07) together with significant higher steroid-sparing effects (OR, 10.49, 95%CI: 3.44-31.93) in patients with active moderate-to-severe UC[63]. In this regard, Domènech *et al*[53] showed that the addition of 7 weekly sessions of GMA to a conventional course of oral prednisolone did not increase the proportion of steroid-free remissions in patients with active steroid-dependent UC. On the other hand, Tominaga *et al*[64] showed that GMA produced efficacy equivalent to prednisolone and was without safety concern. Although they also showed that the average medical cost was 12739.4€/patient in the GMA group and 8751.3€ in the prednisolone group ($P < 0.05$), they concluded that the higher cost of GMA *vs* prednisolone should be compromised by good safety profile of GMA.

In summary, our study showed that CAP was effective in inducing steroid-free remission and maintained sustained steroid-free remission in both steroid-dependent and steroid-refractory UC patients. Additionally, our study also showed that CAP re-induced high-rate steroid-free remission repeatedly in patients who achieved steroid-free remission in the first course of CAP, namely, patients potentially having a high

sensitivity to CAP. Therefore, considering the high level of safety of CAP, we suggest that CAP should be one of the first-line therapies for steroid-dependent and steroid-refractory UC patients. We also suggest that CAP should be chosen as a first-line therapy for patients who achieve steroid-free remission in the first course of CAP and thereafter experience relapses during the disease course.

However, this study has some limitations; that is, this study is a retrospective study with small sample size that was conducted only in two medical institutions. Thus, a multicenter prospective study with large sample sizes is required to warrant our results.

CONCLUSION

In conclusion, our results suggest that CAP effectively induces and maintains steroid-free remission in refractory UC and re-induces high-rate steroid-free remission repeatedly in patients achieving steroid-free remission after the first course of CAP.

ARTICLE HIGHLIGHTS

Research background

Management of refractory ulcerative colitis (UC) patients is a crucial issue, and the goal of the treatment for such patients should be steroid-free remission. Although several breakthrough treatments, including biologics, have been developed for refractory UC, clinical studies focused on the achievement of steroid-free remission in refractory UC patients are insufficient.

Research motivation

Cytapheresis (CAP) is an effective therapeutic strategy for patients with active UC with fewer adverse effects. However, to date, the number of studies focused on the efficacy of CAP in both steroid-dependent and steroid-refractory UC has been limited. It is also important to assess the re-efficacy of CAP in patients who experience relapse after the first course of CAP.

Research objectives

The main objective of the study was to clarify the efficacy and re-efficacy of CAP in achieving steroid-free remission in refractory UC patients.

Research methods

We retrospectively reviewed the collected data from 55 patients with refractory UC treated with CAP. We analyzed the efficacy of the first course of CAP, efficacy of the second, third, and fourth courses of CAP, and long-term efficacy of CAP.

Research results

The rates of clinical remission, steroid-free remission after CAP were 69.1%, 45.5%, respectively, and the rates of steroid-free remission after the second, third, and fourth courses of CAP in patients who achieved steroid-free remission after the first course of CAP were 83.3%, 83.3%, and 60%, respectively. The rates of sustained steroid-free remission were 68.0%, 60.0%, and 56.0% at 12, 24, and 36 mo after the CAP. These results showed that CAP effectively induced steroid-free remission in refractory UC patients and that patients achieving steroid-free remission after the first course of CAP responded to CAP repeatedly after that and had good long-term efficacy.

Research conclusions

Our results suggest that CAP effectively induces and maintains steroid-free remission in both steroid-dependent and steroid-refractory UC patients and re-induces high-rate steroid-free remission repeatedly in patients achieving steroid-free remission after the first course of CAP. Considering the high level of safety of CAP, we suggest that CAP should be one of the first-line therapies for refractory UC patients and should be chosen as a first-line therapy for patients achieving steroid-free remission in the first course of CAP and thereafter experience relapses.

Research perspectives

A multicenter prospective study with large sample sizes is required to warrant our results.

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ORIGINAL ARTICLE

Observational Study

Risk perception and knowledge of COVID-19 in patients with celiac disease

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Abstract

BACKGROUND

We recently demonstrated that the odds of contracting coronavirus disease 2019 (COVID-19) in patients with celiac disease (CeD) is similar to that of the general population. However, how patients with CeD perceive their COVID-19 risk may differ from their actual risk.

AIM

To investigate risk perceptions of contracting COVID-19 in patients with CeD and determine the factors that may influence their perception.

METHODS

We distributed a survey throughout 10 countries between March and June 2020 and collected data on demographics, diet, COVID-19 testing, and risk perceptions of COVID-19 in patients with CeD. Participants were recruited through various celiac associations, clinic visits, and social media. Risk perception was assessed by asking individuals whether they believe patients with CeD are at an increased risk of contracting COVID-19 when compared to the general population. Logistic regression was used to determine the influencing factors associated with COVID-19 risk perception, such as age, sex, adherence to a gluten-free diet (GFD), and comorbidities such as cardiac conditions, respiratory conditions, and diabetes. Data was presented as adjusted odds ratios (aORs)

RESULTS

A total of 10737 participants with CeD completed the survey. From them, 6019 (56.1%) patients with CeD perceived they were at a higher risk or were unsure if they were at a higher risk of contracting COVID-19 compared to the non-CeD population. A greater proportion of patients with CeD perceived an increased risk of contracting COVID-19 when compared to infections in general due to their CeD (56.1% *vs* 26.7%, $P < 0.0001$). Consequently, 34.8% reported taking extra COVID-19 precautions as a result of their CeD. Members of celiac associations were less likely to perceive an increased risk of COVID-19 when compared to non-members (49.5% *vs* 57.4%, $P < 0.0001$). Older age (aOR: 0.99; 95%CI: 0.99 to 0.99, $P < 0.001$), male sex (aOR: 0.84; 95%CI: 0.76 to 0.93, $P = 0.001$), and strict adherence to a GFD (aOR: 0.89; 95%CI: 0.82 to 0.96, $P = 0.007$) were associated with a lower perception of COVID-19 risk and the presence of comorbidities was associated with a higher perception of COVID-19 risk (aOR: 1.38; 95%CI: 1.22 to 1.54, $P < 0.001$).

CONCLUSION

Overall, high levels of risk perceptions, such as those found in patients with CeD,

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may increase an individual's pandemic-related stress and contribute to negative mental health consequences. Therefore, it is encouraged that public health officials maintain consistent communication with the public and healthcare providers with the celiac community. Future studies specifically evaluating mental health in CeD could help determine the consequences of increased risk perceptions in this population.

Key Words: Celiac disease; Gluten; Risk; Infection; Knowledge; Perception; Coronavirus; COVID-19

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Core Tip: Risk perceptions describe an individual's perceived susceptibility to a threat and directly influence their behavior. We conducted an international cross-sectional study to evaluate risk of contracting contracting coronavirus disease 2019 (COVID-19) in celiac disease and evaluated risk perception. Patients with celiac disease perceive they are at an increased risk of contracting COVID-19 due to their condition, which is opposite to current scientific evidence. A higher risk perception may have a negative impact in mental health, and therefore, we encourage healthcare providers, patient care groups, and public health officials to discuss the implications that COVID-19 may have on patients in relation to their specific conditions.

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INTRODUCTION

Coronaviruses, such as severe acute respiratory syndrome coronavirus and middle east respiratory syndrome coronavirus which arose in 2003 and 2012, respectively, represent a family of positive-stranded RNA viruses that infect the respiratory system[1]. Later, the Chinese city of Wuhan reported an outbreak of a novel infectious agent causing severe cases of pneumonia and alerted the World Health Organization (WHO) of its presence on December 31, 2019[2]. The disease caused by this infectious agent was later named coronavirus disease 2019 (COVID-19). Since the WHO declared COVID-19 a global pandemic in March 2020, there has been over 105 million confirmed cases of COVID-19 across 216 countries and territories and the disease has killed over 2300000 people worldwide[3].

Due to the rapid spread and detrimental health consequences of COVID-19, there is an urgent need to determine which groups of individuals may have an increased susceptibility to infection and understand the perceptions they have regarding their susceptibility. A particular group of interest are patients with celiac disease (CeD), a chronic immune-mediated gastrointestinal disease that is triggered by dietary gluten intake in genetically predisposed individuals. Numerous studies suggest that CeD is associated with an increased risk of respiratory infections, particularly pneumonia, tuberculosis, and influenza[4-6]. However, we have shown that the odds of contracting COVID-19 in patients with CeD is similar to that of the general population[7].

As a result of the discrepancies between the risks of contracting different infections in patients with CeD, patient-specific risk perceptions of COVID-19 are of particular interest. Risk perceptions describe an individual's perceived susceptibility to a threat and directly influence their health behaviors[8-10]. Further, risk perception is a complex, psychological construct that varies markedly between individuals and is influenced by their emotional, social, cultural, geographical, and cognitive state[11].

The concept of risk perception is especially important in the context of a pandemic because a group's perception of their susceptibility to infection influences their

willingness to cooperate with and adopt preventative safety measures such as travel restrictions, hand washing, social distancing, and personal protective equipment (PPE) use[11]. Although strict implementation of infection control measures, such as social distancing, can reduce infection rate, they may also increase the risk for mental health conditions[12]. These mental health risks are even more likely to occur in individuals who are or believe they are more vulnerable to COVID-19[13]. Patients with CeD, especially those with a higher risk perception, may be more vulnerable to the negative mental health consequences of COVID-19 due to the high rates of mood disorders commonly associated with CeD[14]. However, studies of risk perception in patients with CeD have been limited to an Italian study of 276 patients which found that 26.1% of their patients either felt neutral or felt they were at an increased risk of COVID-19 because of their CeD[15].

To validate and explore this further, we conducted an international, cross-sectional survey investigating the COVID-19 risk perceptions of patients with a self-reported diagnosis of CeD and examined the factors that may influence their perceptions.

MATERIALS AND METHODS

The study was approved by the Hamilton Integrated Research Ethics Board (Hamilton, Ontario), No. HIREB# 5414. The methods of this study were previously described[8].

Study design

This observational, cross-sectional study included participants of all ages with a self-reported diagnosis of CeD and non-celiac population residing in either Argentina, Australia, Canada, Italy, Mexico, New Zealand, Spain, Uruguay, or the United States. The survey was open to participants from other countries, but extensive distribution of the survey was limited to the above-mentioned countries.

We designed a web-based survey consisting of 41 items. Participants were offered a different link to the survey depending on whether they reported a diagnosis of CeD or not. The study questionnaire was divided into specific sections to capture information on their demographics, adherence to a gluten-free diet (GFD), symptomatology, comorbidities, medications, COVID-19 testing, and patient knowledge/perception of the relationship between COVID-19 and CeD[16]. Individuals who believed they were at an increased risk or were unsure if they are at an increased risk of contracting COVID-19 due to their CeD were considered to have high COVID-19 risk perceptions. Patient knowledge and perception was only assessed in the CeD population. After piloting and testing by the authors, the English survey was placed into the secure online electronic case report platform, Research Electronic Data capture[17], and later translated into Italian and Spanish by the authors. We further collected information on country-specific COVID-19 control and safety measures implemented during the study period.

Participants were recruited from March 2020 to June 2020. Recruitment of self-reported CeD patients was performed through national celiac associations (*via* electronic newsletter and social media) and at clinic visits.

Statistical analysis

Statistical analyses were carried out using IBM-SPSS (IBM-SPSS Inc, Version 25.0, Armonk, NY, United States) and STATA (Stata version 13.0 Corp, College Station, TX, United States). Graphics were created using Microsoft Excel and GraphPad Prism (GraphPad Software, Version 8.4 San Diego, CA, United States). Categorical variables were reported as frequencies and percentages, whereas continuous variables were reported as mean (SD) or median and interquartile range when applicable. Comparisons of categorical variables between groups were performed using χ^2 test. Haldane corrections were applied to χ^2 tests when necessary. A two-sided test was used and P values of < 0.05 were considered statistically significant. Logistic regression was used to assess the predictors of high COVID-19 risk perceptions. The model included COVID-19 risk perception as a dependent variable and factors including age, sex, adherence to a GFD, comorbidities, and use of corticosteroids, as independent variables.

RESULTS

Participant characteristics

Overall, out of the 18022 participants who completed the survey, 10737 participants self-reported a diagnosis of CeD. The demographics for the included population can be found in Table 1. Missing data constituted less than 3% for each variable and thus were not replaced.

The median age of the participants was 41 years, of which 1575 (14.8%) were male. The highest proportions of respondents were from Argentina and Canada followed by Australia, New Zealand, and the United States. The detailed geographical distribution of participants by country, states, provinces and departments can be found in Supplementary Table 1.

The majority of self-reported patients with CeD had been diagnosed *via* CeD-specific serology [anti-tissue transglutaminase immunoglobulin (Ig) A and/or anti-deaminated gliadin peptide IgG] and confirmed *via* duodenal biopsy (n = 7506; 69.9%). The median time since diagnosis was 7 years. Out of all the participants with CeD, 25.8% were affiliated with a regional/national celiac association.

The majority of patients with CeD reported following a strict GFD (65.7%) with 33.4% adopting a GFD with some transgressions and 0.9% following a diet without gluten restriction. Of the patients following a GFD, the median time of gluten restriction was 7 years. Further, 24.1% of patients with CeD reported having household members who were also following a GFD. The majority of participants with CeD reported having their symptoms well-controlled (68.3%), while 31.7% had persistent symptoms.

Risk perceptions for contracting COVID-19 in patients with CeD

Patients with CeD obtained information about the relationship between COVID-19 and CeD through the internet (n = 2942; 27.4%) or through celiac association websites (n = 2465; 23%) (Table 1). Only a small proportion of patients reported learning through their physicians or other healthcare team members (n = 604; 5.6%). When asked to comment on their understanding regarding the relationship between COVID-19 and CeD, the majority of participants with CeD (n = 8815; 63.6%) reported that they did not have a very good understanding of their risk of contracting COVID-19 in relation to their condition. Consequently, 63.6% of patients requested more information on how COVID-19 may affect them. Further, while only 26.7% of participants with CeD believed they either were or were unsure whether they were more susceptible to infections because of their CeD, this proportion increased significantly when asked about their susceptibility to contracting COVID-19 in particular (26.7% *vs* 56.1%, P < 0.0001) (Figure 1). Participants who were members of their local celiac associations had lower rates of perceiving an increased risk of contracting COVID-19 compared to non-members (49.5% *vs* 57.4%, P < 0.0001) (Table 2). There was a stepwise decline in the proportion of patients with high-risk perceptions of contracting COVID-19 as the pandemic progressed (Figure 2).

Country-specific COVID-19 risk perceptions were highest in the United States (73.1%), Australia (67.3%), New Zealand (65.0%), and Argentina (62.9%) and lowest in Spain (19.1%) and Uruguay (23.3%) (Table 3).

There were 3745 participants (34.8%) who reported that they were taking extra precautions for COVID-19 as a result of being diagnosed with CeD. The most common precautions included at least one of the following: Extensive isolation/social distancing (68.6%), extended PPE use (gloves, face masks) before widespread recommendations (32.7%), and consistent hand washing/sanitization (23.4%). Further infection control measures included paying extra attention to maintaining a GFD (6.1%), getting grocery/food delivery (2.9%), strict adherence to public health recommendations (2.4%), implementing vitamins, supplements, and healthy foods into their diet (2.0%), showering/washing clothes after returning home (1.4%), getting the influenza vaccine (0.8%), and one participant noted that they stopped their immunosuppressant medication use. Information on the country-specific infection control/safety measures implemented in the general population during the study period is shown in the Supplementary Material (Supplementary Table 2).

Factors influencing the risk perception for contracting COVID-19 in patients with CeD

Older age [odds ratios (aORs): 0.99; 95%CI: 0.99 to 0.99, P < 0.001], male sex (aOR: 0.84; 95%CI: 0.76 to 0.93, P = 0.001), and adherence to a strict GFD (aOR: 0.89; 95%CI 0.82 to 0.96, P = 0.007) were associated with a lower perception of COVID-19 risk. However,

Table 1 Demographic characteristics of study population

Demographic	CeD, *n* = 10737
Age (years), median (IQR)	41 (28-57)
Gender, *n* (%)	10646 (99.2)
Male	1575 (14.8)
Female	9017 (84.7)
CeD diagnosis, *n* (%)	10570 (98.4)
Bloodwork[1]	1304 (12.1)
Biopsy	1334 (12.4)
Bloodwork + biopsy	7506 (69.9)
Unsure	426 (4.0)
Years since diagnosis, median (IQR)	7 (3-13)
Member of celiac association, *n* (%)	2766 (25.8)
Diet, *n* (%)	
Unrestricted	61 (0.6)
Other restrictions-non gluten	29 (0.3)
GFD-sometimes	118 (1.1)
GFD-most of the time	283 (2.6)
GFD-rare intentional gluten	418 (3.9)
GFD-rare accidental gluten	1566 (14.6)
GFD-possible cross-contamination	1136 (10.6)
Strict GFD	7052 (65.7)
Years diet restriction, Median (IQR)	7 (3-12)
Any household member following GFD, *n* (%)	2688 (25.0)
Some members	1949 (18.2)
All members	409 (3.8)
Other	221 (2.1)
Management of CeD symptoms, *n* (%)	
Well controlled	7336 (68.3)
Symptoms < 2 mo	2362 (22.0)
Symptoms > 2 mo	938 (8.7)
Travel outside of the country[2], *n* (%)	202 (1.9)
Contact with COVID-19 positive, *n* (%)	175 (1.6)
Tested for COVID-19, *n* (%)	478 (4.5)
Fever[2], *n* (%)	252 (2.3)
Respiratory symptoms[2], *n* (%)	1124 (10.5)
Hospitalizations for respiratory infection[2], *n* (%)	21 (0.2)
Comorbidities, *n* (%)	
Chronic lung condition	708 (6.6)
Chronic heart condition	376 (3.5)
Diabetes	413 (3.8)
Receiving steroids	322 (3.0)
Receiving immune suppressive medications	393 (3.7)

Pregnancy, *n* (%)	96 (0.9)
Information about COVID-19 in celiac disease	
Internet	2942 (27.4)
My doctor	291 (2.7)
Other healthcare providers	313 (2.9)
Celiac association website	2465 (23.0)
Other	731 (6.8)

[1]Bloodwork includes celiac disease-specific serology anti-tissue transglutaminase immunoglobulin (Ig) A, and/or anti-deaminated gliadin IgA/IgG, and/or anti-endomysial antibodies IgA.
[2]In the last month. CeD: Celiac disease; IQR: Interquartile range; GFD: Gluten-free diet; COVID-19: Coronavirus disease 2019.

Table 2 Relationship between celiac association membership and coronavirus disease 2019 risk perceptions in patients with celiac disease

Member of a celiac association	CeD respondents, *n* = 7296	Respondents with high COVID-19 risk perceptions	*P* value
Yes	2766	1368 (49.5)	< 0.0001
No	4530	2600 (57.4)	

CeD: Celiac disease; COVID-19: Coronavirus disease 2019.

Table 3 Country-specific risk perceptions of contracting coronavirus disease 2019 in patients with celiac disease

Country	Infection rate[1]	ORs for contracting COVID-19 (CeD *vs* controls)	95%CI	CeD patients believing they are more susceptible to COVID-19, *n* (%)
Argentina	0.14	1.41	0.48-4.12	2637 (62.9)
Canada	0.04	0.80	0.31-2.01	1962 (52.1)
Australia	0	1.92	0.03-99.21	449 (67.3)
New Zealand	0	0.88	0.01-43.32	295 (65.0)
Spain	0.21	0.73	0.21-2.57	85 (19.1)
United States	0.16	3.28	0.61-17.44	304 (73.1)
Uruguay	0	0.24	0.01-6.68	78 (23.3)
Italy	0	0.27	0.01-6.37	85 (41.5)
Mexico	0.6	1.50	0.15-14.42	62 (42.8)
Other[2]	0.17	0.70	0.08-6.22	62 (68.1)

[1]Infection rate: Positive coronavirus disease 2019 test over total tested.
[2]Full list of other countries can be found in Supplementary Table 1 in the Supplementary material. ORs: Odds ratios; CeD: Celiac disease; COVID-19: Coronavirus disease 2019.

the presence of comorbidities such as chronic lung conditions, chronic heart conditions (including hypertension), and diabetes, was associated with an increased perception of COVID-19 risk (aOR: 1.38; 95%CI: 1.22 to 1.54, $P < 0.001$). The use of corticosteroids or immunosuppressants did not change risk perception levels for contracting COVID-19 (aOR: 0.86; 95%CI: 0.68 to 1.08, $P = 0.19$) (Table 4).

Table 4 Logistic regression analysis of coronavirus disease 2019 risk perception in patients with celiac disease

	Risk perception			
	Crude [OR (95%CI)]	*P* value	Adjusted[1] [OR (95%CI)]	*P* value
Older age	0.99 (0.99-0.99)	0.012	0.99 (0.99-0.99)	< 0.001
Sex (M)	0.84 (0.76-0.93)	0.001	0.84 (0.75-0.93)	0.001
Strict GFD	0.88 (0.81-0.95)	0.002	0.89 (0.82-0.96)	0.007
Comorbidities[2]	1.29 (1.17-1.43)	< 0.001	1.37 (1.22-1.54)	< 0.001
Use of corticosteroids or immunosuppressants	1.11 (0.91-1.37)	0.28	0.86 (0.68-1.08)	0.19

[1]By all variables listed.
[2]Comorbidities includes chronic lung conditions, chronic heart conditions, and diabetes. GFD: Gluten-free diet; OR: Odds ratio.

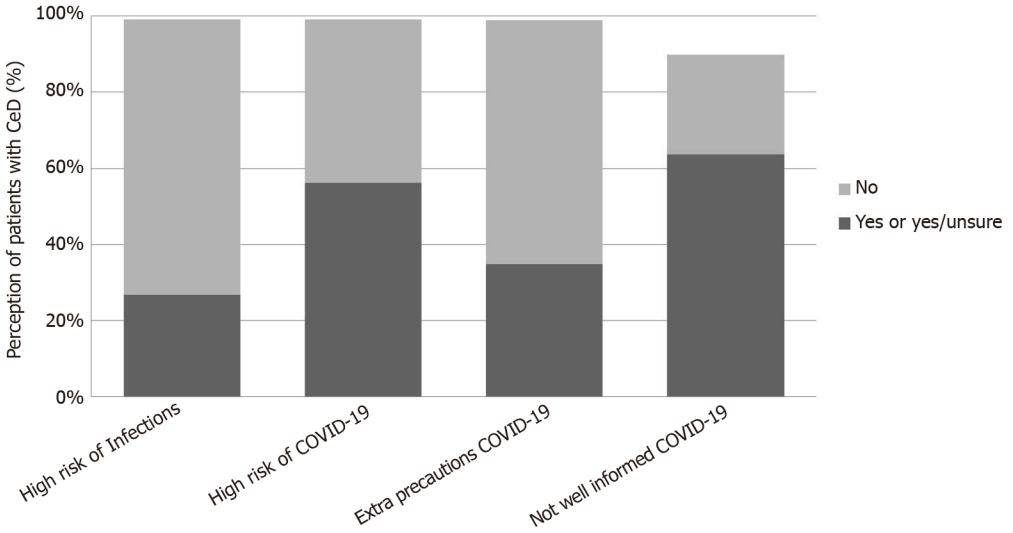


Figure 1 The risk perception of infections, contracting coronavirus disease 2019, need of taking extra precautions and obtaining information related to coronavirus disease 2019 in patients with celiac disease during the coronavirus disease 2019 pandemic. CeD: Celiac disease; COVID-19: Coronavirus disease 2019.

DISCUSSION

This study included over 10500 CeD patients and to our knowledge, is the first large-scale, international study to examine COVID-19 risk perceptions in patients with CeD. Despite demonstrating that the odds of contracting COVID-19 in patients with CeD is similar to that of the non-CeD population in our previous study[7], the majority of patients with CeD either believed they were at an increased risk or were uncertain of whether they were at an increased risk of contracting COVID-19 when compared to the general population.

A significant number of patients (44.0%) reported that their knowledge of the relationship between COVID-19 and CeD is poor or very poor with the majority of patients requesting more information. Further, while many patients learn about COVID-19 and CeD through the internet, very few learn about the relationship from their healthcare team. This is consistent with previous studies on CeD suggesting that patients are dissatisfied with the information offered by their physicians and feel like their general knowledge about CeD is inadequate[18]. Accordingly, previous reports suggest that many physicians have inadequate knowledge/awareness of the features associated with CeD which consequently has a direct impact on their patients' education regarding their condition[19,20]. As a result, while studies have demonstrated that CeD is associated with an increased risk of general infections, we found that only 26.7% of patients with CeD in our study believed they were at an increased risk. This supports the view that patients are generally uninformed about the potential consequences of CeD. This also represents a potential area for improvement as physicians and healthcare providers should be encouraged to thoroughly discuss the

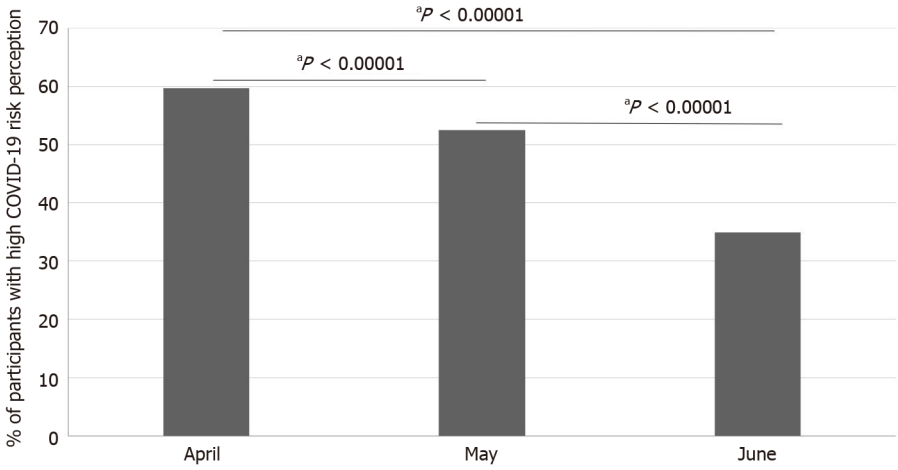


Figure 2 The risk perception of contracting coronavirus disease 2019 in patients with celiac disease decreased as the pandemic progressed. COVID-19: Coronavirus disease 2019.

implications of COVID-19 in relation to their patients' conditions based on the emerging evidence.

Patient perceptions are particularly relevant during the COVID-19 pandemic as high rates of depression and mood disorders have been associated with patients with higher risk perceptions of COVID-19[21]. Notably, in contrast to the generally low risk-perception that patients with CeD had regarding infections overall, more than half of our participants with CeD perceived they were at an increased risk or were unsure whether they were at a higher risk of contracting COVID-19 compared to the general population. As there are major uncertainties related to the novel coronavirus, this drastically affects the ability to properly and accurately inform patients of its potential implications. A recent study identified the lack of information regarding the virus as one of the major elements that contribute to fear and its associated high risk perception related to COVID-19[22]. Conversely, the study conducted by Siniscalchi *et al*[15] in March 2020, found that the majority of their patients with CeD (56.6%) did not feel more vulnerable to COVID-19 due to their condition[15]. One potential consideration contributing to the discrepancy between our results could be related to differences in study populations, as their study was limited to an Italian population. However, our results show the same trend when the analysis is sub-grouped by country and demonstrated similar results within our Italian participants (Table 3). These results suggest that risk perceptions vary markedly depending on the geographical area of the participants, as suggested by others[11]. In particular, country-specific differences in risk perception may be attributed to a variety of factors such as differences in culture, political climate, government communication, phase/timing of the pandemic, country-specific impacts of COVID-19, rates of infection, testing amounts and indications, and infection control measures (Supplementary Table 2). Notably, patients with CeD from Spain and Uruguay were found to have generally low risk perceptions for COVID-19. This aligns with previous studies that have found that the impact of COVID-19 has been relatively small in Uruguay as a result of swift lockdowns[23] and that individuals from Spain have been noted to have low personal concern about COVID-19[24]. Additionally, as the time of data collection was early in the pandemic for the study conducted by Siniscalchi *et al*[15], it is possible that patient perceptions may have changed as the pandemic progressed. Our analysis of risk perceptions by month found a stepwise decline in the proportion of participants with high COVID-19 risk perceptions as the pandemic progressed. This may be a result of timing because later in the study period, many countries have already passed the first wave of the pandemic and it may be due to the release of information regarding the link between COVID-19 and CeD from national celiac associations later on in the pandemic. It is also possible that this could be a result from "COVID-19 fatigue" as people become less concerned and more inclined to return to normal life as they recognize that the pandemic will persist for long periods of time.

We further investigated the different factors that we anticipated may modify the odds of having high risk perceptions for contracting COVID-19 in patients with CeD. Our results demonstrate a small, although significant, association between both younger age and female sex with higher risk perceptions of contracting COVID-19.

These results align with a study conducted by Rimal and Juon[25] which noted that younger, more educated individuals, have a higher risk perception of breast cancer. Further, our sex-specific findings are consistent with studies investigating both COVID-19 risk perceptions in the general population[11,26] and in patients with CeD[15].

Importantly, CeD has been associated with a large number of concomitant conditions such as cardiovascular conditions including hypertension, coronary artery disease, and arrhythmias, respiratory conditions such as asthma[27], and type 1 diabetes mellitus[28]. Further, it has been noted that the above-mentioned comorbidities may also predispose individuals to contracting COVID-19 and may contribute to a more severe disease course and mortality[29]. Accordingly, in our regression analysis, we noted that the presence of comorbidities such as chronic lung conditions, chronic heart conditions, and diabetes, increased the odds of patients believing they are at a higher risk of contracting COVID-19.

Notably, we found the use of corticosteroids or immunosuppressive therapies did not influence the odds of having high risk perceptions of COVID-19. This may potentially be attributed to nearly universal guidelines suggesting the continuation of immunosuppressive treatment during the pandemic and studies suggesting that the morbidity and mortality rates of patients who are immunosuppressed or have an autoimmune condition may be similar to that of the general population[30]. However, it is also possible that there was a selection bias if patients who perceived themselves to be at higher risk of contracting COVID-19 decided to stop the use of immunosuppressive therapies; as expressed in a comment by one participant. It also is possible that this action was taken by other participants; however, this was not systematically investigated in our study. Further, while it has been hypothesized that patients with active CeD (unmanaged or incompletely managed) may be at an increased risk of infection, our results demonstrate that individuals who follow a strict GFD have lower odds of perceiving themselves to be at an increased risk of contracting COVID-19.

Overestimation of risk can lead to being overly anxious, overly cautious, negative mental and physical health consequences[8], and not visiting a healthcare provider even when they believe they should[15,31]. Studies investigating healthcare use during the COVID-19 pandemic found significant reductions in emergency department visits, hospital admissions, and non-urgent healthcare visits[32,33]. Researchers have suggested that this decrease may be due to a perceived fear of contracting COVID-19 in high-risk areas such as hospitals. As a result, virtual patient care has been rapidly adopted to minimize this risk. However, patients in low-resource areas, such as those without technology or internet, are unable to access these alternate forms of healthcare and may be unequally affected by the pandemic[34]. Therefore, the role of governmental and non-governmental organizations in promoting awareness and knowledge in underserved/underdeveloped communities is especially important during the current health crisis.

In our participants with CeD, we found that 34.8% were taking extra COVID-19 precautions as a result of their condition. Importantly, we found that patients who were members of national celiac associations had overall lower risk perceptions for contracting COVID-19. Nearly a quarter of our patients reported learning about the relationship between COVID-19 and CeD through celiac association websites, which are often responsible for distributing patient-centred educational resources[35]. Previous studies have suggested that membership in a patient association has been correlated to increased physical/psychological well-being and social adjustment[36]. As over 60% of our participants reported that they would like more information on how COVID-19 may affect patients with CeD patient associations, such as national celiac associations, represent a promising avenue to help effectively disseminate health information, educate patients, and encourage healthy social relationships.

We acknowledge the presence of limitations associated with our study. First, this study did not assess risk perceptions in the general population and in the CeD group, there may have been potential selection/referral bias towards patients belonging to celiac associations as these associations acted as our primary mode of recruitment. Further, the cross-sectional nature of this study design only allowed us to evaluate COVID-19 risk perceptions during our study period which may change over time. Therefore, future prospective longitudinal studies may help assess changes over different time periods of the pandemic. In addition, although we investigated several dimensions of risk perception, some potential factors were not assessed. For example, we did not assess risk perceptions related to mortality or concerns of infecting family and friends. We also did not assess the mental health outcomes related to high levels of risk perception. As a result, future studies investigating additional factors and potential mediators of COVID-19 risk perception in patients with CeD will inform physicians and celiac associations on how to best design and communicate risk

mitigation strategies to support a potentially vulnerable patient population.

CONCLUSION

In conclusion, this international survey of patients with self-reported CeD demonstrates that a large proportion of patients with CeD perceive themselves to be at a high or unknown risk of contracting COVID-19. This association is more evident in females, those with comorbidities, and those who are not following a strict GFD. As a result of the uncertainty surrounding COVID-19, particularly at the start of the pandemic, and lack of information regarding the link between COVID-19 and CeD, patients typically have high levels of COVID-19 risk perceptions. Therefore, efforts should be made towards improving communication with patients with CeD and educating them based on emerging scientific evidence.

ARTICLE HIGHLIGHTS

Research background

We recently demonstrated that the odds of contracting coronavirus disease 2019 (COVID-19) in patients with celiac disease (CeD) is similar to that of the general population. However, how patients with CeD perceive their COVID-19 risk may differ from their actual risk.

Research motivation

Risk perceptions are important in the context of a pandemic because a group's perception of their susceptibility to infection influences their willingness to cooperate with preventative safety measures such as travel restrictions, hand washing, social distancing, and personal protective equipment use. However, overestimation of risk can contribute to negative mental and physical health consequences

Research objectives

The aim of this study was to investigate risk perceptions of contracting COVID-19 in patients with CeD and determine the factors that may influence their perception.

Research methods

We distributed an international survey throughout 10 countries and collected data on demographics, diet, COVID-19 testing, and risk perceptions of COVID-19 in patients with CeD. Risk perception was assessed by asking individuals whether they believe patients with CeD are at an increased risk of contracting COVID-19 when compared to the general population. Logistic regression was used to determine the influencing factors associated with COVID-19 risk perception.

Research results

A total of 10737 participants with CeD completed the survey. The majority of patients with CeD perceived they were at a higher risk or were unsure if they were at a higher risk of contracting COVID-19 compared to the non-CeD population. A greater proportion of patients with CeD perceived an increased risk of contracting COVID-19 when compared to infections in general due to their CeD. Consequently, 34.8% reported taking extra COVID-19 precautions as a result of their CeD. Members of celiac associations were less likely to perceive an increased risk of COVID-19 when compared to non-members. Older age, male sex, and strict adherence to a GFD were associated with a lower perception of COVID-19 risk and the presence of comorbidities was associated with a higher perception of COVID-19 risk.

Research conclusions

Overall, high levels of risk perceptions, such as those found in patients with CeD, may increase an individual's pandemic-related stress and contribute to negative mental health consequences. Therefore, it is encouraged that public health officials maintain consistent communication with the public and healthcare providers with the celiac community.

Research perspectives

Future studies specifically evaluating mental health in CeD could help determine the consequences of increased risk perceptions in this population.

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ORIGINAL ARTICLE

Observational Study

Risk stratification and geographical mapping of Brazilian inflammatory bowel disease patients during the COVID-19 outbreak: Results from a nationwide survey

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Abstract

BACKGROUND

Coronavirus disease 2019 (COVID-19) pandemic is still evolving globally, and Brazil is currently one of the most affected countries. It is still debated whether

patients with inflammatory bowel disease (IBD) are at a higher risk for developing COVID-19 or its complications.

AIM

To assess geographical distribution of IBD patients at the highest risk and correlate these data with COVID-19 mortality rates in Brazil.

METHODS

The Brazilian IBD Study Group (Grupo de Estudos da Doença Inflamatória Intestinal do Brasil) developed a web-based survey adapted from the British Society of Gastroenterology guidelines. The included categories were demographic data and inquiries related to risk factors for complications from COVID-19. Patients were categorized as highest, moderate or lowest individual risk. The Spearman correlation test was used to identify any association between highest risk and mortality rates for each state of the country.

RESULTS

A total of 3568 patients (65.3% females) were included. Most participants were from the southeastern and southern regions of Brazil, and 84.1% were using immunomodulators and/or biologics. Most patients (55.1%) were at moderate risk, 23.4% were at highest risk and 21.5% were at lowest risk of COVID-19 complications. No association between the proportion of IBD patients at highest risk for COVID-19 complications and higher mortality rates was identified in different Brazilian states (r = 0.146, P = 0.467).

CONCLUSION

This study indicates a distinct geographical distribution of IBD patients at highest risk for COVID-19 complications in different states of the country, which may reflect contrasting socioeconomic, educational and healthcare aspects. No association between high risk of IBD and COVID-related mortality rates was identified.

Key Words: Crohn's disease; Colitis, Ulcerative; COVID-19; Inflammatory bowel disease; Brazil

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Core Tip: The coronavirus disease 2019 pandemic is still evolving globally, and Brazil is currently one of the most affected countries. The Brazilian Inflammatory Bowel Diseases Study Group developed a web-based survey of 3568 patients that was adapted from the guidelines of the British Society of Gastroenterology. This study indicates a distinct geographical distribution of patients with inflammatory bowel disease at higher risk for coronavirus disease 2019 complications in different states of the country, which may reflect contrasting socioeconomic, educational and health aspects. No association between high risk and coronavirus disease 2019-related mortality rates has been identified.

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Informed consent statement: As this is a cross-sectional survey analyzing anonymous data, and information used derived from an unidentified database, informed consent from each individual was waived.

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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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INTRODUCTION

The World Health Organization declared coronavirus disease 2019 (COVID-19) a pandemic on March 11, 2020. Although many countries are already registering a reduction in the incidence of infections and starting vaccination programs, Brazil is

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currently one of the leaders in the world on a daily basis for both numbers of new cases and deaths. As of November 28, 2020, we have confirmed 6238250 infected patients and 171998 COVID-related deaths[1].

Available data suggests that patients with inflammatory bowel disease (IBD) are not at a higher risk for severe acute respiratory syndrome coronavirus-2 infection or the development of COVID-19 complications[2]. Moreover, the evolution of COVID-19 does not seem to be worse in patients with IBD, irrespective of their treatment. A recent analysis of 525 IBD patients from the Surveillance Epidemiology of Coronavirus Under Research Exclusion for Inflammatory Bowel Disease revealed that increasing age, comorbidities and corticosteroids are associated with worse outcomes of COVID-19 and that treatment with tumor necrosis factor inhibitors (TNFi) was not associated with severe COVID-19. The number of reported patients exposed to other medical options was insufficient to drive conclusions regarding risk for severe outcomes in this population[3]. Recently, another report from the Surveillance Epidemiology of Coronavirus Under Research Exclusion for Inflammatory Bowel Disease registry aimed to evaluate the association of IBD medications and their combinations on the risk of adverse COVID-19 outcomes. Based on data from over 1400 IBD patients, it was demonstrated that the combination of thiopurines with TNFi and thiopurine monotherapy are associated with a significantly increased risk of severe COVID-19 as compared with TNFi monotherapy. Anti-interleukins and anti-integrins were not associated with a significantly different risk than TNFi monotherapy[4]. Regardless of the risk of IBD medications, it is noteworthy that maintaining patients in remission with steroid-sparing treatments may be crucial during the pandemic period.

As the effect of immunosuppressive agents in IBD patients remains unclear during the pandemic, the British Society of Gastroenterology has issued guidance on risk stratification for IBD patients depending on medications in use, age, comorbidities and other risk factors[5]. Patients at highest risk for COVID-related complications are those who have a comorbidity and/or are over 70-years-old and are on any immunosuppressant therapy for IBD. Those of any age that are receiving ≥ 20 mg of prednisolone, undergoing new induction therapy with biologics and immunomodulators (combination therapy) within 6 wk with moderate to severe active disease with short gut syndrome or patients requiring parenteral nutrition are also considered at highest risk for severe COVID-19 outcomes. Patients receiving biological therapy, thiopurines, calcineurin inhibitors, Janus kinase inhibitors or combination therapy are stratified as moderate risk and the remaining IBD patients as lowest risk. Depending on the risk stratification, recommendations can be suggested. Patients at highest risk are advised to self-isolate, while patients at moderate risk or lowest risk should follow strict social distancing or social distancing as for the general population, respectively. Specific guidance on instructions for self-isolation (shielding education) and social distancing measures to reduce spread of the disease within the population and to protect vulnerable groups has been recently issued[6].

In order to identify Brazilian IBD patients who could be at high risk for COVID-19 complications, a taskforce group from the Brazilian IBD Study Group (Grupo de Estudos da Doença Inflamatória Intestinal do Brasil, GEDIIB) developed an anonymous web-based survey that allows self-identification risk assessment adapted from the British Society of Gastroenterology guidelines. Through a decision tree, patients were self-identified into different groups according to their risk of developing COVID-19 complications and received updated information according to their respective group (Table 1).

Currently, Brazil still has one of the fastest growing severe acute respiratory syndrome coronavirus-2 outbreaks in the world with one of the highest mortality rates, just behind the United States. Given that a higher absolute number of deaths in the context of an epidemic may reflect a strained healthcare system and economy, mapping IBD patients at highest risk for COVID-19 complications could help public authorities to delineate protection strategies to this possibly vulnerable population. Our study aimed to assess geographical distribution of IBD patients at highest risk and correlate these data with COVID-19 mortality rates in different states of Brazil.

MATERIALS AND METHODS

Data collection and participants

The GEDIIB COVID taskforce members in collaboration with experts in the field developed a questionnaire and established a decision tree to evaluate IBD patients pertaining to their risk of serious complications from COVID-19. The national survey

Table 1 Recommendations for inflammatory bowel disease patients according to their risk level

Highest risk
Stay home at all times and do not leave, even to buy food, medicine or to do exercises
Maintain attendance to infusions (only time to leave home) and the use of prescribed IBD medications
Stay at least 2 m (3 steps) from other people, including family members at home, whenever possible
Delivered products must be left outside the house by the courier
Anyone entering home must wash their hands thoroughly with soap for at least 20 s
Do not receive any visitors, unless help is needed
Moderate risk
Avoid contact with people with symptoms of COVID-19
Avoid using public transportation, crowds, public spaces and meetings with friends or family
Home office whenever possible
Use smartphones or virtual technology to contact physicians or other essential services
Lowest risk
All risk groups must follow the recommendations of the World Health Organization
Wash hands thoroughly with soap for at least 20 s, frequently
Use 70% alcohol gel if soap or water is not available
Avoid touching the face
Clean objects and surfaces that are frequently touched (such as door handles and phones)
Stay home to help prevent the spread of the virus
Leave home for very limited purposes: buying food and medication, exercise once a day (running, walking or cycling) alone or with a family member, help a vulnerable person or donate blood
Travel for professional purposes only if strictly necessary
When leaving home, minimize the time spent away and keep 2 m away from others

Adapted with permission from Queiroz *et al*[6]. COVID-19: Coronavirus disease 2019; IBD: Inflammatory bowel disease.

was available on the website (diicovid.com.br). Data was collected from April 14, 2020 to June 2, 2020. An informative article concerning this survey was posted at the official GEDIIB website, official mailing lists for patients and in national IBD patient association communication medias. All identified erroneous reports were removed for higher accuracy of the data.

The questionnaire consisted of fourteen questions (Supplementary Table 1). The included categories were demographic data (state and city of residence in Brazil, age, sex, smoking status) and questions related to the risk factors for complications from COVID-19 in IBD populations according to current previously published guidance[5,6]. The questions included IBD medications in use, self-reported comorbidities (hypertension, diabetes, cardiovascular disease and chronic pulmonary disease) and abdominal surgery for IBD performed in the past 30 d. Through a decision tree, patients were categorized as highest, moderate or lowest individual risk for the potential to develop serious complications from COVID-19 and received updated recommendations according to their risk level. Respondents were distributed according to their respective domiciliary states.

States of the national federation with a proportion of respondents at the highest risk for COVID-19 infection that were higher than the median rate of the overall country were considered for the analysis. Additionally, states with higher COVID-related cumulative mortality rates than the median of the country (as of June 2, 2020, the last available date of the survey) were also grouped. A possible correlation of these findings would suggest if there could be any coincidental relation between highest risk for COVID-19 complications and mortality in the same states.

Statistical analysis

Demographic and clinical characteristics of the study population were summarized by

descriptive statistics. Categorical variables were expressed as absolute counts and percentages and continuous variables as means and standard deviations. Data was presented initially for the total study population. Thereafter, it was organized according to the five Brazilian regions and respective states to evaluate their geographical distribution. The frequencies of patients using each therapeutic IBD regimen in combination with oral steroids were analyzed as subcategories.

Categorical variables were compared using a Chi-squared test, and continuous data was analyzed using Student's *t*-test. A two-tailed *P* value of 0.05 was used for statistical significance. The Spearman correlation test was performed to study a possible correlation between the proportion of highest risk patients and COVID-19 cumulative mortality in states with higher rates compared with the median national cutoffs for each variable. Data was exported and analyzed in SPSS Statistics 23 (IBM Corporation, Armonk, NY, United States).

Data regarding COVID-19 cumulative death rates from March 3 (first death registered in Brazil) to June 2 were obtained from the Brazilian Ministry of Health COVID-19 website, (https://covid.saude.gov.br/). We computed the COVID-19 mortality per 100000 people using the estimated populational data of 2019 available at the Statistical and Geographical Brazilian Institute for each of the Brazilian states and the federal district (https://datasus.saude.gov.br/populacao-residente/). In order to represent the mortality of COVID-19, we used classification into deciles. ArcMap 10.3® was used to generate the map representation.

Ethical considerations

The study was approved by the GEDIIB ethical review board under the protocol No. 002/2020 on October 28, 2020. Informed consent was waived because the survey recruitment was self-selective. In addition, data were de-identified. Individual participant data were not published, which maintained confidentiality in all steps of study analysis. This study was conducted in compliance with regulations stated in the 1975 Declaration of Helsinki.

RESULTS

A total of 3568 IBD patients participated in the national web-based survey and had data included. Six patients were excluded from the analysis due to inconsistent reported data. Overall demographic and baseline characteristics of respondents are illustrated in Table 2. Most respondents (55.6%) were 20-39-years-old, and 65.3% were females. Current smoking status was reported by 5.1% of the participants. The states with the highest response rates to the survey were São Paulo (29.6%), Rio de Janeiro (9.4%) Santa Catarina (7.7%), Paraná (7.7%), Bahia (6.0%) and the Federal District (5.3%). Details of the distribution of respondents per state are described in Supplementary Table 2.

The proportion of patients presenting with at least one self-reported comorbidity was 21.6%, and the most prevalent was hypertension (11.3%) followed by chronic pulmonary disease (4.6%) and recent (< 30 d) IBD-related abdominal surgery (3.8%). Most patients (84.1%) were on immune-mediated therapy (biologics 51.3% and/or immunomodulators 32.8%), 34.2% of respondents were using aminosalicylates and 13.3% had been recently treated with corticosteroids. Demographic, clinical and treatment characteristics by states is presented in Tables 3 and 4. The uptake of biological therapy was slightly higher among patients from the southeastern (52.9%) and southern (52.2%) regions compared with those from the northern region (33.3%). Demographic, clinical and treatment characteristics by regions are described in detail in Supplementary Table 3.

Overall, the majority (55.1%) of patients were at moderate risk, 23.4% were at highest risk and 21.5% were at lowest risk of developing COVID-19 complications. The proportion of IBD patients at highest, moderate and lowest risk for each state is represented in Figure 1A-C and for each county/city in Supplementary Figure 1. Thirteen states had higher proportional rates of patients at highest risk than the national median cutoff of 22.1% (Amapá, Rio Grande do Norte, Rio de Janeiro, São Paulo, Paraná, Amapá, Federal District, Santa Catarina, Ceará, Goiás, Espírito Santo, Acre and Paraíba). Paraíba was the state with the greatest proportion of IBD patients at highest risk (44.4%), followed by Acre (37.5%), Espírito Santo (29.9%) and Goiás (28.6%).

Geographical distribution of cumulative deaths from COVID-19 in Brazil as of June 2, 2020 by state (per 100000 people) is represented in Figure 1D. The national mortality

Table 2 Demographic, clinical and treatment characteristics from the whole sample of patients

Characteristics	*n* = 3568 (%)
Age (yr)	38.1 ± 12.3
0-9	10 (0.3)
10-19	120 (3.4)
20-29	769 (21.6)
30-39	1214 (34)
40-49	848 (23.8)
50-59	379 (10.6)
60-69	177 (5.0)
≥ 70	51 (1.4)
Sex	
Male	1238 (34.7)
Smoking	183 (5.1)
Clinical risk factors	
Hypertension	402 (11.3)
Diabetes	119 (3.3)
Cardiovascular disease	107 (3.0)
Chronic pulmonary disease	164 (4.6)
Recent abdominal surgery for IBD (< 30 d)	136 (3.8)
Overall IBD medications	
No medication	339 (9.5)
Oral steroids	473 (13.3)
5-ASA	1221 (34.2)
AZA/6-MP/MTX	1169 (32.8)
Biologics	1832 (51.3)
Therapeutic regimen	
Oral steroids monotherapy[1]	83 (2.3)
5-ASA monotherapy[1]	758 (21.2)
5-ASA + oral steroids[2]	115 (15.2)
AZA/6-MP/MTX monotherapy[1]	556 (15.6)
AZA/6-MP/MTX + oral steroids[2]	90 (16.2)
Biologic monotherapy[1]	1219 (34.2)
Biologic + oral steroids[2]	100 (8.2)
Combo therapy[3]	613 (17.2)
Combo therapy[3] + oral steroids[1]	85 (13.9)
COVID-related complications risk	
Highest	768 (23.4)
Moderate	1965 (55.1)
Lowest	836 (21.5)

[1]Monotherapy indicates no concomitant biologics or immunomodulator.
[2]These subcategories represent the frequency of patients in each monotherapy regimen requiring oral steroids.
[3]Combo therapy refers to biologics plus aminosalicylates/6-mercaptopurine/methotrexate.

5-ASA: Aminosalicylates; AZA: Azathioprine; 6-MP: 6-Mercaptopurine; MTX: Methotrexate; COVID: Coronavirus disease; IBD: Inflammatory bowel disease.

rate median cutoff in June 2 was 9.7/100000. The 13 states with mortality rates above the national median were Rio Grande do Norte, Maranhão, Alagoas, Espírito Santo, São Paulo, Acre, Roraima, Amapá, Pernambuco, Rio de Janeiro, Pará, Ceará and Amazonas. The states with higher mortality rates were Amazonas (50.7), Ceará (37.5), Pará (35.3), Rio de Janeiro (32.9) and Amapá (28).

When crossing overall proportion of highest risk patients with cumulative mortality rates, the Spearman rank correlation coefficient was not statistically significant (r = 0.146, P = 0.467). These data are illustrated in Figure 2.

DISCUSSION

This web-based survey analyzed important patient and treatment characteristics that could influence the IBD-related risk of having COVID-19 complications at a national level. Brazil is a continental country with different socioeconomic realities between its five different geographic regions (*i.e.* Northern, Northeastern, Southern, Southeastern and Midwestern). Most patients who participated in the survey were from the southeastern (n = 1886) and southern (n = 738) regions, which are more developed areas of the country. This may reflect patients who more often follow official mailing lists from the study group (as the call for participation in the survey) and might be treated in IBD tertiary referral centers. This could also mirror a higher prevalence of IBD in these regions of Brazil as compared to others as stated in a systematic review and some population-based studies[7-10]. Indeed, the findings of our study may not reflect a full national reality, as patients from the northern and northeastern regions may have a different IBD treatment profile. In the same line, it is noteworthy that the northern region had the highest proportion of patients with no current IBD medication (17.6%), and the southern and southeastern regions the highest proportion of patients under biological therapy (52.2% and 52.9%, respectively).

Our study suggests a different geographical distribution of IBD patients at highest risk for COVID-19 complications in different states of the country, which may reflect different socioeconomic, educational and healthcare issues that could potentially have affected our findings. Despite the fact that the states of Paraíba, Acre, Espírito Santo and Goiás had the greatest proportion of patients at highest risk, this was not reflected in higher COVID-related mortality according to official data from the Ministry of Health. Higher mortality for COVID-19 as of June 2, 2020 (last available date capturing responses in the survey) was observed in the states of Amazonas, Ceará and Rio de Janeiro.

Nevertheless, a clear correlation between the risk of COVID-19 complications in IBD patients and mortality was not demonstrated according to the Spearman test. This important finding is in tune with other previously published data from IBD cohorts, which did not identify worse COVID-19 disease courses in IBD patients[3,11]. This analysis underscores the important finding that the majority of respondents (55.1%) were classified at moderate risk, which means that they were currently having adequate IBD treatment with no increased rates of steroid therapy. Despite being treated with immunosuppressant agents, this population may not be as vulnerable as expected[12]. Although this still needs to be proven, speculation is undertaken if the reduction of the COVID-related "cytokine storm" can be achieved with adequate medical therapy for IBD[13]. More studies in this field are warranted. It is important to state that no longitudinal follow-up of these patients was evaluated in this study. The exact number of infected patients with IBD within our sample was out of the focus of the survey.

When data is analyzed by regions, the proportion of IBD patients at highest risk was 20%-25%, and the majority of patients comprised those using immunomodulators or biologics with no active disease, did not undergo recent IBD-related surgery and were not under steroid therapy. This may reinforce the fact that the survey reached more patients who were likely under regular follow-up with their specialists, a common practice in more developed areas. Another important point is that no significant difference between the regions in the low, moderate or high risk of COVID-related complications was demonstrated (P = 0.118; Supplementary Table 2). This may also be demonstrated by the fact that even in the same region, a particular state could have a

Table 3 Demographic, clinical and treatment characteristics by states in Brazil

Characteristics	São Paulo	Rio de Janeiro	Minas Gerais	Santa Catarina	Paraná	Bahia	Distrito Federal	Rio Grande do Sul	Espírito Santo	Ceará	Pernambuco	Maranhão	Goiás
	n = 1057 (%)	*n* = 336 (%)	*n* = 329 (%)	*n* = 278 (%)	*n* = 277 (%)	*n* = 217 (%)	*n* = 190 (%)	*n* = 183 (%)	*n* = 144 (%)	*n* = 107 (%)	*n* = 74 (%)	*n* = 57 (%)	*n* = 49 (%)
Clinical risk factors													
Age ≥ 70 yr	7 (0.7)	2 (0.6)	2 (0.6)	7 (2.5)	9 (3.2)	1 (0.5)	2 (1.1)	-	8 (5.6)	5 (4.7)	2 (2.7)	-	-
Hypertension	133 (12.6)	43 (12.8)	34 (10.3)	27 (9.7)	31 (11.2)	29 (13.4)	19 (10.0)	11 (6.0)	13 (9.0)	14 (13.1)	6 (8.1)	2 (3.5)	4 (8.2)
Diabetes	33 (3.1)	14 (4.2)	7 (2.1)	8 (2.9)	13 (4.7)	9 (4.1)	9 (4.7)	-	8 (5.6)	6 (5.6)	2 (2.7)	-	3 (6.1)
Cardiovascular diseases	23 (2.2)	10 (3.0)	4 (1.2)	10 (3.6)	12 (4.3)	9 (4.1)	9 (4.7)	3 (1.6)	8 (5.6)	6 (5.6)	2 (2.7)	3 (5.3)	1 (2.0)
Liver diseases	52 (4.9)	15 (4.5)	12 (3.6)	17 (6.1)	11 (4.0)	8 (3.7)	10 (5.3)	11 (6.0)	5 (3.5)	1 (0.9)	3 (4.1)	2 (3.5)	2 (4.1)
Abdominal surgery for IBD (< 30 d)	38 (3.6)	12 (3.6)	7 (2.1)	11 (4.0)	16 (5.8)	5 (2.3)	8 (4.2)	1 (0.5)	16 (11.1)	5 (4.7)	4 (5.4)	1 (1.8)	2 (4.1)
Overall IBD medications													
No medication	112 (10.6)	40 (11.9)	26 (7.9)	22 (7.9)	28 (10.1)	16 (7.4)	16 (8.4)	15 (8.2)	15 (10.4)	6 (5.6)	5 (6.8)	7 (12.3)	1 (2.0)
Oral steroids	137 (13.0)	50 (14.9)	52 (15.8)	32 (11.5)	30 (10.8)	34 (15.7)	27 (14.2)	23 (12.6)	10 (6.9)	16 (15.0)	11 (14.9)	6 (10.5)	7 (14.3)
5-ASA	357 (33.8)	90 (26.8)	124 (37.7)	110 (39.6)	90 (32.5)	88 (40.6)	68 (35.8)	73 (39.9)	20 (13.9)	21 (19.6)	37 (50.0)	18 (31.6)	19 (38.8)
AZA/6-MP/MTX	308 (29.1)	101 (30.1)	130 (39.5)	93 (33.5)	95 (34.3)	71 (32.7)	51 (26.8)	68 (37.2)	62 (43.1)	52 (48.6)	27 (36.5)	17 (29.8)	21 (42.9)
Biologics	575 (54.4)	164 (48.8)	156 (47.4)	145 (52.2)	153 (55.2)	95 (43.8)	93 (48.9)	87 (47.5)	92 (63.9)	64 (59.8)	28 (37.8)	29 (50.9)	29 (59.2)
Therapeutic regimen													
Oral steroid monotherapy[1]	17 (1.6)	14 (4.2)	8 (2.4)	5 (1.8)	2 (0.7)	8 (3.7)	12 (6.3)	4 (2.2)	1 (0.7)	1 (0.9)	3 (4.1)	-	-
5-ASA monotherapy[1]	212 (20.1)	57 (17.0)	67 (20.4)	67 (24.1)	49 (17.7)	68 (31.3)	45 (23.7)	46 (25.1)	11 (7.6)	12 (11.2)	23 (31.1)	12 (21.1)	14 (28.6)
5-ASA + oral steroids[2]	39 (18.4)	4 (7.0)	9 (13.4)	12 (17.9)	5 (10.2)	11 (16.2)	5 (11.1)	6 (13.0)	1 (9.1)	1 (8.3)	1 (4.3)	2 (16.7)	6 (42.9)
AZA/6-MP/MTX monotherapy[1]	141 (13.3)	61 (18.2)	72 (21.9)	39 (14.0)	45 (16.2)	30 (13.8)	24 (12.6)	31 (16.9)	25 (17.4)	24 (22.4)	15 (20.3)	9 (15.8)	5 (10.2)
AZA/6-MP/MTX + oral steroids[2]	28 (19.9)	12 (19.7)	11 (15.3)	2 (5.1)	10 (22.2)	6 (20.0)	3 (12.5)	2 (6.5)	2 (8.0)	6 (25.0)	2 (13.3)	2 (22.2)	-
Biologic monotherapy[1]	408 (38.6)	124 (36.9)	98 (29.8)	91 (32.7)	103 (37.2)	54 (24.9)	66 (34.7)	50 (27.3)	55 (38.2)	36 (33.6)	16 (21.6)	21 (36.8)	13 (26.5)
Biologic + oral steroids[2]	21 (5.1)	17 (13.7)	18 (18.4)	6 (6.6)	9 (8.7)	2 (3.7)	5 (7.6)	5 (10.0)	4 (7.3)	2 (5.6)	3 (18.8)	1 (4.8)	1 (7.7)
Combo therapy[3]	167 (15.8)	40 (11.9)	58 (17.6)	54 (19.4)	50 (18.1)	41 (18.9)	27 (14.2)	37 (20.2)	37 (25.7)	28 (26.2)	12 (16.2)	8 (14.0)	16 (32.7)

Combo therapy[3] + oral steroids[2]	32 (19.2)	3 (7.5)	6 (10.3)	7 (13.0)	4 (8.0)	7 (17.1)	2 (7.4)	6 (16.2)	2 (5.4)	6 (21.4)	2 (16.7)	1 (12.5)	-
Risk classification													
Low	221 (21.0)	71 (21.1)	67 (20.4)	65 (23.4)	53 (19.1)	51 (23.5)	50 (26.3)	46 (25.1)	15 (10.4)	16 (15.0)	20 (27.0)	11 (19.3)	11 (22.4)
Medium	579 (54.8)	184 (54.8)	193 (58.7)	139 (50.0)	156 (56.3)	118 (54.4)	91 (47.9)	108 (59.0)	86 (59.7)	62 (57.9)	40 (54.1)	40 (70.2)	24 (49.0)
High	256 (24.8)	81 (24.1)	69 (21.0)	74 (26.6)	68 (24.5)	48 (22.1)	49 (25.8)	29 (15.8)	43 (29.9)	29 (27.1)	14 (18.9)	6 (10.5)	14 (28.6)

[1]Monotherapy indicates no concomitant biologics or aminosalicylates/6-mercaptopurine/methotrexate.
[2]These subcategories represent the frequency of patients in each therapeutic regimen requiring oral steroids.
[3]Combo therapy refers to biologics plus aminosalicylates/6-mercaptopurine/methotrexate.
5-ASA: Aminosalicylates; AZA: Azathioprine; 6-MP: 6-Mercaptopurine; MTX: Methotrexate; IBD: Inflammatory bowel disease.

different frequency of high risk in comparison to its neighbors. As an example, the proportion of patients at highest risk was 28.6% in the state of Goiás and only 14.3% in Tocantins and 16.7% in Mato Grosso do Sul, all states from the midwestern region. This can also be justified by different types of IBD care and patient profiles in terms of comorbidities between the states, which demonstrates the complexity of analyzing data in a heterogeneous country such as Brazil.

Regarding COVID-related mortality rates in Brazil, it seems clear that the higher rates found in the states of Amazonas, Ceará, Pará, Rio de Janeiro and Amapá reflect difficulties in healthcare in these specific areas. Most of these states belong to less developed regions of the country (northern and northeastern). These are states with chronic difficulties in the public health system over the last decades, with limited resources, reduced numbers of hospitals and consequently disproportional intensive care unit beds per 100000 inhabitants[14]. Recent data revealed that Brazil has 15.6 intensive care unit beds per 100000 inhabitants. Considering only intensive care unit beds from the public health system, the average drops to 7.1 per 100000 inhabitants, and there are significant differences between the regions of the country. Among the population exclusively dependent on the public health system, 30.5% of the Northeast, 22.6% of the North and 21% of the Midwest regions live in places without intensive care unit beds[15].

Socioeconomical limitations may also be illustrated by the assessment of the Human Development Index (HDI) in these states because it is based on three aspects: Health, as measured by life expectancy at birth; Knowledge, as measured by the adult literacy rate; and A decent standard of living, as measured by gross domestic product per capita. Concerning the five geographic regions of Brazil, the first five states with the highest HDI are from the South, Southeast and Midwest regions and the last five states with the lowest HDI are from the North and Northeast regions[16]. Despite the northern and northeastern regions having a lower prevalence of IBD patients, the significant percentage of patients at highest risk for COVID-19 complications might reflect a similar healthcare system limitation, possibly with few available IBD specialists in the

Table 4 Demographic, clinical and treatment characteristics by states in Brazil (continuation of Table 3)

Characteristics	Piauí	Amazonas	Pará	Alagoas	Rio Grande do Norte	Mato Grosso	Paraíba	Acre	Sergipe	Mato Grosso do Sul	Tocantins	Rondônia	Roraima	Amapá
	n = 41 (%)	*n* = 38 (%)	*n* = 32 (%)	*n* = 31 (%)	*n* = 25 (%)	*n* = 22 (%)	*n* = 18 (%)	*n* = 16 (%)	*n* = 13 (%)	*n* = 12 (%)	*n* = 7 (%)	*n* = 6 (%)	*n* = 6 (%)	*n* = 3 (%)
Clinical risk factors														
Age ≥ 70 yr	-	-	-	-	-	-	3 (16.7)	2 (12.5)	-	1 (8.3)	-	-	-	-
Hypertension	5 (12.2)	4 (10.5)	1 (3.1)	4 (12.9)	1 (4.0)	2 (9.1)	5 (27.8)	3 (18.8)	1 (7.7)	3 (25.0)	-	3 (50.0)	3 (50.0)	1 (33.3)
Diabetes	-	2 (5.3)	-	1 (3.2)	-	-	1 (5.6)	2 (12.5)	-	1 (8.3)	-	-	-	-
Cardiovascular diseases	1 (2.4)	-	-	-	1 (4.0)	1 (4.5)	-	1 (6.3)	-	2 (16.7)	-	-	1 (16.7)	-
Liver diseases	2 (4.9)	2 (5.3)	5 (15.6)	1 (3.2)	1 (4.0)	2 (9.1)	2 (11.1)	-	-	-	-	-	-	-
Abdominal surgery for IBD (< 30 d)	2 (4.9)	4 (10.5)	2 (6.3)	1 (3.2)	-	-	-	1 (6.3)	-	-	-	-	-	-
Overall IBD medications														
No medication	5 (12.2)	5 (13.2)	5 (15.6)	1 (3.2)	-	2 (9.1)	-	2 (12.5)	1 (7.7)	3 (25.0)	2 (28.6)	1 (16.7)	3 (50.0)	-
Oral steroids	8 (19.5)	7 (18.4)	6 (18.8)	-	3 (12.0)	5 (22.7)	3 (16.7)	3 (18.8)	1 (7.7)	1 (8.3)	-	1 (16.7)	-	-
5-ASA	11 (26.8)	15 (39.5)	21 (65.6)	8 (25.8)	6 (24.0)	12 (54.5)	10 (55.6)	4 (25.0)	7 (53.8)	3 (25.0)	2 (28.6)	4 (66.7)	2 (33.3)	1 (33.3)
AZA/6-MP/MTX	9 (22.0)	11 (28.9)	7 (21.9)	13 (41.9)	5 (20.0)	5 (22.7)	7 (38.9)	4 (25.0)	4 (30.8)	2 (16.7)	3 (42.9)	1 (16.7)	-	2 (66.7)
Biologics	20 (48.8)	19 (50.0)	6 (18.8)	19 (61.3)	21 (84.0)	5 (22.7)	8 (44.4)	6 (37.5)	7 (53.8)	6 (50.0)	2 (28.6)	1 (16.7)	1 (16.7)	1 (33.3)
Therapeutic regimen														
Oral steroid monotherapy[1]	5 (12.2)	-	1 (3.1)	-	-	-	-	2 (12.5)	-	-	-	-	-	-
5-ASA monotherapy[1]	9 (22.0)	9 (23.7)	15 (46.9)	4 (12.9)	3 (12.0)	10 (45.5)	8 (44.4)	4 (25.0)	3 (23.1)	2 (16.7)	1 (14.3)	4 (66.7)	2 (33.3)	1 (33.3)
5-ASA + oral steroids[2]	-	3 (33.3)	4 (26.7)	-	-	4 (40.0)	1 (12.5)	-	-	-	-	1 (25.0)	-	-
AZA/6-MP/MTX monotherapy[1]	2 (4.9)	5 (13.2)	5 (15.6)	7 (22.6)	1 (4.0)	5 (22.7)	2 (11.1)	2 (12.5)	2 (15.4)	1 (8.3)	2 (28.6)	-	-	1 (33.3)
AZA/6-MP/MTX + oral steroids[2]	-	1 (20.0)	-	-	-	1 (20.0)	-	1 (50.0)	-	1 (100)	-	-	-	-
Biologic monotherapy[1]	13 (31.7)	13 (34.2)	4 (12.5)	13 (41.9)	17 (68.0)	5 (22.7)	3 (16.7)	4 (25.0)	5 (38.5)	5 (41.7)	1 (14.3)	-	1 (16.7)	-
Biologic + oral steroids[2]	1 (7.7)	3 (23.1)	-	-	1 (5.9)	-	-	-	1 (20.0)	-	-	-	-	-
Combo therapy[3]	7 (17.1)	6 (15.8)	2 (6.3)	6 (19.4)	4 (16.0)	-	5 (27.8)	2 (12.5)	2 (15.4)	1 (8.3)	1 (14.3)	1 (16.7)	-	1 (33.3)
Combo therapy[3] + oral steroids[2]	2 (28.6)	-	1 (50.0)	-	2 (50.0)	-	2 (40.0)	-	-	-	-	-	-	-

Risk classification														
Low	10 (24.4)	7 (18.4)	13 (40.6)	4 (12.9)	3 (12.0)	9 (40.9)	6 (33.3)	4 (25.0)	4 (30.8)	3 (25.0)	2 (28.6)	3 (50.0)	2 (33.3)	-
Medium	24 (58.5)	22 (57.9)	14 (43.8)	22 (71.0)	16 (64.0)	11 (50.0)	4 (22.2)	6 (37.5)	7 (53.8)	7 (58.3)	4 (57.1)	2 (33.3)	3 (50.0)	3 (100)
High	7 (17.1)	9 (23.7)	5 (15.6)	5 (16.1)	6 (24.0)	2 (9.1)	8 (44.4)	6 (37.5)	2 (15.4)	2 (16.7)	1 (14.3)	1 (16.7)	1 (16.7)	-

[1]Monotherapy indicates no concomitant biologics or aminosalicylates/6-mercaptopurine/methotrexate.
[2]These subcategories represent the frequency of patients in each therapeutic regimen requiring oral steroids.
[3]Combo therapy refers to biologics plus aminosalicylates/6-mercaptopurine/methotrexate.
5-ASA: Aminosalicylates; AZA: Azathioprine; 6-MP: 6-Mercaptopurine; MTX: Methotrexate; IBD: Inflammatory bowel disease.

respective regions.

It is not being an easy task for Brazilian health authorities to deal with the COVID-19 pandemic. The country is facing important economic and political challenges that likely contribute to the significant increase of the number of cases and deaths throughout the country. The Brazilian government has been a recurrent target for scientific and regular media worldwide[17]. We truly hope this manuscript can raise awareness and call the attention from national health authorities with respect to vulnerability of specific populations during this critical period our country is facing.

The present study is associated with some limitations, which must be considered for adequate interpretation of the results. First, as previously mentioned, the higher proportion of respondents coming from the southeastern and southern regions may not reflect the reality in other states, mostly from the northern and northeastern regions, which have more rural areas and lower HDI. Another important point is that the survey was simple, objective and analyzed a limited number of variables. As an example, even the simple diagnostic difference between ulcerative colitis and Crohn's disease was not evaluated. Disease activity at the time of the survey was not captured, which could have influenced the results. Data is also derived from self-reported personal and treatment-related characteristics, which may be biased by individual intellectual issues. Important additional limitations of our study include the absence of follow-up of the patients who replied to the survey. By not having this information, we could not describe in detail if patients who had COVID-19 infection continued their medications, possible differences between ulcerative colitis and Crohn's disease or common manifestations of severe acute respiratory syndrome coronavirus-2 among included patients due to methodological issues. Despite these limitations, the study's strength is based in the large number of patients who responded to the survey from all states of Brazil, and this represents one of the largest databases regarding COVID-19 risk for complications in IBD patients globally.

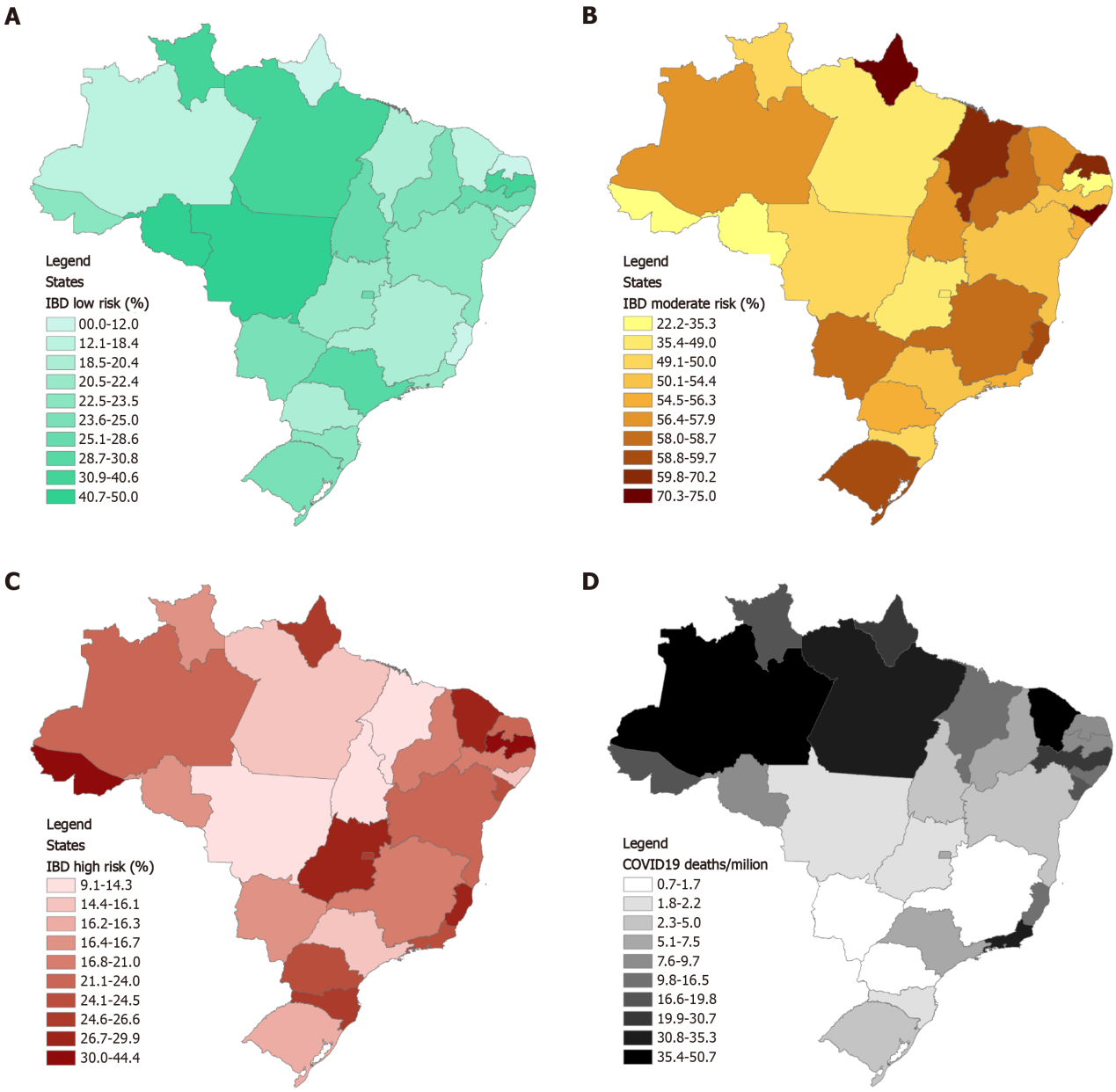


Figure 1 Inflammatory bowel disease patients at low, moderate and high risk for complications of coronavirus disease 2019 and coronavirus disease-related deaths in Brazil by state (per 100000 people). A: Low risk; B: Moderate risk; C: High risk; D: Coronavirus disease 2019 deaths. COVID: Coronavirus disease; IBD: Inflammatory bowel disease.

CONCLUSION

In summary, no correlation between the proportion of IBD patients at highest risk for COVID-19 complications and higher mortality rates was identified among Brazilian states. This can be related to the fact that the majority of the IBD patients are at moderate risk, which could possibly reflect adequate treatment and controlled disease. More epidemiological data comparing IBD and COVID-19 outcomes are suggested in large countries such as Brazil to properly position which IBD patients are more vulnerable in this pandemic period.

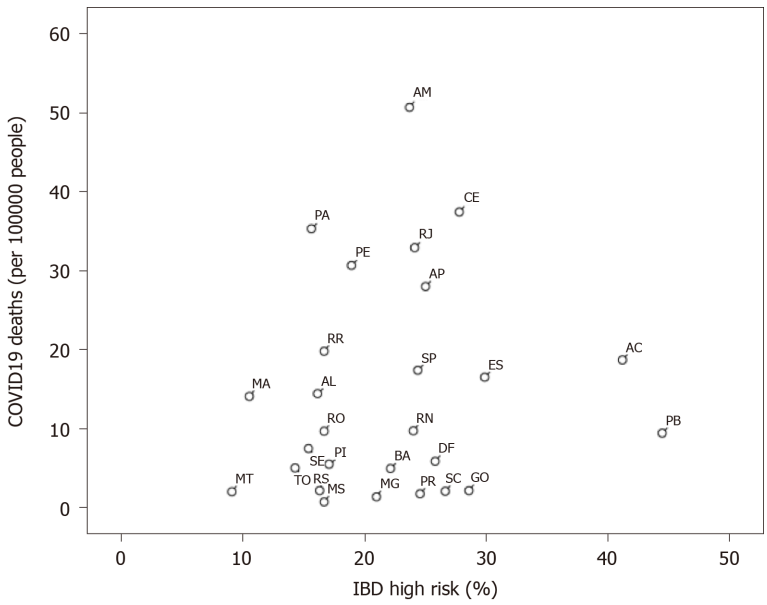


Figure 2 Spearman correlation test between the 27 states and cumulative coronavirus disease 2019 mortality rates. No significant correlation was identified (r = 0.146, P = 0.467). COVID-19: Coronavirus disease 2019; IBD: Inflammatory bowel disease.

ARTICLE HIGHLIGHTS

Research background

The coronavirus disease 2019 (COVID-19) pandemic is a public health emergency of international concern, and Brazil is currently one of the most affected countries.

Research motivation

It is uncertain whether patients with inflammatory bowel disease (IBD) are at a greater risk for developing COVID-19 or its complications. There are scarce data in large countries correlating IBD patients, the risk of COVID-19 complications and mortality.

Research objectives

This study aimed to evaluate geographical distribution of IBD patients at highest risk and correlate these data with COVID-19 mortality rates in different states of Brazil.

Research methods

It was a web-based survey adapted from the British Society of Gastroenterology guidelines. We included demographic data and risk factors for complications from COVID-19. Patients were categorized as highest, moderate or lowest individual risk.

Research results

The proportion of IBD patients at highest risk for COVID-19 complications depends on individual aspects and can vary in specific regions. No correlation between patients with IBD at highest risk and COVID-related mortality rates was demonstrated in different regions of the country.

Research conclusions

This is one of the largest studies analyzing the risk of patients with IBD during COVID-19 pandemic globally.

Research perspectives

These data can be important to large countries such as Brazil, United States, Russia and India, which are currently facing significant problems in terms of controlling the pandemic. Even European countries, facing a second wave of COVID-19 infection, can base future research or decisions using these data as an example.

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META-ANALYSIS

Hepatitis E in solid organ transplant recipients: A systematic review and meta-analysis

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Abstract

BACKGROUND

Hepatitis E virus (HEV) infection is underdiagnosed due to the use of serological assays with low sensitivity. Although most patients with HEV recover completely, HEV infection among patients with pre-existing chronic liver disease and organ-transplant recipients on immunosuppressive therapy can result in decompensated liver disease and death.

AIM

To demonstrate the prevalence of HEV infection in solid organ transplant (SOT) recipients.

METHODS

We searched Ovid MEDLINE, EMBASE, and the Cochrane Library for eligible articles through October 2020. The inclusion criteria consisted of adult patients with history of SOT. HEV infection is confirmed by either HEV-immunoglobulin G, HEV-immunoglobulin M, or HEV RNA assay.

RESULTS

Of 563 citations, a total of 22 studies (n = 4557) were included in this meta-analysis. The pooled estimated prevalence of HEV infection in SOT patients was 20.2% [95% confidence interval (CI): 14.9-26.8]. The pooled estimated prevalence of HEV infection for each organ transplant was as follows: liver (27.2%; 95%CI: 20.0-35.8), kidney (12.8%; 95%CI: 9.3-17.3), heart (12.8%; 95%CI: 9.3-17.3), and lung (5.6%; 95%CI: 1.6-17.9). Comparison across organ transplants demonstrated statistical significance (Q = 16.721, P = 0.002). The subgroup analyses showed that the prevalence of HEV infection among SOT recipients was significantly higher in middle-income countries compared to high-income countries. The pooled estimated prevalence of de novo HEV infection was 5.1% (95%CI: 2.6-9.6) and the pooled estimated prevalence of acute HEV infection was 4.3% (95%CI: 1.9-9.4).

CONCLUSION

HEV infection is common in SOT recipients, particularly in middle-income countries. The prevalence of HEV infection in lung transplant recipients is considerably less common than other organ transplants. More studies examining the clinical impacts of HEV infection in SOT recipients, such as graft failure, rejection, and mortality are warranted.

Key Words: Hepatitis E virus; Hepatitis E virus infection; Solid organ transplant; Prevalence

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Core Tip: Hepatitis E virus (HEV) infection among patients with pre-existing chronic liver disease and organ-transplant recipients on immunosuppressive therapy can result in decompensated liver disease and death. The prevalence of HEV infection in solid organ transplant (SOT) recipients varies by countries and transplanted organs. This meta-analysis, demonstrates the prevalence of HEV infection in SOT recipients is 20.3% (highest in liver transplant recipients and lowest in lung transplant recipients). The prevalence of HEV infection is two-fold more common in middle-income countries compared to high-income countries. Our findings encourage future studies to describe the clinical impacts of HEV infection on patient and allograft outcomes.

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INTRODUCTION

Hepatitis E virus (HEV) results in approximately 20 million infections each year[1]. This virus is endemic to mostly developing countries in Asia, Africa, and Central America. There are additional cases of the disease manifesting in developed countries without patients having traveled to endemic areas[1,2]. As HEV is transmitted by the fecal-oral route, infection is more prevalent in areas with poor water quality and food contamination[1]. Patients typically demonstrate symptoms of fevers, gastrointestinal complaints, and jaundice within weeks of infection that self-resolve with supportive care[1]. Although most patients with HEV recover completely, HEV infection among patients with pre-existing chronic liver disease, pregnant women and organ-transplant recipients on immunosuppressive therapy can result in fulminant hepatitis with a 10%-30% mortality rate[3].

HEV has been noted to impact solid organ transplant (SOT) recipient outcomes. HEV infection has been cited to cause graft cirrhosis and subsequent failure in liver graft recipients secondary to chronic infection[4]. Furthermore, heart transplant recipients have been noted to have secondary liver infection and subsequent fibrosis[5]. In contrast, renal transplant allografts were found to have similar rejection and two-year graft survival between HEV seropositive and negative recipients, thus demonstrating HEV does not always impact non-liver allografts[6]. HEV reactivation from infected SOT allografts remains a risk as well, with case reports describing this occurrence in liver transplant recipients who receive an allograft with latent disease[7]. However, little evidence has demonstrated cases of HEV reactivation in renal transplant patients[7]. HEV viremia has also been found in non-SOTs such as hematopoietic stem cell transplant[8]. This suggests possible transmission of the virus through bone marrow products as well as SOT. Once infected with the virus, transplant recipients are at risk for developing chronic liver disease, especially with HEV genotype 3[9].

With the majority of this evidence being limited to case reports and some retrospective studies, there is very limited conclusive evidence illustrating the true HEV association, its related risk profile, and the clinical outcomes in transplant patients. Pooling the aggregate data of current studies will help elucidate the extent of risk and help stratify the clinical outcomes. We conducted this systematic review and meta-analysis to describe the prevalence of HEV infection in SOT patients. Our study is the first meta-analysis to emphasize the burden of HEV infection in SOT recipients.

MATERIALS AND METHODS

Search strategy

This manuscript follows the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA)[10] statement as well as Meta-analysis of Observational Studies in Epidemiology (MOOSE)[11] guidelines. A systematic search was conducted through the Ovid MEDLINE, EMBASE, and Cochrane Library from database inception to October 2020 using the following search terms: ("hepatitis E" OR "HEV") AND ("transplant" OR "transplantation") AND ("outcome*" OR "mortality" OR "graft loss" OR "graft function" OR "incidence" OR "death"). The detailed search strategy for each database is summarized in Supplementary search strategy. No language restrictions were applied.

Inclusion criteria

The eligibility of each study was determined by the following inclusion criteria: (1) The nature of study is observational or conference abstract; (2) Study population consisted of SOT recipients; and (3) The prevalence of HEV infection was reported as one of the outcomes of interest. Exclusion criteria consisted of pediatric patients, hematopoietic stem cell transplant recipients, and studies with a total sample size of less than 50 patients. The latter was to avoid inter-study variance. Study eligibility was independently evaluated by two investigators (PH and AT). Any disagreements were resolved by mutual consensus. The quality of each study was appraised using the Newcastle–Ottawa quality scale[12], which assesses six components including (1) Representativeness of the subjects; (2) Ascertainment of the exposure; (3) Demonstration of outcome of interest was not present at start of study; (4) Assessment of outcome; (5) Follow-up duration period was long enough for outcome to occur; and (6) Adequate follow-up duration.

Review process and data extraction

The titles and abstracts of all discovered studies were screened (PH and AT) prior to a full-text review. The full-text of the screened articles was reviewed to determine their eligibility for inclusion into the systematic review and meta-analysis. We created a standardized data collection form to extract the following information from the included studies: First author's name, year of publication, country of origin, study design, subject(s), sample size, transplanted organ (heart, lung, liver, kidney, and undifferentiated), age, male sex, ethnicity, prevalence of HEV, confirmatory test used to diagnose HEV infection, prevalence of acute HEV infection, death, other reported outcomes and follow-up duration. Country of research origin was classified into high-income and middle-income based on the definition by the World Bank[12]. De novo HEV infection is defined by post-transplant HEV infection in patients with negative pre-transplant HEV-immunoglobulin G (IgG), HEV-immunoglobulin M (IgM) or HEV-RNA. Acute HEV infection is determined by positive post-transplant HEV-IgM with or without positive HEV-RNA.

Measurements

The prevalence of HEV infection, prevalence of de novo HEV infection, and prevalence of acute HEV infection underwent meta-analysis and the results were reported in percentage along with 95% confidence interval (CI). Forest plot of each analysis was created. Results were presented in percentage for categorical data and in mean ± SD or median (interquartile range) for continuous data.

Network association

The prevalence of HEV infection in each organ transplant (heart, lung, liver, kidney, and undifferentiated) were individually compared using mixed-effects model. The association of each couple comparison was assessed with Cochrane's Q-test and its P value. P values less than 0.05 were considered statistically significant.

Subgroup analysis, meta-regression analysis, and publication bias

Subgroup analyses based on the following variables were performed: study year, country of origin, study design, sample size, mean age, male proportion, number of confirmatory tests used in the study, antibody assay, and follow-up duration. Mixed-effects model of analysis was used in subgroup analyses. Publication bias was evaluated by (1) Funnel plot (if the total number of studies was greater than ten[13]); and (2) Egger's regression intercept. An intercept P value of less than 0.05 was considered significant for potential publication bias. The quality of each study was appraised using the Newcastle-Ottawa quality scale[14].

Statistical analysis

All statistical analyses were performed by the Comprehensive Meta-analysis version 3 software (Eaglewood, NJ, United States) and SPSS version 23.0 (IBM Corp., Armonk, NY, United States). Statistical heterogeneity of the included studies was assessed using the Cochran's Q-test and I^2 statistics. An I^2 value of ≤ 25% represents insignificant heterogeneity, 25%-50% represents low heterogeneity, 50%-75% represents moderate heterogeneity, and > 75% represents high heterogeneity[15]. For analyses with I^2 > 50%, the results were analyzed by random-effects model to minimize the heterogeneity and external variance[16]. A P value of less than 0.05 represents statistical significance.

RESULTS

Study characteristics

Of 563 citations, a total of 20 studies consisting of 5842 subjects were included in this meta-analysis and systematic review. Figure 1 provides a flowchart of the literature search and study selection for this meta-analysis. Included studies were published from 2011 to 2020. The study design was retrospective (66.7%) and prospective (33.3%). The median age was 52.0 (11.9) years, 68.5% were male, and 27.7% were Caucasian. The median duration of follow-up was 13.7 (14.0) mo.

Prevalence of HEV infection

For the prevalence of HEV infection, a total of 18 studies (n = 4557) were included in the meta-analysis. Erken *et al*[17] was excluded as the authors only reported the prevalence of de novo HEV infection while Reekie *et al*[18] was excluded because of the

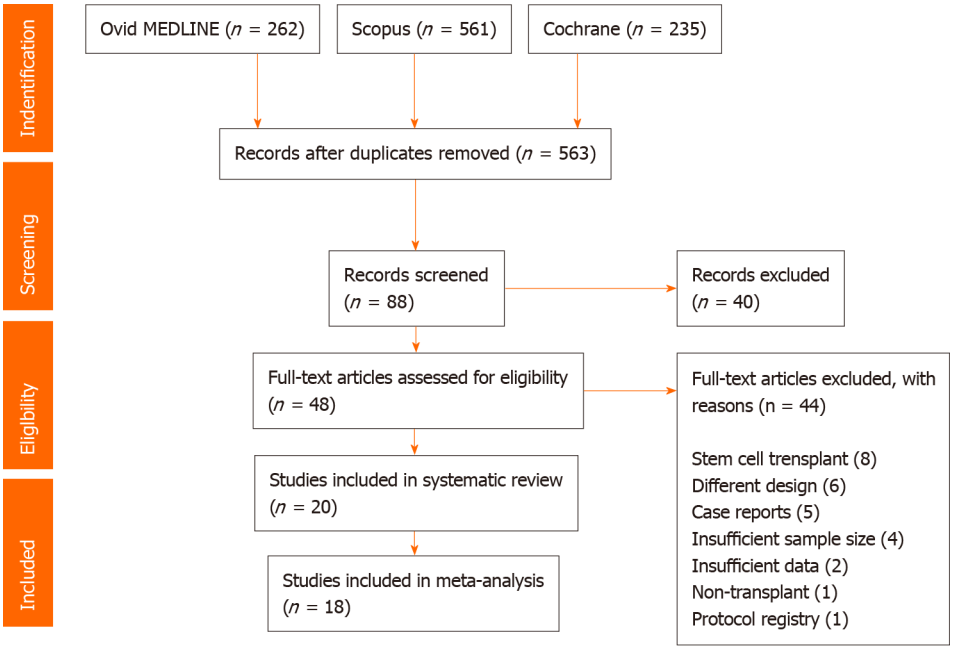


Figure 1 PRISMA flowchart of article search and selection.

potential risk of bias. All other articles had acceptable NOS scores (low risk of bias) for inclusion into meta-analysis for prevalence of HEV infection.

The pooled estimated prevalence of HEV infection in SOT recipients was 20.2% (95%CI: 14.9-26.8; I^2 = 95.3%; Figure 2A). The pooled estimated prevalence of HEV infection in each transplanted organ was: Liver (27.2%; 95%CI: 20.0-35.8; *n* = 11; *n* = 1887), kidney (15.3%; 95%CI: 6.6-31.5; *n* = 4; *n* = 1137), heart (12.8 %; 95%CI: 9.3-17.3; *n* = 1; *n* = 274), lung (5.6%; 95%CI: 1.6-17.9; *n* = 3; *n* = 625), and undifferentiated (29.6%; 95%CI: 10.1-61.1; *n* = 3; *n* = 634).

De novo HEV infection

A total of seven studies (*n* = 2004) were included in the meta-analysis of de novo HEV infection. The pooled estimated prevalence of de novo HEV infection in SOT recipients was 5.1% (95%CI: 2.6-9.6; I^2 = 90.8%). The forest plot is illustrated in Figure 2B.

Acute HEV infection

A total of nine studies (*n* = 1925) were included in the meta-analysis of acute HEV infection. The pooled estimated prevalence of acute HEV infection in SOT recipients was 4.3% (95%CI: 1.9-9.4; I^2 = 90.7%). The forest plot is illustrated in Figure 2C.

Network association analysis

We used subgroup analysis to compare the pooled estimated prevalence of HEV infection from two solid organs of interest at a time. Figure 3 depicts a diagram of the network association analysis. In brief, the prevalence of HEV infection in lung transplant was significantly lower than liver transplant patients (Q = 7.033, *P* = 0.008) and undifferentiated patients (Q = 4.322, *P* = 0.038). There were no statistically significant associations across all other comparisons.

Subgroup analyses

Subgroup analysis results are depicted in Table 1. Here, we analyzed the pooled estimated prevalence of HEV infection based on study characteristics. We applied mixed-effects model to minimize inter-study variance in the subgroup analyses. In brief, we found that the pooled prevalence of HEV infection was similar after adjustment for study year (< 2015 *vs* ³2015), study design (prospective *vs* retrospective), sample size (< 400 *vs* ³400), age (£ 50 years *vs* > 50 years), male proportion (£ 65% *vs* > 65%), number of confirmatory tests (> 1 marker *vs* single marker), and follow-up duration (£ 12 mo *vs* > 12 mo). Interestingly, we found that the prevalence of HEV infection was significantly higher in middle-income countries compared to high-income countries (41.8% *vs* 18.9%; Q = 22.375, *P* < 0.001). The seroprevalence of positive anti-HEV antibodies was significantly higher in studies that

Table 1 Subgroup analyses of all variables

Subgroup	*n* (%)	Incidence (%)	95%CI	
Year				
< 2015	10	17.1	9.9-27.9	
≥ 2015	8	26.8	19.5-35.7	Q = 2.248, *P* = 0.134
Country				
High-income	16	18.9	13.1-26.4	
Middle-income	2	41.8	37.6-46.1	Q = 22.375, *P* < 0.001[c]
Study type				
Prospective	6	22.3	13.4-34.7	
Retrospective	12	20.3	12.8-30.7	Q = 0.077, *P* = 0.782
Sample size				
n < 400	14	25.4	18.9-33.1	
n ≥ 400	4	10.4	3.2-28.8	Q = 2.613, *P* = 0.106
Mean age				
≤ 50 yr	3	17.7	5.4-44.8	
> 50 yr	10	20.2	13.4-29.3	Q = 0.051, *P* = 0.821
Male proportion				
≤ 65%	7	16.0	7.7-30.4	
> 65%	5	23.0	12.5-38.5	Q = 0.631, *P* = 0.427
Diagnostic test				
More than one marker	11	25.9	19-34.4	
Single marker	7	14.1	5.5-31.5	Q = 1.806, *P* = 0.179
Antibody assay				
Wantai assay	8	28.4	21.4-36.6	
Other assays	6	12.3	7.7-19.1	Q = 10.134, *P* = 0.001[b]
Follow-up				
≤ 12 mo	7	23.8	23.8	
> 12 mo	4	26.8	26.8	Q = 0.072, *P* = 0.789

[b]*P* < 0.01.
[c]*P* < 0.001. CI: Confidence interval; *n*: Number of studies.

utilized Wantai assay compared to studies with other assays (28.4% *vs* 12.3%; Q = 10.134, *P* = 0.001).

Evaluation for publication bias

The p-value of Egger's regression intercept for the analysis of pooled total prevalence of HEV infection, de novo HEV infection and acute HEV infection was 0.060, 0.054, and 0.136, respectively. These values indicated no potential publication bias. The funnel plot for the HEV pooled prevalence infection analysis in undifferentiated SOT recipients is illustrated in Supplementary Figure 1.

Systemic review

Table 2 illustrates study characteristics and outcomes included in this systematic review. Kamar *et al*[19] showed that the use of tacrolimus (OR 1.89; 95%CI: 1.49-1.97) and low platelet count (OR 1.02; 95%CI: 1.00-1.10) were associated with chronic HEV infection in SOT patients. Additionally, cirrhosis (OR 7.6; 95%CI: 4.4-13.1), liver transplantation (OR 3.1; 95%CI: 1.8-5.4) and Human immunodeficiency virus (HIV)

Table 2 Characteristics of studies included in the systematic review

Ref.	Country	Type	*n* (%)	Organ	Age	Male	White	HEV	Confirmed test	Acute HEV	Death	Outcome	Follow-up
Kamar *et al*[19], 2011	Monaco	R	85	UD	-	-	-	65.9% (total; 56/85). 32.1% (18/85) had clearance. 0% (0/18) reactivation	Persistently elevated LFTs and positive HEV-RNA	0%	-	Factors associated with chronic HEV infection: Tacrolimus (OR 1.89; 1.49-1.97). Low platelet (OR 1.02; 1.00-1.10)	6 mo
Legrand-Abravanel *et al*[35], 2011	France	R	700	Liver (*n* = 171), kidney (*n* = 529)	52	65.6%	-	15.1% (total; 106/700). 14.6% (25/171) for LTx. 15.3% (81/529 for KTx. 5.6% (de novo; 34/601). 0% (0/17) reactivation	Positive HEV IgG, IgM (Adaltis assay) or HEV-RNA	2.8% (17/611). 2.7% (4/150) for LTx. 2.8% (13/461) for KTx	-	-	22 mo
Pischke *et al* [22], 2012	Germany	P	274	Heart	57	80%	-	12.8% (total; 35/274). 7.3% (de novo; 20/274)	Positive HEV IgG (MP assay) or HEV-RNA	-	-	Heart transplant recipients had significantly higher seroprevalence of HEV-IgG than healthy individuals	8 mo
Crossan *et al*[21], 2014	Scotland	P	317	UD	56.4	93.4%	-	14.2% (total; 45/317)	Positive HEV IgG or IgM (Wantai assay)	0.9% (3/317)	-	Factors associated with chronic HEV infection: HBV coinfection (OR 7.4; 1.4-37). IgG positive is associated with HCC (OR 2.3; 1.1-4.8)	-
Mallet *et al* [36], 2013	France	R	267	Liver	-	-	-	31% (total; 83/267)	Positive HEV IgG (Wantai assay)	-	-	-	-
Pischke *et al*[37], 2014	Germany	R	95	Lung	-	-	-	5.3% (total; 5/95)	Positive HEV IgG (MP assay)	-	-	-	-
Riezebos-Brilman *et al*[38], 2013	Netherlands	R	468	Lung	40	60%	-	2.1% (total; 10/468)	Positive HEV-RNA	-	0.4%	-	6.5 mo
Abravanel *et al*[39], 2014	France	P	263	Liver (*n* = 52), kidney (*n* = 211)	53	64.3%	-	38.4% (total; 101/263). 42.3% for LTx (22/52). 37.4% for KTx (79/211). 1.9% (de novo; 3/162)	Positive HEV IgG, IgM (Wantai assay) or HEV-RNA	-	-	-	12 mo
Buffaz *et al* [40], 2014	France	R	206	Liver	41.1	63.0%	-	36.4% (total; 75/206). 5.3% (de novo; 11/206)	Positive post-transplant HEV IgG, IgM (Wantai assay) or HEV-RNA	-	-	-	32.8 mo
Riveiro-Barciela *et al*[20], 2014	Spain	R	625	Liver (*n* = 332), kidney (*n* = 296), dual (*n* =	54.5	60.8%	-	5.8% (total; 36/625). 3.7% (11/296) for KTx. 7.4% (25/332) for	Positive HEV IgG (MP assay)	-	-	Risk factors associated with HEV infection: Cirrhosis (OR 7.6; 4.4-13.1).	-

				6)				LTx				Liver transplantation (OR 3.1; 1.8-5.4). HIV infection (OR 2.4; 1.3-4.4)	
De Nicola *et al*[41], 2015	Italy	R	79	Liver	55	-	-	33% (total; 26/79). 5.7% (de novo; 3/53)	Positive HEV IgG, IgM (Wantai assay), HEV-RNA	0%	-	-	12 mo
Magnusson *et al*[42], 2015	Sweden	P	62	Lung	55	37.5%	100%	14.8% (total; 8/54). De novo N/A	Positive HEV IgG (recomWell assay)	25% (2/8)	17.8%	-	12 mo
Sherman *et al*[43], 2017	United States	P	171	Liver (*n* = 70), kidney (*n* = 101)	-	-	-	19.9% (total; 34/171). 21.4% (15/70) for LTx. 18.8% (19/101) for KTx	Positive HEV IgG (Wantai assay)	5.5% (3/55) for LTx. 0% for KTx	-	HIV-infected transplant recipients	24 mo
Erken *et al* [17], 2018	Netherlands	R	677	Kidney	-	-	-	0.7% (de novo; 2/300)	Positive HEV-RNA	-	-	Subjects are patients with ALT elevations	-
Reekie *et al* [18], 2018	United Kingdom	R	611	Liver (*n* = 262), kidney (*n* = 349)	-	-	-	0.5% (total; 3/611)	Positive HEV-RNA	-	-	-	36 mo
Samala *et al*[44], 2018	United States	R	232	Liver (*n* = 208), kidney (*n* = 10), both (*n* = 10), intestine (*n* = 4)	58	65%	70%	19.4% (total; 45/232)	Positive IgG, IgM (Wantai assay) or HEV-RNA	-	-	HEV seroprevalence was associated with older age and patients with the diagnosis of alcohol- or NAFLD-associated liver failure	-
Darstein *et al*[45], 2020	Germany	R	74	Liver	55	62.2%	-	28.8% (total; 21/73)	Positive HEV IgG (recomWell assay) or HEV-RNA	-	-	-	-
Komolmit *et al*[46], 2020	Thailand	P	108	Liver	58	69%	0%	44% (total; 48/108)	Positive IgG, IgM (Wantai assay) or HEV-RNA	2% (1/48)	0.9%	-	12 mo
Wang *et al* [23], 2020	China	R	408	Liver	50	81.1%	0%	41.2% (total; 168/408). 16.9% (de novo; 69/408)	Positive HEV-RNA more than 6 months	13.7% (56/408)	-	Alcoholic cirrhosis (OR 5.324; 1.36-20.98). Liver failure (OR 23.76; 2.78-203.08). Graft rejection (OR 0.217; 0.06-0.74)	13.7 mo
Zanotto *et al*[47], 2020	Italy	R	120	Liver	-	-	-	19.2% (total; 23/120)	Positive HEV IgG, IgM (N/A assay) or HEV-RNA	-	-	-	-

UD: Undifferentiated; HEV: Hepatitis E virus; LFTs: Liver function test; OR: Odds ratio; LTx: Liver transplant; IgG: Immunoglobulin G; MC: Multicenter; IgM: Immunoglobulin M; KTx: Kidney transplant; HCC: Hepatocellular carcinoma; HIV: Human immunodeficiency virus; ALT: Alanine aminotransferase; R: Retrospective; NAFLD: Non-alcoholic fatty liver disease; MP: Methylparaben.

infection (OR 2.4; 95%CI: 1.3-4.4) were significant risk factors for HEV infection in the Spanish cohort[20]. Another study[21] demonstrated that HBV coinfection was associated with chronic HEV infection in SOT recipients (OR 7.4; 95%CI: 1.3-37.0), and patients with positive HEV-IgG had higher odds of developing hepatocellular carcinoma (OR 2.3; 95%CI: 1.1-4.8). Pischke *et al*[22] emphasized the prevalence of HEV infection in

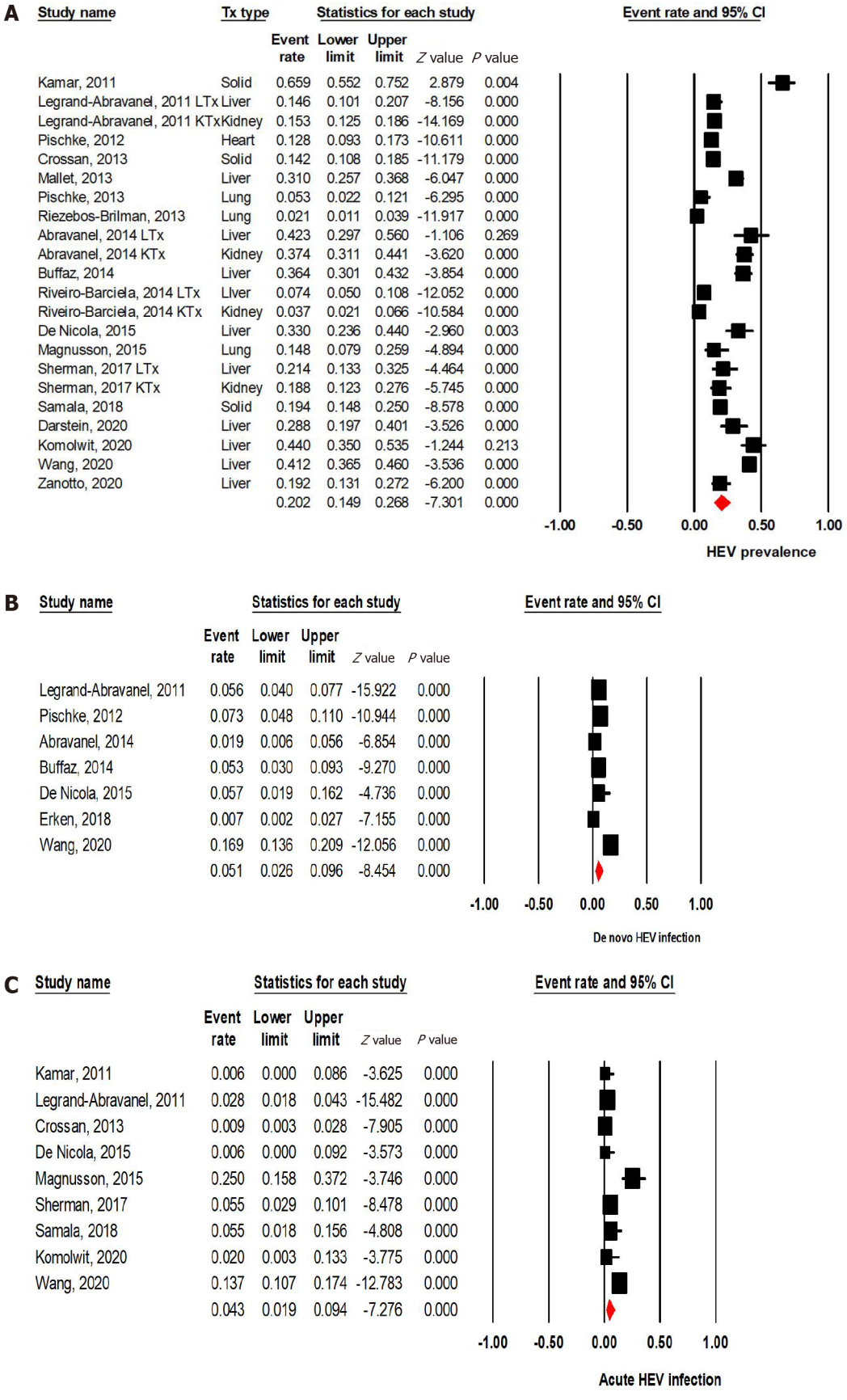


Figure 2 Forest plots of meta-analysis. A: The pooled prevalence of hepatitis E virus (HEV) infection (I^2 95.3%; Egger's intercept 0.060); B: The pooled prevalence of de novo HEV infection (I^2 90.8%; Egger's intercept 0.054); C: The pooled prevalence of acute HEV infection (I^2 90.7%; Egger's intercept 0.136). CI: Confidence interval; HEV: Hepatitis E virus.

heart transplant patients by demonstrating that these patients had a significantly higher seroprevalence of HEV-IgG than healthy individuals. Interestingly, in a Chinese cohort of 408 Liver transplant recipients[23], alcoholic cirrhosis (OR 5.3; 95%CI: 1.4-21.0) and liver failure (OR 23.8; 95%CI: 2.8-203.1) were associated with increased de novo HEV infection during a follow-up of 3 years while graft rejection (OR 0.22; 95%CI: 0.06-0.74) was surprisingly a protective factor.

DISCUSSION

The meta-analysis revealed prevelance of HEV in SOT recipients is 20%. De novo HEV infection and acute HEV infection accounted for less than 5% of infections. A recent meta-analysis of 419 studies comprised of 519,872 individuals showed an estimated global seroprevalence of anti-HEV IgG of 12.5% and a pooled estimated anti-HEV IgM seroprevalence of 1.5%[24]. Although our study did not provide a direct comparison to non-transplant patients, it can be extrapolated that the prevalence of HEV infection is higher in SOT patients (20.1% *vs* 12.5%). The prevalence of acute HEV infection was also higher in SOT patients compared to non-transplant patients (4.3% *vs* 1.5%). These findings emphasize the burden of HEV infection in SOT patients. To date, the United States has not issued national guidelines for the management of hepatitis E in SOT. However, recent guidelines from the British Transplantation Society have recommended screening for HEV infection in individuals with elevated liver enzymes (evidence 1D)[25]. Unfortunately, the evidence for this recommendation is relatively weak due to a lack of studies supporting the association between HEV infection and adverse post-transplant clinical outcomes. More studies on this particular topic are needed. Furthermore, our study indicated a high burden of de novo HEV infection and acute HEV infection in SOT patients. Whether these infections affect the post-transplant clinical outcomes different from chronic HEV infection is yet to be investigated.

It is possible that the seroprevalence of anti-HEV IgG could be affected by the assays used for antibody testing. Rossi-Tamisier *et al*[26] compared the positive rates of two different commercial microplate enzyme-immunoassays and found that the prevalence of seropositive IgG against HEV was higher in the Wantai assay compared to Adaltis assay[26]. Similarly, Li *et al*[24] conducted a meta-analysis and described that the seroprevalence of anti-HEV IgG was highest with the Wantai assay in comparison with other commercial assays[24]. In our subgroup analysis, we also observed that the seroprevalence of anti-HEV antibodies from studies that utilized the Wantai assay was significantly higher than other assays. Thus, the type of assay test should be taken into consideration when interpreting positive anti-HEV IgG or IgM results.

We also found that the prevalence of HEV infection was significantly higher in middle-income countries *vs* high-income countries. This finding is consistent with previously published. Li *et al*[24] suggested that the seroprevalence of anti-HEV IgG was at least two-fold higher in Africa and Asia in comparison to Europe and North America[24]. As HEV route of transmission *via* the fecal-oral route is similar to hepatitis A virus, patients with poor hygiene are predisposed to both hepatitis A and hepatitis E infection. Consumption of raw meat, exposure to soil, contact with dogs, residing in rural areas, and an education level attained less than elementary school is known risk factors for HEV infection[24]. However, our study did not include any articles that originated from low-income countries where the prevalence of HEV infection is anticipated to be high. This may be due to the lower rate of SOTs within this demographic. More studies from low- and middle-income countries are encouraged to reliably determine the global burden of HEV infection in SOT recipients.

We found that the prevalence of HEV infection was lowest in lung transplant recipients. It is unclear why lung transplant recipients had less HEV infection compared with liver transplant recipients. It is possible that the prevalence of HEV infection in lung transplant recipients is under-reported in the literature, given the smaller number of lung transplants annually, at least in the United States. The total number of lung transplants is three times fewer than the total number of liver transplants from the United States Organ Procurement and Transplantation Network[27]. However, it is also possible that lung transplant recipients may be predisposed to receiving ribavirin therapy for other indications, such as respiratory syncytial virus or hepatitis C virus infection. Ribavirin and interferon-a are two main antivirals that have been used to treat cases of HEV infection. There are several reports of successful use of ribavirin in chronic HEV infection to achieve overall sustained virologic response of up to 80%[28-30]. The underlying mechanism by which lung

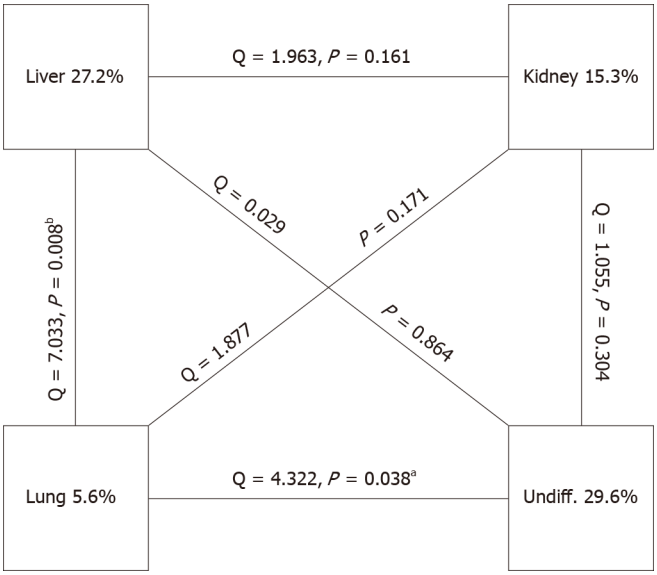


Figure 3 Network association analysis. [a]*P* < 0.05; [b]*P* < 0.01.

transplant patients had lower HEV infection should be investigated in future clinical studies.

Several risk factors for HEV infection in SOT patients have been identified from our systematic review. The use of tacrolimus (versus cyclosporine), low platelet count, cirrhosis, liver failure, human immunodeficiency virus (HIV) coinfection, and hepatitis B virus (HBV) coinfection are all significant risk factors for HEV infection. Hypothetically, tacrolimus generally delivers more immunosuppressive property than cyclosporine, which could predispose patients to contract HEV. This statement, however needs more supporting clinical evidence. Liver disease and associated manifestations including cirrhosis, liver failure, and low platelet count, are not specific to HEV infection; they may be attributed to HEV infection or one of many other etiologies of chronic liver failure. HIV and HBV coinfection raises concern for transfusion-associated HEV transmission, which has been reported in several studies worldwide[31-34].

Our study is subjected to certain limitations. First, all studies were observational in design, making them susceptible to selection bias. We attempted to minimize this bias by performing risk of bias assessment prior to inclusion of studies into our meta-analysis and systematic review. Second, the clinical impact of HEV infection was not meta-analyzed due to limited information from the original articles. More studies investigating the association between HEV infection and clinical outcomes are needed. Third, the genotype of HEV was not reported. Although it is well perceived that HEV genotype 3 and 4 are more common in immunocompromised patients[2], the prevalence of HEV genotype 3 and 4 infection in SOT patients remains inconclusive from our study. Fourth, only the status of recipients was evaluated in our study. HEV infection profile in donors was not taken into consideration due to the limited data in the original articles. HEV transmission *via* transplanted liver has been reported and would potentially impact the prevalence of HEV infection in the recipients. Fifth, generalization of our findings to heart transplant patients is limited because only one study included heart transplant patients. Finally, the majority of included studies were from high-income countries. Additional cohorts from low-income and middle-income countries are highly encouraged.

The future prospects include evaluation of the impact of HEV on SOT patients and graft analysis by meta-analysis or meta-regression analysis. Once the association between HEV infection and adverse clinical outcomes is conclusive, the role of ribavirin therapy for HEV eradication should be investigated in future clinical trials. The ultimate objective of this study is to help contribute to the core knowledge of improving the clinical outcomes of SOT recipients.

CONCLUSION

In conclusion, HEV infection is common in SOT recipients and accounts for 20.2%. It is at least two-fold higher in middle-income countries compared to high-income countries. The prevalence of HEV infection in lung transplant recipients is considerably less common than other organ transplants. More studies demonstrating the clinical impacts of HEV infection in SOT recipients, such as graft failure, rejection, and mortality, are warranted.

ARTICLE HIGHLIGHTS

Research background

Hepatitis E virus (HEV) infection among patients with pre-existing chronic liver disease and organ-transplant recipients on immunosuppressive therapy can result in decompensated liver disease and death.

Research motivation

The prevalence of HEV infection in solid organ transplant (SOT) recipients varies from one organ to another. The disease burden and clinical outcomes of HEV infection in such patients are under-investigated.

Research objectives

To demonstrate the prevalence of HEV infection in SOT recipients.

Research methods

Eligible articles were searched through Ovid MEDLINE, EMBASE, and the Cochrane Library. The inclusion criteria are adult patients with history of SOT. HEV infection is confirmed by either HEV-immunoglobulin G, HEV-immunoglobulin M, or HEV RNA assay.

Research results

Of 563 citations, a total of 22 studies (n = 4557) were included in the meta-analysis. The pooled estimated prevalence of HEV infection in SOT patients was 20.2% (95%CI: 14.9-26.8). The pooled estimated prevalence of HEV infection in each organ transplant was as followed: liver (27.2%; 95%CI: 20.0-35.8), kidney (12.8%; 95%CI: 9.3-17.3), heart (12.8%; 95%CI: 9.3-17.3), and lung (5.6%; 95%CI: 1.6-17.9). The comparison across all organ transplant was statistically significant (Q = 16.721, P = 0.002). The subgroup analyses showed that the prevalence of HEV infection among SOT recipients was significantly higher in middle-income countries compared to high-income countries. The pooled estimated prevalence of de novo HEV infection was 5.1% (95%CI: 2.6-9.6) and the pooled estimated prevalence of acute HEV infection was 4.3% (95%CI: 1.9-9.4).

Research conclusions

HEV infection is common in SOT recipients, especially in middle-income countries. The prevalence of HEV infection in lung transplant recipients is considerably less common than other organ transplants.

Research perspectives

The results of this study offer a preliminary perspective on the magnitude of disease burden from HEV infection, especially in middle-income countries. In the future, large-scale observational studies investigating these associations between HEV infection, patient outcomes, and allograft outcomes are needed to help guide the management of HEV infection in SOT recipients. We also highlight the need for studies from low-income and middle-income countries, as the prevalence of HEV infection from these countries is under-reported.

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REVIEW

Coronavirus disease–2019 and the intestinal tract: An overview

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Abstract

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection can progress to a severe respiratory and systemic disease named coronavirus disease–2019 (COVID-19). The most common symptoms are fever and respiratory discomfort. Nevertheless, gastrointestinal infections have been reported, with symptoms such as diarrhea, nausea, vomiting, abdominal pain, and lack of appetite. Importantly, SARS-CoV-2 can remain positive in fecal samples after nasopharyngeal clearance. After gastrointestinal SARS-CoV-2 infection and other viral gastrointestinal infections, some patients may develop alterations in the gastrointestinal microbiota. In addition, some COVID-19 patients may receive antibiotics, which may also disturb gastrointestinal homeostasis. In summary, the gastrointestinal system, gut microbiome, and gut-lung axis may represent an important role in the development, severity, and treatment of COVID-19. Therefore, in this review, we explore the current pieces of evidence of COVID-19 gastrointestinal manifestations, possible implications, and interventions.

Key Words: COVID-19; SARS-CoV-2; Gastrointestinal; Microbiota; Antibiotics

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Core Tip: Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection can progress to a severe respiratory and systemic disease named coronavirus disease–2019 (COVID-19). Nevertheless, SARS-CoV-2 can also generate a gastrointestinal infection. In this review, we explore the impact of COVID-19 on the gastrointestinal system, gut microbiome, and the gut-lung axis and the severity and possible implications and interventions in COVID-19 patients.

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INTRODUCTION

The coronavirus disease–2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Coronaviruses (CoVs) are a family of single-stranded ribonucleic acid (RNA) viruses; currently six subtypes of CoVs can infect humans. Two CoVs, the Middle East respiratory syndrome coronavirus and severe acute respiratory syndrome coronavirus-1 (SARS-CoV-1), are the etiologic agents of the previous epidemics and have caused over 2000 deaths worldwide[1,2].

COVID-19 can cause a systemic and respiratory infection that can lead to death[3]. Since November 2019, SARS-CoV-2 has infected over 80 million people and killed over 1.5 million people worldwide, being declared a pandemic by the World Health Organization[4]. Several comorbidities have been postulated as risk factors for severe COVID-19, such as high age[5], smoking, chronic obstructive pulmonary disease[6], obesity[7], pregnancy[8], and co-infections[9].

SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) to invade the host's cells. ACE2 and TMPRSS2 are expressed in many different organs in the human body, such as the lungs, heart, liver, kidney, and brain, intestine luminal cells, colonic epithelial cells, and small intestinal enterocytes[10-13]. In addition, SARS-CoV-2 infection has been described in multiple organs, including the lungs, pharynx, heart, liver, brain, kidneys, and gastrointestinal tract[14,15].

COVID-19 AND THE GASTROINTESTINAL TRACT

Gastrointestinal infections have been reported[16] (Table 1), with a lower frequency in comparison with the previous SARS-CoV-1 infection[17]. Nevertheless, SARS-CoV-2 can remain positive in fecal samples after nasopharyngeal clearance and may remain infective[18].

The most frequent gastrointestinal symptoms in COVID-19 are diarrhea, nausea, abdominal pain, and lack of appetite[19]. After a viral gastrointestinal infection, some patients may develop alterations on the gastrointestinal microbiota such as an increase of Proteobacteria and a simultaneous reduction of Bacteriodetes[20]. A recent report by Zuo *et al*[21] identified alterations in the intestinal microbiota of hospitalized patients with COVID-19, with a reduction in beneficial commensals bacteria and an increase in opportunistic pathogens[21]. In addition, Gupta *et al*[18] verified a reduction in the community richness and microbial diversity in COVID-19 patients with and without diarrhea. The intestinal tissue and feces may also be acting as a reservoir; recent reports have identified that even after negative nasopharyngeal and oropharyngeal swabs test for SARS-CoV-2 RNA, patients could still possess SARS-CoV-2 RNA in the stool samples[16,22]. Another report identified that the duration of COVID-19 symptoms was prolonged in patients with diarrhea and that the stool samples from patients with diarrhea were more frequently positive for virus RNA[23].

Although is not clear the mechanisms responsible for the development of diarrhea in COVID-19, the current hypothesis is that the direct viral infection on the intestinal tissue and local immune response to the virus may be involved. In fact, the detection

Table 1 Manuscripts describing patients with severe acute respiratory syndrome coronavirus-2 ribonucleic acid detection in rectal swabs or fecal samples

COVID-19 respiratory manifestations	COVID-19 gastrointestinal clinical manifestations	Percentage of patients with gastrointestinal clinical manifestations	Rectal swabs or fecal samples positive for SARS-CoV-2	Ref.
Yes	Yes	65.38%	53.42%	[16]
Yes	Yes	60%	50%	[122]
Yes	Yes	80%	90%	[123]
Yes	Yes	11%	22%	[124]
Yes	Yes	33%	80%	[125]
Yes	Yes	No data	39%	[126]
Yes (89 only respiratory/69 respiratory and gastrointestinal)	Yes (48 only gastrointestinal/69 respiratory and gastrointestinal)	56%	54% of a cohort of 22 individuals	[127]
Yes	Yes	No data	29%	[128]
Yes	Yes	31%	55%	[24]
Yes	Yes	No data	83%	[129]
Yes	Yes	No data	25%	[130]

COVID-19: Coronavirus disease–2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2.

of SARS-CoV-2 RNA in stool COVID-19 patients may indicate the possibility of fecal-oral transmission[24].

In addition to the SARS-CoV-2 impact on the gut immune response, bacterial co-infections and secondary infection could also occur in COVID-19 patients, implicating the necessary usage of antibiotics[25]. Nevertheless, even in the absence of bacterial co-infections in COVID-19 patients some reports highlighted that the usage of antibiotics is a common clinical practice in COVID-19 patients[25,26], which could also disrupt the gastrointestinal microbiome. Bacterial communities are present in numerous body sites such as the gut, the respiratory system, and skin; therefore, the unnecessary usage of antibiotics may predispose COVID-19 patients to opportunistic infections inside and outside of the gastrointestinal environment.

GUT MICROBIOTA

The microbiota is influenced by environmental factors, food, drugs, and infections[27]. Each microbiota possesses unique characteristics[28,29], differing its composition according to the site in the human body. Many factors influence the microbiome composition such as local pH, temperature, and nutrients[30]. Microorganisms can be found in nearly every niche of the human body[31], but the gastrointestinal tract is the largest interface between the host and microorganisms in the human body. There are approximately 10^{13}-10^{14} microorganisms in the gastrointestinal tract, with greater genomic content than in the human genome[32].

Microbes and humans have a symbiotic relationship. Commensal microbes are crucial for human health, regulating many physiological functions, degradation of substances, production of metabolites, and immune response[33]. Microorganisms can activate and stimulate the differentiation of T helper cells (Th) 1, Th2, Th17, and T regulatory cells (Treg), which in consequence can regulate the immune response[34,35]. A low-diversity in the intestinal microbiota can increase the susceptibility to local[36] and pulmonary disorders[37].

The microbiome's environment is in constant regulation, modulated by external microorganisms and other non-bacterial compounds, for example, food in the intestinal microbiota and viruses. An abrupt change in the microbiota can generate an imbalance in the commensal bacteria and/or increase opportunistic microbes, increasing the susceptibility to diseases[38,39].

The microbiota is essential for the development of the human immune system and can influence both local and non-local immune responses, such as the gut-lung axis[40].

It is well established that alterations in the gut microbiota can modulate the development of respiratory disease[41].

In patients with gut dysbiosis such as patients with an established intestinal inflammatory disease or obese patients, the intestinal microbiota may be a secondary risk factor for the development of severe COVID-19[7]. Besides, patients with COVID-19 may develop a dysbiosis in the gastrointestinal microbiota[21] and a reduction in short-chain fatty acid-producing bacteria[42].

GUT MICROBIOTA DYSBIOSIS

Disruption of the gut microbiota can trigger inflammatory events that are associated with metabolic dysfunction, obesity, cancer, and neurological disorders[43,44]. The proliferation or reduction in certain microorganisms can increase the stimulation of innate immune receptors, like nucleotide-binding oligomerization domain-like receptors and Toll-like receptors[45]. The stimulation of this receptor triggers several pro-inflammatory signals and the production of cytokine and chemokine, which modulate the adaptive immune system, influencing both local and systemic immune response[43,44].

The activation of Toll-like receptors on immune cells by the microbiota can generate a low-grade systemic inflammation in the host that is associated with a change in metabolic and immunological responses[46]. Alterations in the microbiome are related to the development of diseases such as obesity, inflammatory bowel disease, and cancer[47]. Therefore, SARS-CoV-2 gastrointestinal infections and alteration of gut homeostasis may be implicated in the development of disease and impact immune response to oral vaccines and medicines and the pathogen immune response[48,49].

NUTRITIONAL INTERVENTION

Several reports have highlighted the potential role of nutrients in the modulation of the immune response to SARS-CoV-2 or a direct anti-viral and/or anti-SARS-CoV-2 properties[50-53]. During this pandemic, nutritional aspects such as obesity[7,54], malnutrition[55], and micronutrient deficiency[56,57] have been postulated as risk factors for severe COVID-19. Nevertheless, the composition of the human microbiota is influenced by many factors, including dietary components[41]. Some bacteria can ferment nondigestible carbohydrates (prebiotics), like soluble fibers, to produce short-chain fatty acids (SCFAs). SCFAs can stimulate the growth and/or activity of commensal bacteria and are associated with health benefits. SCFAs can induce the regulation of the intestinal barrier, reduce oxidative stress, control diarrhea, and modulate intestinal motility and also induce a local and systemic anti-inflammatory effect[58,59].

High-fiber diets can induce the proliferation of beneficial commensal microbes, such as *Lactobacillus* spp. and *Bifidobacterium* spp. in the gastrointestinal tract[60]. In fact, high-fiber diets may increase immunoglobulin A production and modulate the secretion of interferon-gamma and interleukin (IL)-10[61-63], which could aid in the control of gastrointestinal infections.

Prebiotics may alter the microbiota composition by a mechanism called cross-feeding, when the product of a prebiotic's fermentation by a microorganism in the microbiota can be used as a substrate by another microorganism[64-66]. Another mechanism that prebiotics can alter the microbiota is through pH alterations. The fermentation products are predominantly acids, which may cause a decrease in the intestinal pH, restraining the growth of acid-sensitive bacteria, such as *Bacteroides* spp., and promoting butyrate-producing bacteria[67].

SCFAs are divided into acetate, propionate, and butyrate. All SCFAs have potential anti-inflammatory effects with the reduction of prostaglandin E2 and inflammatory cytokines[68]. Acetate can curb the activation of the NLR family pyrin domain containing 3 inflammasome[69]; propionate can inhibit histone deacetylase and reduce lipopolysaccharide-induced inflammation[70]. Butyrate has been associated with anti-cancer properties and reduces pulmonary inflammation[71,72].

Overall, research has demonstrated a potential anti-inflammatory role for SCFAs in both local (intestinal) and non-local inflammation *via* direct anti-inflammatory effects or modulation of the microbiota[71,73,74].

SCFAs can induce the release of anti-inflammatory cytokines such as IL-10[75,76], promoting the regulation of Th cells and inflammatory diseases[77], including

inflammatory bowel disease[78].

Another intervention for the modulation of the gastrointestinal microbiome is *via* the consumption of probiotics. Probiotics are bacteria that can be ingested and provide a beneficial interaction to the host[79]. Several studies have investigated the effects of probiotics on the gut microbiota[80], with conflicting results involving their ability to graft on the commensal microbiota[81-84]. However, probiotics can produce metabolites that can modify and influence the commensal microbiota, intestinal barrier, and immune system[85,86].

Probiotics can also aid in the prevention or treatment of bacterial[87] and viral infections[88]. The administration of probiotics increases the survival of mice infected with the influenza virus[87]. Besides the influenza virus, studies have demonstrated beneficial protection against respiratory syncytial virus infection[89].

The heath benefit of probiotics in respiratory viral infections is due to the modulation of cytokine production and oxidative stress[90]; therefore, they may possibly be an adjuvant treatment for the aberrant release of pro-inflammatory cytokines, chemokines, and oxidative stress during severe COVID-19[91].

The most used probiotics are *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*[92]. Although there is extensive research demonstrating their health benefits, currently there is a gap in knowledge involving the ideal dosage and comparison among strains of probiotics[93].

DISCUSSION

COVID-19 is a potentially deadly disease, which can infect intestinal cells[13]. SARS-CoV-2 gastrointestinal infections[16] can generate diarrhea, pain, and vomiting[19]. To date, few reports have investigated the possible consequences of gastrointestinal infection by SARS-CoV-2; nevertheless, viral infections can alter the gastrointestinal microbiota[20]. A report by Xu *et al*[94] identified a reduction in *Lactobacillus* and *Bifidobacterium* in fecal samples from COVID-19 patients[94]. Also, the microbiome of COVID-19 patients can be disturbed by the necessary or unnecessary use of antibiotics[34].

Yeoh *et al*[95] identified that the alteration on the gastrointestinal microbiome in COVID-19 patients was independent of medications. The alterations on the gut microbiome included a reduction in *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and *bifidobacteria* for up to 30 d after SARS-CoV-2 clearance[95].

The microbiota dysbiosis in COVID-19 may be involved in the inflammatory response and may be a persistent problem after COVID-19 resolution, indicating a possible role for nutritional interventions to curb the inflammatory response and reestablish the gastrointestinal homeostasis of COVID-19 patients.

Dietary and nutritional intervention can modulate the immune response, increasing or dampening the anti-viral response[59-62]. Western-style diets (low fiber content) can increase *Bacteroidetes* and reduce *Firmicutes*[96] and are linked to the development of obesity[97], a risk factor for severe COVID-19[7]. Although reports have identified an increase in SCFAs in fecal samples from obese individuals[96], SCFAs have been associated with control of appetite[98] and increase energy expenditure[99].

In addition, very low fiber-diets can lower mucus production on the intestine and increase the susceptibility to gastrointestinal infections[39]. Importantly, a change in diet can modify the microbiota composition[100]. The microbial communities are in constant change and are also affected seasonally by food consumption[101]. In fact, a reduction in the consumption of fiber can change the microbiota in as little as 1 d, reducing SCFAs production[102].

In opposition, high fiber-diets increase *Firmicutes* and *Actinobacteria* on the gut microbiota[103] and increase the production of SCFAs, which can aid in the reduction of pulmonary inflammation, *via* the gut-lung axis[41,104,105] and promote a local and systemic anti-inflammatory response *via* IL-10 production and Treg cells[75,76,106]. The ingestion of probiotics may stabilize or alter the gastrointestinal microbiome, especially after a perturbation of the microbiota such as post usage of antibiotics or gastrointestinal infections[107].

Probiotic treatments with *Bacillus subtilis* and *Enterococcus faecalis* have been demonstrated to reduce ventilator-associated pneumonia[108]. Treatment with *Lactobacillus rhamnosus* can reduce ventilator-associated pneumonia and *Clostridium difficile*-associated diarrhea in mechanically ventilated patients[109], making it a possible addition to the treatment of patients with severe COVID-19 in intensive care units with assisted mechanical ventilation. Treatment with *Lactobacillus* may be of particular

importance, because respiratory infections may cause a reduction in *Lactobacillus*, and an increase in *Enterobacteriaceae* and intestinal IL-17 inflammation[110].

Targeting IL-17 has been postulated as a treatment for COVID-19[111] because of the increase in IL-17 in severe COVID-19 patients compared to moderate COVID-19 patients[111]. IL-17 and IL-17-producing T helper cells (Th17), type three innate lymphoid cells, invariant natural killer cells, and γδ T cells are involved in the immune response of COVID-19[112]. IL-17 receptor is expressed on the surface of many different cells such as neutrophils, eosinophils, epithelial cells, keratinocytes, and fibroblasts[112]. In addition, IL-17 can directly influence the expression of ACE2, SARS-CoV-2 entry's receptor[113].

The usage of IL-17 blockade, such as monoclonal antibodies against IL-17A and/or IL-17 receptor A, may represent a possible therapeutic option for COVID-19[112]. Nevertheless, IL-17 is an important cytokine in the immune response against *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, common pathogens in respiratory and intestinal tract infections[112,114]. Secondary bacterial infections can occur in the respiratory system following SARS-CoV-2 infection, especially in patients with invasive mechanical ventilation[34]. IL-17 is especially important for intestinal homeostasis[115]. Therefore, treatment with anti-IL-17 should consider the possible risk for an increase in susceptibility for bacterial infections both respiratory and intestinal.

COVID-19 patients may also develop a cytokine storm syndrome, which may induce multi-organ failure and lead to death or long-term consequences[91]. In this context, probiotics or prebiotics treatment have been previously demonstrated anti-inflammatory effects in respiratory infections *via* the increase in SCFAs[71,73,74,88,116].

SCFAs production and health benefit can be increased by the ingestion of highly fermentable fiber diets[117], probiotics[73], oral administration of drugs like tributyrin (a prodrug of butyrate)[118], or SCFAs directly[119-121].

In this context, the intake of prebiotics and/or probiotics can represent a significant prophylactic intervention and/or recovery of COVID-19 patients.

CONCLUSION

The SARS-CoV-2 infection on the gastrointestinal tract and the long-term consequences of COVID-19 in gastrointestinal homeostasis still needs further investigations. It is clear that SARS-CoV-2 can infect the gastrointestinal tract and impact the intestinal immune response and the gut microbiome. Currently, there is no specific treatment for COVID-19, but investigations on the impact of nutritional intervention *via* modulation of the immune response or *via* microbiota are been investigated and may represent a significant prophylactic intervention and/or recovery of COVID.

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REVIEW

Metabolic complications of hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) infection is a systemic disease that is implicated in multiple extrahepatic organ dysfunction contributing to its protean manifestations. HCV is associated with diverse extrahepatic disorders including atherosclerosis, glucose and lipid metabolic disturbances, alterations in the iron metabolic pathways, and lymphoproliferative diseases over and above the traditional liver manifestations of cirrhosis and hepatocellular carcinoma. The orchestration between HCV major proteins and the liver-muscle-adipose axis, poses a major burden on the global health of human body organs, if not adequately addressed. The close and inseparable associations between chronic HCV infection, metabolic disease, and cardiovascular disorders are specifically important considering the increasing prevalence of obesity and metabolic syndrome, and their economic burden to patients, the healthcare systems, and society. Cellular and molecular mechanisms governing the interplay of these organs and tissues in health and disease are therefore of significant interest. The coexistence of metabolic disorders and chronic hepatitis C infection also enhances the progression to liver fibrosis and hepatocellular carcinoma. The presence of metabolic disorders is believed to influence the chronicity and virulence of HCV

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leading to liver disease progression. This comprehensive review highlights current knowledge on the metabolic manifestations of hepatitis C and the potential pathways in which these metabolic changes can influence the natural history of the disease.

Key Words: Chronic hepatitis C infection; Insulin resistance; Metabolic syndrome; Cardiovascular diseases; Fatty liver; Diabetes mellitus

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Core Tip: Available evidence proves a strong association between hepatitis C virus (HCV) and metabolic complications such as hyperlipidemia, hepatic steatosis, insulin resistance, metabolic syndrome, and diabetes mellitus. *De novo* development of insulin resistance and hepatic steatosis in chronic HCV infection influences the disease progression in the liver and enhances overall morbidity and mortality. The influence of metabolic diseases on HCV infection can increase disease severity. The interplay between HCV major proteins and the liver-muscle-adipose axis is complex and still not fully elucidated. Coexistence of metabolic diseases such as diabetes mellitus and HCV infection are also known to result in adverse outcomes of both disorders. There is evidence that successful treatment halts the progression of liver disease, but more studies are required on how treatment influences the metabolic manifestations.

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INTRODUCTION

Hepatitis C virus-beyond a hepatotropic infection

Hepatitis C virus (HCV) is a major cause of liver disease worldwide, with 130-170 million people infected according to the World Health Organization[1]. Approximately 10%-20% of chronically infected patients experience persistent inflammation and develop liver cirrhosis and eventually hepatocellular carcinoma[1]. Well recognized extrahepatic manifestations include but not limited to; glucose and lipid metabolic disorders, atherogenic disease, mixed cryoglobulinemia, lymphoproliferative disorders, renal disease, insulin resistance (IR), type 2 diabetes (T2DM), sicca syndrome, rheumatoid arthritis-like polyarthritis, and autoimmune diseases[2,3]. Moreover, patients with chronic HCV are at an increased risk of developing metabolic bone disease and osteopenia as it was observed in more than 50% of infected subjects[4]. The deleterious effects of metabolic complications as a result of HCV infection is mainly due to impairments in glucose and lipid metabolism[5]. Independent of the stage of hepatic fibrosis, IR and DM are more prevalent in the course of HCV infection and post liver transplantation in chronic HCV (CHC) infected patients[6-8]. Interestingly, prevalence of HCV infection among diabetic patients is higher than in the age-matched general population[9]. CHC induces systemic and hepatic inflammation that contribute to the development of atherosclerosis through elevated levels of pro-atherogenic cytokines and chemokines[10]. Atherosclerosis is also found to be related to the high tumor necrosis factor alpha (TNF-α)/adiponectin ratio that is found in HCV-infected patients and is related to IR[11].

The systemic burden of hepatitis C infection surpasses its liver disease burden due to its metabolic spectrum and it is plausible that, the virus, through disruptions of glucose and lipid homeostasis, stimulates other mechanisms of liver damage and contributes to the pathogenesis of extrahepatic disorders[12]. Therefore, it is imperative to have thorough understanding on the metabolic impact of this enigmatic disease on human body for optimal management of the disease and its consequences. This evidence-based review highlights the current understanding on CHC infection and its metabolic complications.

IMPACT OF CHRONIC HEPATITIS C ON MAJOR METABOLIC ORGANS

Most HCV infected people are unaware of their condition due to its asymptomatic nature. About one-third of infections resolve spontaneously in the first year, the remaining infections persists and become chronic. CHC infection can progress to end-stage liver disease, including cirrhosis and hepatocellular carcinoma (HCC). HCV can also cause serious problems in organ systems other than the liver, including cryoglobulinemic vasculitis, metabolic bone disease, kidney disease, cardiovascular disease, and hematologic malignancies. In the United States, HCV is one of the leading causes of end-stage liver disease requiring liver transplantation, and the mortality attributed to HCV is expected to continue rising during the next 10 years. Figure 1 illustrates the HCV-related metabolic disturbances mainly targeting the liver, skeletal muscle and adipose tissue as current evidence suggests[13].

METABOLIC EFFECTS OF CHRONIC HEPATITIS C INFECTION ON LIVER

Hepatic steatosis in the setting of HCV viral infection is considered as a distinct entity with specific clinical and prognostic implications. The prevalence of hepatic steatosis in patients with HCV infection is 55.54%, which remains higher than that in non-infected individuals[14]. Contrary to non-alcoholic fatty liver disease being associated with hyperlipidemia, CHC is linked with hypolipidemia including hypo-cholesterolemia, hypo-triglyceridemia and lower low-density lipoprotein (LDL) cholesterol levels. Co-existence of non-alcoholic fatty liver disease in HCV infected patients is associated with features of metabolic syndrome (MetS), and is an independent risk factor for advanced liver fibrosis[15-17].

In patients with CHC, genotype 3 has the highest prevalence of hepatic steatosis (40%-86%) while patients with other genotypes is around 50%[18,19]. A direct association exists between genotype 3 HCV (G3-HCV) and the development of steatosis, while in non-genotype 3 CHC, IR plays a key role in the pathophysiology of hepatic steatosis[20,21].

The term "viral steatosis" is used particularly with G3-HCV infection when hepatic steatosis is related with viral load and not MetS and "metabolic steatosis" occurs secondary to IR/MetS in the genotypes G1, G2, and G4[22-24]. Studies in patients infected with G3-HCV have demonstrated that steatosis can improve and even disappear following successful antiviral treatment with interferon and ribavirin; but data from directly acting antivirals (DAAs) are limited[25,26]. The risk of progression of fibrosis is increased with pre-existing steatosis, and a reduced rate of response to antiviral treatment[27-29]. CHC is implicated in the development and progression of non-alcoholic steatohepatitis (NASH). NASH is a chronic state of liver injury that can be caused by CHC infection or coexist with HCV and biopsies have proven that the association leads to advanced fibrosis and is a predictor of liver disease progression in patients with CHC regardless of the genotype[30,31]. Both "viral" and "metabolic" steatosis, stimulates the progression of fibrosis and liver disease influenced by actions of increased insulin levels and inflammatory cytokines on hepatic stellate cells (Figure 2).

HEPATITIS C VIRUS AND IRON METABOLISM

Iron is a central component for HCV virus replication and translation, though it is debatable whether iron promotes or suppresses HCV viral replication. High serum ferritin levels, associated with hepatic iron overload, were evident in CHC patients and considered an independent risk factor for advanced liver fibrosis[32,33]. Alterations of iron metabolism in CHC is believed to be caused by a reduction in the level of hepcidin. Hepcidin, a peptide hormone, is critical in the strict regulation of iron levels under homeostatic states. Though the underlying mechanisms remains unclear, the current opinion propose that all HCV proteins regulates hepcidin expression through signal transducer and activator of transcription 3, mitogen-activated protein kinase, or bone morphogenetic protein/Sma and Mad proteins signaling pathways, and the altered expression of other related genes[34]. Due to the ability of different factors influencing the levels of serum ferritin levels, it serves as an important indicator of hepatic iron overload but only a liver biopsy can confirm the diagnosis. It is vital to further our understanding on how HCV affects iron metabolism and whether it can be used as a therapeutic tool to prevent disease progression.

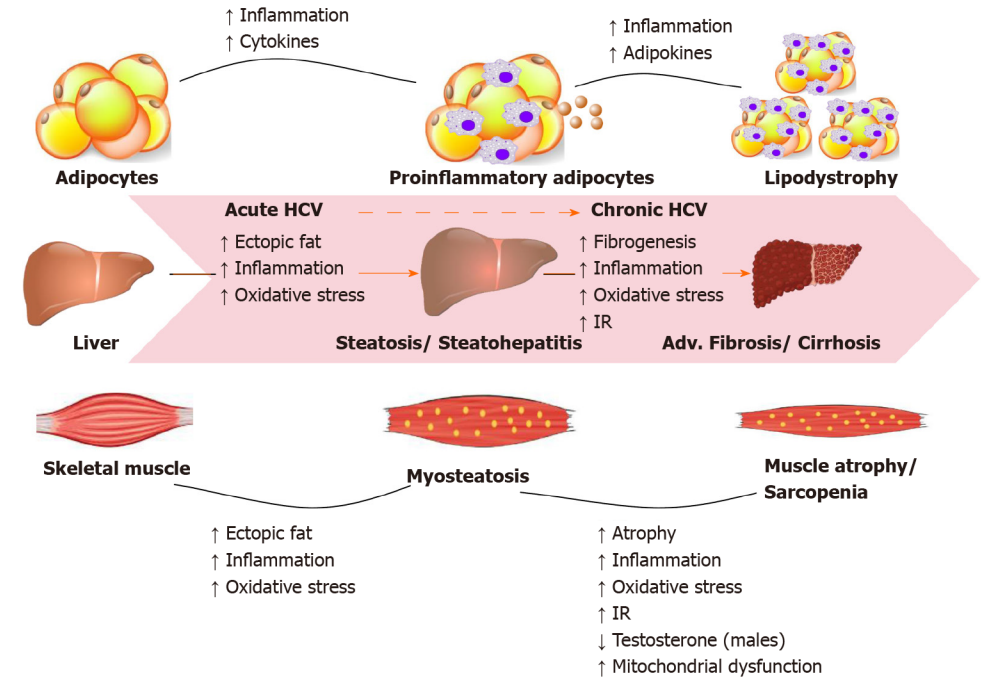


Figure 1 Changes in liver, adipose tissue, and muscle with hepatitis C virus infection. Adv: Advanced; HCV: Hepatitis C virus; IR: Insulin resistance.

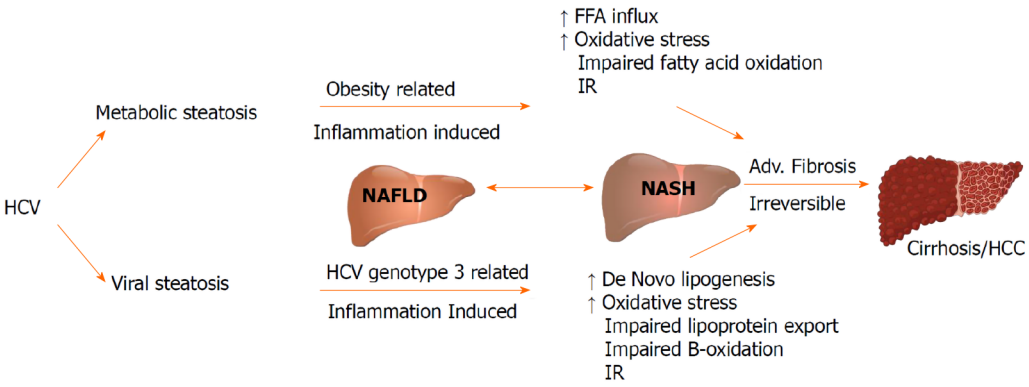


Figure 2 Hepatic steatosis development in hepatitis C virus infection. Adv: Advanced; HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease; FFA: Free fatty acid; IR: Insulin resistance; HCC: Hepatocellular carcinoma.

EFFECTS OF CHRONIC HEPATITIS C INFECTION ON SKELETAL MUSCLE

CHC infection is associated with skeletal muscle mass loss, sarcopenia, increased intramyocellular lipid deposition, myosteatosis, and low muscle strength[35-37]. A recent Japanese study by Fukui *et al*[38] demonstrated that skeletal muscle mass decreases in accordance with liver disease progression in male patients with CHC. Sarcopenia, loss of muscle mass and function, has a high incidence rate in patients with cirrhosis (30%-70%) favoring atrophy of type II fast-twitch glycolytic fibers[39,40]. Myosteatosis, is characterized by increased lipid accumulation within muscle fibers and is a complication of hepatitis C Cirrhosis predisposing individuals to muscle atrophy over time[41]. High BMI, IR, diabetes, steatosis, inflammation, increased oxidative stress and lipotoxicity are all independent risk factors that predispose CHC patients to skeletal muscle disorders[42-47]. Therefore, a comprehensive sarcopenia evaluation assessing skeletal muscle mass, muscle strength and physical performance can provide clinicians the optimal risk assessment tools and therapeutic strategies for improving patient's quality of life and to halt the disease progression.

It is widely recognized that testosterone is highly associated with increased muscle

strength and mass[48]. Chaudhury *et al*[49] demonstrated that male CHC patients with hepatic dysfunction/cirrhosis suffered from low levels of free and total testosterone regardless of the treatment regimens (interferon and ribavirin (6%); interferon, ribavirin, and DAAs (18%); ribavirin and DAAs (14%); and DAAs only (62%)) received by patients[50]. The decrease in testosterone was sustained after viral clearance suggesting that testosterone metabolism in the liver is most likely impaired by liver dysfunction in these patients. Neff *et al*[51] was able to show that administration of testosterone hormone to HCV-patients suffering from liver failure increases muscle strength, enhance albumin synthesis, and improve survival post-liver transplant[49,52-54].

The association between CHC infection of the liver as well as hepatic dysfunction and muscle loss is well documented, though not fully understood. The triad complex association can be linked through increased inflammation, oxidative stress, alterations in the endocrine system (IR, testosterone levels), hepatic steatosis, and multiple factors involved in muscle depletion. A better understanding of the etiology of sarcopenia and skeletal muscle mass loss in HCV infected patients and the appropriate management strategies are expected to improve patient outcomes.

HEPATITIS C VIRUS INFECTION AND ADIPOSE TISSUE

Following liver and skeletal muscles, adipose tissue is another major site of IR in CHC infections[51]. Adipose tissue is considered as an important endocrine organ which releases biologically active polypeptides including adipose tissue specific adipokines like leptin and adiponectin and non-adipose tissue specific adipokines such as plasminogen activator inhibitor I (PAI-1)[55]. Adiponectin (an insulin-sensitizing hormone in muscle and liver) and leptin are most abundant in subcutaneous fat while PAI-1 is found in high levels in extracellular matrix[56,57]. Together, leptin, adiponectin and PAI-1 are the abundant adipokines which regulate the body lipid and glucose metabolism *via* the adipo-insular axis[58]. Alterations in adipokine levels/function are a major culprit for multiple complications including the risk of developing T2DM, cardiovascular disease and neurodegenerative disorders.

An interplay between HCV virion and adipocytes is suggested by a strong relationship between HCV viral load and subcutaneous (not visceral) fat[59]. Patients with CHC have significant subcutaneous adipose tissue insulin resistance in comparison with BMI-matched controls. Lim *et al*[58] suggested that that viral eradication improves global, hepatic, and adipose tissue insulin sensitivity. Serum levels of adiponectin and leptins even though associated with IR, HCV associated IR is predominantly a cytokine-independent direct virus-specific effect[60].

Though data is limited, new adipokines have an important role in the regulation of insulin sensitivity in CHC. Vaspin (visceral adipose tissue-derived serpin; seroinA12) has been found to improve insulin sensitivity and glucose tolerance and down regulate TNF-α synthesis[61-63]. The levels of serum vaspin are significantly decreased in chronic HCV infected patients without advanced fibrosis and increased in cases of advanced fibrosis suggesting vaspin to be a compensatory mechanism switch in HCV associated IR. However, no such association was found between serum vaspin level and viral load or homeostatic model assessment of IR values[64]. The level of another adipokine, visfatin, also increases in CHC and is inversely associated with inflammatory activity[65]. Visfatin exerts insulin like effect stimulating insulin receptor substrate-1 (IRS-1) phosphorylation and the peroxisome proliferator-activated receptors (PPARγ) expression but it also potentiates expression of interleukin-6 and TNF-α like adhesion molecules[65,66]. Chimerin, another adipokine thought to be related to IR in CHC infection, inhibits the synthesis of TNF-α and interleukin-6 and ameliorates IRS-1 phosphorylation, to improve insulin sensitivity of adipocytes and to enhance adiponectin synthesis[67,68].

HEPATITIS C VIRUS AND LIPID METABOLISM

HCV regulates the host factor diacylglycerol acyltransferase-1 to promote the biogenesis of lipid droplets, and the HCV core protein recruit nonstructural protein 5A (NS5A) that carries HCV ribonucleic acid from the replication complex on the endoplasmic reticulum-derived membranous web to lipid droplets[69]. The enveloped viral particles may be simultaneously packaged into endoplasmic reticulum luminal lipid droplets in the very low-density lipoprotein cholesterol (VLDL) precursor and eventually the VLDL-dependent pathway secretes it into the circulation as lipo-viral

particles[70,71]. Therefore, HCV infection is implicated in disrupted lipoprotein homeostasis due to impairment of the VLDL-releasing pathway which is one of the driving mechanisms of hepatic steatosis[72]. Derangement of lipid metabolism in HCV infection plays a role in hepatic steatosis, dyslipidemia, and hypobetalipoproteinemia. The HCV core and NS5A proteins interact with apolipoprotein (apo) A, apo A II and apo E. An increased levels of ApoC-III has been found in HCV lipo-viral particles that may inhibit the activity of lipoprotein lipase, disturbing the intravascular catabolism of triglyceride-rich lipoproteins[73-75]. HCV may modulate lipid metabolism and promote the synthesis of saturated fatty acids by regulating the enzymes involved in lipid synthesis[76,77]. Poly-unsaturated fatty acids supplementation counteracts the effects of HCV-induced lipid alterations and inhibits HCV formation[78]. Previous treatment with interferon-based antiviral therapy could reverse the hypocholesterolaemia induced by HCV. It has been shown that cholesterol and low-density lipoprotein cholesterol (LDL-C) levels rapidly recover following viral clearance or sustained virologic response indicating that large amounts of lipids released from the liver into blood circulation may increase levels of cholesterol and LDL-C, resulting in the hydrolysis of triglycerides[79]. More studies are needed to delineate the pathways and potential therapeutic targets.

CHRONIC HEPATITIS C VIRUS AND GLUCOSE METABOLISM

CHC infection is closely associated with alterations of glucose metabolism from the early stages of infection, prior to the development of significant hepatic fibrosis[80]. In patients infected with HCV, total serum fasting blood glucose levels are found to be higher than that in healthy controls[81]. Clinical studies, both prospective and retrospective, showed a two-fold increased risk of developing insulin resistance and T2DM in HCV-infected patients even after correction of confounding factors[82].

The mechanism by which HCV causes glucose metabolism derangement resulting in T2DM is not fully understood. The proposed mechanisms from experimental studies in mice suggest downregulation of glucose transporter 2 receptors causing impaired hepatic glucose uptake, down-regulation of insulin receptor substrate 2 expression [suppression of cytokine signaling (SOCS)-3-dependent mechanism] causing altered hepatic insulin receptor cascade and impaired insulin-driven hepatic neoglucogenesis switch to glycolysis, as well as a defective insulin-driven shutdown of gluconeogenesis resulting in higher endogenous glucose production. Also alterations of the forkhead box O1 phosphorylation and nuclear exclusion by HCV proteins is implicated in T2DM development[83].

There is some evidence to suggest that HCV infected individuals are at risk of developing Type 1 diabetes mellitus through autoimmunity with some studies pointing towards pancreatic autoimmunity developing after Interferon treatment[84]. Many epidemiologic studies have found an association between T2DM and HCV infection possibly due to an interplay of multiple factors including direct viral effects, cytokine milieu, IR *etc*.[85] T2DM can accelerate the natural course of HCV-induced liver disease causing higher risk of fibrosis, cirrhosis, and hepatocellular carcinoma[86]. In patients with CHC infection and T2DM, significant improvement was noted in their diabetes status after HCV eradication with treatment[87-90]. The patients who did not achieve sustained virological response had a higher risk of T2DM[91-96]. Figure 3 illustrates the deleterious effects of HCV on glucose and lipid metabolism.

HEPATITIS C VIRUS AND INSULIN RESISTANCE: CENTRAL AND PERIPHERAL PATHWAYS

HCV proteins that are mainly involved in IR include core proteins, Serine protease and nonstructural viral proteins NS5A and NS5B that contribute to the formation of capsid, down-regulation of interferon-stimulated genes and polymerase activity. HCV induces IR both through multiple pathways and appears to depend on viral load and specifically genotypes G 1, 2 and 4[97].

In the direct mechanism, HCV proteins interact with various components of the insulin signaling pathway disrupting the signaling process of insulin in the hepatocytes. This leads to overexpression of protein phosphatase 2A and SOCS-3, and down regulates the expression of PPAR and IRS causing IR directly. HCV infection enhances hepatic gluconeogenesis through forkhead box O1-dependent pathway and

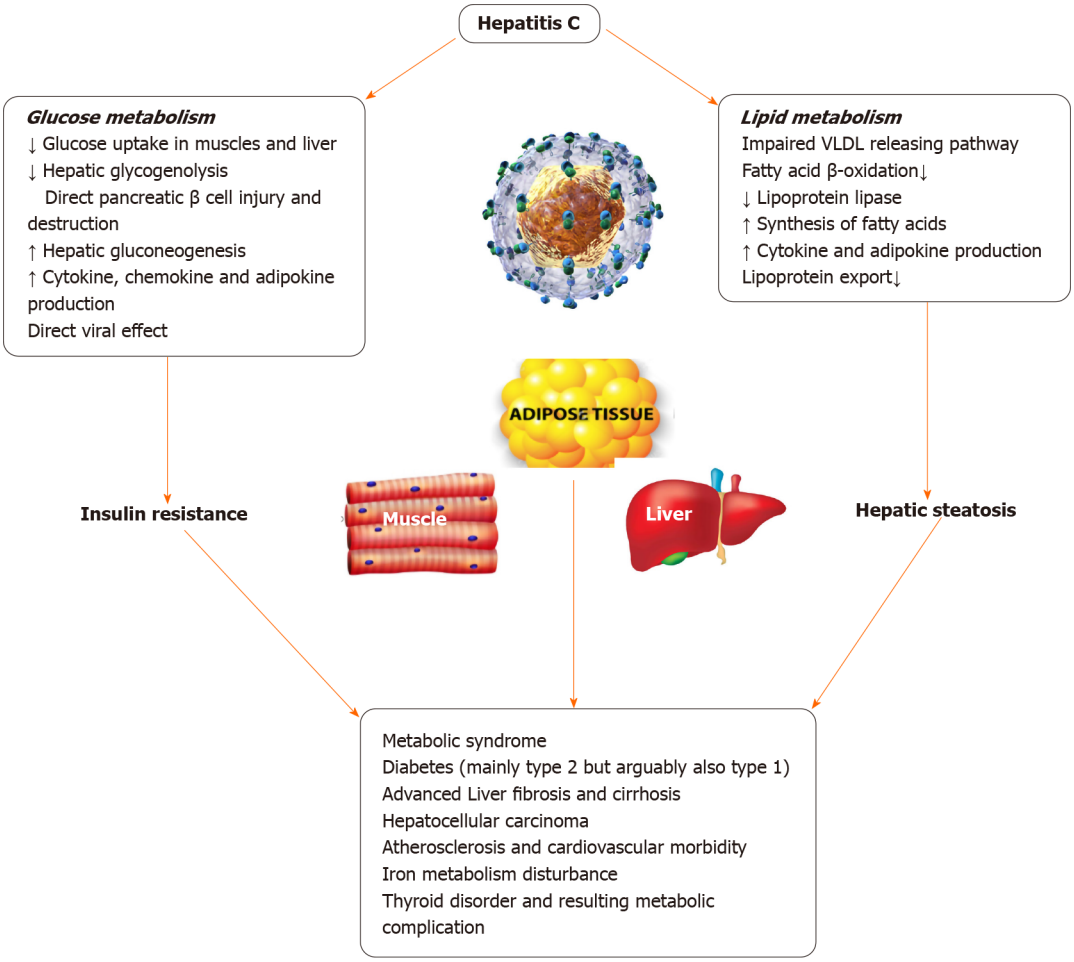


Figure 3 Impact of hepatitis C virus on glucose and lipid metabolism.

suppresses the cell surface expression of glucose transporter 2, resulting in reduced glucose uptake with the help of HCV NS5A protein that plays important roles in these two independent pathways. Any disruption in insulin signaling results in various pathophysiological changes, including glucose intolerance, obesity, dyslipidemia, hypertension, and development of T2DM[98-100]. The mechanism of insulin impairment appears HCV genotype specific, as core protein expression of G3 led to upregulation of SOCS-7 and downregulation of PPARγ, while the core protein from G1 activated mammalian target of rapamycin and induced phosphorylation of IRS-1 at inhibitory serine residues[101]. However, activation of SOCS family appeared to be common mechanism for all major genotypes to induce IR, including genotype 1 as the mammalian target of rapamycin activating variant appeared to be infrequent among known isolates[102-107].

Peripheral IR is the deficits in insulin induced glucose uptake into target tissues (adipose and muscle tissues). IR in CHC infection is thought to primarily affect muscle tissue making it a notable paradigm of IR. Mangia *et al*[106] showed that peripheral insulin resistance and resulting T2DM does not develop for at least first 5 years of infection with HCV suggesting the role of chronic infection in the development of IR rather than acute infection[107]. The pathways of lipid metabolism derangements and IR induced by HCV proteins and host cells opens up potential future treatment options scope for more studies[107,108].

The main PPAR nuclear receptors expressed in the liver that regulates glucose and lipid metabolism, influence cellular differentiation and proliferation as well as regulate the inflammatory process, include PPARα and PPARγ alongside retinoid X receptor. PPARα gene expression in the liver is decreased by 86% in the course of CHC infection[109]. Studies demonstrated a sharp decrease in the hepatic PPARγ expression in G3 CHC patients compared to those with G1 HCV[109-114].

The major pathways by which CHC infection induce insulin resistance are shown in the Table 1.

Table 1 The role of major hepatitis C virus proteins in insulin resistance development

HCV core protein	Nonstructural protein 3 (NS3)	Nonstructural protein 5 (NS5)
Activates members of SOCS family; Genotype 1: Activates mTOR and induces phosphorylation of IRS-1; Genotype 3: Upregulates SOCS-7 and downregulates PPARγ	NOX2 activation	Increasing the ROS within the mitochondria
↓ PPARα gene expression in the liver	↑ ROS	Induces ER stress → ↑ protein phosphatase 2A (PP2A)
↓ Assembly of VLDL	Through ROS → Activation of transcriptional factors such as NF-κB and STAT-3 → more advanced stages of chronic hepatitis → oncogenesis	Dephosphorylation and inactivation of Akt
Induces lipogenesis and gluconeogenesis		Stimulates the NF-κB-mediated increase in proinflammatory cytokines
↓ IFN-α production; ↓ IFN-α stimulated genes		Up-regulation of PP2Ac → hypomethylation of STAT-1 → ↑ association of STAT-1 with PIAS1PIAS1 → impairing the transcriptional activation of IFN-stimulated genes
Activation of the pattern-recognition receptor TLR2 → ↑ production of profibrotic factors. *i.e.* TGF-β, procollagen 1, and MMPs		Stimulates the NF-κB → increase in proinflammatory cytokines (*i.e.* IL-6, TNF-α)
Activates the PA28γ → ↓ IRS-1 tyrosine phosphorylation and IRS-2 expression and TNF-α promoter activation		Dephosphorylation of PKB/Akt → inhibition of insulin signaling
Induces TNF α → portal or periportal inflammation		
Impedes insulin-mediated FoxO1 translocation affecting glucose metabolism		

Akt: Protein kinase B; PKB: Protein kinase B; ER: Endoplasmic reticulum; FoxO1: Forkhead box protein O1; IFN-α: Interferon alpha; IL-6: Interleukin-6; IRS-1: Insulin receptor substrate 1; IRS-2: Insulin receptor substrate 2; MMPs: Matrix metalloproteinases; mTOR: Mammalian target of rapamycin; NF-κB: Nuclear factor kappa light chain; NOX2: NADPH oxidase 2; NS3: Serine protease; NS5: Nonstructural viral proteins NS5A and NS5B; PA28γ: Proteasomal activator; PIAS1: Protein inhibitor of activated STAT 1; PP2Ac: Catalytic subunit of protein phosphatase 2A; ROS: Reactive oxygen species; SOCS: Suppressors of cytokine signaling; SOCS-7: Suppressor Of cytokine signaling 7; STAT-1: Signal transducer and activator of transcription 1; TGF-β: Transforming growth factor beta; TNF-α: Tumor necrosis factor a; TLR2: Toll-like receptor 2; VLDL: Very low density lipoprotein.

HEPATITIS C VIRUS AND METABOLIC SYNDROME

According to the Joint Scientific Statement in 2009, patients who exhibit three of the five following characteristics are diagnosed with the metabolic syndrome (MetS): (1) Abnormal waist circumference as population and country-specific definitions; (2) Systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg or currently taking blood pressure-lowering agents; (3) High-density lipoprotein of cholesterol < 40 mg/dL in males or < 50 mg/dL in females; (4) Fasting blood sugar ≥ 100 mg/dL or currently taking diabetes medications; and (5) Triglyceride ≥ 150 mg/dL[115,116]. Both CHC infection and the MetS have common basic pathogenesis which is glucose metabolic derangement and IR resulting in close association and overlapping manifestations[117]. Amongst HCV infected patients, particularly older subjects, the prevalence of MetS ranges from 13.2% to 31.5%[118]. The prevalence increases in patients with CHC, older than 60 years of age, having an odds ratio of eight times of developing MetS than those under 40 years of age. A study by Lonardo *et al*[118] exploring the association between HCV genotypes and MetS prevalence found no significant difference compared to controls[119]. This could be due to the small sample size and patient selection criteria.

More studies show that African Americans infected with HCV are more prone to develop MetS than other ethnic groups[120,121]. An aggressive and severe liver disease is common in CHC patients with MetS. Liver fibrosis is notably more advanced in CHC patients with MetS than those without[122]. The prevalence of liver cirrhosis in CHC-MetS patients was 30% compared to 18.4% in CHC patients without MetS[123,124]. As MetS may worsen the progression of liver diseases in HCV infected patients, further research is needed to assess the impact of viral clearance and sustained virologic response on MetS.

IMPACT OF METABOLIC DISEASE ON CHRONIC HEPATITIS C DISEASE PROGRESSION

It is crucial to understand the impact of underlying metabolic diseases on the natural history of CHC infection as much as to understand the risk of developing metabolic complications from CHC. Given the rising prevalence of obesity and metabolic syndrome observed in the United States and globally, recognition of the impact of obesity and IR in disease progression among patients with CHC is particularly important[125].

In a recent systemic review by Dyal *et al*[124] 20 cohort studies were identified to evaluate the impact of metabolic diseases on disease progression among patients with CHC[125]. The authors evaluated the effect of obesity, diabetes, and steatosis as risk factors for developing advanced fibrosis in patients with chronic HCV infection and demonstrated that the presence of concurrent diabetes among patients with CHC infection was associated with a significantly higher risk of developing advanced fibrosis, with effect measures ranging from odds ratios of 2.25 to 9.24. Hepatic steatosis was also strongly associated with an increased risk of developing advanced fibrosis, with odds ratios of 1.80 to 14.3[126]. However, further studies are needed to assess the association between obesity and advanced fibrosis in CHC patients.

Available evidence aimed to better clarify the impact of concurrent metabolic diseases on disease progression and the natural history of chronic HCV infection; however, several limitations while interpreting the results must be acknowledged. The standard method for exploring the effects of metabolic diseases on disease progression would be to recognize patients with pre-existing metabolic diseases who eventually acquired HCV infection. However, a number of these studies were observational in nature and either utilized a case-control or retrospective cohort study design, which inherently limit the true ability to understand causal links. Significant advancements in HCV therapy were introduced subsequent to these studies which may influence the metabolic milieu and disease progression differently and we need more evidence before we extrapolate the current data to the presently available regimens.

The presence of metabolic diseases may influence the sequela of HCV infection. Patients with diabetes, hepatic steatosis, NASH, and insulin resistance were at more risk of developing CHC compared to patients without underlying metabolic disorders[127,128]. These patients were also found to have a faster progression to liver fibrosis through the same mediators that are inducing inflammation. A study by Paradis *et al*[129] investigated the risk of developing HCC in patients with underlying metabolic syndrome disorders and found there is a profound role of MetS in HCC development and progression. This can be further aggravated by the presence of HCV. The presence of NASH is a major contributor to liver cirrhosis in the presence/absence of HCV[130,131]. These findings elucidate the strong association between MetS disorders, HCV and their complex involvement in liver disease progression. This evidence contributes to a shift in HCC etiology which signifies further research.

MANAGEMENT OF METABOLIC COMPLICATIONS IN CHRONIC HEPATITIS C

Advances in treatment options with highly effective DAAs therapy for CHC aimed for eradication of HCV will not only improve HCV-related liver disease but will most likely impact the incidence and prevalence of HCV-related metabolic diseases, given the strong association between the two conditions. The efficacy of current regimens for CHC has improved the sustained virologic response reaching almost > 95%-100%. It is important to know whether the patient is treatment naïve or experienced, the genotype, the fibrosis level, and underlying comorbidities for personalized medicine and best tailored treatment options.

Concurrent management of coexistent metabolic disorders is also important for optimal control of CHC related liver injury and complications such as HCC as mentioned earlier. However, a detailed discussion of management of these conditions is beyond the scope of this review.

CONCLUSION

Hepatitis C infection has emerged as a systemic infection with impacts beyond the primary site of infection, causing a wide range of clinical manifestations in patients. It is crucial to understand the systemic effects of CHC along with its hepatic complications as it relates to metabolic manifestations and complications. HCV virion exploits the multiple functions of lipid droplets to sustain its multiplication and replication within host cells. The orchestration between HCV major proteins and the liver-muscle-adipose axis, poses a major burden on all the health systems of human body if not adequately addressed. The close and inseparable associations between chronic HCV infection, metabolic disease, and cardiovascular disorders are specifically important considering the increasing prevalence of obesity and metabolic syndrome and their economic burden to patients, the healthcare systems, and society. Cellular and molecular mechanisms governing the interplay of these three organs in health and disease are therefore of significant interest. As indicated by the extent of the metabolic comorbidities discussed, HCV increases the disruption in the liver–muscle–adipose triangle resulting in myriad clinical outcomes in patients. Further studies are needed to assess the association between metabolic derangement and patient quality of life. Physicians are encouraged to pay close attention to the metabolic parameters in HCV patients especially cirrhotics and patients listed for liver transplant.

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MINIREVIEWS

Artificial intelligence for early detection of pancreatic adenocarcinoma: The future is promising

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a worldwide public health concern. Despite extensive research efforts toward improving diagnosis and treatment, the 5-year survival rate at best is approximately 15%. This dismal figure can be attributed to a variety of factors including lack of adequate screening methods, late symptom onset, and treatment resistance. Pancreatic ductal adenocarcinoma remains a grim diagnosis with a high mortality rate and a significant psychological burden for patients and their families. In recent years artificial intelligence (AI) has permeated the medical field at an accelerated pace, bringing potential new tools that carry the promise of improving diagnosis and treatment of a variety of diseases. In this review we will summarize the landscape of AI in diagnosis and treatment of PDAC.

Key Words: Pancreatic adenocarcinoma; Artificial intelligence; Neural network; Future perspectives; Early diagnosis; Improved performance

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Core Tip: Pancreatic adenocarcinoma is one of the deadliest malignancies in the world. Several factors are responsible for this but delayed diagnosis is one of the most important. Despite improvements in diagnostic methods, early lesions are still missed in clinical practice. Artificial intelligence (AI)-assisted diagnostic methods have the potential of improving the clinical outcomes of these patients. However, major improvements in AI technology and its implementation need to occur before potential

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INTRODUCTION

Current modalities for the diagnosis and treatment of pancreatic ductal adenocarcinoma (PDAC) remain disappointing. With an overall survival rate of 3%-15%, it is one of the deadliest malignancies in the world[1]. Although currently it is the 7th leading cause of cancer death worldwide, recent trends suggest that in North America and Europe PDAC will soon become 2nd and 3rd respectively[2,3]. Several factors contribute to the poor survival statistics of PDAC: Lack of adequate screening tests, delayed diagnosis, and sub-optimal treatment options. Consequently, improvements in all these areas are desperately needed.

Recent technological advances have led to the increased application of artificial intelligence (AI) in different disciplines. Because computers can store and analyze larger amounts of data than the human brain, AI has the potential to achieve unmet needs in medicine. Since improving outcomes for PDAC is an area of urgent need, this review will provide a summary of current and future applications of AI in the diagnosis and management of PDAC.

AI, MACHINE LEARNING, AND ARTIFICAL NEURAL NETWORKS

AI is a branch of computer science dedicated to developing models aimed at performing functions comparable to those accomplished by the human brain. Machine learning (ML) is the area of AI that deals with developing computer models capable of learning specific tasks through the repetition of calculations derived from large amounts of data[4]. These computer models analyze data through repetitive calculations using mathematical self-derived algorithms that are constantly adjusted until the model produces the desired outcome. Once the combination of adjustments necessary to achieve the outcome has been discovered, the computer then "learns" how to perform that specific task[5].

Broadly speaking, ML can be either supervised or unsupervised. The difference lies in whether the desired outcome of interest is previously known by the computer. In supervised learning, a computer is first introduced to a training dataset (the "input") as well as the desired outcome of interest (the "output"). The computer then analyzes the input making the necessary adjustments to the algorithm until it consistently produces the desired output[6]. This type of learning requires large amounts of training data that has been pre-labeled ("curated") by a human operator. Once the training of the machine is completed, a different dataset is used to test its performance (testing data). In unsupervised learning, the computer is introduced to unlabeled data. The machine then sorts it using the algorithm to identify features within it that can be grouped and analyzed further to reach a specific outcome[7]. Because the data in unsupervised ML is not curated, larger amounts of training data are required than for supervised ML.

To date, most of the ML used in medicine has been supervised; and it has been made possible due to the emergence of a relatively new discipline called radiomics. Radiomics studies the conversion of digital medical images into data that can be then subjected to statistical analysis[8]. Using computer technology, predefined quantitative features are extracted from computed tomography (CT), magnetic resonance Imaging (MRI) or other imaging modalities. An essential step prior to this data extraction however, is lesion "segmentation". Segmentation is in simple terms, delineation of the lesion within an image. In supervised ML a human operator delineates the lesion prior to the algorithm operating on the data. In unsupervised ML, the algorithm learns how to segment the lesion of interest by itself.

Once the quantitative information of the lesion in the image is extracted, it is

analyzed utilizing artificial neural networks (ANN). An ANN is a group of interconnected computers with a structure similar to a neural network in the human brain. Each computer represents a neuron or "node", and each connection a synapse or "weight" (Figure 1). ANNs are organized in "layers" (groups of nodes) and the most basic model contains three: (1) Input layer (which receives the data), (2) Hidden layer (which performs the calculations and analyses), and (3) Output layer (which produces the final output)[9]. Deep neural networks contain more than one hidden layer and therefore can learn to analyze data with higher complexity levels; this is termed "deep learning"[10]. The different layers work in a hierarchical structure to produce the desired output.

AI-ASSISTED ANALYSIS OF ENDOSCOPIC ULTRASOUND IMAGES

Endoscopic ultrasound (EUS) is currently one of the most useful imaging modalities in the diagnosis of PDAC. The overall sensitivity of EUS guided biopsies for the diagnosis of PDAC reaches 98%, but its specificity can be as low as 20%[11]. The accuracy of EUS depends on both operator and lesion-related factors. The most important operator-related factor is the amount of experience performing EUS. On occasions, variations in gastroduodenal anatomy, (*i.e.* after partial gastric resection, or presence of a duodenal stricture) can significantly limit the ability of operator to visualize the pancreas. On the other hand, lesion-related factors include patient's body habitus, the presence of acute inflammation (when EUS is done immediately after an episode of acute pancreatitis) or the presence of chronic pancreatitis (CP), particularly in the presence of parenchymal calcifications. Sensitivity of EUS for PDAC in the presence of CP can be as low as 54%[12,13]. In addition, CP and autoimmune pancreatitis can occasionally form pseudotumors, which may complicate image analysis by the endosonographer.

Several studies have reported on the application of AI in the analysis of EUS images of pancreatic diseases[14-22] (Table 1). For the most part, these studies have focused on evaluating the accuracy of ANNs in differentiating CP from PDAC. Norton *et al*[14] analyzed still EUS images previously selected by experts who did not perform the procedure and were blinded to the final diagnosis using an ANN. A total of 21 patients with PDAC and 14 with CP were included. Four features were analyzed in each image by the ANN, achieving an overall accuracy of 89%.

In a similar study, Das *et al*[17] retrospectively analyzed the performance of an ANN in differentiating PDAC from normal pancreas and CP. A total of 56 patients (22 normal, 12 CP and 22 PDAC) were studied. Their AI algorithm identified PDAC with an area under the curve of 0.93. The differences in accuracy in this study may have been secondary to the more stringent criteria in the definition of CP and the higher number of image features analyzed by the ANN. A larger study performed by Zhu *et al*[18], analyzed 262 patients with PDAC and 126 with CP and reported that their algorithm reached an overall accuracy of 94%.

Elastography is an imaging method developed to establish the differences in consistency ("strain") between normal and abnormal tissue during EUS. Such differences are portrayed in a color-coded overlay on the EUS image, with red correlated with softer tissue and blue with harder[19]. A multicenter study reported an overall accuracy, sensitivity and specificity of 84%, 88% and 83% respectively in differentiating PDAC from CP[20]. This study used ANN analysis of histograms from elastography images previously selected by experts blinded to the patients' diagnoses. Another similar small study of 68 patients reported an accuracy rate of 90%[21].

Contrast agents have also been developed to aid in the differentiation between PDAC and CP. Săftoiu *et al*[22] reported that ANN analysis of contrast-enhanced EUS images could establish a difference with an area under the curve of 94%. Few studies have focused on using standard EUS B mode images to diagnose pancreatic tumors in the absence of CP. These have reported accuracies up to 99%[15,16].

AI-ASSISTED ANALYSIS OF COMPUTERIZED TOMOGRAPHY IMAGES

Computerized tomography (CT) is perhaps the most common medical imaging modality being explored with AI. The analysis of CT images of neoplastic lesions involves three main steps: detection, characterization and monitoring of change over time[23]. Most of the available studies have focused on AI-assisted characterization of lesions, which is equivalent to the previously defined concept of segmentation. Three

Table 1 Studies exploring artificial intelligence in the diagnosis of pancreatic ductal adenocarcinoma

Ref.	Study design	Data source	AI instrument	Patient	Aim	Accuracy
Norton *et al*[14], 2001	Retrospective	Standard EUS	ANN	21	PDAC *vs* CP	89%
Ozkan *et al*[15], 2015	Retrospective	Standard EUS	ANN	332	PDAC *vs* Nl	89%-92%
Zhang *et al*[16], 2010	Retrospective	Standard EUS	ANN	216	PDAC *vs* Nl	98%
Das *et al*[17], 2008	Retrospective	Standard EUS	ANN	56	PDAC *vs* Nl *vs* CP	93%
Zhu *et al*[18], 2013	Retrospcective	Standard EUS	ANN	388	PDAC *vs* CP	94%
Săftoiu *et al*[20], 2012	Prospective	EUS w/ elastography	ANN	258	PDAC *vs* CP	91%
Săftoiu *et al*[21], 2008	Prospective	EUS w/elastography	ANN	68	PDAC *vs* CP	90%
Săftoiu *et al*[22], 2015	Prospective	EUS w/contrast	ANN	167	PDAC *vs* CP	95%[1]
Fu *et al*[24], 2018	Retrospective	CT	ANN	59	Pancreatic tumor segmentation	76%[1]
Chu *et al*[25], 2019	Retrospective	CT	Computer derived forest algorithm	380	PDAC *vs* Nl	99%
Liu *et al*[26], 2019	Retrospective	CT	ANN	338	PDAC *vs* Nl	76%
Chu *et al*[29], 2019	Retrospective	CT	ANN	456	Segmentation of PDAC *vs* Nl	94%
Devi *et al*[32], 2019	Retrospective	MRI	ANN	168	Nl *vs* Abnormal pancreas	96%
Gao *et al*[33], 2020	Retrospective	MRI	ANN	504	Identify pancreatic disease	77%
Liang *et al*[34], 2020	Retrospective	MRI	ANN	27	Segmentation of panc tumors	Not explicitly stated
Muhammad *et al*[42], 2019	Retrospective	Clinical variables	ANN	800114	PDAC prediction	85%
Klein *et al*[43], 2013	Retrospective	Clinical variables	Computer derived model	7003	PDAC risk	61%
Hsieh *et al*[45], 2018	Retrospective	Clinical variables	ANN	> 1 million	NOD predicting PDAC	72%
Zhao *et al*[46], 2011	Retrospective	Clinival variables + Pubmed data	Bayesian network inference	N/A	PDAC prediction	85%
Sanoob *et al*[47], 2016	Retrospective	Clinical variables	ANN	120	PDAC detection	Not explicitly stated
Momeni-Boroujeni *et al*[56], 2017	Retrospective	FNA samples	ANN	75	PDAC diagnosis	77%
Bhasin *et al*[58], 2016	Retrospective	PDAC genes	Computer vector model	5[2]	PDAC detection	92%
Almeida *et al*[59], 2020	Retrospective	PDAC genes	ANN	40[2]	PDAC detection	86%

[1]Sensitivity.

[2]Genes. AI: Artificial intelligence; ANN: Artificial Neural Network; CT: Computerized tomography; EUS: Endoscopic ultrasound; MRI: Magnetic resonance imaging; FNA: Fine needle aspiration; PDAC: Pancreatic ductal adenocarcinoma; CP: Chronic pancreatitis; NOD: New onset diabetes.

studies have applied AI to the analysis of CT images in PDAC for diagnostic purposes[24-26]. In a small study of 15 healthy patients and 44 with a variety of pancreatic tumors, Fu *et al*[24] reported that their algorithm achieved an overall sensitivity of 76%. In a retrospective case control study of 380 patients (190 cases and 190 controls) Chu *et al*[25] reported an accuracy of 99% in their computer-derived algorithm. Meanwhile, a prospective study by Liu *et al*[26] reported an accuracy of 76%. AI has also been utilized to establish correlations between the CT images of PDAC and their subsequent biological behaviors[27,28].

Two important ongoing projects are worth mentioning. The Felix Project funded by the Lustgarten Foundation is a multidisciplinary study carried out by a group at Johns Hopkins University. Using deep learning computer models with manually segmented images from 156 PDAC cases and 300 normal controls, the group reported a sensitivity

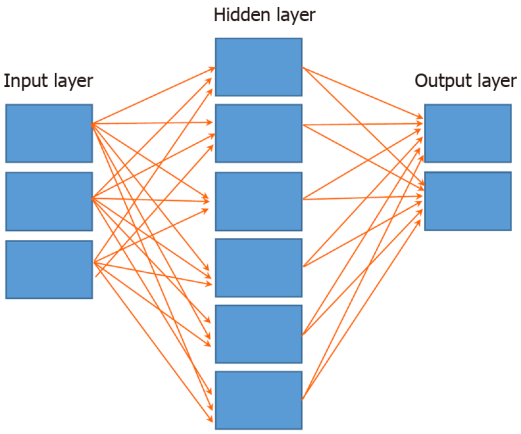


Figure 1 Basic anatomy of an artificial neural network. Input layer, hidden layer (may have more than one) and output layer. All nodes are interconnected through wieghts (arrows).

and specificity of 94% and 99% respectively in their initial report[29]. Based on these encouraging results, the group's next step is to expand their analysis to 575 normal and 750 PDAC patients. The group's ultimate goal is to fine tune the performance of their algorithm prior to expanding it to larger externally derived datasets.

The second ongoing study is being conducted by the Alliance of Pancreatic Cancer Consortium Imaging Working Group (2). Their objective is to collect pre- and post-diagnosis CT, magnetic resonance imaging (MRI) and transabdominal ultrasound images from patients ultimately diagnosed with PDAC. These images will be used to create a repository that will later be shared and analyzed. The ultimate goal of the project is to develop AI models that can predict the appearance of PDAC and diagnose the disease in its early stages.

AI-ASSISTED ANALYSIS OF MAGNETIC RESONANCE IMAGES

Segmentation of MRI images by AI has been reported to be more technically challenging than CT images[30,31]. A few studies have reported that ML models can be trained to accurately identify pathology in the pancreas, although the literature on PDAC is scarce. Devi *et al*[32] reported that an ANN could accurately identify a variety of abnormal pancreatic findings with a 96% accuracy. Similarly, Gao *et al*[33] reported that an AI model differentiated normal from abnormal pancreas at a level comparable to humans (77% *vs* 82% respectively). In the only published study aimed specifically at identifying PDAC in MR images, Liang *et al*[34] reported that a convoluted neural network (a variety of ANN) performed similarly to humans in identifying the lesion.

Artificial Intelligence processing of MRI images has been also applied in the context of PDAC therapy. Spieler *et al*[35] reported that an ANN accurately delineated pancreatic tumors prior to radiotherapy. Zhao *et al*[36] reported similarly positive results. In a study utilizing AI aiming to automatically calculate the dose of stereotactic body radiation therapy, Campbell *et al*[37] demonstrated that an ANN-calculated dose was comparable to a human-calculated one. By applying radiomics to MRI images, investigators have reported that their quantitative data can be correlated with aspects such as tumor subtype, survival and response to chemotherapy[38,39]. This same technology has made possible other studies showing that MR images data can be used to predict relapse after PDAC treatment[40,41].

AI ANALYSIS OF CLINICAL DATABASES

The evolution of AI has resulted in models with the capacity of analyzing data beyond quantitative image features. This type of ML requires a higher complexity in the architecture of the neural networks given the broad range of variables analyzed at any given point in time. These models have been applied in the development of algorithms that can accurately identify patients with or at risk of developing PDAC based on several clinical variables.

Muhammad *et al*[42] utilized an ANN to analyze a large patient population derived from the National Health Interview Survey and the Prostate, Lung, Colorectal and Ovarian trial. The authors developed and trained the ANN with > 800000 patients of which 898 had PDAC. Analyzing variables such as demographics, comorbidities, race and family history, the model predicted the development of PDAC with an AUC of 0.85. In a similar manner, albeit with a lower accuracy, Klein *et al*[43] utilized data from the PanScan Consortium to develop a model that predicted high risk of PDAC among patients of European ancestry with an AUC of 0.61.

New onset diabetes has been adopted as a marker for patients with high risk of developing PDAC within the following 3 years[44]. As such, it has been the subject of analysis by AI techniques. Hsieh *et al*[45] compared the PDAC prediction accuracy of new onset diabetes when analyzed by a regular logistic regression or an ANN. A total of 3092 PDAC cases were identified from a population of > 1000000 patients. Interestingly, the logistic regression slightly outperformed the ANN (AUROC 0.7 and 0.64% respectively). In a complex study combining PubMed data and clinical information, Zhao *et al*[46] utilized an innovative weighted Bayesian network that accurately predicted PDAC with an AUROC of 0.91. In a similar but simpler study, Sanoob *et al*[47] reported that an ANN can accurately diagnose PDAC based on a combination of signs and symptoms[47]. ANNs that analyze clinical data have also been used in determining patient survival and performance after PDAC treatment[48,49].

AI-ASSISTED ANALYSIS OF PATHOLOGICAL AND MOLECULAR FEATURES OF PDAC

Currently, pathologists are responsible for interpretation of histology specimens, and this process is dependent on their previous training, level of experience and individual skills. In an attempt to standardize interpretation and reduce human bias, AI techniques have been applied to pathology specimen analysis[50].

Application of AI in pathology is dependent on creation of a high-resolution digital image from the glass slide. This step is called "whole slide imaging" (WSI). WSI is accomplished by use of glass slide scanners and the technology to support them[51]. WSI and the necessary IT infrastructure to support its clinical use is referred to as "digital pathology". It is expected that eventually, pathology workflow will move away from pathologists looking at glass slides through a microscope to pathologists reviewing digital images of slides on high resolution computer screens. Food and Drug Administration clearance for these devices is relatively new (2017), and widespread adoption of digital pathology technology is still in its early phases. There is an increasing amount of data on validation of WSI compared to microscope viewing of glass slides, for example a recent study showed good concordance of slide interpretation of frozen sections done with glass slides/microscope compared to WSI/digital pathology workstation[52] Similar excellent intraobserver concordance between glass slides and digital pathology has been shown for routine clinical workload in surgical pathology[53].

There is already significant work and available commercial devices that can bring AI computing power to aid in the interpretation and screening of biopsies, although commercial expansion of this is in its infancy[54]. Much of the work regarding AI in pathology concerns prostate and breast malignancy, since the incidence of these malignancies is fairly high, and the clinical need and potential commercial applications present a more attractive corporate opportunity for device sales. However, with continued digitization of glass slides, particularly of pancreatic malignancy (both FNA specimens and surgical pathology) it is hoped that an enlarging curated group of cases can serve as a training set for AI analysis regarding pancreatic cancer.

There has been only a limited number of investigations of AI in pathology for the diagnosis of pancreatic cancer. Although the sensitivity and specificity of EUS guided FNA samples is in average > 90%[55], some specimens still fall under the "atypical cells" category, posing a considerable diagnostic dilemma. In a recent study, Momeni-Boroujeni *et al*[56] studied the performance of an ANN in reclassifying EUS-FNA specimens originally labeled as "atypical" by pathologists. Among a group of 31 patients in whom the final diagnosis had been previously established by other diagnostic methods, the ANN's overall accuracy for adequately reclassifying the specimen as malignant or benign was 77%.

Two of the main factors driving the high mortality of PDAC are suboptimal understanding of its malignant behavior and its unpredictable treatment response rate.

Advances in AI-assisted genetic and molecular profiling of PDAC have recently broadened insight on these factors[57]. Recent data showed that early diagnosis could be possible through AI analysis of the transcription products of certain PDAC genes. These studies have reported sensitivity and specificity ranging from 88%-95% and 83%-95% respectively[58,59]. AI has also been utilized to match PDAC biological information with chemical properties of specific drugs in order to develop models capable of predicting response to these specific agents[60,61].

FUTURE CONSIDERATIONS

Technological advances in the last 50 years have exponentially increased the amount and quality of data available for medical decision making. Hence, it is becoming increasingly evident that new methods for storage and analysis of it are necessary. Although the concept of AI or its applications in medicine may still seem foreign for most practitioners, it is rapidly positioning itself as an indispensable tool to reduce human error. As sophisticated and elegant our diagnostic and therapeutic capacities may be currently, they remain inevitably limited by our subconscious and conscious bias, as well as our wide range of intellectual and technical skills. William Osler's famous quote: "medicine is a science of uncertainty and an art of probability" will likely never be proven false.However the degree of uncertainty and probability considered tolerable in modern medicine is constantly shrinking. The advent of AI brings, in theory, the promise of reducing and even eliminating these shortcomings

Nevertheless, this promise is one that needs to be taken cautiously. There are several hurdles that must be overcome before AI can see widespread adoption in medical care. One limitation is the current lack of adequate standardization. Uniform protocols for data collection, processing, storage, reproduction and analysis must be established and standardized. Furthermore, different types of data may require different AI technologies. For example, ANNs trained to adequately classify histologic slides of pancreatic biopsies which have been fixed and stained with a specific method, may underperform, or not perform at all, when presented with slides prepared in a different manner. Creating such universal protocols, although possible, will be laborious and expensive.

Another concern is with the ethical handling of information. AI systems require vast amounts of data, and therefore, its implementation demands reliable methods of patient data de-identification. This is indispensable to ensure patient confidentiality, since one of the pillars of AI is data sharing. On the other hand, de-identified data needs to maintain its traceability, in order to allow individual practitioners to retrieve it and make the necessary decisions at the bedside. Three different models have been developed for data sharing in AI, and all have their advantages and disadvantages[2,62-65].

Centralized models require sharing of large amounts of data by different sources (*i.e* institutions). This data is uploaded into a central server that carries out the algorithmic adjustments. Once trained, the central server shares the finalized algorithm with the individual sources for internal use. The main drawback of this model is that centralization of the information in the server may increase the risk of a security breach, as the individual source no longer controls the information (Figure 2). In distributed or federated models, each source develops and adjusts its own algorithm with internal data. Once each source has fine-tuned their algorithm, they share its parameters with a central server. The central server then utilizes all the individual parameters to develop a centralized algorithm that later gets returned to the source for internal use (Figure 3). The main advantage of this model is that data is not shared with the central server. In hybrid models features of both models are present. A data repository is created to be shared by both data providers and the central server. The data repository then develops an algorithm using each individual institution's data before it gets sent to the central server. The central server then updates the master algorithm before it gets sent back to the data repository (Figure 4).

The quality of data currently utilized in AI needs to be improved. Most of the AI systems used in data analysis so far have been trained and tested with rather small datasets originating from within local institutions. This raises the issue of information bias. These datasets lack the degree of diversity necessary to mirror the scenarios human providers face during routine clinical practice. For AI systems to perform adequately, the datasets need to be sufficiently diverse in all the possible variables that come into play when making clinical decisions (demographics, medical and/or family history, physical and laboratory findings and others). Therefore, datasets need to

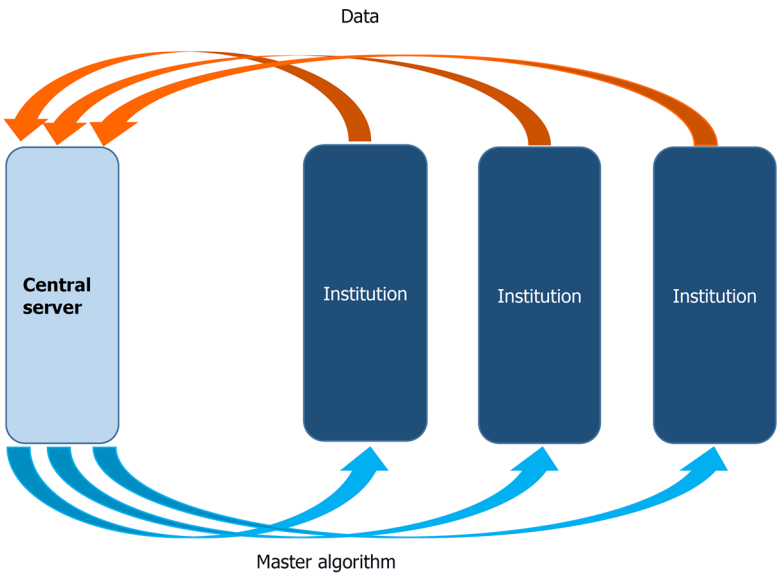


Figure 2 Centralized artificial intelligence information sharing system. Each individual institution provides data to the central server. The server analyzes all the data and develops and algorithm that is sent to each institution. This algorithm is then used by each institution to analyze its own internal data in the future.

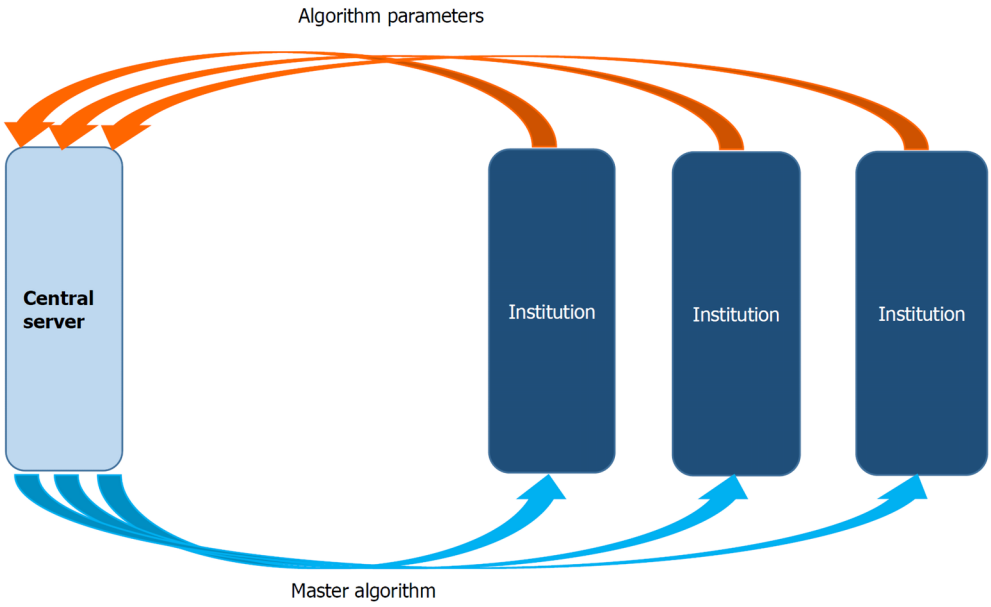


Figure 3 Federated artificial intelligence information sharing system. Each individual institution develops its own algorithm with internal data. Once the algorihms are developed, their parameters are shared with the central server. The server then develops a master algorithm using all the individual parameters. The master algorighm is sent back to the institutions for its internal use.

originate from a variety of sources for them to be representative and inclusive, not from a limited number of large academic medical centers or research institutions.

Another shortcoming is the fact that the average ANN functions as a "black box"[66]. As such, how a specific variable in a dataset is weighted by specific nodes in the network is currently uninterpretable. When evaluating the performance of any ANN, clinicians, mathematicians and computer scientists need to understand the "reasoning" that occurs within the hidden layers. Although questioning of ANNs is possible through mathematical reasoning, it does not reflect clinical decision making. Understanding the way in which ANNs analyze information is paramount for improving their performance and correcting errors that can lead to fatal consequences.

Finally, much of the promise of applying AI in medical image analysis depends on the ability to get actionable results rapidly. The current need for multiple intricate post-processing steps prior to its analysis, indicates that much more work must be

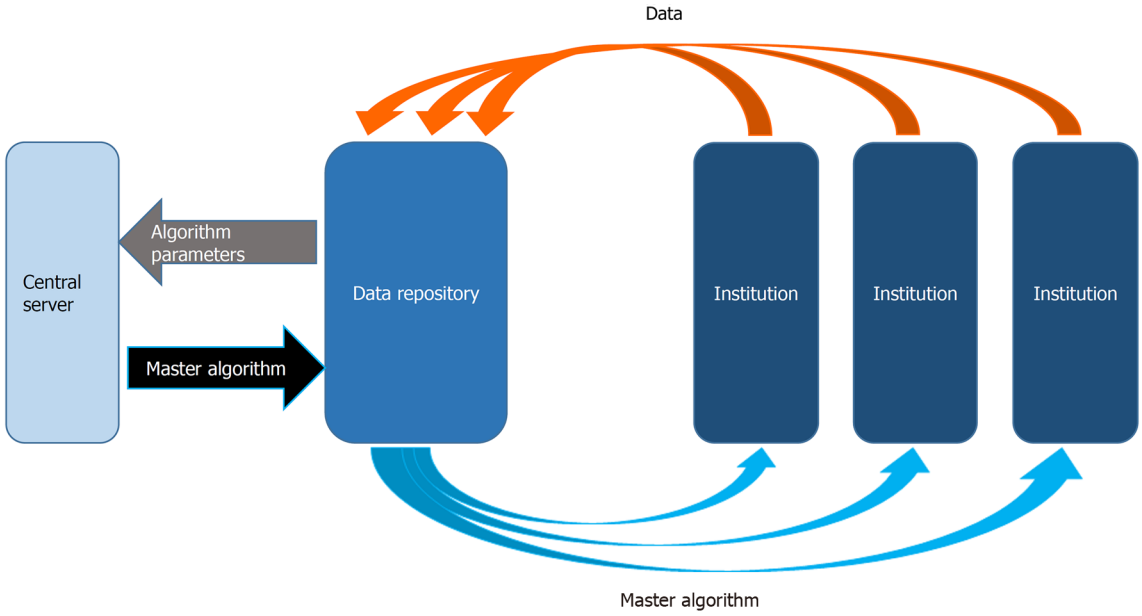


Figure 4 Hybrid artificial intelligence infromation sharing system. A data repository is created as an intermediary between the institutions and the central server. The data repository develops one or more algorithm(s) with the data. The parameters of these algorithms are then shared with the central server to create a master algorithm which is then returned to the repository. New data coming from the institutions is then used by the repository to create new parameters that are then sent to the central server to renew the master algorithm.

done to develop this into a technology that provides "on-the-fly" results[63].

Because of the limitations enumerated above, it is clear that major improvements to the technology need to occur before AI can support everyday activities in clinical practice. It is evident however, that medicine has reached a "point of no return" regarding application of AI. Overcoming these hurdles will require collaboration between academic centers, industry, computer scientists, venture capitalists, regulating organizations and governments (Figure 5). The main goals of this collaboration should be streamlining development of standard data platforms, lowering technology costs and making the technology more "user friendly" so that any provider can use it in real time.

CONCLUSION

With further research, AI could have a large impact on the diagnosis and treatment of PDAC in the future. Novel screening methods are needed, and AI analysis of large comprehensive clinical datasets may yield opportunity for early detection or even predict development of PDAC before a visible lesion can be seen on imaging. An AI protocol which prescreens computed tomography or magnetic resonance imaging prior to a radiologist reading the study could ensure that lesions will not be missed due to human error. "On the fly" AI assistance with endoscopic ultrasound imaging could help the endosonographer optimally target a needle biopsy of a mass. The pathologist can be assisted by an AI algorithm that analyzes the biopsy on the slide that is being read. And AI could prove very useful for following response to treatment and even in suggesting optimal treatment regimens, with personalized treatment strategies based on biological profiling. While AI applications in PDAC are still in the very early stage of development, further investment in research could lead to substantial improvements in screening, early diagnosis, and treatment.

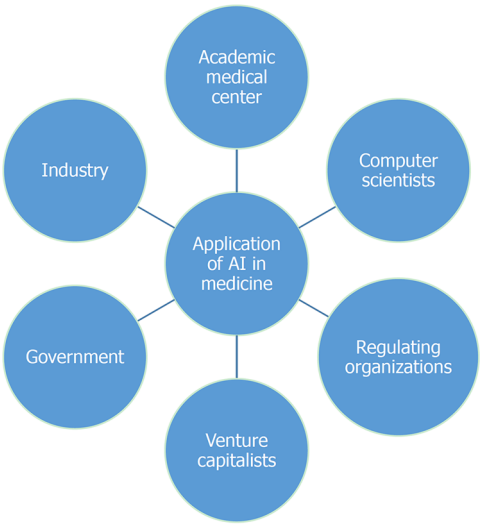


Figure 5 Collaboration to expedite broad artificial intelligence application in medicine. AI: Artificial intelligence.

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MINIREVIEWS

COVID-19 and comorbidities of hepatic diseases in a global perspective

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Abstract

The worldwide outbreak of coronavirus disease 2019 (COVID-19) has challenged the priorities of healthcare system in terms of different clinical management and infection transmission, particularly those related to hepatic-disease comorbidities. Epidemiological data evidenced that COVID-19 patients with altered liver function because of hepatitis infection and cholestasis have an adverse prognosis and experience worse health outcomes. COVID-19-associated liver injury is correlated with various liver diseases following a severe acute respiratory syndrome-coronavirus type 2 (SARS-CoV-2) infection that can progress during the treatment of COVID-19 patients with or without pre-existing liver disease. SARS-CoV-2 can induce liver injury in a number of ways including direct cytopathic effect of the virus on cholangiocytes/hepatocytes, immune-mediated damage, hypoxia, and sepsis. Indeed, immediate cytopathogenic effects of SARS-CoV-2 *via* its potential target, the angiotensin-converting enzyme-2 receptor, which is highly expressed in hepatocytes and cholangiocytes, renders the liver as an extra-respiratory organ with increased susceptibility to pathological outcomes. But, underlying COVID-19-linked liver disease pathogenesis with abnormal liver function tests (LFTs) is incompletely understood. Hence, we collated COVID-19-associated liver injuries with increased LFTs at the nexus of pre-existing liver diseases and COVID-19, and defining a plausible pathophysiological triad of COVID-19, hepatocellular damage, and liver disease. This review summarizes recent findings of the exacerbating role of COVID-19 in pre-existing liver disease and vice versa as well as international guidelines of clinical care, management, and treatment recommendations for COVID-19 patients with liver disease.

Key Words: Liver disease; COVID-19; Pathophysiology; Epidemiology; Prophylaxis

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Core Tip: The clinical menace of coronavirus disease 2019 (COVID-19)-related comorbidities of hepatic diseases and severe acute respiratory syndrome-coronavirus type 2 (SARS-CoV-2) tropism for the liver result in liver impairment with increased liver injury markers and cytokine storm. SARS-CoV-2 aggravates liver injury *via* coagulative and fibrinolytic pathways, cytokine-mediated liver injury, ischemia-hypoxia, and immune-mediated cell death pathways owing to adverse outcomes of liver disease such as nonalcoholic steatohepatitis, drug-induced liver injury, nonalcoholic fatty liver disease, metabolic associated fatty liver disease, and hepatocellular carcinoma. This review summarizes diagnostic approaches, therapeutics, clinical guidelines, and vaccines for COVID-19 and liver disease comorbidities.

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INTRODUCTION

Coronavirus belongs to family *Coronaviridae*, subfamily *Orthocoronavirinae*, and order Nidovirales[1]. All over the world, coronaviruses are responsible for causing enteric, neurologic, and hepatic diseases in humans, animals, and other mammals[2]. On the basis of genome and phylogenetic analysis, the subfamily contains four genera named *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*[3]. Coronaviruses caused epidemics of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012[2]. The novel coronavirus 2019 (SARS-CoV-2)- belongs to genus *Betacoronavirus*. It is an enveloped virus with a positive-sense, single-stranded RNA genome. SARS-CoV-2 has affected more than 65.8 million people with 1.5 million deaths globally.

In early December 2019, the first documented pneumonia case of unknown origin in Wuhan city of China was confirmed by high-throughput sequencing analysis as a novel *Betacoronavirus* that is currently named SARS-CoV-2. A sudden worldwide outbreak of this virus was declared as health emergency and pandemic of international concern by the World Health Organization (WHO)[4]. In previous epidemics of SARS-CoV and MERS-CoV, bats were considered as the natural host and potential reservoir. Different studies evidenced bats as a reservoir of SARS-CoV-2, however, pangolin species were also considered as natural reservoir of SARS-CoV-2[5].

In the SARS pandemic, the virus was found in patient's stool sample[6], which indicates that fecal samples could be a possible source of transmission of SARS-CoV-2[7]. After clinical recovery, prolonged shedding of SARS-CoV-2 in feces highlighted the possibility of fecal-oral transmission[8]. Positive semen samples containing SARS-CoV-2 have also been observed in two patients who were in recovery and in four who were in the acute stage of infection[9]. Recently, an ocular route of transmission has also been identified in SARS-CoV-2[10].

Symptoms of coronavirus disease 2019 (COVID-19) vary from mild respiratory symptoms to acute respiratory distress syndrome along with multiple organ failure[11] and death, mainly in elderly patients having several comorbidities. The WHO provided timely, effective, and safe supportive management guidelines for COVID-19 patients, which revealed that clinical syndromes associated with SARS-CoV-2 include uncomplicated illness, mild-to-severe pneumonia, acute respiratory distress syndrome, sepsis, and septic shock[12]. However, gastrointestinal symptoms[13] and several extra-pulmonary signs such as liver injury have also been associated with COVID-19[14].

In COVID-19 patients, markers of liver injury may be abnormal[15]. As shown in Table 1, disturbed alanine transaminase (ALT), aspartate transaminase (AST) levels and increased bilirubin have been seen in 14%-53% of COVID-19 patients[16]. A recent study of 1100 patients reported that 18% patients with nonsevere and 56% with severe COVID-19 had elevated serum AST levels. Increased ALT levels were observed in 20% of patients with nonsevere and 28% with severe COVID-19 infection[17]. Moreover, it has been noted that COVID-19 patients with gastrointestinal symptoms showed a high

Table 1 Prevalence of coronavirus disease 2019 patients with altered manifestations of hepatic injury markers

Salient findings	Country	Ref.
Of 417 COVID-19 patients, 76.3% had altered values of liver function tests and 21.5% had liver injury during hospitalization. The use of lopinavir/ritonavir increased the risks of liver injury by 4-fold.	China	Cai *et al*[32], 2020
The prevalence of patients with GI symptoms and elevated level of liver enzymes was 18.6%. The severity of disease increased in patients with digestive symptoms.	China	Pan *et al*[81], 2020
Abnormal liver function tests are common in COVID-19 patients. Of 115 patients, 9.57% had increased ALT levels and 14.78% had increased AST levels.	China	Zhang *et al*[82], 2020
Liver dysfunction at an early stage increases the mortality risk in COVID-19 patients. A total of 151 patients (42.5%) were reported with cholestasis and 101 (28.5%) had hepatocellular injury. Liver dysfunction was more common in critically ill patients.	China	Fu *et al*[83], 2020
About 48.4% of patients with normal liver function had abnormal liver function tests after receiving lopinavir/ritonavir. Liver injury biomarkers (LDH, ALP, GGT, TBiL, prealbumin, and albumin) were dysregulated in a cohort of 288 COVID-19 patients, suggestive of potential as markers of liver injury and a prognosis of severe of COVID-19 disease.	China	Fan *et al*[37], 2020 and Fan *et al*[84], 2020
The presence of acute liver injury was linked with high risk of COVID-19 morbidities and admission to an ICU.	United States	Hajifathalian *et al*[85], 2020
Serum liver enzymes were increased in from 14% to 53% of hospitalized COVID-19 patients.	United States	Fix *et al*[68], 2020
Increased bilirubin level was seen in 16.7% and increased ALT and AST were seen in 15% of COVID-19 patients.	United States	Sultan *et al*[86], 2020

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; COVID-19: Coronavirus disease 2019; GGT: Gamma-glutamyl transferase; GI: Gastrointestinal; ICU: Intensive care unit. LDH: Lactate dehydrogenase; TBiL: Total bilirubin.

prevalence of liver injury compared with patients with no gastrointestinal symptoms. Correspondingly, the prevalence of chronic liver disease (CLD) was high in patients with gastrointestinal symptoms of COVID-19[18].

Liver cancer is the fourth most common cause of cancer-associated death worldwide[19]. The most common type is hepatocellular carcinoma (HCC), and patients with HCC have underlying CLD, which includes alcoholic liver injury, nonalcoholic fatty liver disease (NAFLD), and chronic hepatitis B or C virus infection[19]. It has been reported that cancer patients are at high risk of COVID-19. A study of three hospitals in Wuhan reported 1276 confirmed cases of COVID-19, among which 28 patients had different types of cancer and two of them had HCC[20]. NAFLD, also called metabolic associated fatty liver disease (MAFLD), is highly prevalent globally[21]. A study reported that patients with NAFLD had increased ALT levels following COVID-19 infection[22]. A study from China showed that patients with NAFLD had an increased risk of COVID-19 compared with patients without it[23]. However, more studies are required to understand the mechanism of liver injury caused by NAFLD and COVID-19. It has been observed that use of immunosuppressant drugs in post liver transplant patients more prone them to SARS-CoV-2 infection. Additionally, the use of these drugs were found to enhance the cytokine storm in COVID-19 patients[18,24].

COVID-19-associated liver injury is manifested by entry of SARS-CoV-2 into the liver that can progress during COVID-19 treatment in patients with or without pre-existing liver disease[25]. SARS-CoV-2-related liver injury can occur by multiple factors, including direct cytopathic effect of the virus on cholangiocytes/hepatocytes, immune-mediated damage, hypoxia[19], sepsis[26], and drugs (*e.g.*, acetaminophen, ritonavir/lopinavir, interferon, Chinese herbs, and antibacterial agents) used in treating COVID-19[27]. However, there is no proof that patients with chronic hepatitis are at risk of COVID-19 unless other comorbidities (*e.g.*, cardiovascular disease, diabetes, and hypertension) that can increase the risk of liver disease are present[26]. The angiotensin-converting enzyme 2 (ACE2) receptor is associated with entry of SARS-CoV-2 and is highly expressed (about 80%) in alveolar cells of the lungs[28], the gut, and the kidneys[29]. The expression of ACE2 receptor has also been observed in myocardial cells, nephron proximal tubule cells, absorptive enterocytes of the ileum and colon, bladder urothelial cells; and the oral, nasal and nasopharyngeal mucosal epithelia [30]. It has been reported that ACE2 cell surface receptors are highly expressed in cholangiocytes and hepatocytes, which indicates tropism of SARS-CoV-2 in the liver[31].

COVID-19-ASSOCIATED LIVER INJURY AND ITS CLINICAL IMPLICATIONS IN A GLOBAL SCENARIO

Abnormal levels of liver injury markers such as AST and ALT or abnormal liver function tests (LFTs) in COVID-19 patients place an additional disease burden on clinicians as well as to scientists in terms of treatment regimens and defining the association of COVID-19-linked hepatic diseases. The data that document liver impairment in COVID-19 patients reveal variable levels of liver injury markers across the globe, as shown in Figure 1[32-36]. In this context, the preliminary data were reported by Cai *et al*[32] from Shenzhen, China from January 11 to February 21, 2020 and followed-up to March 7, 2020. The data in the report was from a local hospital that found 318 of 417 COVID-19 patients (76.3%) with abnormal LFT values and 90 of 417 (21.5%) with liver injury during hospitalization. The incidence of liver injuries in Asia was further evaluated by Vespa *et al*[34] in a cohort of 292 COVID-19 patients until March 30, 2020. Compared with Cai *et al*[32], a cholestatic pattern of liver injury with an increased level of alkaline phosphatase (ALP) > 150 U/L was observed in 9.6% of the selected population. Another study of cholestatic liver injury conducted from January 20 to 31, 2020 at the Shanghai Public Health Clinical Center reported abnormal liver functions as increased levels of ALT and AST, gamma-glutamyl transferase (GGT), ALP, and total bilirubin[37]. Thus, the documented reports describe data related to predictive markers of COVID-19 and associated liver injury, but the underlying causes of liver injury associated with worse outcomes are still elusive.

The clinical significance of COVID-19-associated liver disease needs to be described. Some studies have addressed the clinical challenges. Of relevance is a report by Vespa *et al*[34] of an increase in ALP values because of SARS-CoV-2 liver tropism *via* the ACE2 receptor on hepatocytes and cholangiocytes. Furthermore, they correlated the ALP elevation as a marker of patient frailty or as representing an increased systemic inflammatory response to SARS-CoV-2 infection[34]. The prevalence of altered liver injury markers is highly associated with drug-induced liver injury (DILI) during the COVID-19 pandemic. Several studies[32,38-40] have reported potential harms related to pharmacotherapy with lopinavir/ritonavir in COVID-19 patients and the vulnerability of patients in developing severe pneumonia *via* increased LFTs. Moreover, the pathological characteristics of liver injury *via* autopsy analysis of COVID-19 patients provides evidence in support of DILI. A study revealed moderate microvesicular steatosis with mild hepatic inflammation, indicating the possibility of hepatic injury. However, this pattern of histological injury could be correlated with either DILI or SARS-CoV-2 infection[41].

Several risk factors can be linked to the incidence of COVID-19-associated liver injury. A retrospective study in a French population found a marked prevalence of obesity in confirmed COVID-19 patients. The study included 340 COVID-19 patients, 230 (68%) with noncritical COVID-19 and 110 (32%) with critical COVID-19. It was found that 85 of 340 patients with severe COVID-19 (25%) were obese compared with 15.3% of the general French population. Following standardization by age and sex, the prevalence rates of obesity were 1.35% and 1.89% times higher in patients with severe COVID-19 and in those admitted to intensive care units, respectively, than in the general French population[42]. Likewise, Zheng *et al*[43] reported the key association of obesity with the severity of COVID-19 in MAFLD patients, which provides a rationale for the likelihood of the importance of obesity-related comorbidities of liver diseases[44,45].

PRE-EXISTING LIVER DISEASE AND COVID-19: AN INTERLINKED SETUP AND ITS RELATED CONSEQUENCES

CLDs are an existing threat that accounts for the leading causes of liver-related mortality worldwide[46-48]. The major CLDs, including hepatitis B virus or C virus infection, alcohol-induced liver damage (ALD), and NAFLD, lead to prolonged liver damage and increased incidence of CLD-associated cancers, particularly, HCC[49,50]. Underlying liver diseases were one of the crucial causative factors in the previous SARS outbreak, with high mortality rates in adults and elders[51]. The Centers for Disease Control and Prevention recently included liver diseases as a comorbidity and predisposing factor for contracting SARS-CoV-2 infection. Moreover, the American Association for the Study of Liver Diseases (AASLD) endorses COVID-19 testing on a priority basis for patients who manifest symptoms of liver disease[52]. The COVID-19

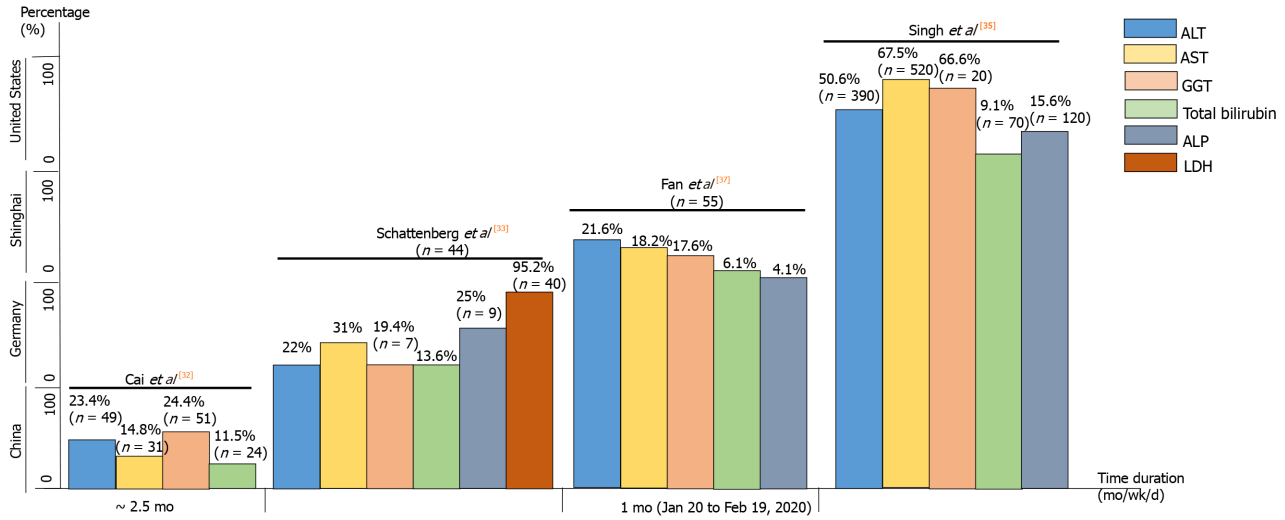


Figure 1 Overview of a global perspective related to incidence of coronavirus disease 2019-associated alterations in liver function tests.
The figure presents only data published in peer-reviewed journals. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase; LDH: Lactate dehydrogenase.

pandemic together with the global prevailing menace of CLD further complicates the care of pre-existing liver disease patients as a result of failure of screening, and follow-up. Therefore, the intricate link between pre-existing liver disease and COVID-19 requires additional study and specific disease management. It has to be expected that patients with underlying liver diseases are more vulnerable to exacerbating COVID-19-related effects and vice versa[53] that may account for the high morbidity and mortality rates in the current COVID-19 pandemic.

Relevant to pre-existing liver diseases, alcohol use disorder or ALD is the CLD with the highest hospitalization burden and has seen a more than 2-fold increase in China. The effect is likely to be repeated in the United States, with 12.7% estimated hospitalization rate associated with COVID-19. A gradual increase in the incidence of ALD was accompanied by an increase in patients indicated for liver transplantation prior to COVID-19 pandemic. The direct clinical implications of COVID-19 on ALD are still unknown, but it can be suspected that SARS-CoV-2 infection can serve as a major host-compromising factor with underlying ALD and subsequently results in acute-on-chronic liver failure. The high proportion of ALD among CLD patients is reflected in the number of patients with decompensated ALD during COVID-19 pandemic[52].

Growing evidence of COVID-19-related liver disease comorbidities suggests that MAFLD patients are at higher risk of COVID-19 disease progression[43,54,55]. A retrospective study conducted by Fondevila *et al*[56] described a mechanistic approach in the context of a higher risk of SARS-CoV-2 infection in obese patients. They assessed the hepatic mRNA expression of SARS-CoV-2 cell entry molecules, ACE2 and the cellular transmembrane protease serine 2 (*TMPRSS2*) in obese patients with NAFLD and/or diabetes mellitus type-2. Based on liver mRNA expression of both ACE2 and *TMPRSS2* in obese patients, the results revealed that SARS-CoV-2 entry factors are differently affected in diabetes and NAFLD. Moreover, major alterations in the expression of SARS-CoV-2 entry molecules in in men and women suggest a lower susceptibility of women to liver injury. While obese women with diabetes have unexpectedly lower levels of ACE2 and *TMPRSS2* than obese normoglycemic women, obese patients with nonalcoholic steatohepatitis had a higher expression of those genes, suggesting that advanced stages of NAFLD might predispose to COVID-19.

COVID-19 patients with comorbidities of advanced hepatic complications are generally at an increased risk of infection because of cirrhosis-associated immune dysfunction[57]. A retrospective study by Iavarone *et al*[58] documented a substantial 30-d mortality rate of 34% in a cohort of 50 cirrhotic patients with COVID-19, which was higher than the rate in cirrhotic patients with bacterial infections. Overall COVID-19 mortality from the medical consequences of respiratory failure was correlated with the worsening of liver dysfunction. Other categories of COVID-19 patients with hepatic diseases that are of great concern are liver transplant recipients and patients with autoimmune liver diseases receiving immunosuppressant drugs[57]. However, COVID-19-associated effects in recipients of living donor allografts are still unclear[59]. A case reported by The American Society of Transplantation and the American Society of

Transplant Surgeons described the impact of COVID-19-associated hepatitis during liver transplantation. A patient underwent ABO-incompatible living donor liver transplantation without knowing that the liver donor was infected with COVID-19 during the donation procedure. In that case, donor-derived transmission to the recipient was not identified, and the liver donor was found to be recovering from COVID-19 infection. Donor-derived transmission was not identified[60].

A PLAUSIBLE PATHOPHYSIOLOGICAL TRIAD OF COVID-19, HEPATOCELLULAR DAMAGE AND LIVER DISEASES

Epidemiological studies have described liver dysfunction and the effects of SARS-CoV-2 infection on the liver cells in COVID-19 patients by elevated levels of liver injury markers such as ALT, AST, and bilirubin[32,33,57,61,62]. It has been stated previously that the SARS-CoV and MERS-CoV viruses primarily affect the upper respiratory tract but also affect the liver[62,63]. Despite the immediate and direct action of SARS-CoV-2 *via* potential targets on the epithelial cells of lung alveoli as well as the respiratory tract, emerging evidence suggests that the high expression of ACE2 receptors in the liver renders it susceptible to the pathogenicity of SARS-CoV-2[36,64]. Pathological examination of COVID-19 patients has confirmed cytopathic injury in the lungs[41] and has recently confirmed SARS-CoV-2 infection as an etiology of liver disease. The proposed mechanisms associated with COVID-19-induced liver injury include direct viral insult-linked hepatic derangements, cytokine storm-prompted liver injury, and ischemia related to COVID-19-induced hypoxia as shown in Figure 2[52,65-68].

Liver impairment in COVID-19 might be directly correlated with SARS-CoV-2 infection of liver cells. Approximately 2%-10% COVID-19 patients with diarrhea have confirmed SARS-CoV-2 RNA in stool and blood samples, which implies the possibility of viral exposure in the liver. The affinity of SARS-CoV-2 and SARS-CoV for the ACE2 receptor indicates respective target sites, mainly the upper respiratory tract, lung tissue, and cholangiocytes of the liver, where the virus replicates and manifests COVID-19 associated symptoms[61]. In that context, Zhao *et al*[69] studied a SARS-CoV-2 infection model in human liver ductal organoids. They reported genomic evidence that SARS-CoV-2 virus infection resulted in dysregulation of barrier and bile-acid transporting functions of the cholangiocytes. The study found that altered cholangiocyte functions of could have been the result of a direct SARS-CoV-2 cytopathogenic effect on target cell that expressed ACE2 and *TMPRSS2*.

Cytokine storm is one of the hallmarks of infectious and noninfectious diseases that are capable of causing severe multiple organ injuries. Establishment of the cytokine environment is a multifactorial network that involves an immunological response to an invading antigen along with interplay of activated host immune and inflammatory cells. In line with the concept of cytokine storm in COVID-19 patients, Han *et al*[65] performed a prospective cohort study at a local hospital in Wuhan, China that enrolled 102 COVID-19 confirmed patients and 45 healthy control volunteers. They analyzed the serum profiles of inflammatory cytokines, including tumor necrosis factor-, interferon-, interleukin (IL)-2, IL-4, IL-6, IL-10, and C-reactive protein (CRP) by immunoassays. Significant increases in the levels of the inflammatory markers and CRP were seen in COVID-19 patients compared with the healthy volunteers. Moreover, the levels of IL-6 and IL-10 were significantly higher in critical than severe or moderate COVID-19 patients, suggesting that increased IL-6 and IL-10 may allow rapid diagnosis of patients with increased risk of lethal disease. Furthermore, Wang *et al*[70] reported COVID-19-associated conspicuous cytopathy. The severity of SARS-CoV-2 infection is associated with disturbed levels of liver enzymes, increased alveolar-arterial oxygen gradient and GGT level, and decreased albumin and circulating $CD4^+$ T cells and B lymphocytes. The predominant histological features of COVID-19 liver infection are substantial apoptosis and binuclear hepatocytes[70].

Hepatic dysfunction in severe COVID-19 is accompanied by aberrant activation of the coagulative and fibrinolytic pathways[71], moderately decreased platelet counts, increased neutrophil counts and neutrophil-to-lymphocyte ratios, and high ferritin levels. Such laboratory findings are perceived as nonspecific inflammatory markers, but the altered levels can coincide with a failure of innate immune regulation during progression of severe COVID-19. Indeed, alteration of immune balance activates coagulation and NETosis, and subsequently affects systemic iron metabolism secondary to macrophage activation[38].

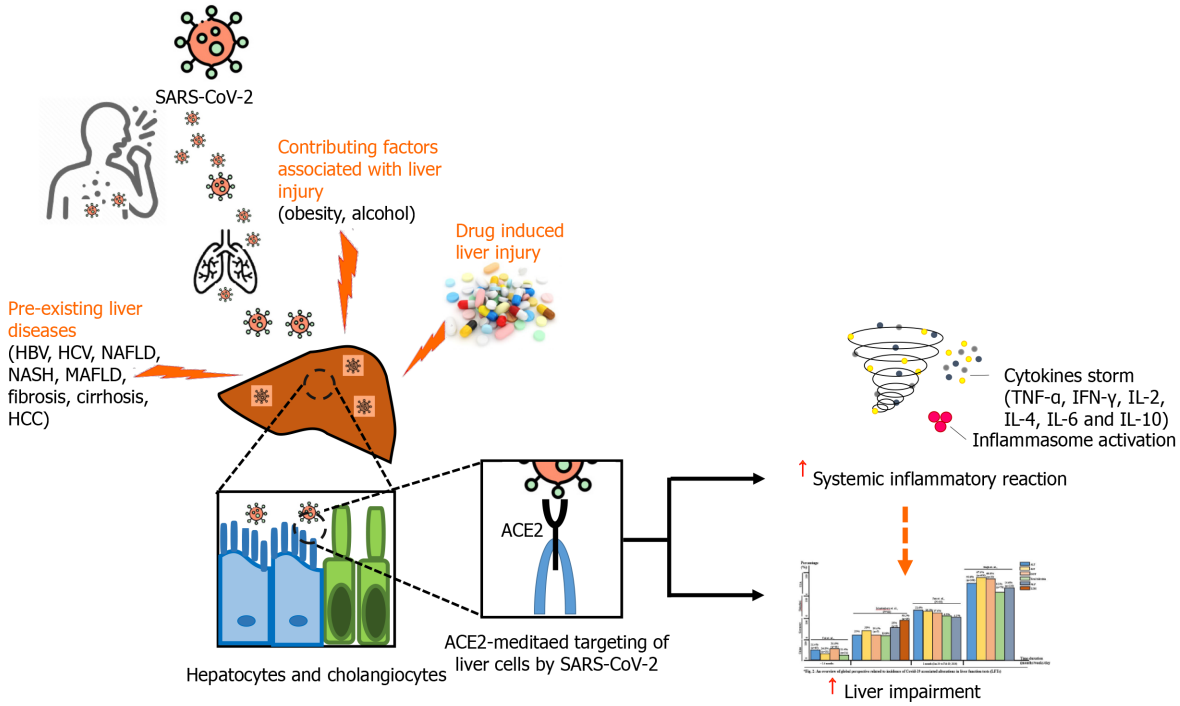


Figure 2 Severe acute respiratory syndrome-coronavirus type 2-associated pathogenesis and immunological response in the liver. ACE2: Angiotensin-converting enzyme 2; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; IFN: Interferon; IL: Interleukin.; MAFLD: Metabolic associated fatty liver disease; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; SARS-CoV-2: Severe acute respiratory syndrome-coronavirus type 2; TNF: Tumor necrosis factor.

DIAGNOSTIC APPROACHES FOR COVID-19

SARS-CoV-2 infection is confirmed by serologic tests that measure the antibody response or antibody titer in the patient. In COVID-19 patients, antibodies are produced in the days to weeks after SARS-CoV-2 infection. Thus, the presence of antibodies indicates that a person was infected with SARS-CoV-2, irrespective of whether the infection caused severe or mild disease or even an asymptomatic infection. Surveillance of antibody seropositivity allows inferences to be made about the extent of infection and the cumulative incidence of infection in the population. Serologic Enzyme-linked immunosorbent assays can be used for COVID-19 serosurveillance and to determine the extent of infection in the population. A confirmed case of SARS-CoV-2 infection is declared by a positive result from nasal and pharyngeal swab specimens by high-throughput sequencing or real-time reverse transcriptase polymerase chain reaction (RT-PCR)[17]. Molecular testing (RT-PCR) confirms infection, often in patients with severe disease, as they are individuals who seek and require health care. A percentage of patients with mild or asymptomatic infections who do not require medical attention may not be tested, and as a result, the full spectrum of the disease will not be known[72]. SARS-CoV-2 infection is associated with epidemiological characteristics, clinical signs, and symptoms of COVID-19 that can be accessed from electronic medical records and laboratory findings. Radiological assessments of COVID-19 patients include chest X-rays or computed tomography. Whereas, laboratory assessments that help to indicate the prognosis of COVID-19 include complete blood counts, blood chemistry, coagulation tests, assays of liver and renal function markers, electrolytes, CRP, procalcitonin, lactate dehydrogenase, and creatine kinase[17].

RECOMMENDATIONS OF THERAPEUTICS FOR COVID-19: VACCINES, LABEL AND OFF-LABEL MEDICATIONS FOR PATIENT CARE

The COVID-19 pandemic has prompted the scientific community and clinicians to develop COVID-19-related therapeutics and vaccines to mitigate as well as control the pathogenicity of SARS-CoV-2. Previous investigations on genomic sequencing of

SARS-CoV and MERS have contributed to vaccination strategies in developing current vaccines against SARS-CoV-2[73]. The characteristics of vaccines in clinical testing are based upon inactivated or live-attenuated viruses, protein subunits, virus-like particles, replicating and nonreplicating viral vectors, and DNA and RNA that may provoke protective immunity to SARS-CoV-2 infection[74,75]. Since July 2, 2020, the global landscape of SARS-CoV-2 vaccine development reported 163 vaccine candidates, 135 of the 163 are in preclinical or exploratory stages of development. Currently, mRNA-1273 (Moderna), Ad5-nCoV (CanSino Biologicals), INO-4800 (Inovio, Inc.), LV-SMENP-DC, a Pathogen-specific aAPC (ShinzenGeno-Immune Medical Institute), ChAdOx1 (Oxford University) BioNtech (Pfizer), Sputnik V (Gamaleya, Russia), and Sinovac (China) have been approved by the WHO under emergency use authorization[76]. India has started mass vaccination with its locally produced Covaxin vaccine. A prolonged time period for approval of vaccines is needed for validation of efficacy and adverse effects in target populations before post-market surveillance. However, adverse reactions to vaccines including fatigue, chills, aches, skin rashes, muscle pain, fever, and joint pain could be a barrier in the global rollout of COVID-19 vaccines[77]. In April, 2020, the Access to COVID-19 Tools accelerator was launched by the WHO and its partners to cooperate in fighting against the COVID-19 pandemic. Moreover, a global collaboration aims to accelerate development, production, and equitable access to COVID-19 tests, treatments, and vaccines among countries, particularly low-to-middle income countries (SAGE; http://www.who.int/).

In terms of therapeutics, COVID-19 medications fall into two categories: Those that target the viral replication cycle and those that aim to control the symptoms of the disease. The aminoquinolines chloroquine and hydroxychloroquine are polymerase inhibitors classically used as antimalarial medications. In malaria, it inhibits heme polymerase, causing the accumulation of toxic heme in the parasite that leads to its death. In COVID-19, it is thought that the drugs keep the virus out of host cells by blocking glycosylation of host receptors and blocking the production of viral proteins by inhibiting endosomal acidification. The WHO recommends the off-label use of hydroxychloroquine or chloroquine and lopinavir/ritonavir for treatment of COVID-19 with any disease severity and any duration of symptoms. Remdesivir and systemic corticosteroids are potential candidates for conditional recommendation of use in hospitalized COVID-19 patients for usual care regardless of disease severity (WHO/2019-nCoV/therapeutics/2020.1). The WHO considers off-label use of medication as country-specific. In many countries, doctors are giving COVID-19 patients medicines that have not been approved for this disease (MEURI; http://www.who.int/). Thus, COVID-19 patients have received off-label and compassionate-use therapies, such as interferon- combined with the repurposed drug Kaletra, an approved combination of the human immunodeficiency virus protease inhibitors ritonavir and lopinavir, chloroquine, azithromycin, favipiravir, remdesivir, steroids, and anti-IL-6 inhibitors, based on either *in vitro* antiviral or anti-inflammatory properties[78]. It is unclear if COVID-19 therapeutics protect against liver injury or disease. It would be interesting to look into protective effects in future.

RECOMMENDATIONS FOR MANAGEMENT OF COVID-19 PATIENTS WITH PRE-EXISTING LIVER DISEASES

The WHO has approved comprehensive guidelines to strengthen the care and management of COVID-19 patients and to provide up-to-date guidance to clinicians and physicians. That report addresses the best practices to manage severe acute respiratory infection (SARI), including infection prevention and control measures and supportive care for COVID-19 patients. Furthermore, the prime considerations focus on recognizing and treating patients with SARI through appropriate diagnosis, early supportive therapy, management of acute respiratory distress and septic shock, prevention of complications, and use of specific COVID-19 treatments[79]. Indeed, patients with CLD do not appear to be over-represented in cohorts of patients with COVID-19, where they make up less than 1% of reported cases. These observations suggest that patients with CLD may have a decreased risk of contracting severe SARS-CoV-2. However, the risk of infection and/or the risk of a severe course of COVID-19 may be different depending on the nature of the CLD and the presence or absence of advanced fibrosis or cirrhosis. In that context, the European Association for the Study of the Liver, European Society of Clinical Microbiology and Infectious Diseases and AASLD provided comprehensive guidance for physicians and clinicians for the care of

patients with CLD during the early stages of the COVID-19 pandemic[68,80]. The salient guidelines are summarized in Table 2 for clinical relevance and management of COVID-19 and liver patients.

CONCLUSION

The chaotic conditions of the global spread of COVID-19 necessitate clinical care and management of patients with pre-existing morbidities, especially highly prevalent liver diseases in developed and developing countries to decrease the economic and health losses globally. Understanding the pathophysiological mechanisms of COVID-19 and its associated adverse effects in hepatic diseases is indispensable for the development of therapeutics and vaccines as well as mitigation of risks factors of disease. The WHO guidelines and associations for the study of liver diseases are timely and helpful in decreasing burden of disease and health education. The WHO needs to play a vital role in equitable and global availability (rollout) of COVID-19 vaccines to low-to-middle income countries to prevent COVID-19 pandemic and associated comorbidities.

Table 2 Summary of specific European Association for the Study of the Liver, European Society of Clinical Microbiology and Infectious Diseases and American Association for the Study of Liver Diseases guidelines and recommendations for the clinical care and management of patients with liver diseases during coronavirus disease 2019 pandemic

	Hospitalization and severe COVID-19	Alterations to standard treatment strategies		Progression of liver disease
	Early administration, laboratory findings and risk of SARS-CoV-2 infection	Treatment of higher risk groups	Resumption of targeted treatment and surveillance	Patient education and intensive lifestyle advice
NAFLD	High prevalence risk of SARS-CoV-2 infection in NAFLD patients with COVID-19 suggest an early admission to the hospital	No side effects related to ACE inhibitors or AR blockers to date, thus, arterial hypertension treatment should continue in accordance to prescribed guidelines	Not well known	Intensive lifestyle interventions including nutritional guidance, weight loss and diabetes management may prevent the risk of severe COVID-19 complications
Chronic Viral Hepatitis	Patients on chronic HBV or HCV medications with poor compliance should observed treatment protocols, directly	(1) In HBV and COVID-19 patients, an alternative agent should be considered rather than interferon-α therapy; (2) COVID-19 patients with high risk of severe acute HCV should consider for an appropriate antiviral therapy on case-by-case basis under the full consultation; and (3) COVID-19 patients with resolved HBV infection, receiving corticosteroids, tocilizumab, or other immunosuppressant agents should be considered for appropriate antiviral therapy to prevent viral reactivation under full consultation	(1) Without COVID-19, the patients should continue the HBV or HCV medications in accordance to general guidelines; and (2) in COVID-19 patients, initiation of HBV or HCV medication should be deferred until full recovery from COVID-19 or on case-by-case basis under the full consultation	Use of telemedicine for patients of on-going chronic HBV or HCV treatment without COVID-19
Autoimmune hepatitis	(1) Immunocompromised patients on corticosteroid treatment during COVID-19 requires respiratory support; And (2) patients on respiratory support may be considered for addition of, or conversion to, dexamethasone treatment	(1) Patients on high doses of corticosteroid may show more susceptibility to SARS-CoV-2 infection or severe COVID-19; (2) Low doses may be considered under special circumstances (*e.g.*, drug-induced lymphopenia, or bacterial/fungal superinfection with severe COVID-19) under consultation with specialist; (3) or may consider budesonide as an alternative first line agent in patient without cirrhosis to induce remission who have a flare of autoimmune hepatitis	Immunocompromised patients with COVID-19 may be considered for dosing of corticosteroid, sufficient for adrenal insufficiency	All patients should receive vaccination of *Streptococcus pneumoniae* and influenza
Alcohol-related liver hepatitis	Alcohol-induced severe hepatitis patients on corticosteroid treatment with COVID-19 require respiratory support	Not well known	Not well known	Increased probability of higher alcohol consumption during social distancing, so, preemptive strategies including patient outreach and telephone alcohol liaison, should be considered
Cirrhosis	(1) Cirrhotic patients with COVID-19 should be considered for early hospitalization; and (2) to avoid admission and to prevent decompensation, guidelines on prophylaxis of spontaneous bacterial peritonitis, gastrointestinal hemorrhage and hepatic encephalopathy should be followed	Vasoconstriction therapy should be considered with great caution for critically ill cirrhotic patients with COVID-19	Cirrhotic patients are vulnerable to both SARS-CoV-2 infection and altered standards of patient care during pandemic. Thus, the best efforts should be made for care of cirrhotic patients according to general guidelines	All patients should receive vaccination of *Streptococcus pneumoniae* and influenza
Hepatocellular carcinoma	Specific risk of HCC patients with COVID-19 remains undefined	In COVID-19 patients, initiation of HBV or HCV medication should be deferred until full recovery from COVID-19 or on case-by-case basis under the full consultation	Full HCC surveillance should resume under specific circumstances	Consider virtual patient visits to discuss diagnosis and management of HCC and other liver tumors
Liver	Patients on the liver transplant	Precautions should be followed to make	Not well known	Patients should

transplant candidates	waiting list with decompensated cirrhosis are at high risk of severe COVID-19 and death following SARS-CoV-2 infection	COVID-19 free liver transplantation process		avoid attending in-person community recovery support meetings, such as Alcoholics Anonymous, and provide alternative telephone or online resources
Liver transplant recipients	Early admission should be considered for all liver transplant recipients who develop COVID-19	Drug levels of calcineurin inhibitors and mechanistic target of rapamycin inhibitors should be closely monitored on administration with COVID-19 medications, particularly hydroxychloroquine, protease inhibitors or new trial drugs for COVID-19	Reduction of immunosuppressant dosing may be considered under special circumstances (*e.g.*, drug-induced lymphopenia, or bacterial/fungal superinfection with severe COVID-19) under consultation with specialist	All patients should receive vaccination of *Streptococcus pneumoniae* and influenza

ACE: Angiotensin-converting enzyme; AR: Adrenoreceptor; COVID-19: Coronavirus disease 2019; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma. HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; SARS-CoV-2: Severe acute respiratory syndrome-coronavirus type 2.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Non-responsive celiac disease in children on a gluten free diet

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Abstract

BACKGROUND

Non-responsive celiac disease (NRCD) is defined as the persistence of symptoms in individuals with celiac disease (CeD) despite being on a gluten-free diet (GFD). There is scant literature about NRCD in the pediatric population.

AIM

To determine the incidence, clinical characteristics and underlying causes of NRCD in children.

METHODS

Retrospective cohort study performed at Boston Children's Hospital (BCH). Children < 18 years diagnosed with CeD by positive serology and duodenal biopsies compatible with Marsh III histology between 2008 and 2012 were identified in the BCH's Celiac Disease Program database. Medical records were longitudinally reviewed from the time of diagnosis through September 2015. NRCD was defined as persistent symptoms at 6 mo after the initiation of a GFD

Gastroenterologues du Quebec; and Phase 2 Award from the Fonds de Recherche Sante Quebec.

Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of Boston Children's Hospital.

Informed consent statement: Patients were not required to give informed consent to the study because this study presented no more than minimal risk to patient privacy and confidentiality.

Conflict-of-interest statement: Leffler DA is employed by Takeda Pharmaceuticals International Co. Kelly CP has acted as a scientific advisor to companies attempting to develop new diagnostic and management approaches for Celiac disease including Cour Pharma, Glutenostics, Innovate, Immunogenx and Takeda. He also acts as Principal Investigator on a research grant on Celiac disease supported by Aptalis. Silvester JA has served on an advisory board for Takeda Pharmaceuticals and has received research funding from Biomedal S.L., Cour Pharma and Glutenostics.

Data sharing statement: No additional data are available.

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and causes of NRCD as well as symptom evolution were detailed. The children without symptoms at 6 mo (responders) were compared with the NRCD group. Additionally, presenting signs and symptoms at the time of diagnosis of CeD among the responders and NRCD patients were collected and compared to identify any potential predictors for NRCD at 6 mo of GFD therapy.

RESULTS

Six hundred and sixteen children were included. Ninety-one (15%) met criteria for NRCD. Most were female (77%). Abdominal pain [odds ratio (OR) 1.8 95% confidence interval (CI) 1.1-2.9], constipation (OR 3.1 95%CI 1.9-4.9) and absence of abdominal distension (OR for abdominal distension 0.4 95%CI 0.1-0.98) at diagnosis were associated with NRCD. NRCD was attributed to a wide variety of diagnoses with gluten exposure (30%) and constipation (20%) being the most common causes. Other causes for NRCD included lactose intolerance (9%), gastroesophageal reflux (8%), functional abdominal pain (7%), irritable bowel syndrome (3%), depression/anxiety (3%), eosinophilic esophagitis (2%), food allergy (1%), eating disorder (1%), gastric ulcer with *Helicobacter pylori* (1%), lymphocytic colitis (1%), aerophagia (1%) and undetermined (13%). 64% of children with NRCD improved on follow-up.

CONCLUSION

NRCD after ≥ 6 mo GFD is frequent among children, especially females, and is associated with initial presenting symptoms of constipation and/or abdominal pain. Gluten exposure is the most frequent cause.

Key Words: Celiac disease; Non-responsive celiac disease; Children; Gluten-free diet; Constipation; Abdominal pain

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Core Tip: This is a retrospective chart review study of children < 18 years of age diagnosed with celiac disease (CeD) by positive serology and biopsies showing Marsh III histology to characterize non-responsive CeD (NRCD). NRCD was attributed to a wide variety of diagnoses with gluten exposure (30%) and constipation (20%) being the most common causes. Most (64%) patients improved on follow-up. Our study highlights the importance of performing a diligent search for etiologies of NRCD when there are persistent symptoms despite following a gluten-free diet and reinforces the need for close follow up in the first year of the diagnosis of CeD.

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INTRODUCTION

Celiac disease (CeD) is a chronic immune-mediated enteropathy precipitated by exposure to gluten in genetically predisposed individuals[1]. CeD is diagnosed based on clinical symptoms and serological markers in conjunction with specific histological changes found in the duodenum, including villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis[2].

Strict elimination of gluten is currently the only effective treatment for CeD[2,3]. A gluten-free diet (GFD) leads to significant improvement in most people with CeD within weeks[4]. Failure to respond after six to twelve months of a GFD is defined as non-responsive CeD (NRCD), and may occur in both adults and children[5]. NRCD incorporates a range of specific and distinct underlying diagnoses, such as other food intolerance, small intestinal bacterial overgrowth, microscopic colitis, and irritable bowel syndrome[6,7]; nevertheless, the most common etiology of NRCD is continued

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gluten exposure[6,8]. Studies suggest that 7% to 52% of adult CeD patients may have NRCD[2,5,6]. A recent retrospective study showed that 34% of children adhering to a GFD still report at least one symptoms more than 24 mo after their diagnosis[5]; however, little is known about the spectrum and etiologies of NRCD in children.

The objective of this study was to determine the incidence, clinical characteristics and underlying causes of NRCD in children.

MATERIALS AND METHODS

In this retrospective cohort study, we used the Boston Children's Hospital (BCH) Celiac Disease Program database to identify children less than 18 years of age with an initial biopsy-confirmed CeD diagnosis at BCH between January 1, 2008 and December 31, 2012. Celiac antibodies included tissue transglutaminase (tTG) immunoglobulin (Ig) A, deamidated gliadin peptide (DGP) IgG and endomysial antibody (EMA) IgA. Included patients had Marsh III lesions, defined by increased intraepithelial lymphocytes, villous atrophy, and crypt hyperplasia[2]. Children diagnosed elsewhere and seen for a second opinion, and those who were asymptomatic at the time of diagnosis were excluded.

Data extracted from medical records included demographics, clinical presentation, serologic results, comorbidities, family history of CeD and number of dietitian visits at BCH within 120 d of CeD diagnosis. Medical records were reviewed longitudinally from the time of diagnosis through September 2015.

Adherence to the GFD was determined from review of medical records, primarily from dietician documentation/notes of their clinical assessment which included a dietary interview and is reported using a previously published assessment scale graded as follows: (1) Excellent = patient never ate gluten intentionally and/or had rare exposure, (2) Good = inadvertent exposure once per month, (3) Fair = exposure 2-3 times per month, (4) Poor = exposure 1-2 times per week, (5) Noncompliant = not on a GFD, or (6) Unable to assess GFD adherence from the medical record[9].

NRCD was defined as persistent clinical symptoms six months or more after the initiation of a GFD. Children with clinical improvement while on a GFD were considered as responders and compared with the NCRD group. This study was approved by the BCH Institutional Review Board.

Statistical analysis

Categorical data were summarized as frequency (percentage) and comparisons between responders and children with NRCD made with Fisher's exact test. Continuous data were presented as median with interquartile range (IQR) and compared across groups by Wilcoxon rank-sum test. A stepwise logistic regression model was used to investigate symptoms at the time of diagnosis that are independently associated with NRCD signs and symptoms included abdominal pain, constipation (hard or infrequent stools), nausea/vomiting, diarrhea, weight loss or poor weight gain, fatigue, short stature, gassiness, abdominal distension, loss of appetite, arthritis/arthralgia, rash, and mouth ulcers. $P < 0.20$ was required for a symptom to enter the model, and $P < 0.05$ was required for the symptom to remain in the model. All comparisons were two-sided, with statistical significance established a priori as $P < 0.05$. Data analysis was performed with SAS v9.4 (Cary, NC, United States).

RESULTS

A total of 659 children under 18 years of age were diagnosed with biopsy-confirmed CeD at BCH between January 1, 2008 and December 31, 2012 (Figure 1). All had elevated serum tTG IgA except for nine subjects. These nine were diagnosed with CeD according to the presence of other celiac serology, HLA typing compatible with CeD or response to a GFD. Forty-three who did not have symptoms at the time of the diagnosis (evaluated for CeD because of type 1 diabetes, family history of CeD or short stature) were excluded from the primary analysis. Of the remaining 616 children included, 525 (85%) subjects showed complete response as determined by resolution of clinical symptoms in the 6 (± 2) mo following the initiation of the GFD (responders). Ninety-one children (15%) met the criteria for NRCD, with persistent symptoms 6 mo after the diagnosis.

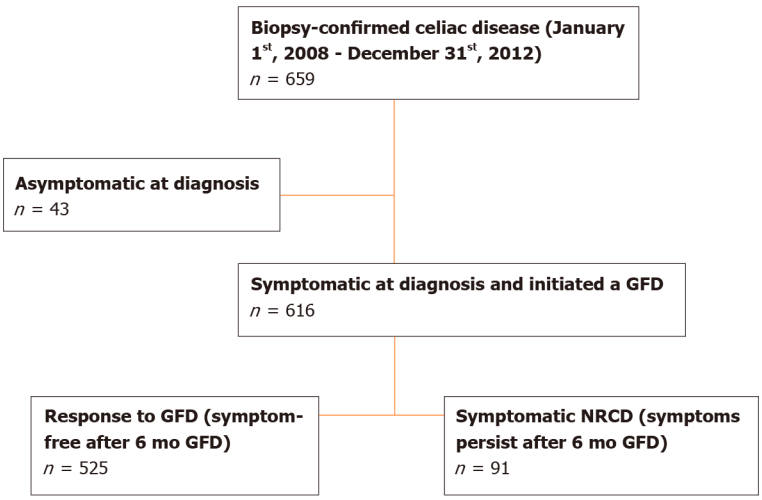


Figure 1 Patient selection flowchart. GFD: Gluten-free diet; NRCD: Non-responsive celiac disease.

Characteristics of responders (n = 525) and NRCD (n = 91) groups are shown in Table 1. Significantly more subjects with NRCD were female (77% *vs* 65%, respectively; P = 0.02). There was no difference in age at time of biopsy, and subjects of all ages were included: < 3 year (9%), 3-6 year (25%), 7-11 year (31%), and 12-17 year (35%). There was a trend towards more Hispanic patients among NRCD subjects compared to responders (5% *vs* 1%, respectively; P = 0.06). Family history of CeD (27%) was less common than family history of other autoimmune disorders (46%), but the incidence for each was similar between NRCD subjects and responders. Finally, at the time of CeD diagnosis, subjects with NRCD had lower body mass index (BMI) z-score compared to responders (median 0.00 (IQR -1.12, 0.58) *vs* 0.18 (IQR -0.48, 0.82); P = 0.02) (Table 1).

Abdominal pain was the most common presenting symptom at diagnosis and was more likely in children with NRCD compared to responders (70% *vs* 54%, P < 0.006). Constipation (hard or infrequent stools) was the second most common presenting symptom and was also more frequent with NRCD (54% *vs* 28%, P < 0.0001). Psychiatric manifestations (anxiety and restrictive eating disorder) were also more prevalent at diagnosis among children with NRCD compared to responders (Table 2).

Symptoms at the time of diagnosis that were determined by stepwise logistic regression to be independently associated with NRCD included constipation, lack of abdominal distension, and abdominal pain. The odds of NRCD were 3.1 (95% confidence interval (CI) 1.9-4.9) times higher for subjects with constipation compared to those with normal bowel movements. Likewise, absence of abdominal distension (odds ratio (OR) for abdominal distension 0.4, 95%CI 0.1-0.98) and presence of abdominal pain (OR 1.8, 95%CI 1.1-2.9) were independently associated with NRCD.

When examining serum tTG IgA antibody levels, 14 (15%) of NRCD patients had a less than 20% decrease in tTG IgA at 6 mo, 11 of whom continued to have tTG IgA levels greater than 1.5 × the upper limit of normal (ULN) at 1 year after diagnosis.

Formal evaluation and counselling by a dietitian within the first 120 d following CeD diagnosis was performed for 75 (82%) of patients with NRCD, compared to 342 (67%) of responders (P = 0.001). Additional nutrition counselling after the first 120 d occurred for 41% (n = 255) children and was more common among NRCD patients than responders (55% *vs* 39%, P < 0.006). GFD adherence was classified as excellent in 54 (66%) of symptomatic NRCD patients and poor in 12 (15%). We were unable to assess adherence to the diet in 9 patients based on medical record review. Seventeen of the 91 patients (19%) had repeat endoscopy as part of their evaluation, a median of 18.9 (IQR 9.5-25.4) months after their initial endoscopy. The majority showed normal villous architecture (82%, n = 14). Two children with persistent villous atrophy had on-going gluten exposure; a third one had follow-up biopsies only 2.8 mo after CeD diagnosis (to follow-up on coexisting eosinophilic esophagitis).

An etiology of NRCD was identified for 88/91 children. Gluten exposure was the most common cause (30%, n = 27) (Figure 2). These children had similar rates of elevated tTG IgA at 6 mo (52% *vs* 46%) and tTG IgA > 1.5 × ULN at one year (15% *vs* 11%) to the rest of the cohort.

Chronic constipation was the underlying cause of NRCD in 18 (20%) patients, and

Table 1 Characteristics of responders to the gluten-free diet and children with non-responsive celiac disease based on persistent symptoms after 6 mo on a gluten-free diet

Characteristic	Responders (*n* = 525)	NRCD (*n* = 91)	*P* value
Female sex, *n* (%)	339 (65)	70 (77)	0.02
Age at biopsy (yr), median (IQR)	9.4 (5.9, 13.4)	9.6 (5.7, 13.4)	0.73
Caucasian race[1] (%)	410/438 (92)	75/85 (91)	1.00
Hispanic[2] (%)	6/438 (1)	4/84 (5)	0.06
Medical history			
Diabetes mellitus, *n* (%)	39 (7)	3 (3)	0.18
Thyroid condition, *n* (%)	20 (4)	1 (1)	0.34
Down syndrome, *n* (%)	12 (2)	0 (0)	0.23
Family history of celiac disease, *n* (%)	144 (27)	22 (24)	0.53
Family history of other autoimmune disease[3], *n* (%)	239 (46)	43 (47)	0.73
BMI at diagnosis[4], median (IQR)	17.0 (15.6, 20.0)	16.4 (15.1, 19.3)	0.03
BMI *z*-score at diagnosis[5], median (IQR)	0.18 (-0.48, 0.82)	0.00 (-1.12, 0.58)	0.02
≥ 1 nutrition visit within first 120 d of GFD, *n* (%)	342 (65)	75 (82)	0.001
Time between diagnosis and first nutrition visit (wk), median (IQR)	4.0 (2.1, 7.0)	3.4 (2.2, 5.6)	0.36

Data unknown:
[1]*n* = 86.
[2]*n* = 84.
[4]*n* = 33.
[5]*n* = 39.
[3]Lupus, rheumatoid arthritis, type 1 diabetes, auto-immune thyroid diseases. *P* value from Fisher's exact test or Wilcoxon rank-sum test. IQR: Interquartile range; GFD: Gluten-free diet; NRCD: Non-responsive celiac disease.

was already present (*n* = 15) and treated with laxatives (*n* = 14) at the time of CeD diagnosis. Other causes for NRCD which were identified are detailed in Figure 2.

Forty-three (64%) of the 67 NRCD children with follow-up had eventual resolution of their clinical symptoms, whereas the remainder (*n* = 24, 26%) were lost to follow-up before symptoms resolved. Of the 11 patients for whom the etiology of NRCD was unclear, 4 were lost to follow-up, and 5 resolved with time. Only 2 children had persistent symptoms of undetermined etiology lasting 3 years or longer. Symptom resolution was frequent in patients with NRCD secondary to gluten exposure (*n* = 14, 67%) and constipation (*n* = 10, 71%). Those with functional abdominal pain (*n* = 6), irritable bowel syndrome (IBS) (*n* = 3), depression or anxiety (*n* = 3) fared worse. Only 1 patient in each group had resolution of symptoms at 3 years of follow-up despite treatment.

DISCUSSION

We report the incidence, etiology, and clinical characteristics of 91 children with NRCD who were diagnosed and treated at BCH between January 2008 and December 2012. The incidence of symptomatic NRCD 6 mo after initiation of a GFD was 15%. Pediatric NRCD was more prevalent in females and associated with a lower BMI *z*-score, greater abdominal pain, constipation and absence of abdominal distension at diagnosis. Etiologies of NRCD mirrored those reported in adults, with gluten exposure as the most common cause; however, there were no cases of refractory CeD.

Although definitions may vary, our results were consistent with previously published data on NRCD in adults of the same geographic region[6]. We found a lower incidence of NRCD than in a recent pediatric cohort study, in which 34% of children had at least one symptom of CeD more than 24 mo after initiating a GFD[5]. It is unclear whether the prevalence of NRCD increases with time, or if the apparently higher rate of NRCD at 24 mo reflects that the responders were lost to follow-up. Our experience

Table 2 Signs and symptoms at diagnosis of celiac disease among responders to the gluten-free diet and non-responsive celiac disease patients

	Responders (*n* = 525)	NRCD (*n* = 91)	*P* value
Abdominal pain, *n* (%)	286 (54)	64 (70)	< 0.006
Hard or infrequent stools, *n* (%)	147 (28)	49 (54)	< 0.0001
Nausea/vomiting, *n* (%)	127 (24)	26 (29)	0.43
Diarrhea, *n* (%)	133 (25)	22 (24)	0.90
Weight loss, poor weight gain, *n* (%)	126 (24)	21 (23)	0.89
Fatigue, *n* (%)	71 (14)	15 (16)	0.51
Short stature, *n* (%)	78 (15)	8 (9)	0.14
Gassiness, *n* (%)	58 (11)	9 (10)	0.86
Abdominal distention, *n* (%)	63 (12)	5 (5)	0.07
Loss of appetite, *n* (%)	53 (10)	14 (15)	0.15
Neurologic[1], *n* (%)	4 (1)	3 (3)	0.07
Psychiatric[2], *n* (%)	0 (0)	2 (2)	0.02
Arthritis, arthralgia, *n* (%)	28 (5)	9 (10)	0.10
Rash, *n* (%)	25 (5)	4 (4)	1.00
Mouth ulcers, *n* (%)	18 (3)	5 (5)	0.37
Other[3], *n* (%)	5 (1)	10 (11)	< 0.0001

[1]Neurologic symptoms include: Headache (*n* = 6), blurry vision (*n* = 1).
[2]Psychiatric symptoms include: anxiety (*n* = 1) and restrictive eating disorder (*n* = 1).
[3]Other signs and symptoms include: Fecal soiling (*n* = 2) dysphagia (*n* = 2), symptomatic anemia (*n* = 2), rectal bleeding (*n* = 3), encopresis (*n* = 2), hair loss (*n* = 2) delayed puberty (*n* = 1), easy bruising (*n* = 1). *P* value from Fisher's exact test. NRCD: Non-responsive celiac disease.

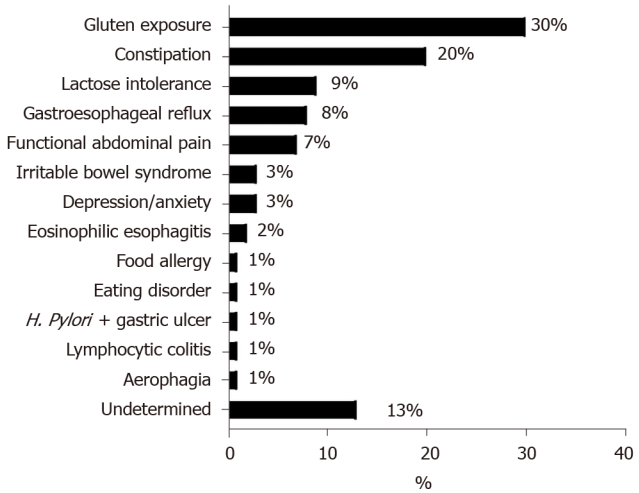


Figure 2 Reasons for non-responsive celiac disease in subjects with persistent symptoms after 6 mo on a gluten-free diet (*n* = 91). *H. Pylori*: *Helicobacter pylori*.

with a pediatric population followed at an academic center that had persisting symptoms six months after the initiation of a GFD may also differ from children who had recurrence of symptoms 12 mo or 18 mo after the diagnosis. Although resolution of symptoms may be slow after starting the GFD, our clinical conclusion is that most children are generally symptom-free after six months on a GFD. Accordingly, 85% of pediatric patients diagnosed at BCH had a good clinical response to the GFD.

Some definitions of NRCD include isolated elevation of tTG[2,6]; however, we did not include such children if they were asymptomatic because tTG is not a reliable proxy

for intestinal histology on a GFD[10]. Coexisting disorders, GFD adherence, the initial degree of tTG elevation and severity of villous atrophy all affect time to normalization of tTG which frequently takes longer than 1 year in children[11,12]. Moreover, the presence of villous atrophy is not a sine qua non for NRCD, since the formal definition is based on signs and symptoms and not histology results[2,6]. On the other hand, by only selecting children with positive serology at the time of the diagnosis of CeD, we may have missed some seronegative NRCD[13]. However, seronegative CD is rare, accounting for less than 1% of cases of pediatric biopsy-confirmed (Marsh III) CD in a recent study, so whether they have a different course than seropositive CD remains unclear[14].

As in previous adult and pediatric studies, females were more likely to be diagnosed with NRCD[5,6]. This trend has also been reported among children with functional abdominal pain, but not constipation[14,15]. Females may be more likely to experience, or report GI symptoms. We did not observe any difference in the age at the time of CeD diagnosis. Intriguingly, an association between longstanding symptoms before the diagnosis of CeD and the risk of NRCD was recently reported[5]. Due to the retrospective design of our study, we unfortunately do not have access to this information; however, we did observe lower BMI *z*-scores at CeD diagnosis among the NRCD group. Lower BMI may be a marker of disease severity, thus explaining a prolonged clinical recovery. This underscores the need to monitor CeD patients after diagnosis because malabsorption due to persistent disease and decreased food intake in relation to GI symptoms may further affect BMI and growth. Moreover, the NRCD group had significantly more symptoms at diagnosis which may reflect visceral hypersensitivity or portend a functional gastrointestinal disorder. Although these manifestations are frequently described among pediatric CeD patients[5], the rate and response to a GFD is variable[16]. Identification of a cause other than gluten exposure for NRCD in most of our patients highlights the importance of considering a coexisting disorder.

Gluten exposure was the most common cause of NRCD in our cohort, as previously reported in adults[6,8]. A strict GFD is the only current treatment for CeD. Consultation with a registered dietitian for specialized GFD education at the time of CeD diagnosis is recommended in clinical practice guidelines and standard practice at our institution[2,3]. Nonetheless, only 65% of the responder group and 82% of the NRCD group had a dietitian visit at our institution within the first 4 mo after diagnosis of CD. Due to the retrospective nature of this study, we were unable to ascertain whether some individuals received GFD education outside of our institution, or if families opted out of dietitian visits because a family member had received GFD education. Nevertheless, additional nutrition counselling after the first 120 days was also more common among NRCD patients than responders, perhaps because ongoing symptoms prompt families to seek out additional education/support. Recent American College of Gastroenterology guidelines recommend reevaluation of diet as a first step in the management of NRCD[2].

Eliminating gluten ingestion, both intentional and unintentional, is a significant challenge for many pediatric CeD patients. Even with specialized GFD education, a sizable number of patients had ongoing gluten exposure. Avoiding gluten cross-contact, which occurs during processing, preparation and serving of foods, is especially difficult. Non-adherence to a strict GFD in children and teenagers has been estimated at 8% when disclosed openly[5], but may be nearer 30% when stools are assessed for gluten immunogenic peptides[17] and may increase with GFD duration[18]. Interestingly, 82% of symptomatic NRCD patients in our study appeared to have excellent or good GFD adherence. This is likely an overestimate due to the inherent challenges associated with assessing GFD adherence through dietary recalls and interviews compared to objective measures such as fecal gluten immunogenic peptides[17].

Fourteen (67%) of the 21 symptomatic NRCD patients with suspected ongoing gluten exposure experienced resolution of their symptoms with improved GFD adherence. It is unclear whether the remaining 7 patients were unsuccessful at fully excluding dietary gluten or if healthcare providers erroneously suspected continued gluten exposure as the cause of NRCD. This cohort was prior to the development of the technology allowing the identification of gluten immunogenic peptides in urine and stools. While these technologies appear promising, currently there is no validated measure of gluten exposure other than dietitian assessment. Although the second step in the management algorithm of NRCD in adults after excluding gluten exposure or food intolerance is to proceed to duodenal biopsies to assess for villous atrophy[2], most patients in this pediatric NRCD cohort did not undergo repeat endoscopy. Moreover, some degree of villous atrophy 6 mo to 12 mo after CeD diagnosis is not uncommon

among patients in clinical remission[19].

The second most common cause of NRCD was constipation, which was also an initial manifestation of CeD for most of these patients. Constipation is a common presenting symptom among children with CeD[5,20]. 196 children included in this cohort were constipated at the time of diagnosis, but only 18 had persisting constipation that was deemed to be the cause of NRCD. The GFD is inherently low in fibers which may precipitate constipation in CeD patients[21]. Thus, some patients with constipation as a cause of NRCD may have had either initially treated constipation that recurred on the GFD after stopping laxative (n = 7), new constipation probably caused by the GFD (n = 3) or persisting constipation despite initial laxative therapy (n = 7) or noncompliance to laxative therapy (n = 1). The latter may have been suffering from functional constipation unrelated to CeD, as it is a quite common disorder in children[22]. Nevertheless, many of these patients subsequently improved, highlighting that early and intensive strategies in addition to the GFD can be effective and may avoid persistent symptoms in children.

Notably, constipation is not typically reported as an etiology of NRCD in adults, possibly because they are diagnosed with IBS. Functional abdominal pain and IBS were the cause of NRCD in 10% of our cohort. In comparison, 18% of adult NRCD patients may have IBS[6]. Other less common etiologies include lactose intolerance, present in 9% of our cohort and 7% of adults with NRCD[6]. Upper GI conditions, including gastroesophageal reflux, peptic ulcer disease, *Helicobacter pylori* infection and eosinophilic esophagitis were also responsible for persistent symptoms in pediatric NRCD. Finally, anxiety and eating disorders were responsible for NRCD and may be associated with gastrointestinal dysmotility. This underscores the need to consider the patient as a whole, and how conditions other than CeD may contribute to symptoms on a GFD.

While most children with NRCD had resolution of their symptoms over the follow-up period, symptoms persisted in 36%. Patients with constipation had higher rates of resolution than those with diagnoses such as functional abdominal pain or IBS. Functional abdominal pain is frequent among children with and without CeD, with a recent multicenter study finding a prevalence of 2.1% to 8.2%[23]. The prevalence of IBS is somewhat lower, from 1.2% to 2.9%[15,24].

Strengths and limitations

This large cohort of children with CeD provides epidemiologic data on the characteristics, causes and evolution of pediatric NRCD thereby addressing an important knowledge gap. Chart reviews conducted by either one of two authors (GV and MD) were reviewed by an expert clinical pediatric gastroenterologist; however, the retrospective nature of this study limited our ability to perform a standardized evaluation of each patient. The large number of clinicians at our institution contributed to variability in documentation and differences in approach to medical evaluation. Also, many etiologies of NCRD are diagnosed based primarily on clinical history. Although inclusion of only children who were diagnosed at BCH reduces referral bias, our cohort may differ from populations in other geographic areas.

CONCLUSION

In summary, NRCD after six months of GFD is frequent among children, especially females. Initial presentation with constipation, abdominal pain and absence of abdominal distension is associated with subsequent diagnosis of NRCD. Although gluten exposure was the most frequent cause, a wide variety of diagnoses are also found among NRCD patients. Our study highlights the importance of performing a diligent search for the above etiologies for NRCD in any celiac child with persistent clinical symptoms despite being on GFD and reinforces the need for close follow up in the first year of a CeD diagnosis.

ARTICLE HIGHLIGHTS

Research background

Non-responsive celiac disease (NRCD) is defined as the persistence of symptoms in individuals with celiac disease (CeD) despite being on a gluten-free diet (GFD). There is scant literature about NRCD in the pediatric population.

Research motivation

Addressing an important knowledge gap, this study examines a large cohort of children with CeD providing data on the characteristics, causes and evolution of pediatric NRCD. By characterizing this sub-population of individuals with CeD, we are better equipped to provide clinical guidance and follow-up in those with persistent symptoms.

Research objectives

Through this retrospective cohort study, we sought to determine the incidence, clinical characteristics, and underlying causes of NRCD in children. Additionally, symptom evolution was detailed and compared to identify any potential predictors for NRCD.

Research methods

Retrospective cohort study performed at Boston Children's Hospital (BCH). Children < 18 years diagnosed with CeD by positive serology and duodenal biopsies compatible with Marsh III histology between 2008 and 2012 were identified in the BCH's Celiac Disease Program database. Medical records were longitudinally reviewed from the time of diagnosis through September 2015. NRCD was defined as persistent symptoms at 6 mo after the initiation of a GFD, and causes of NRCD as well as symptom evolution were detailed and compared to identify any potential predictors for NRCD.

Research results

Six hundred and sixteen children were included in this retrospective study, of which 91 (15%) met criteria for NRCD, and of this, most were female (77%). Abdominal pain [odds ratio (OR) 1.8 95% confidence interval (CI) 1.1-2.9], constipation (OR 3.1 95%CI 1.9-4.9) and absence of abdominal distension (OR for abdominal distension 0.4 95%CI 0.1-0.98) at diagnosis were associated with NRCD. NRCD was attributed to a wide variety of diagnoses with gluten exposure (30%) and constipation (20%) being the most common causes. 64% of children with NRCD improved on follow-up.

Research conclusions

NRCD after ≥ 6 mo of GFD is frequent among children, especially females, and is associated with initial presenting symptoms of constipation and/or abdominal pain. Gluten exposure is the most frequent cause. Our study highlights the importance of performing a diligent search for the etiologies for NRCD in any celiac child with persistent clinical symptoms despite being on GFD and reinforces the need for close follow up in the first year of a CeD diagnosis.

Research perspectives

Although the use of a large pediatric cohort positively contributes to the breadth of knowledge surrounding NRCD, and inclusion of only children who were diagnosed at BCH reduces referral bias, our cohort may differ from populations in other geographic areas. As such, a future direction of note is to extend this project to include pediatric Celiac Disease Programs across the United States, to assess if geographic location is a factor in the manifestation and characterization of NRCD.

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ORIGINAL ARTICLE

Retrospective Study

Endoscopic diagnosis for colorectal sessile serrated lesions

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Author contributions: Nishizawa T analyzed the data, and wrote the manuscript; Yoshida S performed colonoscopy and collected the data; Toyoshima A contributed to the data interpretation; Yamada T reviewed endoscopic images; Sakaguchi Y, Irako T, and Ebinuma H critically revised the manuscript; Kanai T and Koike K supervised the study; Toyoshima O designed the study, and analyzed the data.

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Abstract

BACKGROUND

Hyperplastic polyps are considered non-neoplastic, whereas sessile serrated lesions (SSLs) are precursors of cancer *via* the ''serrated neoplastic pathway''. The clinical features of SSLs are tumor size (> 5 mm), location in the proximal colon, coverage with abundant mucus called the ''mucus cap'', indistinct borders, and a cloud-like surface. The features in magnifying narrow-band imaging are varicose microvascular vessels and expanded crypt openings. However, accurate diagnosis is often difficult.

AIM

To develop a diagnostic score system for SSLs.

METHODS

We retrospectively reviewed consecutive patients who underwent endoscopic resection during colonoscopy at the Toyoshima endoscopy clinic. We collected

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data on serrated polyps diagnosed by endoscopic or pathological examination. The significant factors for the diagnosis of SSLs were assessed using logistic regression analysis. Each item that was significant in multivariate analysis was assigned 1 point, with the sum of these points defined as the endoscopic SSL diagnosis score. The optimal cut-off value of the endoscopic SSL diagnosis score was determined by receiver-operating characteristic curve analysis.

RESULTS
Among 1288 polyps that were endoscopically removed, we analyzed 232 diagnosed as serrated polyps by endoscopic or pathological examination. In the univariate analysis, the location (proximal colon), size (> 5 mm), mucus cap, indistinct borders, cloud-like surface, and varicose microvascular vessels were significantly associated with the diagnosis of SSLs. In the multivariate analysis, size (> 5 mm; $P = 0.033$), mucus cap ($P = 0.005$), and indistinct borders ($P = 0.033$) were independently associated with the diagnosis of SSLs. Size > 5 mm, mucus cap, and indistinct borders were assigned 1 point each and the sum of these points was defined as the endoscopic SSL diagnosis score. The receiver-operating characteristic curve analysis showed an optimal cut-off score of 3, which predicted pathological SSLs with 75% sensitivity, 80% specificity, and 78.4% accuracy. The pathological SSL rate for an endoscopic SSL diagnosis score of 3 was significantly higher than that for an endoscopic SSL diagnosis score of 0, 1, or 2 ($P < 0.001$).

CONCLUSION
Size > 5 mm, mucus cap, and indistinct borders were significant endoscopic features for the diagnosis of SSLs. Serrated polyps with these three features should be removed during colonoscopy.

Key Words: Sessile serrated lesion; Mucus cap; Indistinct borders; Hyperplastic polyp; Endoscopic features; Size

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Core Tip: The features of sessile serrated lesions (SSLs) include tumor size > 5 mm, location in the proximal colon, ''mucus cap'', indistinct borders, cloud-like surface, and varicose microvascular vessels. Our multivariate analysis revealed that size > 5 mm, mucus cap, and indistinct borders were independent predictors for SSLs. The combination of these three features in serrated polyps allowed the diagnosis of SSLs with 75% sensitivity, 80% specificity, and 78.4% accuracy.

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INTRODUCTION

Recent molecular biology studies have supported the classification of colorectal serrated lesions into hyperplastic polyps (HPs), sessile serrated lesions (SSLs), and traditional serrated adenomas (TSAs)[1]. HPs are considered non-neoplastic, whereas SSLs and TSAs are both precursors of cancer *via* the "serrated neoplastic pathway", which is responsible for up to 20% of all sporadic colorectal cancers[2]. The features of serrated neoplastic pathways include high microsatellite instability and the potential for rapid conversion to malignancy[3,4]. This implies that all SSLs should be accurately diagnosed and endoscopically removed[5], whereas HPs do not need to be removed. Therefore, it is essential to differentiate SSLs from HPs.

SSLs typically occur in the proximal colon[6]. Endoscopically, SSLs usually measure > 5 mm, are frequently covered by mucus called ''mucus cap'', and have indistinct borders and a cloud-like surface[7]. The features of SSLs under magnifying narrow-

band imaging (NBI) are varicose microvascular vessels and expanded crypt openings[8]. The feature of SSLs on chromoendoscopy is a type II open-shape pit pattern. In contrast, HPs commonly occur in the distal colon or rectum and are often ≤ 5 mm in size. However, differential diagnoses are often difficult[9]. We hypothesized that well-designed combination of endoscopic features might be useful for the diagnosis of SSLs. Therefore, the present study aimed to develop a diagnostic score system for SSLs.

MATERIALS AND METHODS

Ethics

This retrospective study was approved by the Ethical Review Committee of the Hattori Clinic on September 4, 2020 (approval No. S2009-U04). Written informed consent was obtained from all participants. All clinical investigations were conducted according to the ethical guidelines of the Declaration of Helsinki.

Patients

We retrospectively reviewed consecutive patients who underwent polypectomy or endoscopic mucosal resection during colonoscopy between July and September 2019 and between January and March 2020 at the Toyoshima Endoscopy Clinic. Colonoscopy was performed to evaluate symptoms such as hematochezia, rectal bleeding, abdominal symptoms, abnormal bowel habits, anemia, or positive fecal immunochemical test findings or for colorectal polyp surveillance. We used an electronic endoscopy reporting system (T-File System; STS Medic, Japan) that was integrated into the clinic's patient record systems.

Endoscopic procedures

The colonoscopies were performed using an Elite CF290 endoscopy system (CV-290 and CLV-290, Olympus, Japan) with a 290 series colonoscope (CF-HQ290Z, CF-HQ290, or PCF-H290Z, Olympus, Japan) or a 260 series colonoscope (PCF-PQ260)[10]. The patients underwent colonoscopies while under conscious sedation with midazolam and/or pethidine hydrochloride. Pancolonic chromoendoscopy was performed by spraying 0.05% indigo carmine[10,11]. All polyps detected by white light imaging during colonoscopy were washed with water, and also assessed by NBI with and without magnification. The colonoscopies were performed by three expert endoscopists (Nishizawa T, Sakaguchi Y, and Toyoshima O).

Endoscopic diagnosis was performed based on the Workgroup on Serrated Polyps and Polyposis classification[12]. We removed lesions diagnosed as adenomas or clinically significant serrated polyps. A clinically significant serrated polyps was defined as any SSL, TSA, or HP measuring ≥ 1 cm anywhere in the colon or HP measuring ≥ 5 mm located proximal to the sigmoid colon[13-15]. We included polyps measuring 15 mm or more in diameter.

The location, size, morphology, and endoscopic diagnosis for each detected polyp were recorded. The location of the polyps was specified as the cecum; ascending, transverse, descending, or sigmoid colon; or rectum. The distal side from the splenic flexure was defined as the distal colon, while the proximal side to the splenic flexure was defined as the proximal colon. The polyp size was estimated by comparison with a closed snare or forceps. The morphology and presence of a mucus cap, indistinct borders, cloud-like surface, varicose microvascular vessels, and expanded crypt opening were also recorded. The mucus cap was defined as coverage with abundant mucus (Figure 1A). Indistinct borders were defined as vague demarcations of the lesion border (Figure 1B). A cloud-like surface appeared granular, nodular, or bumpy and like the surface of a cumulus cloud. Varicose microvascular vessels were defined as vessels thicker than meshed capillary vessels that meandered similar to varicose veins. Expanded crypt opening was defined as the heterogeneous expansion of nearby crypts.

The pathological findings were evaluated by hematoxylin and eosin staining and histological diagnoses were made by an expert gastrointestinal pathologist (H. W.). Serrated lesions were classified as HP, SSL, and TSA according to the World Health Organization classification[16,17]. We collected data on polyps with endoscopic or pathological diagnosis of serrated polyps.

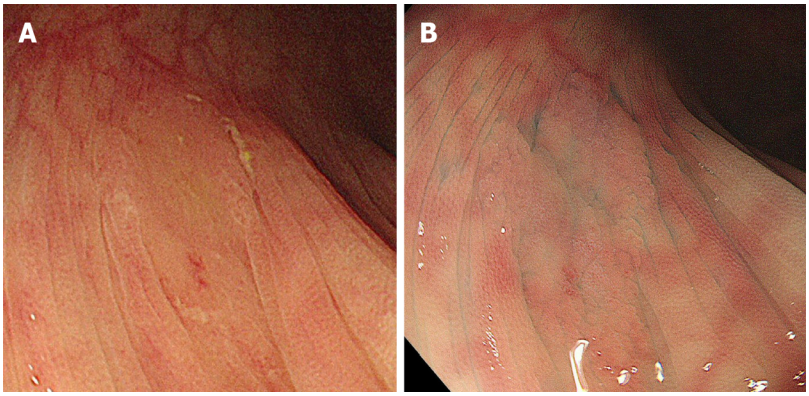


Figure 1 Endoscopic findings regarding sessile serrated lesions. A: "Mucus cap" was defined as coverage with abundant mucus; and B: Indistinct borders were defined as vague demarcations of the lesion border.

Statistical analysis

The significant factors for the diagnosis of SSLs were assessed using logistic regression analysis. The univariate and multivariate analyses included the location (proximal colon) and size (> 5 mm) and presence of mucus cap, indistinct borders, cloud-like surface, varicose microvascular vessels, and expanded crypt opening. Each item that was significant in the multivariate analysis was assigned 1 point and the sum of these points was defined as the endoscopic SSL diagnosis score. The optimal cut-off endoscopic SSL diagnosis score was determined by receiver-operating characteristic (ROC) curve analysis. The calculations were performed using StatMate software, version IV (ATOMS, Tokyo, Japan) and Ekuseru-Toukei 2015 (Social Survey Research Information company, Limited, Tokyo, Japan).

RESULTS

A total of 629 patients underwent colonoscopies with polypectomy or endoscopic mucosal resection. Among 1288 polyps removed from these patients, we identified 232 polyps as endoscopically or pathologically diagnosed serrated polyps. A study flowchart of the included polyps was presented in Figure 2.

The characteristics of the polyps are shown in Table 1. The pathological diagnoses included 72 SSLs, 130 HPs, 1 TSA, 7 adenomas, 20 normal mucosa, and 2 others.

Table 2 shows the results of the univariate and multivariate analyses for the diagnosis of SSL. In the univariate analyses, the location (proximal colon), size > 5 mm, mucus cap, indistinct borders, cloud-like surface, and varicose microvascular vessels were significantly associated with the diagnosis of SSL. On the other hand, expanded crypts opening was not significant in the univariate analysis. In the multivariate analysis, size > 5 mm (P = 0.033), mucus cap (P = 0.005), and indistinct borders (P = 0.033) were independently associated with the diagnosis of SSL. Size > 5 mm, mucus cap, and indistinct borders were each assigned 1 point and the sum of the points was defined as the endoscopic SSL diagnosis score. The minimum and maximum endoscopic SSL diagnosis scores were 0 and 3, respectively. In the ROC curve analysis, the area under the curve was 0.806 and the optimal endoscopic SSL diagnosis score cut-off was 3 (Figure 3A), which corresponded to 75% sensitivity, 80% specificity, and 78.4% accuracy for the prediction of pathological SSL. The ROC curves of the three predictors (size, mucus cap, and indistinct borders) were presented in Figure 3B-D, respectively.

Figure 4 shows the pathological SSL rates for each endoscopic SSL diagnosis score. The pathological SSL rate for an endoscopic SSL diagnosis score of 3 was significantly higher than that for an endoscopic SSL diagnosis score of 0, 1, or 2 (P < 0.001).

DISCUSSION

We found that size > 5 mm, mucus cap, and indistinct borders were independent predictors for SSLs. The combination of these three features in serrated polyps allowed the diagnosis of SSLs with 75% sensitivity, 80% specificity, and 78.4% accuracy.

Table 1 Polyp characteristics

Polyp characteristics	
Size	
Diminutive (≤ 5 mm)	114
Small (6-9 mm)	82
Large (≥ 10 mm)	36
Shape (Paris classification)	
Is	4
IIa	226
IIb	1
IIc	1
Location	
Cecum	33
Ascending colon	61
Transvers colon	71
Descending colon	12
Sigmoid colon	42
Rectum	13
Pathology	
Sessile serrated lesion	72
Hyperplastic polyp	130
Traditional serrated adenoma	1
Adenoma	7
Normal mucosa	20
Others	2

Table 2 Univariate and multivariate analyses on the diagnosis of sessile serrated lesion

Variables	Univariate analysis			Multivariate analysis		
	Partial regression coefficient	95% confidence interval	*P* value	Partial regression coefficient	95% confidence interval	*P* value
Location (proximal colon)	1.094	0.374-1.813	0.003	0.197	-0.685-1.078	0.662
Size (> 5mm)	2.050	1.369-2.731	< 0.001	0.904	0.074-1.733	0.033
Mucus cap	2.548	1.657-3.440	< 0.001	1.520	0.448-2.592	0.005
Indistinct borders	2.067	1.371-2.764	< 0.001	0.926	0.075-1.778	0.033
Varicose microvascular vessel	0.668	0.088-1.248	0.024	-0.133	-0.869-0.602	0.723
Cloud-like surface	1.640	1.015-2.264	< 0.001	0.708	-0.022-1.437	0.057
Expanded crypts opening	0.367	-0.202-0.936	0.206	-0.497	-1.198-0.203	0.164

Various studies have sought to clarify the features of SSLs. Hasegawa *et al*[18] investigated 107 SSLs to discriminate them from other serrated lesions, reporting that the mean lesion size of SSLs was larger than that of HP (14.2 *vs* 6.2 mm; $P < 0.01$). Moreover, SSL was preferentially located in the proximal rather than the distal colon (81.8% *vs* 18.2%), whereas HP was located less often in the proximal colon than in the distal colon.

Hazewinkel *et al*[19] investigated 150 polyps, including 50 SSLs, 50 HPs, and 50

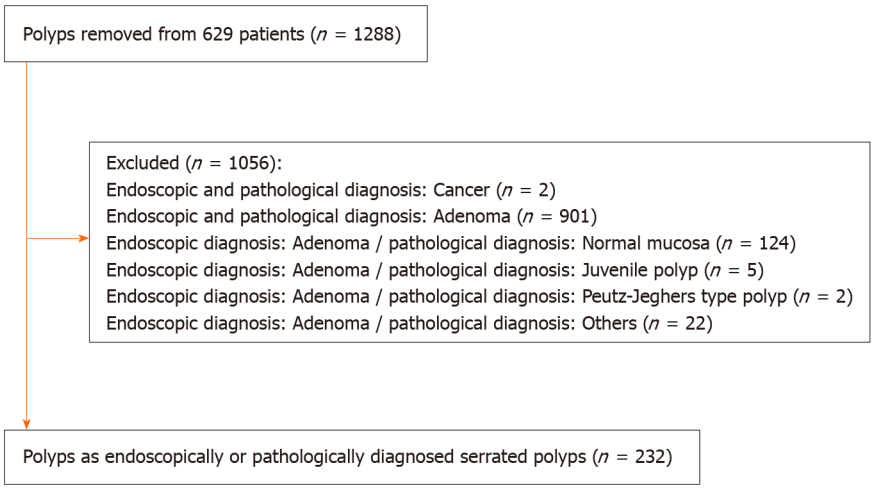


Figure 2 Flowchart of polyp enrollment.

adenomas. Their multivariate analysis demonstrated that indistinct borders [odds ratio (OR): 3.11, P = 0.001] and cloud-like surface (OR: 2.65, P = 0.015) were independent diagnostic factors for SSLs on white-light imaging.

Uraoka *et al*[1] investigated 89 Lesions, including 38 SSLs and 41 HPs. Their multivariate analysis showed that varicose microvascular vessels (OR: 8.2, P = 0.001), size ≥ 10 mm (OR: 7.2, P = 0.002), and proximal location (OR: 6.1, P = 0.004) were independent diagnostic factors for SSLs.

Pereyra *et al*[20] investigated 440 polyps, including 34 SSLs, 135 HPs, and 249 adenomas. Their multivariate analysis showed that flat morphology (OR: 3.81, P = 0.002), red-colored surface (OR: 12.97, P <0.001), right-sided location (OR: 22.21, P <0.001), and mucus cap (OR: 8.77, P < 0.001) were independent diagnostic factors for SSLs.

Murakami *et al*[7] summarized the features of SSLs in their review report, indicating that SSLs were > 5 mm, frequently covered by a mucus cap, and more commonly located in the proximal colon. The features also included small dark spots and varicose microvascular vessels on magnifying NBI and type II open pit patterns on magnifying chromoendoscopy. The small dark spots are nearly synonymous with expanded crypt openings and these are considered to correspond to type II open pit patterns. This phenomenon is likely due to mucin overproduction, which could also lead to the mucus cap[19].

The results of our univariate analysis showed that proximal colon, size > 5 mm, mucus cap, indistinct borders, cloud-like surface, and varicose microvascular vessels were significant factors for the diagnosis of SSLs. Multivariate analysis revealed that size > 5 mm, mucus cap, and indistinct borders were independent predictors for SSLs in our clinical setting.

A combination of endoscopic features has also been previously proposed for the diagnosis of SSL. For instance, Yamada *et al*[21] evaluated the combination of three endoscopic features for SSLs. The combination of dilated and branching vessels (synonymous with varicose microvascular vessels), proximal location, and size (≥ 10 mm) resulted in an area under the curve of 0.7832. The best cut-off point was 2, which corresponded to 79% sensitivity and 81% specificity.

The endoscopic SSL diagnosis score consists of size (> 5 mm), mucus cap, and indistinct borders. The area under the curve in this study was 0.806. The optimal cut-off point was 3, which resulted in 75% sensitivity and 80% specificity. Our score is simple and does not require magnifying NBI. Thus, it may be useful in busy clinical settings or examinations with a scope without a magnifying function.

The present study has several limitations. First, this retrospective study was conducted at a single institution; however, the recording of medical data was well controlled. Second, our study did not perform magnifying chromoendoscopy. A follow-up study is needed to verify the validity of the endoscopic SSL diagnosis score.

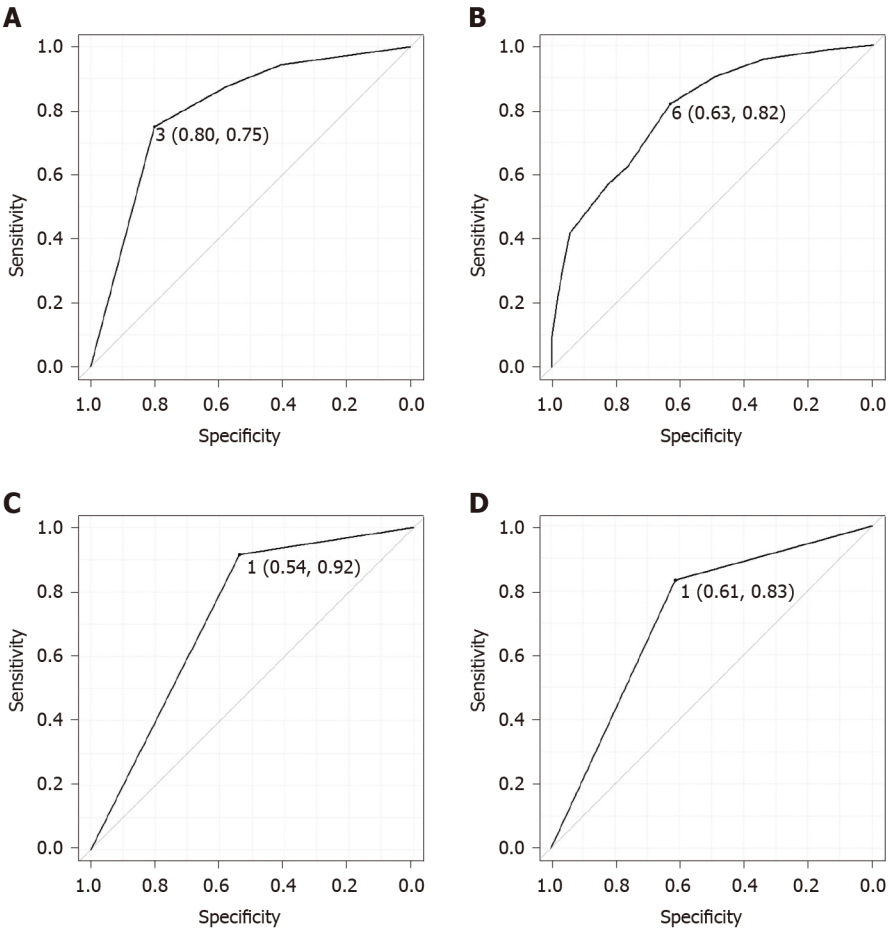


Figure 3 Receiver-operating characteristic curve for predicting sessile serrated lesions. A: Receiver-operating characteristic (ROC) curve for predicting sessile serrated lesion (SSL) based on the endoscopic SSL diagnosis score: The area under the curve (AUC) was 0.806. The optimal cutoff value was 3, for which the endoscopic SSL diagnosis score predicted pathological SSLs with 75% sensitivity and 80% specificity; B: ROC curve for predicting SSL based on polyp size: AUC was 0.801. Size ≥ 6 mm predicted pathological SSLs with 82% sensitivity, and 63% specificity; C: ROC curve for predicting SSL based on mucus cap: AUC was 0.727. The presence of mucus cap predicted pathological SSLs with 92% sensitivity, and 54% specificity; and D: ROC curve for predicting SSL based on indistinct borders: AUC was 0.723. The presence of indistinct borders predicted pathological SSLs with 83% sensitivity, and 61% specificity.

CONCLUSION

In conclusion, size (> 5 mm), mucus cap, and indistinct borders were significant endoscopic features for the diagnosis of SSLs. Serrated polyps with these three features should be removed during colonoscopy.

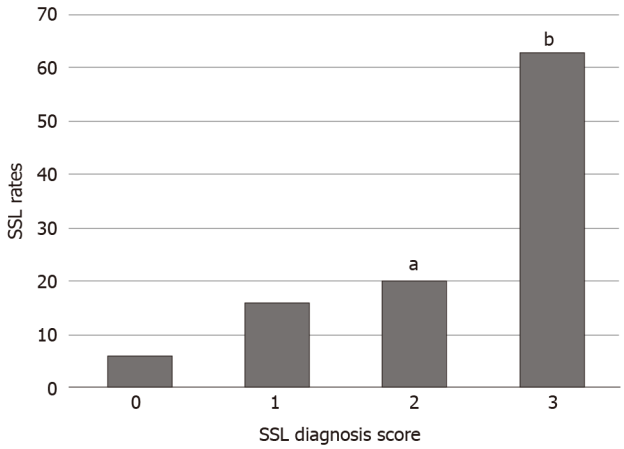


Figure 4 Sessile serrated lesion diagnosis rates based on the endoscopic sessile serrated lesion diagnosis score. [a]*P* < 0.05 compared to an endoscopic Sessile serrated lesion diagnosis score of 0; [b]*P* < 0.001 compared to an endoscopic sessile serrated lesion diagnosis score of 0, 1, or 2. SSL: Sessile serrated lesion.

ARTICLE HIGHLIGHTS

Research background

The serrated neoplastic pathway is responsible for up to 20% of all sporadic colorectal cancers. Sessile serrated lesions (SSLs) should be accurately diagnosed and endoscopically removed.

Research motivation

Various findings have been proposed as features of SSLs. However, accurate diagnosis is often difficult.

Research objectives

This study developed a scoring system to predict the diagnosis of SSLs.

Research methods

We retrospectively reviewed patients who underwent endoscopic resection at the Toyoshima Endoscopy Clinic. We collected data on 232 polyps that were endoscopically or pathologically diagnosed as serrated polyps. The significant factors for the diagnosis of SSLs were assessed using logistic regression analysis.

Research results

In the multivariate analyses, size (> 5 mm; $P = 0.033$), mucus cap ($P = 0.005$), and indistinct borders ($P = 0.033$) were independently associated with a diagnosis of SSL. The endoscopic SSL diagnosis score consisted of three features. An endoscopic SSL diagnosis score of 3 predicted pathological SSLs with 75% sensitivity, 80% specificity, and 78.4% accuracy.

Research conclusions

Size (> 5 mm), mucus cap, and indistinct borders were significant endoscopic features for the diagnosis of SSL. Serrated polyps with these three features should be removed during colonoscopy.

Research perspectives

A follow-up study is needed to verify the validity of the endoscopic SSL diagnosis score.

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ORIGINAL ARTICLE

Retrospective Study

Factors influencing the short-term and long-term survival of hepatocellular carcinoma patients with portal vein tumor thrombosis who underwent chemoembolization

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Abstract

BACKGROUND

The factors affecting the short-term and long-term prognosis of hepatocellular carcinoma (HCC) patients with portal vein tumor thrombosis (PVTT) receiving transarterial chemoembolization (TACE) are still unclear.

AIM

To clarify the predictors correlated with the short-term and long-term survival of HCC patients with PVTT who underwent TACE.

METHODS

The medical records of 181 HCC patients with PVTT who underwent TACE at the Second Affiliated Hospital of Chongqing Medical University from January 2015 to July 2019 were retrospectively analyzed. We explored the short-term and long-term prognostic factors by comparing the preoperative indicators of patients who died and survived within 3 mo and 12 mo after TACE. Multivariate analyses were conducted using logistic regression. The area under the receiver operating characteristic curve (area under curve) was used to evaluate the predictive ability of the factors related to the short-term and long-term prognosis.

RESULTS

The median survival time was 4.8 mo (range: 2.5-8.85 mo). The 3 mo, 6 mo, and 12 mo survival rates were 68.5%, 38.7%, and 15.5%, respectively. In multivariable analysis, total bilirubin, sex, and aspartate aminotransferase (AST) were closely linked to short-term survival. When AST ≥ 87 U/L and total bilirubin ≥ 16.15 μmol/L, the 3-mo survival rate after TACE was reduced significantly ($P < 0.05$). AST had the best predictive ability, followed by total bilirubin, while sex had the worst predictive ability for short-term survival area under curve: 0.763 (AST) *vs*

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0.707 (total bilirubin) *vs* 0.554 (sex)]. The long-term survival outcome was significantly better in patients with a single lesion than in those with ≥ three lesions (P = 0.009). Patients with massive block HCC had a worse long-term survival than patients with nodular and diffuse HCC (P = 0.001).

CONCLUSION
AST, total bilirubin, and sex are independent factors associated with short-term survival. The number of tumors and the gross pathological type of tumor are related to the long-term outcome.

Key Words: Transarterial chemoembolization; Hepatocellular carcinoma; Portal vein tumor thrombosis; Survival; Prognostic factors

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Core Tip: It is unclear which factors affect the short-term and long-term prognosis of hepatocellular carcinoma patients with portal vein tumor thrombosis receiving transarterial chemoembolization. In our research, we clarified the predictors correlated with the short-term and long-term survival of hepatocellular carcinoma patients with portal vein tumor thrombosis who underwent transarterial chemoembolization by analyzing preoperative indicators. Results showed that aspartate aminotransferase, sex, and total bilirubin were independent factors associated with short-term survival. The number of lesions and the gross pathological type of tumor were related to the long-term outcome.

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INTRODUCTION

Primary liver cancer is currently one of the most common malignant tumors, and it is among the top five causes of death and morbidity from malignant tumors around the world[1]. Hepatocellular carcinoma (HCC) is the most common pathological type, accounting for approximately 90% of all primary liver cancers. Because of its biological and anatomical characteristics, HCC easily invades the portal vein during the progression of liver cancer and forms portal vein tumor thrombosis (PVTT). Some reports have proposed that the incidence of HCC with PVTT is approximately 44%-62.2%[2]. HCC accompanied by PVTT is closely associated with a poor prognosis and is likely to lead to intrahepatic metastasis, liver function damage, portal hypertension, upper gastrointestinal bleeding, and other complications. The median overall survival time of HCC patients with PVTT who receive no treatment is only 2.7 mo[3].

There is still controversy about the treatment of HCC with PVTT in Eastern and Western countries. Previous studies have suggested that HCC with PVTT should be classified as stage C of Barcelona Clinic Liver Cancer and is no longer suitable for surgical treatment[4]. Some researchers have mentioned that compared with conservative treatment, TACE is a safe and effective therapy for selected HCC patients with PVTT[5-7]. However, some patients die in the short term after TACE, and patients who have expected postoperative survival times of less than 3 mo may not be suitable for TACE.

At present, the factors affecting the short-term (3 mo) and long-term (12 mo) prognosis of HCC patients with PVTT treated with TACE are still unclear. We aimed to identify the preoperative factors related to the short-term and long-term prognosis by comparing the preoperative clinical data of patients who died in the short-term (< 3 mo) with those who survived into the long-term (> 12 mo) after TACE. The area under the curve (AUC) was utilized to assess the predictability of the factors related to short-term and long-term survival to provide some help for doctors when screening HCC

patients with PVTT to identify those who can benefit from TACE.

MATERIALS AND METHODS

Patient

We enrolled a total of 181 HCC patients with PVTT who received TACE in the Second Affiliated Hospital of Chongqing Medical University from January 2015 to July 2019. The inclusion criteria were as follows: (1) Histopathology confirmed as HCC or clinically diagnosed as HCC; (2) Abdominal color Doppler ultrasound, digital subtraction angiography, contrast-enhanced computerized tomography, or magnetic resonance imaging showed signs of PVTT; (3) No treatment, such as surgery, radiotherapy, chemotherapy, liver transplantation, targeted, or biological therapy, was administered before and after TACE; (4) Age ≥ 18 years; and (5) Complete clinical data were available. Patients with other malignancies or severe heart and lung diseases were eliminated. The follow-up endpoint was July 2020 or the date of death, whichever came first. The follow-up was conducted by telephoning or outpatient visits. During our follow-up, 14 patients received other treatments, such as targeted therapy and High Intensity Focused Ultrasound, and one patient was diagnosed with and treated for a hematologic malignancy. Twenty-six patients were lost to follow-up. The above patients were not included in the study. The study complied with the ethical guidelines of the Declaration of Helsinki in 1964 and passed the review of the Review Committee of the Second Affiliated Hospital of Chongqing Medical University.

Data collection

We collected the basic data of the patients, including sex, age, cause of hepatitis, ascites, hepatoportal arteriovenous fistula, liver cirrhosis, cavernous transformation of the portal vein, Child-Pugh grade, the model for end-stage liver disease score, the Eastern Cooperative Oncology Group score, and albumin-bilirubin grade (ALBI grade). The biochemical parameters included the following indicators: Prealbumin, serum albumin (ALB), total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alpha-fetoprotein, activated partial thromboplastin time, prothrombin time, international normalized ratio, hemoglobin, platelet count, white blood cell count, serum cholinesterase, and gamma-glutamyl transpeptidase. The characteristics of the tumors included the number and size of tumors, the type of PVTT, gross pathological type, invasion of the left and right liver lobes and inferior vena cava tumor thrombus. Cheng's classification of PVTT was applied[8]. The formula for calculating the ALBI score was as follows: ALBI = log10 bilirubin × 0.66 + albumin × (-0.085)[9]. Gross pathological types of liver cancer included the massive block type, the nodular type, and the diffuse type. The clinical diagnostic criteria for patients with HCC are in line with the European Society for Medical Oncology Clinical Practice Guidelines[10].

TACE procedure

The puncture of the artery used Seldinger technology. The right femoral artery was often selected. The catheter was delivered to the celiac trunk and the common hepatic artery, and arteriography was performed to identify the tumor's nutrient artery. The catheter was sent into the tumor-feeding artery and injected with chemotherapy drugs. Chemoembolization drugs commonly included lipiodol, polyvinyl alcohol particles, cisplatin, bleomycin, gelatin sponge particles, *etc*.

Statistical analysis

The mean ± SD or median were used to express the continuous variables. Categorical variables are presented as *n* (%). Two groups of data were compared using independent-samples *t* tests, Mann-Whitney *U* tests, χ^2 tests or Fisher's exact tests. The statistically significant indicators in the univariate analysis ($P < 0.05$) were selected in multivariate analysis in the study. The independent predictors for survival were determined by logistic regression analysis. The predictive ability of the independent predictors for short-term survival was assessed by the AUC. The cutoff value was calculated by the receiver operating characteristic (ROC) curves. The Kaplan-Meier method was used to plot the survival curves. SPSS 25.0 software (Armonk, NY, United States) was used to perform all statistical analyses. When $P < 0.05$, the statistical results were considered significant.

RESULTS

Patient basic characteristics

A total of 181 HCC patients with PVTT who underwent TACE were enrolled in our study. Their baseline data are shown in Table 1. The average age of the patients was 52.16 ± 9.73 years. The 3 mo, 6 mo, and 12 mo survival rates were 68.5%, 38.7%, and 15.5%, respectively. In our study, 159 patients (87.8%) were men and 22 patients (12.2%) were women, hepatitis B patients accounted for 90.1%, and 133 (73.5%) patients had a background of cirrhosis. According to the type of PVTT, 29 (16%) patients, 77 (42.5%) patients, 68 (37.6%) patients, and 7 (3.9%) patients were classified as having type I, II, III and IV PVTT, respectively. For the Child-Pugh grade, 123 (68%) had grade A, 55 (30.4%) had grade B, and 3 (1.6%) had grade C. Patients with greater than or equal to 3 lesions accounted for 48.1%. The median survival time was 4.8 mo.

Short-term survival outcome

Of the 181 patients, 56 patients died within 3 mo after interventional therapy, and 125 patients survived. The clinical data of patients who died and survived within 3 mo after treatment were compared. The results of the univariate analysis are shown in Table 2. Multivariate analysis showed that total bilirubin [odds ratio (OR): 1.027, 95% confidence interval (CI): 1-1.054 P = 0.046), sex (OR: 2.832, 95%CI: 1.025-7.828, P = 0.045] and AST (OR: 1.014, 95%CI: 1.006-1.021, P < 0.01) were significant independent predictors of short-term survival (Table 3).

The AUC was used to evaluate the predictive ability of these indicators for short-term survival. AST (AUC: 0.763, 95%CI: 0.686-0.841) had the best predictive ability, followed by total bilirubin (AUC: 0.707, 95%CI: 0.627-0.788), while sex (AUC: 0.554, 95%CI: 0.461-0.648) had the worst predictive ability (Figure 1).

The cutoff values of the above indicators were calculated by using ROC curves, and the 3-mo cumulative survival rates of patients with AST < 87 U/L and AST ≥ 87 U/L were 84.6% and 40.6% (Figure 2A), respectively. The difference was significant (P < 0.001). The 3-mo cumulative survival rates of the patients with total bilirubin < 16.15 μmol/L and bilirubin ≥ 16.15 μmol/L were 86.9% and 53.6% (Figure 2B), respectively, and the difference was statistically significant (P < 0.001). The 3-mo cumulative survival rates of men and women were 71.1% and 50% (Figure 2C), respectively. The discrepancy was statistically significant (P = 0.019).

Long-term survival outcome

Of the 181 patients, 150 died within 12 mo after interventional therapy, and 31 survived. Univariate analysis showed that the number of tumors, invasion of the left and right liver lobes, pathological type, platelet count, and gamma-glutamyl transpeptidase were correlated with the long-term survival of patients (Table 4), and logistic regression analysis showed that the number of lesions and pathological type were independent predictors of long-term survival (Table 3). The long-term survival outcome was significantly better in patients with a single lesion than in those with ≥ three lesions (OR: 5.809, 95%CI: 1.563-21.594, P = 0.009). Patients with massive block HCC had a worse long-term survival than patients with nodular and diffuse HCC (OR: 0.197, 95%CI: 0.075-0.521, P = 0.001).

We used ROC curves to evaluate the predictability of the number of tumors and pathology for long-term survival. Number of tumors (AUC: 0.665, 95%CI: 0.568-0.763) had the best predictive ability, followed by gross pathology (AUC: 0.620, 95%CI: 0.510-0.730) (Figure 3).

DISCUSSION

In this paper, we found that sex, AST, and total bilirubin were independent influential factors for short-term survival. The incidence and mortality of HCC in men were higher than in women[11]. This was different from the conclusion in our article. The main reason was the imbalance of gender ratio in the article. The AUC of sex was not high (AUC: 0.554, 95%CI: 0.461-0.648, P > 0.05), which showed that although sex was an independent factor influencing short-term prognosis, its predictability was not well.

Total bilirubin was a biochemical indicator of liver metabolic function. Carr *et al*[12] believed that high levels of serum bilirubin can increase the risk of death in HCC patients with PVTT. In our study, it was also found that elevated bilirubin levels were associated with poor short-term survival in HCC patients with PVTT who underwent TACE. According to the AUC of bilirubin, we thought that this indicator had good

Table 1 Characteristic of the 181 patients

Parameters	Patients, *n* = 181
Age in yr	52.16 ± 9.73
Sex, men/women	159/22
Cause of liver disease, hepatitis B/C/B and C/others	163/3/1/14
Tumor size, < 5 cm/≥ 5 cm, < 10 cm/≥ 10 cm	39/86/56
Number of tumors, 1/2/≥ 3	82/12/87
Liver cirrhosis, no/yes	48/133
Ascites, no/small/moderate-massive	91/70/20
CTPV, no/yes	158/23
Invade left and right liver lobes, no/yes	119/62
Type of gross pathology, massive/nodular/diffuse	112/59/10
PVTT type, I/II/III/IV	29/77/68/7
Inferior vena cava tumor thrombus, no/yes	171/10
Arteriovenous fistula, no/yes	125/56
Total bilirubin, μmol/L	17.1 (12.3-24.7)
Prealbumin, mg/L	110 (79-147)
Albumin, g/L	37 ± 4.8
Hemoglobin, g/L	127 (115-142)
WBC, 10^9/L	5.1 (3.925-6.505)
PLT, 10^9/L	116 (81- 179.5)
INR	1.12 (1.055-1.205)
PT, S	14.3 (13.7-15.25)
APTT, S	39.5 (37-42.8)
ALT, U/L	46 (31-71.5)
AST, U/L	65 (46-109.5)
GGT, U/L	211 (124.5-352.5)
Cholinesterase, kU/L	4.5 (3.065-5.78)
Creatinine, μmol/L	68 (57.7-78.1)
AFP, μg/L	1210 (82.905-1210)
Child-Pugh grade, A/B/C	123/55/3
ECOG score, 0/1/2/3	8/142/29/2
ALBI grade, 1/2/3	56/120/5
MELD score	7.67 (6.08-9.54)
Overall survival time, mo	4.8 (2.5-8.85)

AFP: Alpha fetoprotein; ALBI: Albumin-bilirubin; ALT: Alanine aminotransferase; APTT: Activated partial thromboplastin time; AST: Aspartate aminotransferase; CTPV: Cavernous transformation of the portal vein; ECOG: Eastern Cooperative Oncology Group; GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio; MELD: Model of end-stage liver disease; PLT: Platelets; PVTT: Portal vein tumor thrombosis; WBC: White blood cell.

predictive power. When total bilirubin was greater than 16.15 μmol/L, the patients' mortality within 3 mo after TACE was significantly increased. This suggested that for patients with preoperative total bilirubin greater than 16.15 μmol/L, the treatment of lowering the bilirubin level may be beneficial in reducing the short-term postoperative mortality.

AST level is elevated in patients with liver disease, reflecting the degree of liver

Table 2 Univariate analysis of survivors *vs* non-survivors at 3 mo after transarterial chemoembolization

Parameters	Survivors, *n* = 125	Non-survivors, *n* = 56	*P* value
Age in yr	52.56 ± 9.966	51.27 ± 9.212	0.18
Sex, men/women	114/11	45/11	0.04
Cause of liver disease, hepatitis B/C/B and C/others	113/1/0/11	50/2/1/3	0.157
Tumor size, < 5 cm/≥ 5 cm, < 10 cm/≥ 10 cm	29/57/39	10/29/17	0.659
Number of tumors, 1/2/≥ 3	62/10/53	20/2/34	0.06
Liver cirrhosis, no/yes	36/89	12/44	0.299
Ascites, no/small/moderate-massive	68/47/10	23/23/10	0.087
CTPV, no/yes	110/15	48/8	0.67
Invade left and right liver lobes, no/yes	85/40	34/22	0.34
Type of gross pathology, massive / nodular and diffuse	78/47	34/22	0.829
PVTT type, I/II/III/IV	24/52/44/5	5/25/24/2	0.354
Inferior vena cava tumor thrombus, no/yes	119/6	52/4	0.775
Arteriovenous fistula, no/yes	89/36	36/20	0.352
Total bilirubin, μmol/L	15 (11.15-21.75)	22.25 (17.175-32)	< 0.01
Prealbumin, mg/L	119 (87-159.5)	91.5 (67.25-120.5)	< 0.01
Albumin, g/L	36.407 ± 4.749	37.205 ± 4.853	0.758
Hemoglobin, g/L	127 (115-142)	127 (113.25-143)	0.89
WBC, 10^9/L	5.06 (4.06-6.27)	5.09 (3.61-6.72)	0.87
PLT, 10^9/L	119.00 (86.00-180.50)	111.00 (69.25-178.25)	0.15
INR	1.11 (1.06-1.19)	1.12 (1.05-1.26)	0.33
PT, S	14.20 (13.70-15.20)	14.35 (13.70-15.78)	0.38
APTT, S	39.60 (37.25-42.80)	39.35 (36.32-42.85)	0.47
ALT, U/L	41.00 (29.50-62.00)	58.00 (41.25-85.75)	< 0.01
AST, U/L	56.00 (41.00-81.00)	109.00 (72.00-161.75)	< 0.01
GGT, U/L	188.00 (109.00-300.50)	256.00 (186.00-415.25)	< 0.01
Cholinesterase, kU/L	4.88 (3.44-5.84)	3.89 (2.69-5.4675)	0.02
Creatinine, μmol/L	68.60 (58.20-77.25)	66.95 (54.98-79.60)	0.63
AFP, μg/L	1210 (47.86-1210)	1210 (270-1210)	0.34
Child-Pugh grade, A/B/C	94/30/1	29/25/2	< 0.01
ECOG score, 0/1/2/3	8/102/14/1	0/40/15/1	0.016
ALBI grade, 1/2/3	43/79/9	13/41/2	0.31
MELD score	7.53 (5.88-9.02)	9.10 (7.08-11.19)	< 0.01

AFP: Alpha fetoprotein; ALBI: Albumin-bilirubin; ALT: Alanine aminotransferase; APTT: Activated partial thromboplastin time; AST: Aspartate aminotransferase; CTPV: Cavernous transformation of the portal vein; ECOG: Eastern Cooperative Oncology Group; GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio; MELD: Model of end-stage liver disease; PLT: Platelets; PVTT: Portal vein tumor thrombosis; WBC: White blood cell.

damage[13]. In this study, AST was found to be closely related to short-term survival after TACE, while ALT was not found to be associated with short-term survival after interventional treatment.

Xie *et al*[14] suggested that the total cause mortality rate, liver disease mortality and liver cancer mortality rate of those with elevated AST were higher than those of the correspondingly elevated ALT patients. This may mean that patients with

Table 3 Multivariate analysis of short-term and long-term survival

Variables	Short-term survival		Long-term survival	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Total bilirubin, μmol/L	1.027 (1-1.054)	0.046		
AST, U/L	1.014 (1.006-1.021)	$P < 0.01$		
Sex				
Men	1			
Women	2.832 (1.025-7.828)	0.045		
Type of gross pathology				
Massive			1	
Nodular and diffuse			0.197 (0.075-0.521)	0.001
Number of tumors				
1			1	
2			1.365 (0.283-6.581)	0.698
3			5.809 (1.563-21.594)	0.009

AST: Aspartate aminotransferase; CI: Confidence interval; OR: Odds ratio.

preoperative high levels of AST do not obtain good short-term survival from TACE. When AST ≥ 87 U/L, the 3-mo mortality rate after TACE increased significantly, which indicated that correcting high levels of AST before TACE may be an effective measure to reduce short-term mortality.

In long-term survival analysis, we found that patients with only one lesion had better long-term survival than those with ≥ three lesions. Liu *et al*[15] obtained the same conclusion. Second, patients with massive block liver cancer had a worse long-term outcome than patients with nodular and diffuse liver cancer. This study used the gross pathological type of liver cancer because some patients were clinically diagnosed with liver cancer, and it was not possible to obtain liver tissue biopsy results. The gross pathological type could be judged by imaging, and its clinical applicability was better. However, in this research, the sample size of patients with the diffuse type of liver cancer was small, and this conclusion may need to be further validated in a larger sample size.

In addition, most patients had a background of viral hepatitis in the study, which was different from some areas. This may limit the application of our findings in regions where the cause of liver cancer was non-viral hepatitis. Therefore, research in other countries or regions may be needed to make up for the shortcoming of our study. Because patients with viral hepatitis accounted for the majority, model for end-stage liver disease scores in our study were higher than those of patients whose cause of liver cancer was alcohol or cholestasis.

The following limitations existed in this study. First, this study was a single-center, retrospective study, and it was difficult to avoid selection bias. Second, perhaps because of the small sample size, AUC value was not high, but the AUC value was still statistically significant. The larger and multicenter studies are needed to validate further the results in the future.

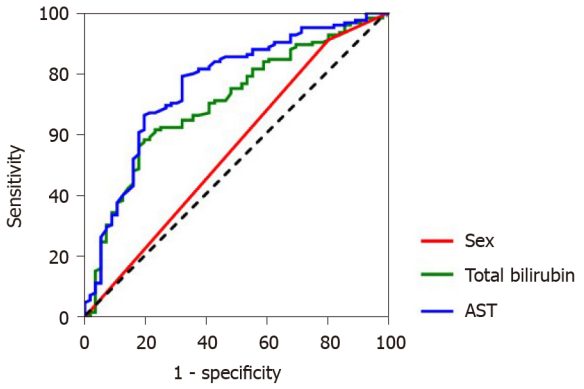
CONCLUSION

In summary, sex, AST, and total bilirubin were associated with the short-term survival outcomes in HCC patients with PVTT who underwent TACE. According to the AUC, AST was the best predictor of short-term survival, followed by total bilirubin. Multiple tumor lesions and massive block types of liver cancer were closely related to long-term adverse survival outcomes in HCC patients with PVTT who underwent TACE. In the future, multi-center, prospective and large sample studies are needed to verify these results.

Table 4 Univariate analysis of survivors *vs* non-survivors at 12 mo after transarterial chemoembolization

Parameters	Survivors, *n* = 31	Non-survivors, *n* = 150	*P* value
Age in yr	54.770 ± 10.724	51.800 ± 9.400	0.225
Sex, men/women	28/3	131/19	0.871
Cause of liver disease, hepatitis B/C/B and C/others	30/0/0/1	133/3/1/13	0.751
Tumor size, < 5 cm/≥ 5 cm, < 10 cm/≥ 10 cm	11/14/6	28/72/50	0.081
Number of tumors, 1/2/≥ 3	21/4/6	61/8/81	0.002
Liver cirrhosis, no/yes	4/27	44/106	0.059
Ascites, no/small/moderate-massive	16/11/4	75/59/16	0.892
CTPV, no/yes	26/5	132/18	0.74
Invade left and right liver lobes, no/yes	26/5	93/57	0.019
Type of gross pathology, massive/nodular and diffuse	13/18	99/51	0.012
PVTT type, I/II, III/IV	7/10/11/3	22/67/57/4	0.155
Inferior vena cava tumor thrombus, no/yes	30/1	141/9	0.854
Arteriovenous fistula, no/yes	21/10	104/46	0.861
Total bilirubin, μmol/L	19.550 (11.200-33.400)	16.600 (12.900-24.000)	0.436
Prealbumin, mg/L	124.500 (86.750-160.500)	105.000 (78.000-142.000)	0.163
Albumin, g/L	37.047 ± 5.635	36.813 ± 4.622	0.669
Hemoglobin, g/L	132.500 (117.500-146.500)	125.000 (115.000-142.000)	0.316
WBC, 10^9/L	4.565 (3.398-5.850)	5.100 (4.050-6.610)	0.284
PLT, 10^9/L	89.000 (63.250-126.000)	119.000 (87.000-182.000)	0.046
INR	1.120 (1.070-1.253)	1.110 (1.050-1.200)	0.281
PT, S	14.400 (13.850-15.875)	14.300 (13.700-15.200)	0.266
APTT, S	39.200 (36.450-42.075)	39.700 (37.100-42.900)	0.541
ALT, U/L	46.500 (38.500-72.000)	45.000 (30.000-72.000)	0.770
AST, U/L	56.500 (41.750-83.750)	71.000 (46.000-115.000)	0.108
GGT, U/L	177.500 (72.250-265.250)	216.000 (132.000-356.000)	0.036
Cholinesterase, kU/L	4.25 (2.775-5.905)	4.51 (3.08-5.79)	0.945
Creatinine, μmol/L	71.300 (61.475-79.500)	67.600 (57.400-78.100)	0.220
AFP, μg/L	568 (15.13~1210)	1210 (97.335-1210)	0.188
Child-Pugh grade, A/B/C	19/11/1	104/44/2	0.373
ECOG score, 0/1/2/3	4/24/3/0	4/118/26/2	0.086
ALBI grade, 1/2/3	13/16/2	43/104/3	0.09
MELD score	7.130 (5.548-9.315)	7.860 (6.070-9.790)	0.348

AFP: Alpha fetoprotein; ALBI: Albumin-bilirubin; ALT: Alanine aminotransferase; APTT: Activated partial thromboplastin time; AST: Aspartate aminotransferase; CTPV: Cavernous transformation of the portal vein; ECOG: Eastern Cooperative Oncology Group; GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio; MELD: Model of end-stage liver disease; PLT: Platelets; PVTT Portal vein tumor thrombosis; WBC: White blood cell.



	AUC (95%CI)	*P* value
Sex	0.554 (0.461-0.648)	0.244
AST	0.763 (0.686-0.841)	*P* < 0.001
Total bilirubin	0.707 (0.627-0.788)	*P* < 0.001

Figure 1 Comparisons of the area under the receiver operating characteristics curves for short-term survival among aspartate aminotransferase, total bilirubin, and sex. AST: Aspartate aminotransferase; AUC: Area under curve; CI: Confidence interval.

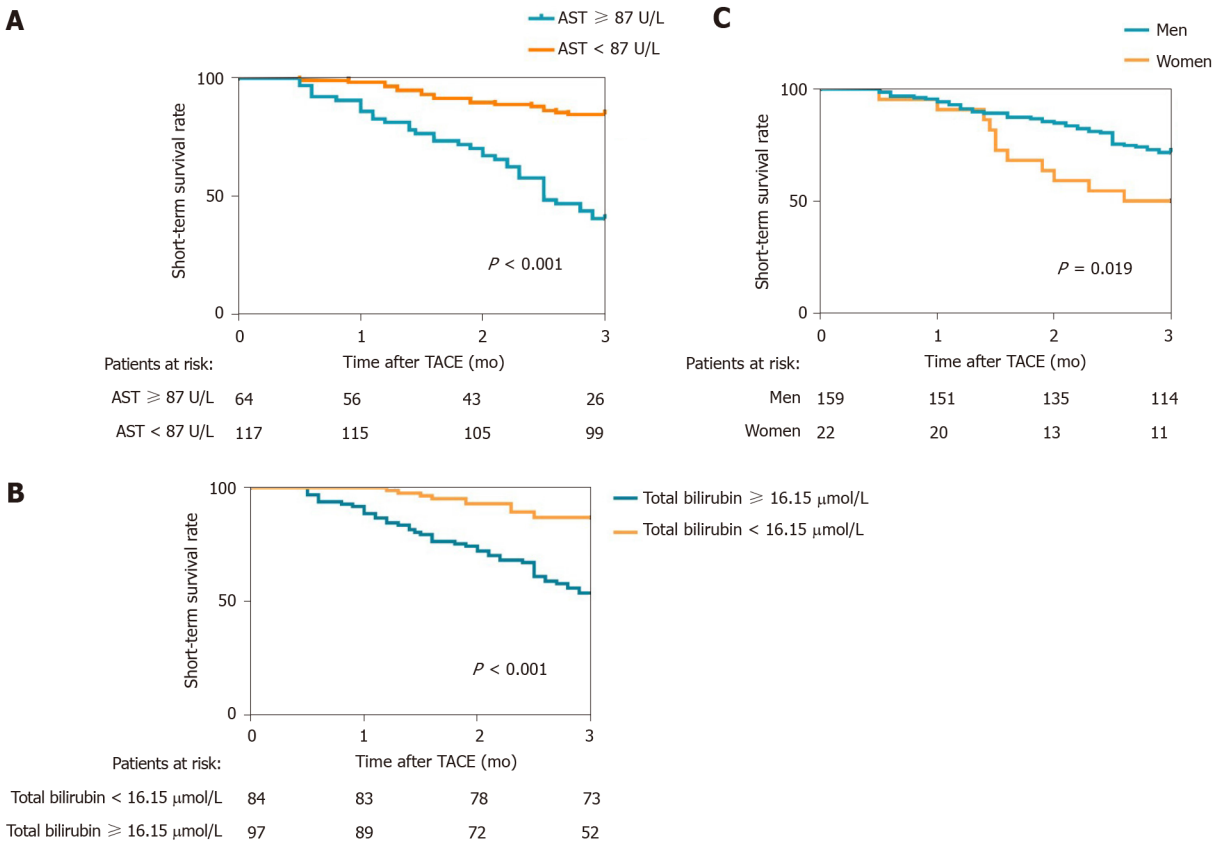
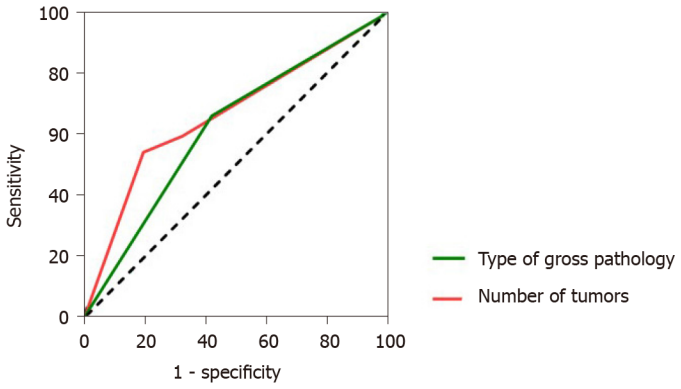


Figure 2 Cutoff values of the indicators were calculated by using receiver operating characteristic curves. A: The 3-mo survival rate curve based on the stratification of aspartate aminotransferase; B: The 3-mo survival rate curve based on the stratification of bilirubin; C: The 3-mo survival rate curve based on the sex. AST: Aspartate aminotransferase; TACE: Transarterial chemoembolization.



Variable	AUC (95%CI)	*P* value
Type of gross pathology	0.620 (0.510-0.730)	0.035
Number of tumors	0.665 (0.568-0.763)	0.004

Figure 3 Comparisons of the area under the receiver operating characteristics curves for long-term survival between pathology and number of tumors. AUC: Area under curve; CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) patients with portal vein tumor thrombosis (PVTT) have poor prognosis. Transarterial chemoembolization (TACE) is an effective treatment for HCC patients with PVTT. The factors affecting the short-term and long-term prognosis of HCC patients with PVTT receiving TACE are still unclear.

Research motivation

The main aim of this study was to clarify the predictors correlated with the short-term and long-term survival of HCC patients with PVTT who underwent TACE.

Research objectives

We can provide some guidance to clinicians for selecting suitable patients for TACE by analyzing preoperative indicators.

Research methods

A total of 181 HCC patients with PVTT who underwent TACE were enrolled in this retrospective study. We explored the short-term and long-term prognostic factors by comparing the preoperative indicators of patients who died and survived within 3 mo and 12 mo after TACE. Multivariate analyses were conducted using logistic regression. The area under the receiver operating characteristic curve was used to evaluate the predictive ability of the factors related to the short-term and long-term prognosis.

Research results

Total bilirubin, sex, and aspartate aminotransferase (AST) were closely linked to short-term survival. When AST ≥ 87 U/L and total bilirubin ≥ 16.15 μmol/L, the 3-mo survival rate after TACE was reduced significantly. In long-term survival analysis, we found that patients with only one lesion had better long-term survival than those with ≥ three lesions. Patients with massive block liver cancer had a worse long-term outcome than patients with nodular and diffuse liver cancer.

Research conclusions

Sex, AST, and total bilirubin were associated with short-term survival outcomes in HCC patients with PVTT who underwent TACE. According to the area under the curve, AST was the best predictor of short-term survival, followed by total bilirubin. Multiple tumor lesions and massive block types of liver cancer were closely related to long-term adverse survival outcomes in HCC patients with PVTT who underwent TACE.

Research perspectives

Larger and multicenter studies are needed to validate further the results in the future.

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SCIENTOMETRICS

Global research trends in the microbiome related to irritable bowel syndrome: A bibliometric and visualized study

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Author contributions: Zyoud SH designed the study, collected the data, analyzed the data, made major contributions to the manuscript's existing literature search and interpretation, and drafted the manuscript; Al-Jabi SW, and Sweileh W participated in the study design; Smale S and Waring WS contributed towards the conception, wrote part of the article; Al-Jabi SW, Sweileh W, Smale S and Waring WS were involved in interpretation of the data, and made revisions to the initial draft; all authors provided critical review and approved the final manuscript before submission.

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Abstract

BACKGROUND

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder. Dysregulation of the gut–brain axis plays a central role in the pathophysiology of IBS. It is increasingly clear that the microbiome plays a key role in the development and normal functioning of the gut–brain axis.

AIM

To facilitate the identification of specific areas of focus that may be of relevance to future research. This study represents a bibliometric analysis of the literature pertaining to the microbiome in IBS to understand the development of this field.

METHODS

The data used in our bibliometric analysis were retrieved from the Scopus database. The terms related to IBS and microbiome were searched in titles or abstracts within the period of 2000–2019. VOSviewer software was used for data

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visualization.

RESULTS
A total of 13055 documents related to IBS were retrieved at the global level. There were 1872 scientific publications focused on the microbiome in IBS. There was a strong positive correlation between publication productivity related to IBS in all fields and productivity related to the microbiome in IBS ($r = 0.951$, $P < 0.001$). The United States was the most prolific country with 449 (24%) publications, followed by the United Kingdom ($n = 176$, 9.4%), China ($n = 154$, 8.2%), and Italy ($n = 151$, 8.1%). The h-index for all retrieved publications related to the microbiome in IBS was 138. The hot topics were stratified into four clusters: (1) The gut–brain axis related to IBS; (2) Clinical trials related to IBS and the microbiome; (3) Drug-mediated manipulation of the gut microbiome; and (4) The role of the altered composition of intestinal microbiota in IBS prevention.

CONCLUSION
This is the first study to evaluate and quantify global research productivity pertaining to the microbiome in IBS. The number of publications regarding the gut microbiota in IBS has continuously grown since 2013. This finding suggests that the future outlook for interventions targeting the gut microbiota in IBS remains promising.

Key Words: Irritable bowel syndrome; Bibliometric; Microbiota; Microbiome; Scopus; Brain gut axis

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Core Tip: This is the first study to evaluate and quantify the global research productivity pertaining to the microbiome in irritable bowel syndrome (IBS). We present a holistic picture of this emerging topic and explore future research directions. A number of abnormalities have been described within the microbiome of patients with IBS. The relationships of these abnormalities to the causality of dysfunction and associated symptomatology have not been clearly elucidated. Our finding, while preliminary, suggests that the future outlook for interventions targeting the gut microbiota in IBS remains promising.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a term used to describe a group of symptoms, including abdominal pain and altered bowel habits, that arises because of disturbances of the interactions between the diet, the brain, the gut, and the microbiome. IBS poses a significant disease burden, with 20% of adults estimated to develop IBS symptoms in any given year. Symptoms arise as a consequence of abnormal visceral sensation and or motility as a result of abnormalities within the enteric or central nervous systems, or both[1]. This usually occurs in the absence of 'organic' disease, but the association with other gastrointestinal diseases is common. Many patients experiencing gastrointestinal inflammation due to infection or inflammatory bowel disease develop IBS symptoms[2]. Nearly a quarter of patients with IBS without identifiable 'organic' disease appear to have low-grade intestinal inflammation as judged by faecal inflammatory markers (calprotectin)[3]. Inflammation may lead to sensitization of peripheral nerves. Most inflammation within the gastrointestinal tract settles, but in susceptible individuals sensitization of peripheral afferents may persist. Such sensitization may be driven by ongoing mast cell activation and increased epithelial

permeability associated with low-level inflammation[4].

The onset of symptoms in IBS is often associated with psychological stressors. A high proportion of people with IBS have psychological comorbidities. Chronic stress is associated with stress-induced hyperalgesia. This hyperalgesia is mediated *via* corticotrophin-releasing hormone and the hypothalamic–pituitary axis (HPA)[5]. Dysfunction within these systems is well documented in patients with functional gastrointestinal disorders and other chronic pain syndromes. Furthermore, many patients have histories of traumatic events in early life. These events may result in changes that profoundly affect the microbiome and brain development, predisposing patients to visceral pain syndromes[6-8]. In addition to direct effects on neurons proximal to the bowel, bacteria and their products have a profound effect on the development and activity of the central nervous system and, consequently, upon the psychological state and behaviors[9]. Within the enteric nervous system, certain bacterial strains may directly promote sensitization or downregulation of peripheral afferents. Of note, low-grade inflammation seen in a proportion of patients with IBS may result from the ability of specific bacteria to enhance the production and the effects of proinflammatory cytokines. Bacteria may also be able to transmit signals *via* the enteric nervous and immune systems to the central nervous system[10].

Although several bibliometric studies have been conducted to evaluate research productivity regarding various research aspects in the medical field[11-18], and some bibliometric studies have focused on microbiota science[19-25], there has not been an assessment of the research output regarding the microbiome in IBS. This study undertakes a bibliometric analysis of the literature within this field to understand the development of the literature pertaining to the microbiome in IBS and to facilitate the identification of specific areas of focus that may be of relevance to future research. This study evaluates data that will help to address the research gaps in this field. The current bibliometric study plays a significant role for researchers interested in the relationship between the microbiome and IBS, because it offers a quick reference guide for interdisciplinary researchers to know how this field has been assessed by scientific experts in previous years.

MATERIALS AND METHODS

The data used in our bibliometric analysis were retrieved from Scopus, which is a multidisciplinary database owned by Elsevier and is considered the largest and most widely used database[26-28]. Most of the publications in this field were published between 2000 and 2019. The following term combinations were searched in the title or abstract within this period: Terms related to IBS, *i.e.*, 'adaptive colitis' OR 'colon spasm' OR 'functional bowel disease' OR 'irritable bowel' OR 'irritable colon' OR 'membranous colitis' OR 'mucous colitis' OR 'spastic colitis' OR 'spastic colon' OR 'spastic bowel' OR 'functional colonic disease' OR 'colon irritable' OR 'colon neurosis' OR 'bowel neurosis' OR 'functional colopathy' OR 'functional colonopathy' OR 'chronic catarrhal colitis' OR 'colica mucosa' OR 'colonic enterospasm' OR 'dyskinesia of the colon' OR 'dyssynergia of the colon' OR 'functional enterocolonopathy' OR 'functional diarrhea' OR 'Glarry enteritis' OR 'glutinous diarrhea' OR 'intestinal croup' OR 'irritable gut syndrome' OR 'lienteric diarrhea' OR 'Membranous catarrh of the intestine' OR 'mucomembranous colic' OR 'myxoneurosis' OR 'nervous diarrhea' OR 'neurogenic mucous' OR 'nonspecific diarrhea' OR 'tubular diarrhea' OR 'unhappy colon' OR 'unstable colon', and terms related to microbiome, *i.e.*, 'microbiome' OR 'microflora' OR 'microbiota' OR 'flora' OR 'probiotic' OR 'Saccharomyces' OR 'Lactobacillus' OR 'Bifidobacterium' OR 'Escherichia coli'. The terms were selected from related reviews on IBS[29,30] and the microbiome[19,20,31-33].

Data analysis

Microsoft Excel was used to analyze the retrieved data to calculate frequencies and percentages. The current bibliometric study included quantitative data about document types, language, country of the publications with their h-index and collaboration pattern, authors of the publications, journals that published papers on IBS, microbiome documents, and citation reports. In addition, we used the Statistical Package for the Social Sciences 16.0 software for Windows to assess the correlation between yearly quantitative distributions of publications related to IBS in all fields and publications related to the microbiome in IBS by using the Pearson correlation test. Analysis with $P < 0.05$ is considered statistically significant. In addition, the VOSviewer program[34,35] was used for mapping and clustering terms according to their

occurrence in both titles and abstracts to determine hot topics in this field. Furthermore, network visualization maps for collaboration among the most productive countries and the most prolific authors in this field were prepared using VOSviewer.

RESULTS

A total of 13055 documents related to IBS in titles and abstracts were retrieved over the period of 2000–2019 at the global level. There were 1872 scientific publications focused on the microbiome in IBS. The research findings showed that English was the main research language of documents indexed in Scopus, accounting for 48.8% of the documents, followed by German (3.9%) and Chinese (2.9%). Articles were the main type of document for publications related to the microbiome and IBS, accounting for 51.9% of documents, followed by reviews (35.3%).

The number of publications related to the microbiome in IBS has increased year by year. The quantity of documents has increased each year, from 9 documents in 2000, to 84 documents in 2009, to 219 documents in 2019 (Figure 1). Published papers during the last 5 years (2015 to 2019) accounted for 47.0% of the total publications. There was a strong positive correlation between the publication productivity related to IBS in all fields and the productivity related to the microbiome in IBS ($r = 0.951$, $P < 0.001$).

In total, authors from 105 countries published their work on the microbiome and IBS topics. Table 1 lists the top 10 most prolific countries. The United States was the most prolific country with 449 (24%) publications, followed by the United Kingdom ($n = 176$, 9.4%), China ($n = 154$, 8.2%), and Italy ($n = 151$, 8.1%). The h-index for all retrieved publications related to the microbiome in IBS was 138. Figure 2 shows the network visualization map for the collaboration among the 40 most productive countries. This includes countries that published more than 5 papers on microbiome and IBS research according to the number of publications in the last two decades (2000–2019). The United States and the United Kingdom can be referred to as central countries for this network because they published research in collaboration with 40 and 31 countries, respectively. In total, 5960 authors published their work on the microbiome and IBS topics. Figure 3 shows the network visualization map for the collaboration among the 32 most productive authors (authors who published more than 10 papers) on the microbiome in IBS research according to the number of publications in the last two decades (2000–2019). EMM Quigley (United States), G Barbara (Italy), JF Cryan (Ireland), and TG Dinan (Ireland) can be considered the prolific authors of this network due to the fact that they published 61, 25, 25, and 23 documents, respectively.

Terms were extracted from the titles and abstracts of all retrieved publications related to the microbiome in IBS and analyzed by the VOSviewer software to identify the current state and hot topics in this field. Terms appearing more than 10 times were included in the map (Figure 4) and were stratified into four clusters: IBS related to the gut–brain axis (yellow cluster), clinical trials related to IBS and the microbiome (red cluster), drug-mediated manipulation of the gut microbiome (blue cluster), and the role of the altered composition of intestinal microbiota in IBS prevention (green cluster).

The top 10 journals accounted for 19.98% of all articles (Table 2). The four journals that published the most studies related to microbiome and IBS were *World Journal of Gastroenterology* ($n = 70$), *Alimentary Pharmacology and Therapeutics* ($n = 54$), *Neurogastroenterology and Motility* ($n = 52$), and the *Journal of Clinical Gastroenterology* ($n = 39$).

The sample presents an average citation rate of 42 citations per publication. However, 16.6% of the publications have never been cited. Table 3 lists the information on the top ranking of publications in terms of the highest number of citations[36-55]. The article 'Prebiotic Effects: Metabolic and Health Benefits" is the most cited article with 1147 citations; it was authored by Roberfroid *et al*[49] and published in the *British Journal of Nutrition* in 2010. This study reported that a substantial number of studies about human intervention have shown that dietary intake of food products/ingredients/supplements results in statistically important improvements in the faecal gut microbiota composition. The second most highly cited article is 'Lactobacillus and Bifidobacterium in IBS: Symptom Responses and Relationship to Cytokine Profiles' by O'Mahony *et al*[44] and published in *Gastroenterology* in 2005. This study showed that intake of *Bifidobacterium infantis* over 8 wk was associated with symptomatic improvement in IBS.

Table 1 Top 10 most productive countries on the microbiome in irritable bowel syndrome research, ranked by the total number of publications in the last two decades (2000-2019)

SCR	Country	Number of documents	%
1st	United States	449	24.0
2nd	United Kingdom	176	9.4
3rd	China	154	8.2
4th	Italy	151	8.1
5th	France	116	6.2
6th	Germany	107	5.7
7th	Canada	103	5.5
8th	Ireland	102	5.4
9th	Australia	72	3.8
10th	Spain	69	3.7

SCR: Standard competition ranking.

Table 2 Top 10 most productive journals on the microbiome in irritable bowel syndrome research, ranked by the total number of publications in the last two decades (2000-2019)

SCR[1]	Journal	Frequency	%	IF[2]
1st	*World Journal of Gastroenterology*	70	3.74	3.665
2nd	*Alimentary Pharmacology and Therapeutics*	54	2.88	7.515
3rd	*Neurogastroenterology and Motility*	52	2.78	2.946
4th	*Journal of Clinical Gastroenterology*	39	2.08	2.973
5th	*American Journal of Gastroenterology*	34	1.82	10.171
6th	*Gastroenterology*	33	1.76	17.373
7th	*Current Opinion in Gastroenterology*	27	1.44	3.225
8th	*PLoS One*	24	1.28	2.740
9th	*Nutrients*	21	1.12	4.546
10th	*Gut Microbes*	20	1.07	7.740

[1]Equal journals have the same ranking number, and then a gap is left in the ranking numbers.
[2]Impact factors based on Journal Citation Reports 2019 from Clarivate Analytics.
SCR: Standard competition ranking; IF: Impact factor.

Table 4 presents the top 10 most productive institutions on the microbiome in IBS research, ranked by the total number of publications. The four institutions that have published the most studies related to microbiome and IBS are the University College Cork (n = 87), APC Microbiome Ireland (n = 84), McMaster University (n = 45), the Mayo Clinic (n = 32), and Alma Mater Studiorum Università di Bologna (n = 38) (Table 4).

DISCUSSION

This bibliometric study was carried out using data from Scopus for the period of 2000–2019. It presents a comprehensive review of research progress on the microbiome and IBS, identifies hot topics within this field, and indicates potential directions for future research. It is apparent that the research productivity in this field has progressively increased due to the increasing recognition of the role of the gut microbiota[56,57]. In addition, this increase is probably linked to the growing number of

Table 3 Top 20 most cited articles on the microbiome in irritable bowel syndrome research in the last two decades (2000-2019)

SCR	Ref.	Title	Year	Source title	Cited by
1st	Roberfroid *et al*[49]	"Prebiotic effects: Metabolic and health benefits"	2010	*British Journal of Nutrition*	1147
2nd	O'Mahony *et al*[44]	"Lactobacillus and Bifidobacterium in irritable bowel syndrome: Symptom responses and relationship to cytokine profiles"	2005	*Gastroenterology*	1042
3rd	Claesson *et al*[37]	"Composition, variability, and temporal stability of the intestinal microbiota of the elderly"	2011	*Proceedings of the National Academy of Sciences of the United States of America*	836
4th	Collins *et al*[38]	"The interplay between the intestinal microbiota and the brain"	2012	*Nature Reviews Microbiology*	735
5th	Kassinen *et al*[40]	"The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects"	2007	*Gastroenterology*	684
6th	Pimentel *et al*[46]	"Rifaximin therapy for patients with irritable bowel syndrome without constipation"	2011	*New England Journal of Medicine*	659
7th	Rinttilä *et al*[48]	"Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR"	2004	*Journal of Applied Microbiology*	658
8th	O'Mahony *et al*[45]	"Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses "	2009	*Biological Psychiatry*	634
9th	Rolfe[50]	"The role of probiotic cultures in the control of gastrointestinal health"	2000	*Journal of Nutrition*	584
10h	Carabotti *et al*[36]	"The gut-brain axis: Interactions between enteric microbiota, central and enteric nervous systems"	2015	*Annals of Gastroenterology*	580
11th	De Vrese and Schrezenmeir[39]	"Probiotics, prebiotics, and synbiotics"	2008	*Advances in Biochemical Engineering/Biotechnology*	575
12th	Rajilić-Stojanović *et al*[47]	"Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome"	2011	*Gastroenterology*	545
13th	Whorwell *et al*[55]	"Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome"	2006	*American Journal of Gastroenterology*	541
13th	Swidsinski *et al*[54]	"Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease"	2005	*Journal of Clinical Microbiology*	541
15th	Malinen *et al*[41]	"Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR"	2005	*American Journal of Gastroenterology*	523
16th	Mayer *et al*[42]	"Gut/brain axis and the microbiota"	2015	*Journal of Clinical Investigation*	519
17th	Simrén *et al*[52]	"Intestinal microbiota in functional bowel disorders: A Rome foundation report"	2013	*Gut*	510
18th	Spiller and Garsed[53]	"Postinfectious irritable bowel syndrome "	2009	*Gastroenterology*	508
19th	Rousseaux *et al*[51]	"Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors"	2007	*Nature Medicine*	500
20th	Nobaek *et al*[43]	"Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome"	2000	*American Journal of Gastroenterology*	498

researchers who are interested in the fields of neurology and gastroenterology [*e.g.*, EMM Quigley (the United States), JF Cryan (Ireland), TG Dinan (Ireland), G Barbara (Italy), Y Ringel (the United States), M Simrén (Sweden), M Camilleri (the United States), A Palva (Finland), M Pimentel (Canada), NJ Talley (Australia), and others]. Over the last two decades, advances in our understanding of the enteric and central nervous systems have led to an evolving understanding of their interactions and how the gastrointestinal microbiome influences their function. Disturbances in the gastrointestinal microbiome have been identified in patients with functional gastrointestinal disease, like IBS, and it is often the case that symptoms may be triggered by events that lead to changes in the microbiome, where the emotional context and the enteric nervous system function in tandem[1].

The results of this study indicate that the United States has been the most prolific country in this field. These findings are consistent with many previous bibliometric studies[12,58]. Research productivity in the United States is likely associated with

Table 4 Top 10 most productive institutions on the microbiome in irritable bowel syndrome research, ranked by the total number of publications in the last two decades (2000-2019)

SCR[1]	Institute	Country	*n*	%
1st	University College Cork	Ireland	87	4.65
2nd	APC Microbiome Ireland	Ireland	84	4.49
3rd	McMaster University	Canada	45	2.40
4th	Alma Mater Studiorum Università di Bologna	Italy	38	2.03
5th	Helsingin Yliopisto	Finland	36	1.92
5th	Mayo Clinic	United States	36	1.92
7th	Inserm (Institut national de la santé et de la recherche médicale)	France	30	1.60
8th	Baylor College of Medicine	United States	27	1.44
9th	Göteborgs Universitet	Sweden	27	1.44
10th	The University of North Carolina at Chapel Hill	United States	27	1.44

[1]Equal institutes have the same ranking number, and then a gap is left in the ranking numbers.
SCR: Standard competition ranking.

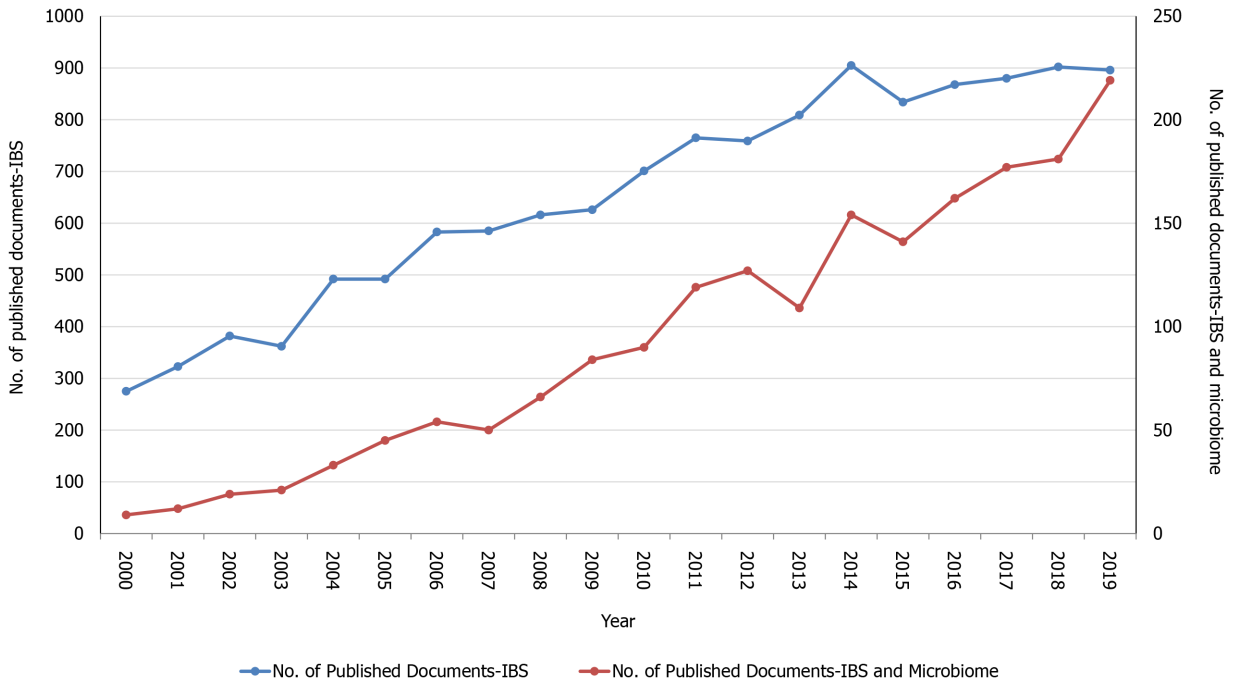


Figure 1 The annual number of publications related to irritable bowel syndrome and the microbiome from 2000 to 2019. IBS: Irritable bowel syndrome.

overwhelming support in terms of research, the wide range of researchers with an interest within this field, several well-resourced research environments, and greater availability of a well-trained workforce. In addition, the economic strength of the United States has led to a substantial amount of financial support for researchers and has enhanced the mobility of researchers[59,60]. Furthermore, in 2013, the United States launched an exceptional research project on the gut microbiota–brain axis[61], thus contributing to the increasing number of publications regarding the microbiome in IBS.

Our study demonstrates that the gut–brain axis has become the most prolific area for research within IBS and the microbiome globally, and several studies in a range of journals are proof of this emerging trend. According to these data, we can infer that central pain amplification occurs through a wide variety of mechanisms within the

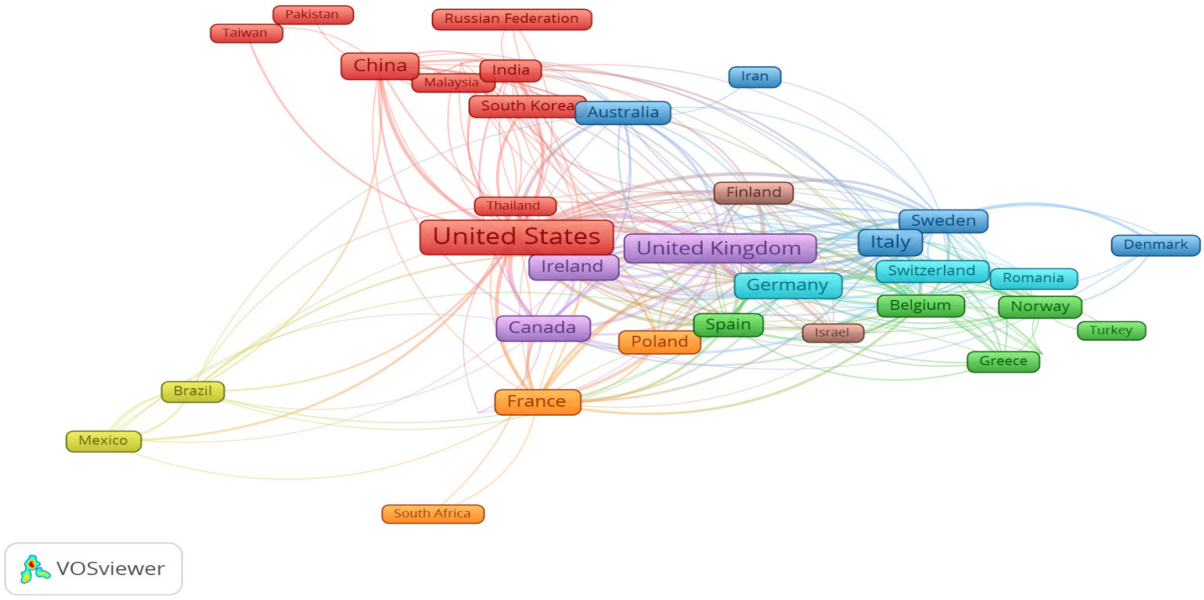


Figure 2 Network visualization map for the collaboration among the 40 most productive countries on the microbiome in irritable bowel syndrome research according to the number of publications in the last two decades (2000–2019).

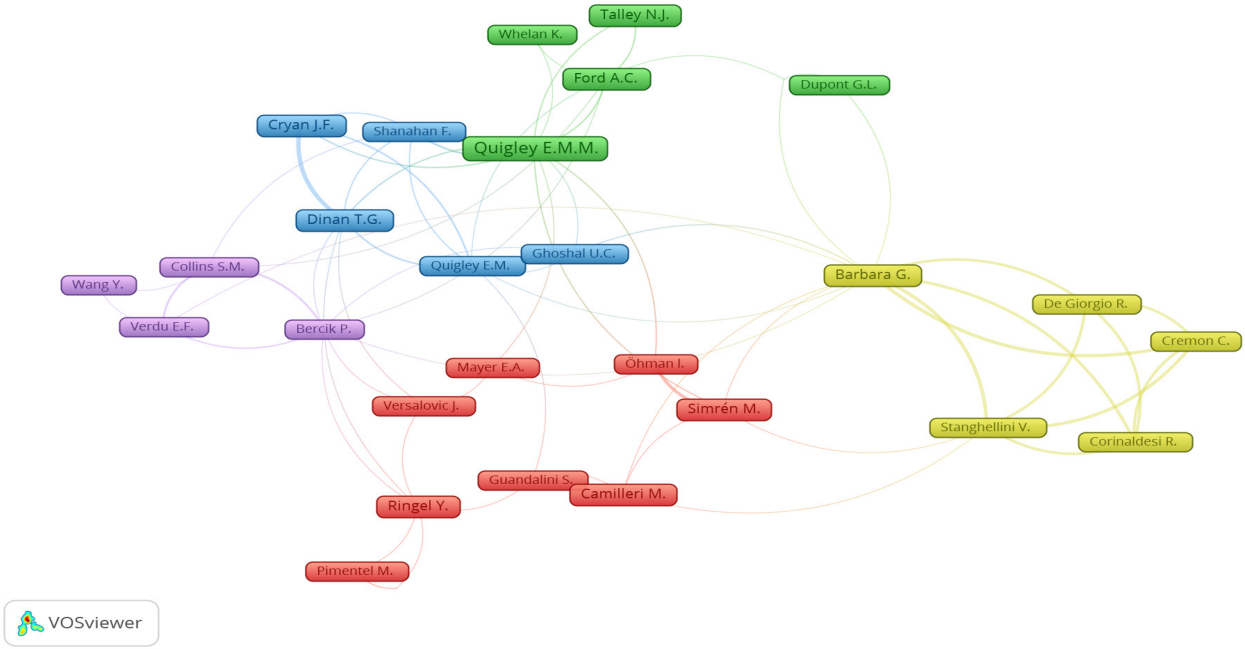


Figure 3 Network visualization map for the collaboration among the 32 most productive authors on the microbiome in irritable bowel syndrome research according to the number of publications in the last two decades (2000–2019).

central nervous system and mediates the effects of mood, emotional context, and environmental circumstances upon our central pain perception. Functional magnetic resonance imaging (fMRI) studies have demonstrated that patients with IBS often display an exaggerated response of arousal circuits associated with the HPA and activation of endogenous pain pathways in response to visceral stimulation. Similarly, studies have shown enhanced anticipation of pain in response to conditioning stimuli in patients with functional gastrointestinal disease[62]. Neuro-immune activation within the central nervous system is associated with altered astrocyte and microglial activity that further enhances central sensitisation.

Early deprivation in patients with IBS can have profound effects on the microbiome, and researchers recognise that these factors may predispose susceptible individuals to more severe IBS[8,63-66]. Changes in central nervous system function arise as a result of

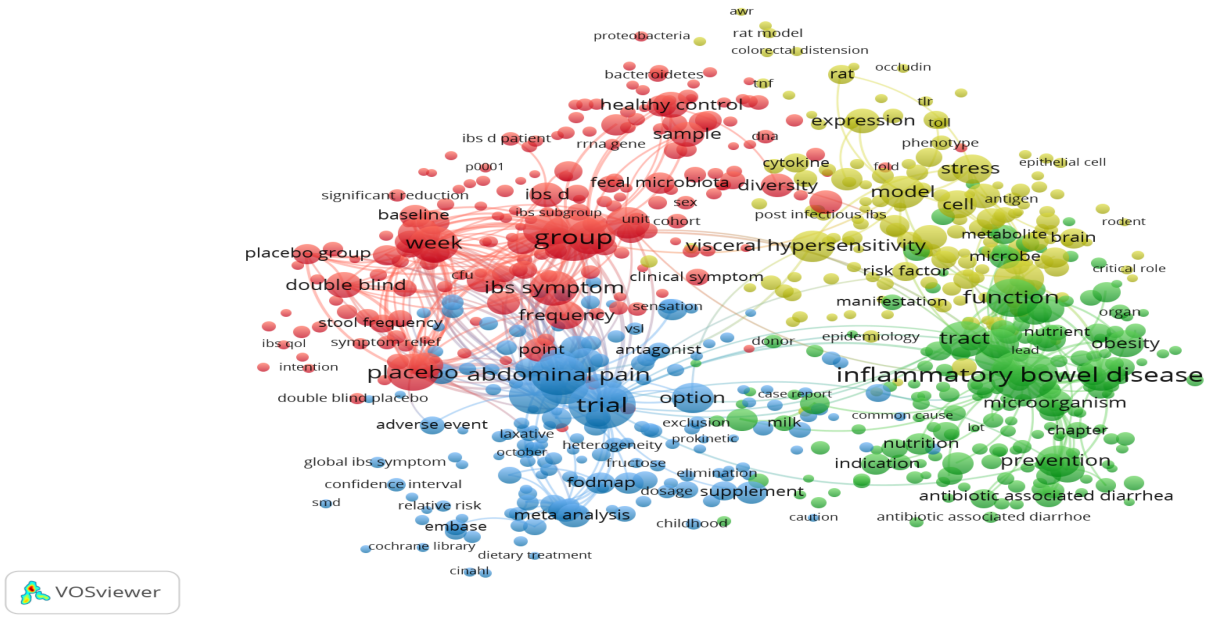


Figure 4 Network visualization map for the terms with high co-occurrence frequencies, based on the titles and abstracts of documents related to the microbiome in irritable bowel syndrome research published in the last two decades (2000–2019).

microbial products, which then influence astrocyte, microglial, and central nervous system inflammatory responses[67]. Certain bacterial strains, possibly as a result of being able to modulate these effects, may influence behaviour. Offspring of mice fed a high-fat diet display disordered behaviour and socialisation. These behavioural changes appear to be manifested by alterations in the microbiome. Correction of 'bacterial deficits' or their metabolic sequelae leads to improvements in behaviour. Specific bacterial strains have been demonstrated to increase resilience in individuals with anxiety, and these behavioural changes have neurological correlates in fMRI studies. Those factors known to provoke IBS have profound effects on the microbiome function; gastroenteritis can deplete the microbiome, and psychological stress can lead to significant changes in the representation of different phyla within the microbial flora. These potentially detrimental changes may facilitate feedback from the gut and, consequently, permit the gut to have effects on mood, behaviour, and cognitive functions.

Limitations

There are some limitations in our study that are similar to previous bibliometric studies. First, although Scopus is the most recognized and the largest database for peer-reviewed literature, our study is restricted to only the Scopus database as a source of data collection. Second, some authors or institutions have different name formats in the Scopus database, and their research count might be scattered; therefore, their names might not show in the active list. Despite these limitations, we still consider that the findings of our analysis were adequate to characterize accurately the state of IBS and microbiome research at the global level.

CONCLUSION

This is the first study to evaluate and quantify the global research productivity pertaining to the microbiome in IBS to present a holistic picture of this emerging topic and explore future research directions. The number of publications regarding the gut microbiota in IBS has continuously grown since 2013. The United States, the United Kingdom, Italy, and Ireland have been the most productive regions. Currently, the main hot topics regarding the gut microbiota in IBS are the gut–brain axis related to IBS, clinical trials related to IBS and the microbiome, drug-mediated manipulation of the gut microbiome, and the role of the altered composition of intestinal microbiota in IBS prevention. A number of abnormalities have been described within the microbiome of patients with IBS. The relationship between these abnormalities and the causality of dysfunction and associated symptomatology have yet to be clearly

elucidated. Our findings, while preliminary, suggest that the future outlook for interventions targeting the gut microbiota in IBS remains promising.

ARTICLE HIGHLIGHTS

Research background

The ability of specific bacteria to boost the development and the effects of proinflammatory cytokines can result in low-grade inflammation seen in a proportion of patients with irritable bowel syndrome (IBS).

Research motivation

The current bibliometric analysis plays an important role for researchers interested in the relationship between the microbiome and IBS. It provides a simple reference guide for interdisciplinary researchers to learn how scientific experts have examined this area in previous years.

Research objectives

This study aimed to carry out a bibliometric review of the IBS and the microbiome literature to explain the growth of this field and assist the identification of unique focus areas that may be important for future research.

Research methods

The information used in our bibliometric research was derived from the Scopus database. Terms related to IBS and the microbiome were searched in titles or abstracts during the period of 2000–2019. For data visualization, VOSviewer software was used.

Research results

Since 2013, the number of publications on gut microbiota in IBS has continuously increased. This result indicates that the future outlook remains optimistic for treatments targeting the gut microbiota in IBS.

Research conclusions

This is the first study to analyze and measure the global research productivity of IBS and microbiome research to provide a holistic view of this evolving subject and explore future research directions. It is evident that, due to the growing understanding of the role of the gut microbiota, research productivity in this area has steadily increased. Currently, the key hot topics are the gut–brain axis related to IBS, clinical trials related to IBS and the microbiome, drug-mediated modulation of the gut microbiome, and the role of the altered composition of the intestinal microbiome in the prevention of IBS.

Research perspectives

Our results indicate that the future outlook for IBS therapies targeting the intestinal microbiota remains promising.

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CASE REPORT

Transperineal core-needle biopsy of a rectal subepithelial lesion guided by endorectal ultrasound after contrast-enhanced ultrasound: A case report

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Abstract

BACKGROUND

Rectal subepithelial lesions (SELs) are commonly seen in endoscopic examination, generally manifested as bumps with a smooth surface. Precise preoperative diagnoses for rectal SELs are difficult because abnormal tissues are not easily to be obtained by regular endoscopic forceps biopsy. Traditional guidance modalities of preoperative biopsy, including endoscopic ultrasound, computed tomography, and transabdominal ultrasound, are often unsatisfactory. An updated, safe, and effective biopsy guidance method is required. We herein report a new biopsy guidance modality—endorectal ultrasound (ERUS) combined with contrast-enhanced ultrasound (CEUS).

CASE SUMMARY

A 32-year-old woman complained of a mass inside the rectovaginal space for 9 years, which became enlarged within 1 year. A rectal SEL detected by endoscopy was suspected to be a gastrointestinal stromal tumor or exophytic uterine fibroid. Pathological diagnosis was difficult because of unsuccessful transabdominal core needle biopsy with insufficient tissues, as well as vaginal hemorrhage. A second biopsy was suggested after multiple disciplinary treatment discussion, which referred to a transperineal core needle biopsy (CNB) guided by ERUS combined with CEUS. Adequate samples were procured and rectal gastrointestinal stromal tumor was proved to be the pathological diagnosis. Imatinib was recommended for first-line therapy by multiple disciplinary treatment discussion. After the

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tumor shrunk, resection of the rectal gastrointestinal stromal tumor was performed through the posterior vaginal wall. Adjuvant therapy was applied and no recurrence or metastasis has been found by the last follow-up on December 13, 2019.

CONCLUSION

Transperineal CNB guided by ERUS and CEUS is a safe and effective preoperative biopsy of rectal SELs yet with some limitations.

Key Words: Transperineal core needle biopsy; Endorectal ultrasound; Contrast-enhanced ultrasound; Rectal subepithelial lesion; Case report

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Core Tip: Rectal subepithelial lesions (SELs) often manifest as bumps with a smooth surface on endoscopy. An efficient biopsy strategy is crucial to sampling for differentiation of pathological types. The transperineal core needle biopsy guided by endorectal ultrasound combined with contrast-enhanced ultrasound overcomes the limitations of previous guidance, such as sampling inadequacy, high risk of complications, and exposure to radiation. In our case, unsuccessful transabdominal biopsy led to failure of pathological diagnosis. The patient underwent this new biopsy modality then, and a diagnosis was finally made. No complications occurred. We recommend this new strategy as a promising tool for rectal SEL biopsy.

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INTRODUCTION

Rectal subepithelial lesions (SELs) vary in types and there have been many studies on the diagnostic value of ultrasound (US). Pelvic lipomas, gastrointestinal stromal tumors (GISTs), carcinoid tumors, leiomyomas, schwannomas, and lymphomas often originate from the anorectum[1,2]. These tumors are apt to manifest as rectal SELs on endoscopy. Traditional biopsy guidance methods include endoscopy, endoscopic US (EUS), transabdominal ultrasound, and computed tomography (CT), which have been reported with many disadvantages[1-4] to achieve successful biopsies. By application of endorectal US (ERUS) in anorectal and pelvic diseases in our daily work, we found a novel guidance method for rectal SELs biopsy, which is safer and more effective. Out of several preoperative biopsy approaches for rectal SELs, this case report focuses on transperineal core needle biopsy (CNB)[5] guided by contrast-enhanced US (CEUS) combined with ERUS. We present the following case in accordance with the CARE reporting checklist.

CASE PRESENTATION

Chief complaints

A 32-year-old woman was admitted to our hospital with a progressively enlarged mass in the rectovaginal space for 9 years.

History of present illness

The mass was found during a routine examination 9 years ago and no obvious progression was seen in following annual examinations until a year ago. The maximum diameter of the mass had significantly grown from 4.0 cm to 9.7 cm in a year, with dents in stools, which was caused by tumor compression. The patient had a

submucosal protrusion demonstrated by endoscopy in a local hospital. However, no absolute tumor tissue but only inflammation was found after several times of endoscopic biopsies in the latest 2 years, which was not consistent with clinical estimation. Therefore, she came to our hospital for further diagnosis (Table 1).

History of past illness

The patient had no previous medical history.

Personal and family history

The patient and family had no history of previous similar illness.

Physical examination

The patient's mental status, appetite, sleep, and weight were normal without any obvious symptoms of abdominal distension, tenesmus, prolapse, diarrhea, or constipation. The anterior rectum wall was plump during digital rectal examination without tenderness or blood stain on the fingertip.

Laboratory examinations

Laboratory examinations were normal, including routine blood analysis, carcinoembryonic antigen, α-fetoprotein, carbohydrate antigen (CA) 19-9, and CA125.

Imaging examinations

Contrast-enhanced CT (CECT) showed a mass with liquefaction necrosis (low density area) inside the rectovaginal space, with an obscure margin (Figure 1A). Transabdominal US was performed. A heterogeneous hypoechoic mass with a maximum diameter of 9.7 cm was also seen in the rectovaginal space (Figure 2A), which was suspected to be a rectal GIST or exophytic uterine fibroid.

Further diagnostic work-up

Transabdominal CNB (MG1522 BARD MAGNUM Biopsy Instrument; Tempe, AZ, United States; disposable core tissue biopsy needle, gauge size and needle length: 16 G and 16 cm) was performed to make a definite diagnosis (Figure 2B). Before the operation, written informed consent was obtained from the patient, and her preoperative regular laboratory examinations were normal, including routine blood test, coagulation function, and blood transfusion set. Some hemorrhage occurred from the vagina, causing premature end of the biopsy, and spontaneously relieved several days later. Strips of greyish shattered tissue (Figure 3A) were obtained, with the largest diameter smaller than 0.3 cm. Pathology indicated inadequate biopsy tissue for diagnosis. Another biopsy after ERUS assessment was recommended by multiple disciplinary treatment (MDT).

First, ERUS was performed with the MyLab Twice US system (Esaote, Genoa, Italy) equipped with a biplane endoscopic probe (TRT33, linear frequency of 4–13 MHz, convex frequency of 3-9 MHz), recording the location, size, stratification, adjacence, and echogenicity of the tumor. Then CEUS was performed with a bolus injection through the elbow vein of 2.4 mL SonoVue (Bracco, Milan, Italy) (Figure 4). Because of the heterogeneity of the tumor, it was difficult to recognize the necrotic from non-necrotic part in B mode, which can be clearly depicted by CEUS as nonenhanced *vs* enhanced area. As previous CECT has pointed out necrosis, CEUS was performed right before puncture to confirm the substantial part of the tumor for biopsy guidance for the second time, avoiding the failed samples inside presumptive solid area, which turned to be non-enhanced after CEUS. This guarantees the precision and efficiency of biopsy samples.

The probe was switched to linear mode. The transperineal puncture site and path were decided, and disinfection as well as drape were also completed. After local anesthesia, a freehand biopsy of the lesion was performed with the guidance of in-plane needle. The needle tip and its movements were continuously monitored in real time by ERUS during the whole puncture procedure[6] to procure the tissue of lesion from enhanced area of the tumor on CEUS (Figure 5). Several strips of greyish tissue were obtained with a length of 0.3-1.2 cm (Figure 3B and C). No complications occurred.

Table 1 Timeline of diagnosis and treatment process

Time	Events
January 2018	A mass in the rectovaginal space found 9 years ago had significantly grown in a year, with several times of negative endoscopy biopsy results
January 31, 2018	Transabdominal biopsy failed to provide qualified tissues to make well-defined pathological result
February 8, 2018	The patient underwent transperineal biopsy guided by ERUS and CEUS, diagnosed as rectal GIST
July 23, 2018	The tumor shrunk after 5-mo imatinib treatment, and was completely resected through the posterior vaginal wall
October 13, 2019	Fifteen months after tumor resection, no obvious sign of recurrence was found

ERUS: Endorectal ultrasound; CEUS: Contrast-enhanced ultrasound; GIST: Gastrointestinal stromal tumor.

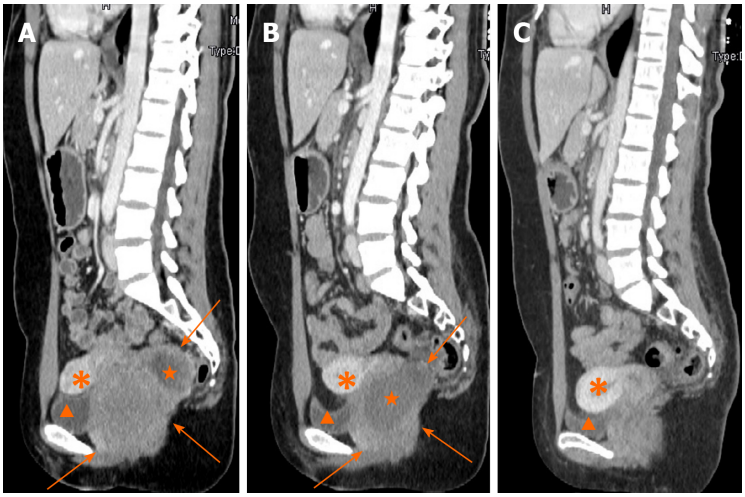


Figure 1 Contrast-enhanced computed tomography in sagittal section. A: A rectovaginal space mass of 8.0 cm in maximum diameter with non-enhancing liquefaction necrosis at the time of admission; B: The mass significantly shrunk to 6.4 cm in maximum diameter after 5 mo of imatinib treatment and non-enhanced liquefaction area enlarged clearly; C: No obvious sign of tumor recurrence in rectovaginal space 15 mo after tumor resection. Arrows: The mass; pentagrams: Liquefaction necrosis area; arrow heads: Bladder; asterisks: Uterus.

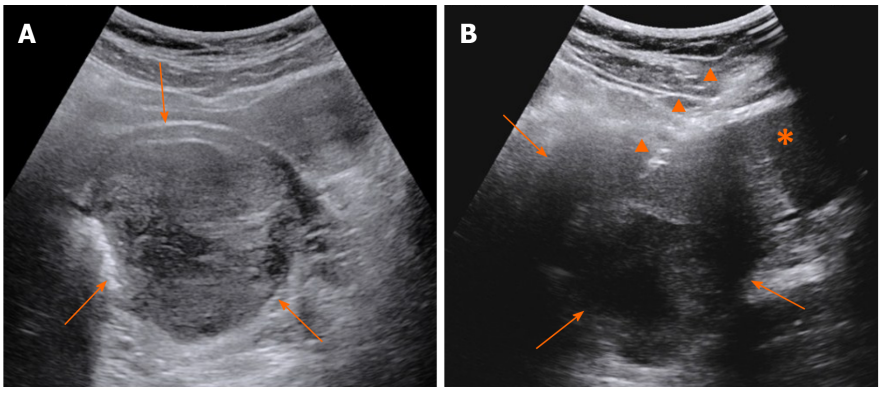


Figure 2 Grey scale ultrasound. A: A mass was demonstrated inside the rectovaginal space in the grey scale images; B: Transabdominal core needle biopsy of the mass. Arrows: The mass; arrow heads: Core needle; asterisk: Uterus cervix.

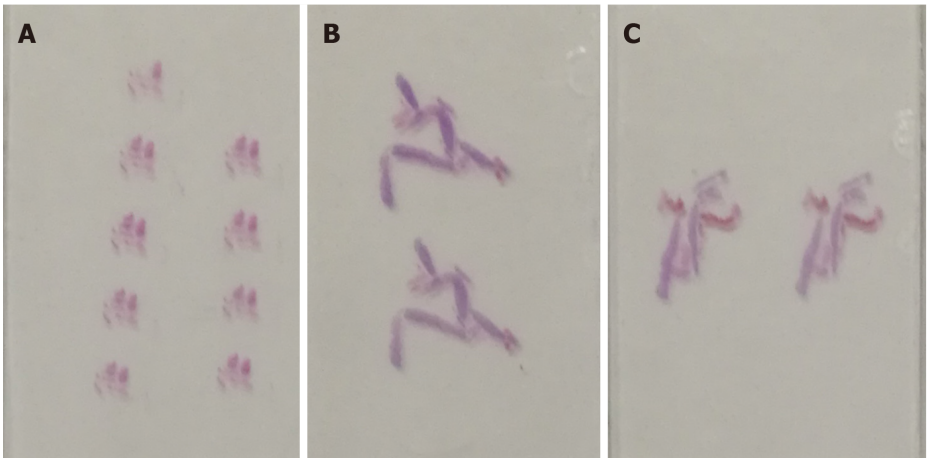


Figure 3 Slide samples. A: The shattered slide samples from transabdominal ultrasound-guided biopsy; B and C: The slide samples from transperineal biopsy guided by endorectal ultrasound, which were abundant and intact.

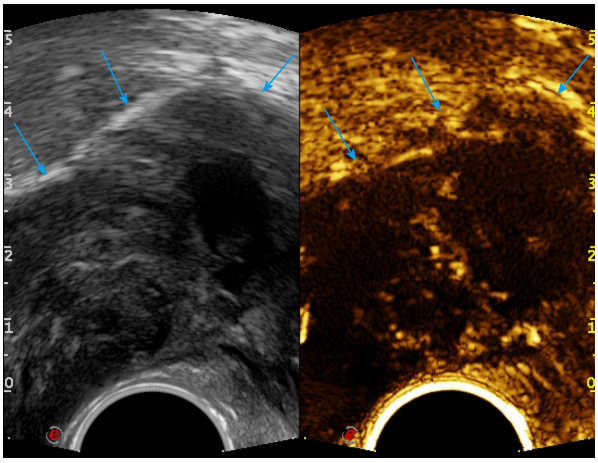


Figure 4 Endorectal ultrasound performed with the convex probe of TRT33. Liquefaction necrosis and cellular appearance were found inside the tumor in transverse sections, and the solid part of the tumor was either hypo- or iso-enhancing. Arrow: Rectal stromal tumor.

FINAL DIAGNOSIS

According to the post-operation immunohistochemical (IHC) staining, CD117, Dog-1, CD34, and succinate dehydrogenase B subunit were positive, and spinal muscular atrophy, Des, and S-100 were negative. The proliferation index of Ki-67 was 2%. The diagnosis was finally settled as GIST with post-therapeutic reaction (Figure 6).

TREATMENT

After the second MDT discussion, imatinib (Gleevec; Novartis, Basel, Switzerland) was decided as first-line therapy. It was suggested to wait for the treatment reaction before making a further therapeutic schedule. After 5 mo, CECT showed that the tumor had significantly shrunk (Figure 1B). The patient underwent complete GIST resection through the posterior vaginal wall. During the surgery, the tumor was found to originate from the rectal muscular layer, close to the posterior vaginal wall, with a smooth capsule, 5.5 cm in maximum diameter. It was greyish white on the cut surface with an interlaced appearance. The postoperative pathology findings were as follows: A suspected spindle cell tumor, interstitial and fibrous tissue proliferation, focal glassy degeneration area accompanied by a few lymphocyte reactions, remote hemorrhage with hemosiderin deposition, and a negative surgical margin.

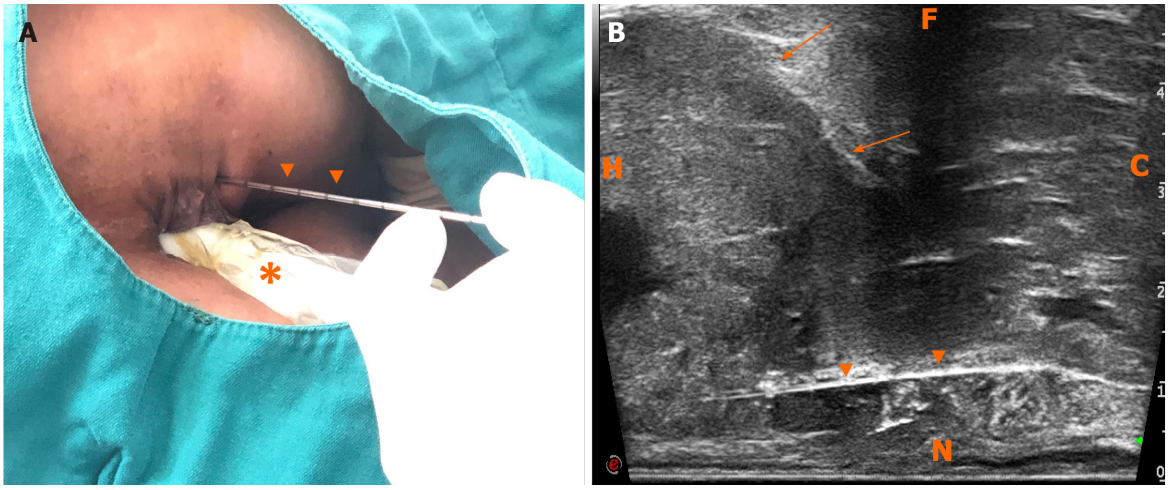


Figure 5 Freehand transperineal biopsy of the rectal subepithelial mass guided by endorectal ultrasound. A: The patient in left lateral decubitus, and the freehand transperineal biopsy was performed, guided by endorectal ultrasound (ERUS); B: Longitudinal sectional image of transperineal core needle biopsy guided by ERUS obtained with the linear probe of TRT33. H: Head; C: Caudal; N: Near field; F: Far field; arrows: Rectal stromal tumor; triangular arrowheads: Core needle; asterisk: TRT33 probe.

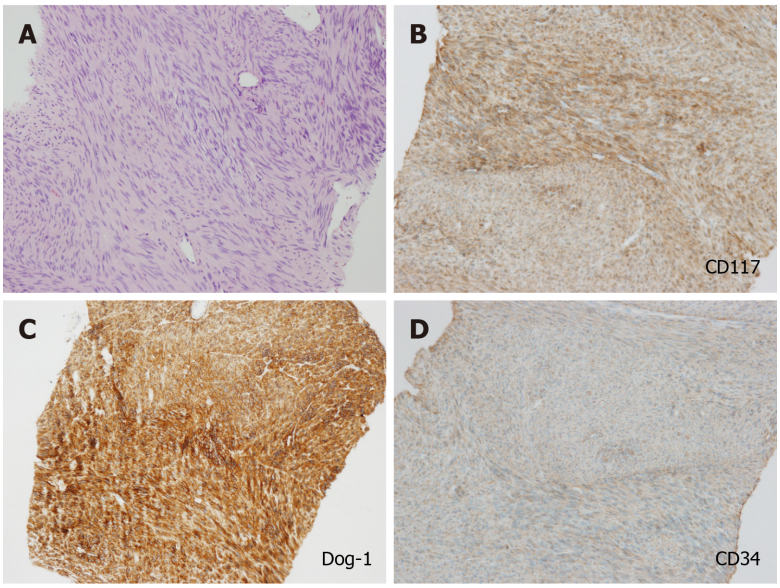


Figure 6 Pathological results. A: A mass consisting of spindle-shaped cells in hematoxylin and eosin staining; B-D: Immunohistochemical staining of the rectal stromal tumor: CD117, Dog-1, and CD34 were strongly positive (low power field).

OUTCOME AND FOLLOW-UP

Fifteen months after resection, CECT of the pelvic cavity on October 26, 2019 (Figure 1C) indicated no obvious sign of tumor recurrence. No recurrence or metastasis has been found either by the last follow-up in December 13, 2019.

DISCUSSION

GISTs are nondirectional differentiated mesenchymal neoplasms that arise anywhere along the digestive tract with an incidence of 3% in the rectum[7]. Approximately 25% of GISTs were found accidently during surgery or by imaging and about 5% at autopsy[8].

Rectal GISTs are often concealed, accompanied by long disease duration, thus symptoms often appear in the late phase of the disease. Common imaging findings include a well-circumscribed tumor with nonannular and exophytic growth pattern, without causing perirectal lymphadenopathy or bowel obstruction in most cases[9].

Rectal GISTs are of increasing concern because of the rare site and post-treatment implications for lifestyle. Targeted therapy has been successful compared with conventional chemotherapy and radiotherapy. Surgery is still the main approach to cure GISTs[10,11]. Preoperative diagnosis is crucial for GISTs, because they differ from other rectal SELs in treatment and prognosis.

GISTs have malignant potential and treatment depends on accurate pathological diagnosis by IHC staining, which requires adequate tissue samples. Endoscopic biopsy only samples mucosal tissue, yet rectal GISTs often present as submucosal growths, which forms a barrier for endoscopic biopsy. EUS-guided fine needle aspiration (EUS-FNA) is considered for preoperative diagnosis of submucosal lesions. In our case, the results of several times of EUS-FNA from the local hospital were negative. Inadequate tissue samples were mentioned by Yegin and Duman[1] in approximately one third of cases. A multicenter retrospective study[3] of EUS-FNA of suspected GISTs gave cytopathological yield, IHC yield, and diagnostic yield of 46%, 41%, and 37%, respectively. In addition, EUS-FNA has been limited in its inability to procure intact and adequate samples that are vital for histopathological and IHC analysis, causing difficulties in pathological diagnosis of GISTs[2,4]. EUS-guided fine needle biopsy (EUS-FNB) is still the commonest biopsy mode for GISTs. However, it requires adequate preoperative bowel preparation, and the risks of infection and hemafecia are higher in transrectal approach than in transperineal approach[12,13]. Also, the technique of EUS-FNB is more difficult, and it requires complex instruments, leading to unavailability in primary hospital.

We performed CNB guided by transabdominal US at first. Post-urination procedure was taken in order to avoid bladder injuries. Compression of the pelvic cavity by the probe was made to protect the surrounding bowel. The needle still went through the vagina and caused vaginal hemorrhage. Eventually, we were not able to obtain a pathological diagnosis because of the conflict between high risk of complications and inadequate sampling.

Then we performed transperineal freehand biopsy under the guidance of ERUS and CEUS, and it succeeded. We consider that ERUS guided transperineal biopsy is a reliable and safe method for preoperative pathological diagnosis of rectal SELs. And CEUS does help in the location of sampling. This method possesses advantages of both high resolution and low risk of infection[14], allowing acquirement of adequate tissue through a short puncture path. And it can be carried out under local anesthesia in outpatient department, with a lower cost of time and money[12]. The needle does not go through the rectal wall, so there is no need for bowel preparation. This method also reduces the application of antibiotics due to a low risk of infection[15].

For patients manifesting as rectal SELs and suspected tumors, to obtain preoperative diagnosis is difficult. ERUS guided transperineal biopsy can be one of the supplementary methods to routine biopsy work-up, especially for the difficult cases after several times of failure for insufficient sampling and risk of puncture complications. In addition, freehand biopsy is more suitable for underdeveloped areas because it does not need needle guide kit and costs less.

There are some limitations of this approach. For patients with stenosis in the rectum or anal tube, or those with high-position lesions beyond the display limit of the probe, the transperineal CNB guided by ERUS combined with CEUS would not be able to carry out.

CONCLUSION

Transperineal CNB guided by ERUS ensures adequate samples with a shorter puncture path, avoiding injuries to the bladder, bowel, uterus, vagina, and prostate. It also protects patients from infection caused by transrectal puncture. CEUS of the lesion can make it clear which points are the most appropriate for sampling. Transperineal CNB guided by CEUS combined with ERUS could be a supplement to traditional biopsy guidance method and it has the potential to be an important tool for preoperative biopsy of rectal SEL. The application could be limited by anorectal stenosis or with high-position lesions.

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OPINION REVIEW

How far along are we in revealing the connection between metformin and colorectal cancer?

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Abstract

Colorectal cancer (CRC) is among the most prevalent cancers worldwide, and its prevention and reduction of incidence is imperative. The presence of diabetes has been associated with a 30% increased risk of CRC, likely through the mechanism of hyperinsulinemia, which promotes tumorigenesis *via* the insulin receptor in the epithelium or by insulin-like growth factor pathways, inflammation, or adipokines, inducing cancer cell proliferation and cancer spread. Metformin, the first-line agent in treating type 2 diabetes, has a chemopreventive role in CRC development. Additionally, preclinical studies suggest synergistic effects of metformin with oxaliplatin in inhibiting *in vitro* models of colon cancer. Although preclinical studies on the post diagnostic use of metformin were promising and suggested its synergistic effects with chemotherapy, the data on the possible effects of metformin after surgery and other CRC treatment in the clinical setting are less conclusive, and randomized controlled trials are still lacking.

Key Words: Metformin; Type 2 diabetes mellitus; Colorectal cancer; Chemoprevention; Recurrence-free survival; Surgery

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Core Tip: Metformin is one of the oldest oral antidiabetic agents used to treat type 2 diabetes mellitus. While there is substantial evidence that metformin may have a chemopreventive role in colorectal cancer (CRC) development, the data on the possible effects of metformin after surgery and other CRC treatment is much less conclusive.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide, irrespective of gender. Factors contributing to the risk of development of CRC include lifestyle, genetics, and chronic diseases such as diabetes mellitus and obesity[1-3]. Although patient survival has increased over the years due to more effective treatments, there is still a problem with adverse effects and therapy costs. The prevention and reduction of CRC incidence is imperative. Several agents with chemopreventive effects have emerged. Cyclooxygenase-2 inhibitors offered the most promising results in CRC prevention, but their use was associated with elevated cardiovascular risk. Considering that CRC patients often have diabetes mellitus and obesity, which also confers cardiovascular risk, novel targets for CRC chemoprevention are needed. Accumulating data indicates that metformin, the first-line treatment for type 2 diabetes mellitus, may be a candidate chemoprevention agent for cancer, including CRC.

METFORMIN-CLINICAL USE AND MODE OF ACTION

Metformin is one of the oldest oral antidiabetic agents used to treat type 2 diabetes mellitus alone or in combination with other oral or injectable agents. It lowers blood glucose levels by reducing hepatic glucose production, stimulating glucose uptake by peripheral tissues (muscle and fat), and lowering intestinal glucose absorption[4]. The presence of diabetes has been associated with a 30% increased risk of CRC. This potentially occurs through the mechanism of hyperinsulinemia accompanying insulin resistance, which promotes tumorigenesis *via* action on the insulin receptor in the epithelium or by influencing insulin-like growth factor pathways, inflammation, or adipokines inducing cancer cell proliferation and metastasis[5-7]. Therefore, metformin might provide preventive effects by reducing insulin resistance and lowering hyperinsulinemia[8] (Figure 1). Additionally, the use of metformin in various cancer models provided anticarcinogenic action by inhibiting angiogenesis or through antimetabolic and radio-chemosensitizer effects[9]. This is mediated by the synergistic regulation of metformin on adenosine monophosphate-activated protein kinase (AMPK), glycogen synthase kinase-3β, and proliferator-activated receptor-γ as observed in the case of pancreatic cancer[10].

The mechanistic target of rapamycin (mTOR) pathway, with complex multiple feedback loops, plays a central role in coordinating cell growth and proliferation in CRC carcinogenesis. Different mTOR inhibitors have been extensively studied for preventing and/or treating CRC, but no single agent has shown a significant therapeutic efficacy towards CRC potentially because many of the genetic pathways involved in CRC development lie upstream of mTOR and elicit the oncogenic effect through the mTOR signaling pathway[11]. According to the contemporary understanding, the mTOR pathway is continuously activated by various hormones, inflammation, and energy-related factors such as glucose, insulin, and insulin-like growth factor 1. The hypothesis to combine inhibition of these pathways seems promising in providing more effective tumor suppression[12]. Metformin inhibits mitochondrial mammalian respiratory chain complex I followed by activation of liver kinase B1 and downstream target AMPK that results in an inhibition of mTOR activity[13,14]. Metformin also exerts its metabolic effects by improving insulin resistance, hyperinsulinemia, and glycemia. Metformin has apoptotic effects on cancer

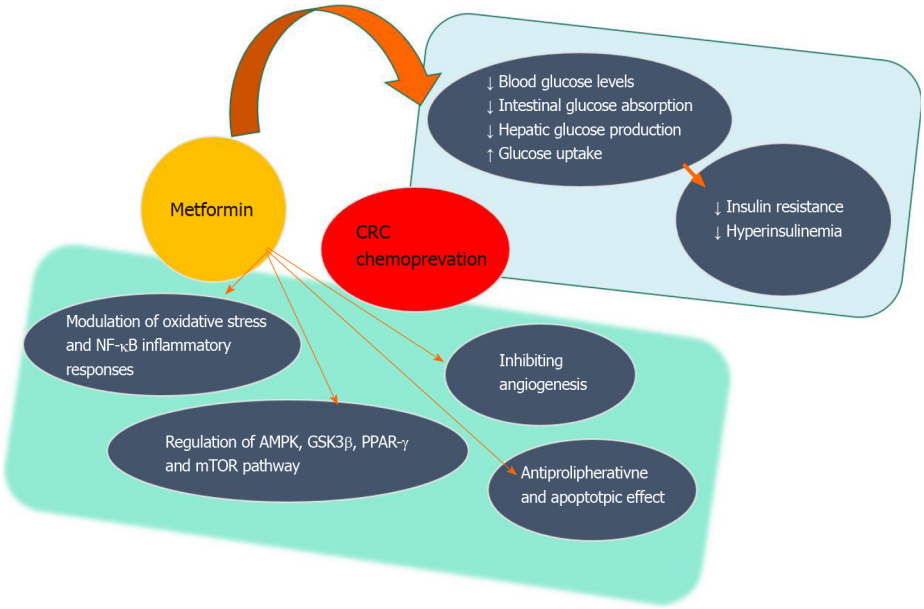


Figure 1 Metformin mode of action including chemopreventive potential in colorectal cancer development. CRC: Colorectal cancer; NF-kB: Nuclear factor kB; AMPK: Adenosine monophosphate-activated protein kinase; GSK3β: Glycogen synthase kinase-3β; PPAR-γ: Proliferator-activated receptor-γ; mTOR: Mechanistic target of rapamycin.

stem cells and potentially has a synergistic effect with other chemotherapeutics[15,16].

Animal and *in vitro* studies indicate that the role of metformin as an antiproliferative agent on CRC cells is through the activation of AMPK[17,18]. One of the first reports on anticancer effect of metformin in a CRC cell model showed the concentration and time-dependent effects on AMPK activation and the reduction of cancer cell proliferation[19]. Other research provided further evidence of the apoptotic effects of metformin by modulating oxidative stress and nuclear factor-κB inflammatory responses[20-22]. In the *in vitro* models, metformin was used either alone or in combination with other agents, primarily fluorouracil (commonly referred to as 5-FU)[20,23-25], which is similar to its use in clinics.

CHEMOPREVENTIVE EFFECTS OF METFORMIN IN CRC TREATMENT

Previous studies have indicated that metformin may decrease the risk of development of colorectal carcinoma[26,27]. Observational data indicates lower tumor incidence in diabetic patients taking metformin, and results from interventional studies show a reduction in the incidence of colorectal adenomas in patients without diabetes taking low-dose metformin[28-30]. Specifically, in a phase 3 randomized trial, a 1-year treatment with low-dose metformin reduced the number and prevalence of premalignant colorectal lesions, such as polyps and adenomas, in patients without diabetes post polypectomy[30]. A recently published retrospective cohort study including 47351 people found an inverse association between long-term (> 5 years) exposure to metformin in diabetic patients and the risk of CRC[31], suggesting its chemopreventive potential. This finding was substantiated with the meta-analysis results of 58 studies showing that metformin usage significantly reduced the incidence of colorectal adenoma and CRC, improving overall and CRC-specific survival rates[32].

On the other hand, data on the role metformin plays in CRC carcinogenesis in nondiabetic patients are not extensive. According to the randomized placebo-controlled trial results, metformin has the chemopreventive potential in nondiabetic patients with a high risk for CRC development[33]. Although metformin therapy was usually well tolerated by patients with different cancers and side effects did not differ from those seen in diabetic patients (dose-related gastrointestinal adverse effects, rarely lactic acidosis, and vitamin B12 deficiency), more data are needed on the optimal dose, schedule, route of administration, treatment duration, and combinations with other chemotherapeutic agents for CRC patients (especially nondiabetic patients) to be able to minimize the potential side effects[34-36].

METFORMIN AND SURVIVAL OF PATIENTS UNDERGOING TREATMENT FOR CRC

While there is substantial evidence that metformin may have a chemopreventive role in CRC development, the data on the possible effects of metformin after surgery and other CRC treatments are much less conclusive.

Lee *et al*[37] first observed a protective effect of metformin after CRC diagnosis in a single institution cohort of 595 patients. Metformin use showed a lower risk of overall mortality [hazard ratio (HR) = 0.66; 95% confidence interval (CI): 0.476-0.923] and CRC-specific mortality (HR = 0.66; 95%CI: 0.45-0.975) in patients with diabetes. However, this analysis suffered from immortal time bias, which may have influenced the results[38]. Fransgaard *et al*[39] examined the association between metformin and overall survival after resection for CRC in patients from the national Danish CRC database. They found better overall survival of patients treated with metformin compared to patients treated with insulin. However, cancer-specific mortality was not differentiated from diabetes-specific mortality, and it is not clear what their respective roles were in all-cause mortality. Therefore, it is impossible to ascertain whether better overall survival in the metformin-treated group can be attributed to metformin or the antitumor effects of other factors. Interestingly, in a recent paper, the same group found no association between metformin use and recurrence-free survival or disease-free survival in patients with surgically treated CRC[40]. The findings of a large population-based analysis from the United Kingdom also do not support a protective association between post-diagnostic metformin use and survival in a cohort of CRC patients with type 2 diabetes mellitus[41].

Several meta-analyses have been published on the topic, and most of them have reported a reduction in CRC-specific mortality with metformin use compared with nonuse in CRC patients[42-45]. However, the studies included in these meta-analyses are very heterogeneous regarding their design and inclusion criteria (*e.g.*, analyzing patients with different CRC stages). Also, information is often not available on the types of surgeries and administered chemotherapy in the included studies.

Evidence exists that metformin in combination with adjuvant therapy may be associated with a better prognosis in CRC patients treated with metformin post-surgery. In preclinical studies, metformin has shown synergistic effects with oxaliplatin in inhibiting *in vitro* models of colon cancer[46]. Based on such evidence, it was hypothesized that metformin combined with adjuvant chemotherapy might be associated with a better prognosis in patients with resected CRC. This was not confirmed in the setting of a randomized study in patients with resected high-risk stage II or stage III CRC who received FOLFOX-4/XELOX adjuvant therapy[47]. Another study exploring the impact of metformin on overall survival and recurrence-free survival in a homogenous group of stage III CRC patients receiving FOLFOX adjuvant therapy failed to find a significant association[48].

These findings stand in contrast to the findings of studies that found an association between metformin use and improved outcomes. However, these were the studies that considered patients with stages I-IV treated with different chemotherapy regimens, including both patients with low stages without adjuvant chemotherapy and patients with advanced stages who received multiple chemotherapeutics[37,49,50]. Therefore, the effect of metformin on overall survival and recurrence-free survival in patients with CRC receiving chemotherapy after resection remains uncertain. Extensive prospective studies that include homogenous patient populations, ideally within randomized trials, with longer follow-up would provide more evidence on the possible association of metformin use and improved survival of patients after CRC treatment.

CONCLUSION

Mounting evidence suggests a chemopreventive role of metformin on CRC development, but the data on its role in diagnosed CRC in adjunct to surgery and chemotherapy are still inconclusive. Additional evidence from prospective, randomized controlled trials are required.

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REVIEW

Intracellular interferon signalling pathways as potential regulators of covalently closed circular DNA in the treatment of chronic hepatitis B

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Abstract

Infection with the hepatitis B virus (HBV) is still a major global health threat as 250 million people worldwide continue to be chronically infected with the virus. While patients may be treated with nucleoside/nucleotide analogues, this only suppresses HBV titre to sub-detection levels without eliminating the persistent HBV covalently closed circular DNA (cccDNA) genome. As a result, HBV infection cannot be cured, and the virus reactivates when conditions are favorable. Interferons (IFNs) are cytokines known to induce powerful antiviral mechanisms that clear viruses from infected cells. They have been shown to induce cccDNA clearance, but their use in the treatment of HBV infection is limited as HBV-targeting immune cells are exhausted and HBV has evolved multiple mechanisms to evade and suppress IFN signalling. Thus, to fully utilize IFN-mediated intracellular mechanisms to effectively eliminate HBV, instead of direct IFN administration, novel strategies to sustain IFN-mediated anti-cccDNA and antiviral mechanisms need to be developed. This review will consolidate what is known about how IFNs act to achieve its intracellular antiviral effects and highlight the critical interferon-stimulated gene targets and effector mechanisms with potent anti-cccDNA functions. These include cccDNA degradation by APOBECs and cccDNA silencing and transcription repression by epigenetic modifications. In addition, the mechanisms that HBV employs to disrupt IFN signalling will be discussed. Drugs that have been developed or are in the pipeline for components of the IFN signalling pathway and HBV targets that detract IFN signalling mechanisms will also be identified and discussed for utility

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in the treatment of HBV infections. Together, these will provide useful insights into design strategies that specifically target cccDNA for the eradication of HBV.

Key Words: Covalently closed circular DNA; Interferons; *APOBECs*; Epigenetic modification; Hepatitis B virus therapeutics

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Core Tip: Hepatitis B virus (HBV) infection remains an incurable disease affecting millions worldwide. Treatment with interferons (IFNs) can eliminate the virus by clearing its persistent genome, covalently closed circular DNA (cccDNA), from infected cells. However, its clinical efficacy is limited as HBV proteins antagonize IFN signalling. Other current therapeutics do not target cccDNA thus cannot eliminate HBV. Therefore, new drugs based on the knowledge of how IFNs cause cccDNA degradation and silencing, as well as insights into how HBV antagonizes IFN-mediated mechanisms are needed. This review summarizes what is known about these processes and highlights drugs and developing therapeutics targeted against them for HBV eradication.

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INTRODUCTION

Chronic hepatitis B (CHB) is a major global health problem affecting about 250 million people worldwide, resulting in 800000 deaths per year[1]. It is the main etiological factor and cause of mortality for liver cirrhosis and hepatocellular carcinoma (HCC). Despite the availability of a prophylactic vaccine and antiviral therapies, the very high numbers of chronic carriers for the causative hepatitis B virus (HBV) indicates that current treatment regimens are inadequate in eliminating HBV. The primary reason for this is due to the stability and persistence of its genomic DNA[2,3], the covalently closed circular DNA (cccDNA), even under long-term therapy. This pivotal viral DNA template is solely responsible for generating all HBV transcripts[4] and viral proteins. Therefore, sub-detection levels of continually present cccDNA in infected cells and tissues act as template reservoirs for HBV to reactivate and persist long after achieving the treatment endpoint of expressing sub-detection levels of HBV antigens in patient sera. Given its importance in maintaining chronicity of HBV infection, experts in the field have come to a consensus that a curative regime can only be achieved through cccDNA elimination or permanently silencing cccDNA[5,6].

Of the 2 treatment options available, oral administration of nucleoside/nucleotide analogues (NAs) that target the HBV polymerase/reverse transcriptase (pol/RT) are better tolerated in patients to achieve reduced HBV titres[7]. However, NAs do not directly target cccDNA and therefore HBV reactivation persists. In contrast, the less tolerated interferon (IFN) treatment has been shown to be directly efficacious in promoting cccDNA clearance or epigenetically silencing cccDNA[8-10]. This is not surprising, as IFNs are a group of cytokines released from host cells as natural defence against external stimuli such as viral pathogens[11]. IFNs bind to their cognate receptors to elicit an intracellular signalling cascade that activates a set of IFN-stimulated genes (ISGs) with antiviral, immunomodulatory and anti-proliferative functions[12]. As such, IFNs are often used to treat viral infections against a range of viruses including HBV[13], hepatitis C virus[14], and West Nile virus[15]. Multiple lines of evidence have shown that IFN treatment effectively inhibits cccDNA function and eliminates cccDNA with great potency *in vitro* and in selected CHB patients[16,17]. However, the underlying mechanisms of its antiviral functions, especially on cccDNA are poorly understood. This is further compounded by the myriad of adverse effects associated with IFN treatments, such as neuropsychiatric disorders and neutropenia[18,19], greatly

limiting its use in HBV therapy. Moreover, HBV has multiple mechanisms that counteract IFN signalling, dampening the antiviral effect of IFN treatment[20,21] to result in poor antiviral response in some patients[22,23]. To overcome and enhance the sub-optimal antiviral response from direct IFN administration, a better understanding of the precise mechanisms of how IFNs target cccDNA and how HBV can overcome these is necessary. By targeting host factors and HBV products that modulate intracellular IFN signalling and its antiviral and anti-cccDNA effectors, curative therapies with fewer undesirable pleiotropic adverse effects can be developed. This review will highlight these mechanisms and targets for which therapeutic agents can be and have been developed.

cccDNA in HBV life cycle

cccDNA is a stable non-integrated mini chromosome formed in the nucleus of HBV-infected cells[24]. HBV enters cells by engaging the sodium taurocholate cotransporting polypeptide (NTCP) receptor[25] and utilizing epidermal growth factor receptor for internalization[26]. Following cell entry, HBV releases its core nucleocapsid into the cytoplasm while the genomic material carried within, the partially double-stranded relaxed circular DNA (rcDNA) is transported into the nucleus where it is converted into cccDNA through a series of biochemical steps involving multiple host proteins[27-29]. cccDNA contains 4 overlapping open reading frames from which all HBV transcripts are transcribed. These include the 0.7 kb mRNA for the HBx protein, 2.4 kb and 2.1 kb mRNAs that encode three different forms (L, M, and S) of envelope surface proteins (HBs), 3.5 kb pre-Core mRNA that codes for the p22 pre-core protein, a precursor for HBe and another multi-functional 3.5 kb pre-genomic RNA (pgRNA) that is both the template for rcDNA synthesis and also encodes components of the core particle, the core protein (HBc) which forms the viral capsid and the HBV pol/RT[30]. pgRNA and pol/RT are encapsidated by HBc to form the core particle, where reverse transcription takes place within to form the full-length (-) strand of rcDNA, followed by the incomplete synthesis of (+) strand to generate the partially double-stranded rcDNA. This newly synthesized rcDNA can then be recycled into the nucleus to generate more cccDNA, maintaining nuclear cccDNA pool and contributing to cccDNA persistence. Alternatively, core particles can be enveloped for secretion to generate progeny virions[31]. Since cccDNA plays a central role in this replicative cycle, it is clear to see that HBV would cease to replicate or persist when cccDNA is eliminated.

cccDNA copy number and persistence

cccDNA exists in low copies and persists long after antiviral treatment, accounting for HBV reactivation after cessation of treatment[32]. cccDNA is found in every phase of the natural course of HBV infection, even in patients who underwent HBs seroconversion to produce protective anti-HBs antibodies after effective antiviral treatment. Seroconversion or the loss of HBs is an important end goal of HBV therapy as it is associated with positive long-term clinical outcomes such as improvement in liver function and reducing the risk of HCC[33]. Surprisingly, there is currently no international standard for HBV cccDNA quantitation, and a universally endorsed HBV cccDNA assay is also lacking. Thus, the kinetics and amount of cccDNA in infected cells are not clearly defined as the cccDNA copy numbers reported merely reflect the assay used in a publication and does not facilitate comparison between studies. Cell culture models suggest that cccDNA persists up to 40 d in infected HepG2 cells, at up to 12.5 copies per infected cell[31]. Studies in human liver biopsies show that the copies of cccDNA per cell varies greatly by >5000-fold, from very low copies of 0.03 copies per cell to very high levels of 173.1 copies per cell, and that this is correlated with HBV reactivation status[3]. Consistent with the correlation between high cccDNA copies and CHB status, HBe-positive patients have a higher level of cccDNA compared to HBe-negative patients. Moreover, very low cccDNA copies of 1.5 copies per infected hepatocyte is sufficient to cause virus persistence[34]. High cccDNA levels has also been shown to be associated with increased risk of liver inflammation[35]. Collectively, high cccDNA levels is associated with greater HBV titres, increased risk of HBV reactivation and higher risk of developing HBV-associated liver diseases, emphasizing the need for cccDNA elimination to achieve a complete cure from HBV infection.

CURRENT HBV TREATMENT STRATEGIES

Nucleoside/nucleotide analogues

The standard of care for hepatitis B is based on two therapeutic strategies: the use of NAs and IFN-α or its pegylated form (PEG-IFN)[36,37]. To date, six NAs have been approved for treatment, including lamivudine, adefovir, entecavir, telbivudine, tenofovir, and tenofovir alafenamide. As NAs are taken orally, they are easy to administer hence promote patient compliance. NAs suppress viral replication by targeting HBV pol/RT activity to disrupt rcDNA synthesis[38]. By suppressing HBV load, NAs can alleviate HBV-associated liver diseases, regress fibrosis[39] and reduce the risk of developing HCC[40]. However, NAs cannot cure HBV infection as loss of virological markers such as HBs is rarely achieved, and seroconversion rates are negligible (Table 1). As a result, HBV reactivation rate is high, with >50% patients showing flares in virological markers (*e.g.* HBV DNA) and biochemical markers for liver damage [*e.g.* alanine aminotransferase (ALT)]. This is often accompanied with irreversible liver decompensation, resulting in death even when re-introduced to lamivudine[41]. Thus, to avoid HBV reactivation, CHB patients are often put on long-term (often >10 years) or even life-long NA therapy.

However, long-term administration of NAs leads to drug resistance, with nearly 65% of CHB patients becoming resistant when treated with lamivudine for 5 years[42]. This is because NAs target the error-prone HBV pol/RT, generating progeny virions that may carry escape mutations such as rtM204V/I, rtL180M, rtA181T/V, or rtL80V/I in the pol/RT protein sequence, preventing the mutant pol/RT to incorporate NAs into the nascent rcDNA hence escape rcDNA chain termination. As a result, prolonged treatment with NAs allows these "fitter" escape mutants to accumulate, generating drug resistant HBV strains[43,44]. As such, new generations of NAs need to be designed to tackle the problem of HBV mutation. Entecavir and tenofovir are such new generation NAs that are currently favoured over first-generation lamivudine and adefovir due to their higher potency and lower occurrence of drug resistance[45]. Only time will tell if the lower drug resistance rates indeed hold true for these newer NAs. It is more important to note that, NAs do not target cccDNA as the established cccDNA pool does not require HBV pol/RT for maintenance. Therefore, NAs cannot cure HBV infection.

IFNs

IFN-α and PEG-IFN are immunomodulators that augment cell-mediated immunity, part of which includes intracellular antiviral activities that can be executed without the aid of immune cells[46]. The use of PEG-IFN has superseded standard IFN-α as pegylation improves IFN-α half-life, requiring less frequent dosing[47]. More importantly, PEG-IFN-α is more effective in reducing cccDNA levels and also leads to greater rates of ALT normalization with lower HBV reactivation rates (Table 1). When compared to NAs, treatment with IFN and PEG-IFN leads to higher rates of HBe and HBs seroconversion with greater reduction in HBV markers indicative of lower HBV replication rates. Of note, HBs seroconversion is rarely achieved with the use of NAs. Recent clinical studies[48,49] have also confirmed that switching from NA therapy to IFN therapy sustains more significant HBe and HBs losses for longer periods, demonstrating the potency of IFNs in the suppression of HBV replication. However, the use of IFNs in the clinical setting is limited due to the need for high dosage and unpredictably variable patient response, which depends on the status of immune cells, HBV titre and type of HBV. It is also less tolerated in patients due to pleiotropic off-target effects from IFN signalling.

At the tissue level, immune tolerance from chronic infection significantly reduces clinical efficacy of IFN treatment. The extracellular arm of the IFN-mediated antiviral response depends on the activation of HBV-specific $CD4^+$ helper T-cells and $CD8^+$ cytotoxic T-cells to produce cytokines such as IFN-γ and tumour necrosis factor-alpha (TNF-α) that lead to the elimination of HBV-infected cells[50]. However, constant exposure to HBV leads to T-cell exhaustion and an immunotolerant environment[51]. While IFN-γ also induces intracellular antiviral properties, its continued elevation upregulates programmed death-1 (PD-1) immune checkpoint protein on T-cells and also induces its ligand PD-ligand 1 on hepatocytes, leading to immune tolerance as HBV-specific T-cells fail to act on infected cells[52,53]. IFN-γ also promotes the secretion chemokines from hepatic macrophages that retain $CD4^+$ T-cells in the liver and induce apoptosis of HBV-specific T-cells, further contributing to HBV evasion of immune clearance. Many other mechanisms of how HBV-specific immune cells' antiviral activities have been augmented in chronic HBV infection have been documented and

Table 1 Treatment outcomes of nucleoside/nucleotide analogue and interferon therapy

Ref.	Treatment		% loss of HBV markers				% seroconvert		% normal ALT	% HBV reactivation
	n	Schedule	HBs	HBe	rcDNA	cccDNA	α-HBs	α-HBe		
Reijnders *et al*[131]	132	NA: 16-43 mo	3	42	-	-	-	35	-	56
Song *et al*[132]	98	NA: 6-22 mo	-	35	-	-	-	35	-	49
Jeng *et al*[133]	691	NA: 1-8 yr	6	n.a.	n.a.	-	4	n.a.	-	79
Liem *et al*[134]	45	NA: ≥ 1 yr	2	n.a.	-	-	-	-	-	71
	22	NA: ≥ 2 yr	4	n.a.	-	-	-	-	-	18
Marcellin *et al*[135]	181	NA: 48 wk	0	n.a.	29	-	0	-	44	-
	177	PEG-IFN: 48 wk	4	n.a.	43	-	3	-	59	-
Lau *et al*[136]	272	NA: 48 wk	0	21	22	-	0	19	28	-
	271	PEG-IFN: 48 wk	3	34	32	-	3	32	41	-
van Zonneveld *et al*[137]	165	IFN: 16 wk	23	33	43	-	-	-	62	13
Niederau *et al*[138]	103	IFN: 4-6 mo	10	51	51	-	-	51	50	-
	53	Untreated	0	13	9	-	-	-	9	-
Liu *et al*[139]	38	IFN: 48 wk	-	-	-	47	-	-	Low	-
	38	PEG-IFN: 48 wk	-	-	-	63	-	-	High	-

HBV: Hepatitis B virus; rcDNA: Relaxed circular DNA; cccDNA: Covalently closed circular DNA; ALT: Alanine aminotransferase; NA: Nucleoside/nucleotide analogue; PEG: Pegylated form; IFN: Interferon; n.a.: Not applicable.

reviewed[54-56], together showing very clearly that IFN treatment alone cannot induce effective clearance of HBV, allowing HBV to persist. Indeed, strategies aimed at restoring extracellular anti-HBV immunity in CHB patients such as with anti-PD-1 therapeutics[57] and adoptive T-cell therapy[58] are being investigated. Since immune tolerance in CHB patients render immune cells non-responsive to IFN therapy, the efficacy of IFN therapy lies heavily on the intracellular arm of IFN-mediated immunity brought about by induction of intracellular antiviral proteins from IFN signaling.

IFNs induce the production of antiviral proteins that specifically disrupt HBV replication and degrade cccDNA. However, HBV has devised multiple strategies that antagonize IFN signalling. These strategies are so effective that even when coupled with NAs, the inability of NAs to reduce the amount of HBV products allows them to continue disrupting IFN signalling, rendering combination therapy efficacious only in selected patients[59,60] but redundant in others[61]. Multiple factors including type of combination therapy, HBV genotype and level of HBV replication greatly affect IFN treatment efficacy[62]. Some studies suggest that sequential NA and IFN therapy is more effective than simultaneous combination therapy[63], a phenomenon which warrants further confirmatory clinical investigation to enhance clinical response rates. HBV itself also significantly affects patient response to IFN therapy, as treatment outcomes are more efficacious in patients carrying the genotype A virus than the genotype D virus[64], and patients are also thrice more responsive to IFN treatment if they carry the genotype B virus than genotype C virus[65]. As further proof to the extent in which HBV alters patient sensitivity to IFN treatment, 30.4% of patients with low end-of-treatment HBs levels (< 10 IU/mL) achieve HBs clearance in a 5-year follow-up, in stark contrast to < 10% of patients achieving HBs clearance[66] when end-of-treatment HBs levels are ≥ 10 IU/mL. This is further supported by the association of greater PEG-IFN response in patients with HBV DNA levels of < 9 Log_{10} copies/mL sera[67]. Clearly, the full potential of IFN therapy has yet to be harnessed and directed towards HBV and its cccDNA. To achieve this, a strong understanding of how IFNs target cccDNA specifically and how HBV overcomes such mechanisms is necessary. New therapeutic agents and strategies can then be developed to prevent HBV from interfering with IFN signalling and enhance the anti-HBV and anti-cccDNA activities of IFNs to bring about HBV elimination.

IFN SIGNALLING

IFNs are key mediators of immunity, comprising a group of cytokines with antiviral properties against a wide range of pathogens. There are 3 types of IFNs (Type I, II, III) (Figure 1) based on the distinct receptors used for signal transduction[68]. Amongst them, only type I IFN-α2a and IFN-α2b are used clinically in the treatment of HBV infections[69,70], although the mechanisms underlying its clinical efficacy remain poorly understood. IFNs from all 3 types have been shown to possess anti-HBV properties (Table 2), but many have poorer patient tolerability than IFN-α. IFNs also differ in anti-HBV potency, but the underlying reasons for such differences are not clear. At high doses, IFNs non-cytolytically purge or silence cccDNA from infected hepatocytes.

Activation of ISGs

The antiviral effects of IFNs are achieved through ISGs[46,68], which are induced through the IFN signalling pathway that activates janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling (Figure 1). After IFNs engage their specific IFN receptors, tyrosine kinase 2 (TYK2) transduces the activating signal to result in phosphorylation of STAT1 and STAT2 transcription factors by protein kinase C-delta (PKC-δ) necessary for nuclear translocation. The heterodimer interacts with IFN regulatory factor 9 (IRF9) to form the ISG factor 3 (ISGF3) transcription factor complex which translocates into the nucleus by importin-dependent mechanisms. ISGF3 can then induce the expression of antiviral ISGs through IFN-stimulated response elements (ISRE) cis regulatory elements with the consensus sequence "AGTTTCNNTTTCN" in ISG gene promoters. Alternatively, phosphorylated STAT1 may homodimerize to form the IFN-gamma-activated factor (GAF) complex that binds to another cis-element, the IFN-gamma activated site with the "TTCN$_{2-4}$GAA" conserved sequence motif and induce ISG expression in the absence of IRF9[71]. As will be further elaborated, each ISG functions differently to bring about the destruction or silencing of cccDNA and other antiviral effects, accounting for pleiotropic effects of IFN treatment. There are a variety of IFN subtypes, each targeting the activation of different sets of ISGs with antiviral properties that may suppress HBV replication (Table 2). By specifically activating these ISGs relevant for cccDNA degradation or silencing, new drugs can be developed that specifically eliminate HBV. In doing so, adverse effects of IFNs that are not well-tolerated in patients may be eliminated as the activation of ISGs that are irrelevant for suppressing HBV replication are by-passed.

Regulators of IFN signalling

As JAK/STAT signalling is critical for the expression of ISGs, it is tightly regulated by several proteins[72]. Suppressor of cytokine signalling (SOCS) 2 and SOCS3 inhibit the IFN signalling cascade by preventing TYK2 activation and hence STAT1 phosphorylation. STAT1 methylation by protein arginine methyltransferase 1 (PRMT1) prevents its binding to the inhibitor protein inhibitor of activated STAT1 (PIAS1), where PIAS1 functions to sequester STAT1 away from its DNA-binding motifs[73]. The methylation of STAT1 is in turn negatively regulated by protein phosphatase 2A (PP2A), which inhibits PRMT1 activity[74]. These factors can be targeted by HBV to disrupt and dampen the IFN signalling and will be discussed later.

ANTI-cccDNA MECHANISMS

Degradation by APOBECs

Many of the IFN subtypes with anti-HBV properties induce APOBEC family genes[75], which are cytidine deaminases that perform RNA editing[76]. They have also been well-documented to act directly on cccDNA to result in degradation. IFN-α, which is used in clinical therapy of CHB, has been shown to reduce the expression of cccDNA, HBV RNA, HBs and HBe in primary human hepatocytes (PHH) and HepRG liver cells. The IFN-α mediated loss of cccDNA was found to last up to 15 d[8]. Induced expression of APOBECs can be seen after IFN treatment in liver biopsies of hepatitis B patients, and in HBV-infected chimpanzees[77]. The importance of APOBECs in mediating cccDNA clearance is supported by the fact that the expression of APOBECs correlates with clinical response to IFN.

Besides IFN-α and IFN-γ, other pathways induced by pro-inflammatory cytokines including TNF-α and lymphotoxin-β (LT-β) have also been shown to induce APOBECs and affect the stability of cccDNA. In agreement with its anti-cccDNA functions[78], APOBECs induced through LT-β signalling with LTβR agonist antibody was shown to

Table 2 Interferons with anti-hepatitis B virus properties

Types of Interferon		Effect on HBV				Remarks	Antiviral ISGs	Ref.
		cccDNA	rcDNA	HBs	HBe			
I	IFN-α2a/PEG-IFN-α2a	↓	↓	↓	↓	Clinical anti-HBV agent	APOBEC3A[1],APOBEC3F[1], APOBEC3G[1],STAT1[1],ISG20[1], TRIM38, MX1, *etc.*	[140-142]
	IFN-α2b/PEG-IFN-α2b	↓	↓	↓	↓	Clinical anti-HBV agent	IFITM1, IFITM3, TRIM14, RNASEL *etc.*	[143-145]
	IFN-α14	↓	-	↓	↓	Activates IFN-α and IFN-γ signalling	GBP4, GBP5	[146]
	IFN-β/PEG-IFN-β	↓	↓	↓	↓	-	MX1, CXCL10	[147]
	IFN-ω	-	↓	↓	↓	-	IRF1, IRF9, ISG15, OAS	[148-150]
II	IFN-γ	-	-	-	↓	-	APOBEC3G[1], OAS, IDO	[151-153]
III	IFN-λ1a	-	↓	↓	↓	Increased HBe seroconversion	PKR, OAS	[154,155]
	IFN-λ2	↓	↓	-	↓	-	APOBEC3A[1],APOBEC3B[1], APOBEC3G[1], MX1, OAS	[156]
	IFN-λ3	-	↓	↓	↓	Increased JAK/STAT signalling	APOBEC3G[1],IRF9[1], IRF7, MX1, OAS, ISG15 *etc.*	[157-159]

↓: Reduced; -: Not determined.

[1]Important for cccDNA elimination or silencing. HBV: Hepatitis B virus; rcDNA: Relaxed circular DNA; cccDNA: Covalently closed circular DNA; JAK: Activate janus kinase; STAT: Signal transducers and activators of transcription; ISGs: Interferon-stimulated genes; PEG: Pegylated form; IFN: Interferon.

clear 90% of cccDNA in HBV-infected cultured liver cells with good *in vivo* tolerability[8]. As the clinical dose of IFN-α used to achieve cccDNA clearance is relatively high, this suggests that inducing expression of APOBECs through LT-β signalling is an alternative means for cccDNA clearance with potentially fewer adverse effects. While its clinical efficacy and safety remain to be studied, upregulation of natural ligands for LTβR in HBV-infected liver tissues have been reported[79]. IFN-γ and TNF-α also exhibit anti-cccDNA effects to degrade cccDNA[80] levels without causing cell death in HBV-infected chimpanzees, PHH and cultured liver cells.

Following IFN receptor or LTβR activation, the upregulated IFN-α induced APOBEC3A (A3A) and LTβR activation-induced APOBEC3B (A3B) are brought to cccDNA by HBc (Figure 2). Similarly, IFN-γ induced the expression of both A3A and A3B while TNF-α induced the expression of A3B only, whereas knockdown of A3A and A3B abrogated the antiviral effects of IFN-γ and TNF-α. HBc amino acids 77-149 are crucial for this interaction, which brings the APOBECs into close proximity with cccDNA for deamination[8]. cccDNA deamination generates apurinic/apyrimidinic (AP) sites that are recognized by endonucleases[81], which ultimately degrade cccDNA. The dependence on AP sites for cccDNA degradation was confirmed by reduced intact DNA amount when DNA extracted from IFN-γ or TNF-α treated cells were digested with recombinant APE1, an AP endonuclease which specifically recognizes and cleaves AP sites[9]. Surprisingly, knockdown of APE1 in IFN-γ and TNF-α treated HBV infected cells did not show a reduction in cccDNA clearance, suggesting the redundancy of endonucleases in clearing cccDNA. While AP sites can be repaired by the host cells' DNA repair machinery, this is not observed with IFN-mediated AP site formation in cccDNA. This is most likely due to the concurrent downregulation of the base excision repair enzymes such as thymine DNA glycosylase and Nei-like DNA glycosylase after IFN treatment[77]. These studies indicate that A3A and A3B are key proteins responsible for IFN and LTβR mediated cccDNA clearance. APOBECs also have other important roles in clearing HBV cccDNA. APOBEC3F (A3F) and APOBEC3G (A3G) were also found to have anti-HBV properties[82]. A3G for example inhibits pgRNA packaging to reduce virion formation[83]. The mechanism of action for A3F has not been well-characterized.

Epigenetic silencing and transcriptional repression

Apart from eliminating cccDNA, permanently silencing cccDNA is another strategy for the development of anti-HBV therapy. Several studies have shown that IFNs control the epigenetic silencing of cccDNA[84,85], spurring growing interest in

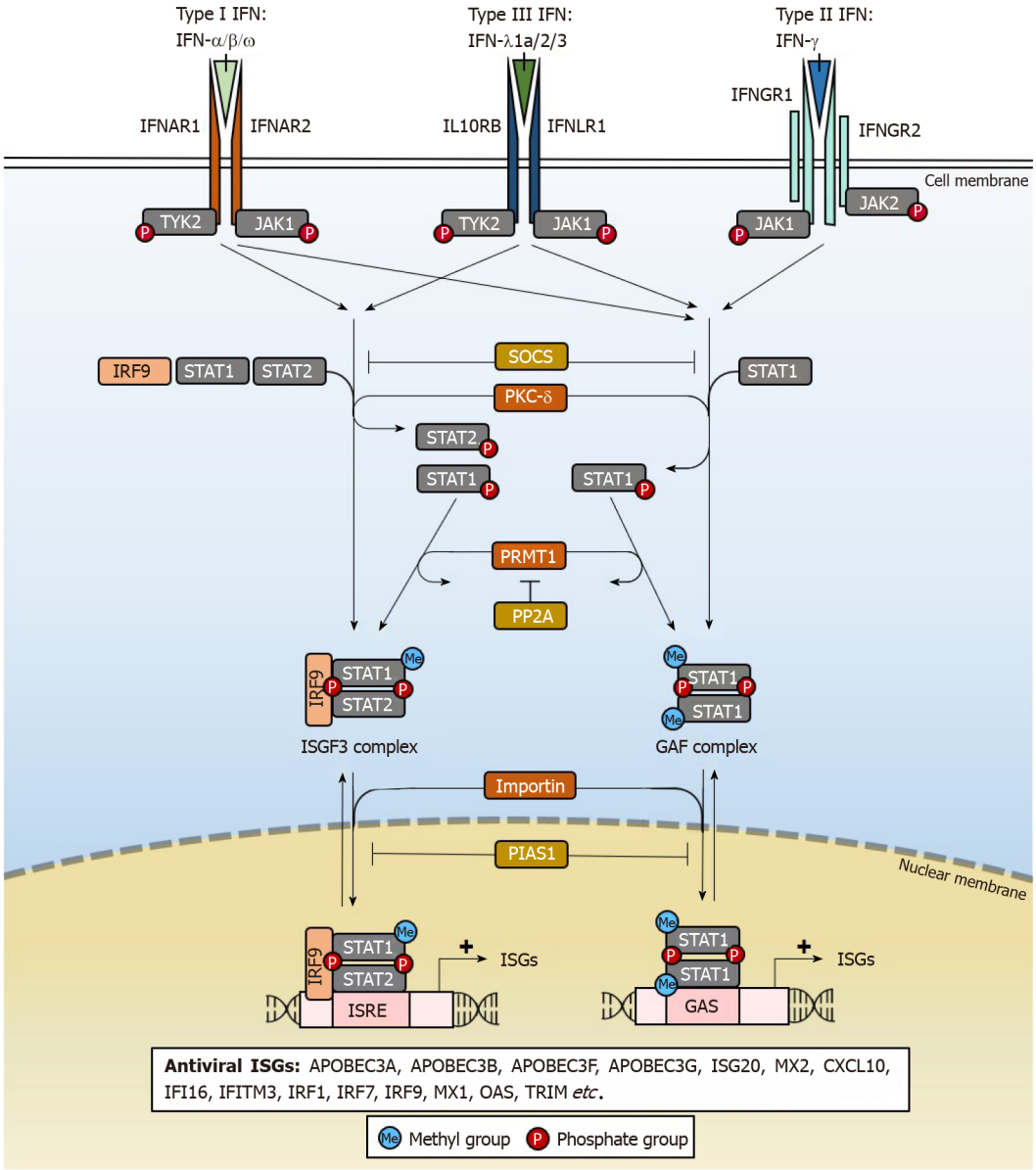


Figure 1 Activation of antiviral interferon-stimulated genes by different interferon subtypes. Different subtypes of interferons (IFNs) bind to their cognate receptors to trigger IFN signalling pathways. The activate janus kinase/signal transducers and activators of transcription-dependent signalling results in the formation of transcription complexes that induce the expression several IFN-stimulated response elements-dependent and gamma activated site-dependent antiviral IFN-stimulated genes (ISGs), which target covalently closed circular DNA (cccDNA) stability and function through a variety of mechanisms. The potency of IFN subtype for cccDNA degradation or silencing is dependent on the types and number of ISGs induced. IFN: Interferon; TYK2: Tyrosine kinase 2; SOCS: Suppressor of cytokine signalling; PKC-δ: Protein kinase C-delta; IRF: Interferon regulatory factor; JAK: Activate janus kinase; STAT: Signal transducers and activators of transcription; ISGF: Interferon-stimulated gene factor; GAF: Gamma-activated factor; ISGs: Interferon-stimulated genes; ISRE: Interferon-stimulated response elements; GAS: Gamma activated site.

controlling cccDNA transcriptional activity through epigenetic modifications[86]. HBV cccDNA exists as episomal chromatin wound around cellular histones that undergo post-translational modifications (PTMs)[87], altering cccDNA chromatin compaction hence accessibility to transcription regulators (Figure 2). Some of the histone marks associated with active cccDNA transcription include H3K4me3, H3K27ac, H3K122ac, and repressive PTMs include H3K27me3. Of note, the distribution of these histone marks varies across different types of HBV-producing samples[10]. In the HepG2-NTCP cell model, histone PTM enrichment is mainly confined to the pre-core/core promoter (CP) region whereas in PHH, they are found throughout the genome with the greatest modifications at the X and pre-S1 promoter regions. In contrast, HBV-infected liver tissues have few PTMs at the CP but accumulate them near pre-S2 and the X promoter regions. The reason and basis for this variability is at present unclear.

cccDNA transcription activity is heavily influenced by the state of epigenetic modification by host cellular factors and PTMs. The cccDNA mini chromosome is

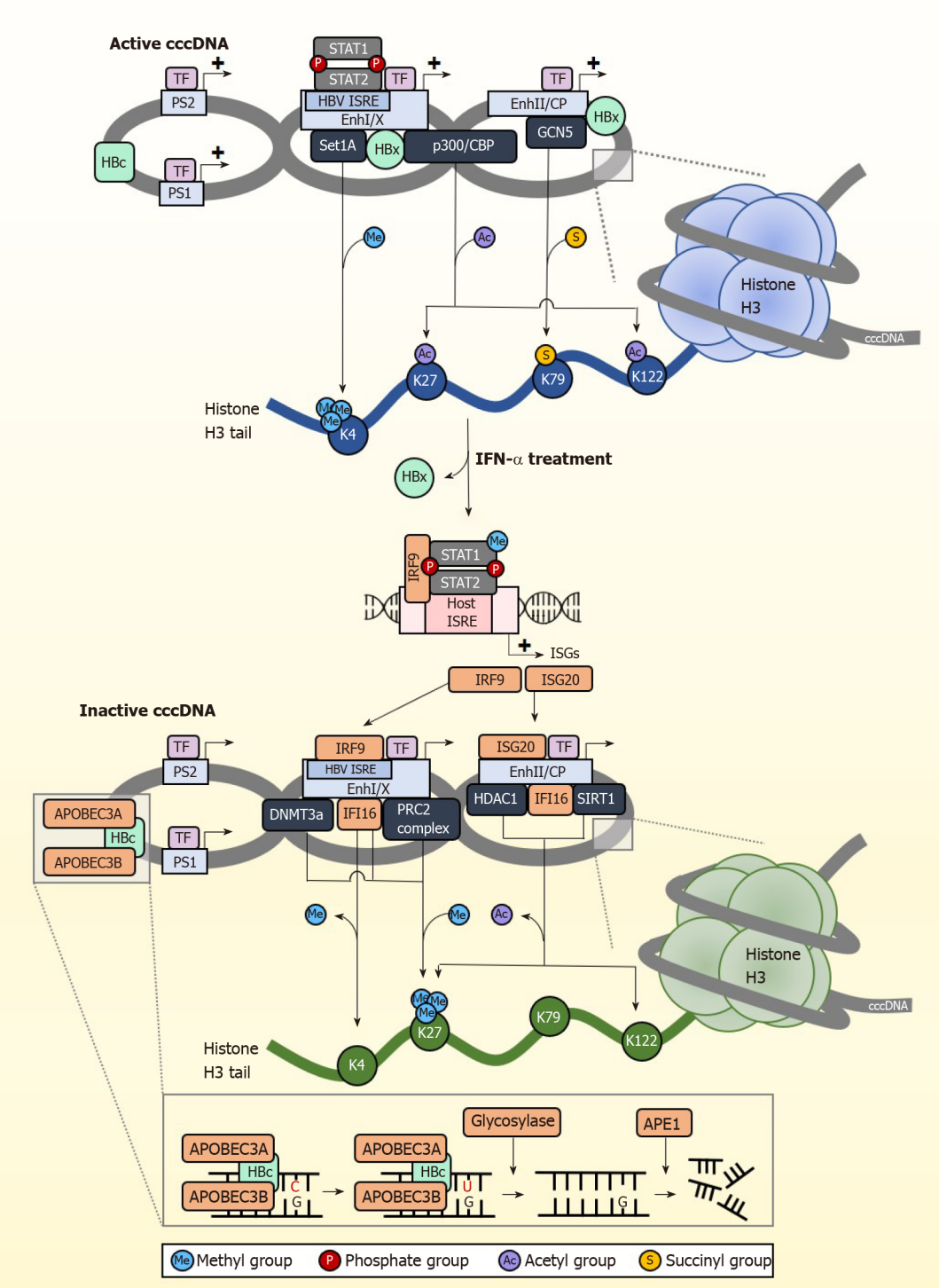


Figure 2 Interferon treatment silences covalently closed circular DNA transcription and recruits APOBECs to actively degrade covalently closed circular DNA. Covalently closed circular DNA (cccDNA) exists as an episomal mini chromosome that is epigenetically modified to support active transcription. Interferon (IFN)-α treatment results in the recruitment of histone modifying complexes that remove activating transcription post-translational modifications (PTMs), and add repressive PTMs that silence cccDNA function. These complexes can be recruited by IFN-stimulated genes (ISGs) such as IFI16. ISGs may also bind directly to cccDNA to repress transcription. Other ISGs such as APOBECs induced by IFN signalling are also recruited by HBc, generating AP sites which lead to cccDNA degradation. HBV: Hepatitis B virus; IFN: Interferon; IRF: Interferon regulatory factor; JAK: Activate janus kinase; STAT: Signal transducers and activators of transcription; ISGs: Interferon-stimulated genes; ISRE: Interferon-stimulated response elements; cccDNA: Covalently closed circular DNA.

heavily modified by various activating PTMs under normal circumstances (Figure 2). H3K4 methyltransferase Set domain containing 1A (Set1A) is recruited to cccDNA promoter sites by HBx, depositing the activating PTM H3K4me3 to drive active transcription[88]. By modulating the expression of Set1A, the relative expression of H3K4me3 can be fine-tuned. IFN treatment disrupts cccDNA transcription activity by

downregulating these epigenetic PTMs that support transcription. In studies using PHH, IFN-α specifically reduced trimethylation of H3K4 and acetylation of H3K27 and H3K122 on cccDNA chromatin to inhibit transcription of HBV RNA, but had negligible effect on epigenetic modification for the control promoters of ACTB and Nanog in the host genome[10].

IFNs also result in cccDNA transcriptional repression through active recruitment of complexes that confer transcription inhibitory epigenetic modifications. The repressive H3K27me3 PTM is induced by IFN-α through increased binding of polycomb repressive complex 2 (PRC2) to cccDNA[84]. The importance of H3K27me3 in inhibiting cccDNA transcription was also confirmed in a separate study showing that upregulation of DNA methyltransferase 3a hypermethylates HBV cccDNA to repress transcription[89]. IFNs have also been recently shown to inhibit succinylation of cccDNA histones, adding on to the transcriptionally repressed state brought about by hypoacetylation and/or methylation[90]. This involves the succinylation of H3K79 by GCN5 histone succinyltransferase (also known as lysine acetyltransferase 2A), which corresponds to higher HBV replication. They further showed that low levels of succinylated H3K79 from GCN5-specific knockdown resulted in significantly reduced cccDNA levels, and that expression of GCN5 and cccDNA correlate well in HBV-infected individuals. More importantly, IFN-α treatment could overcome the effects of overexpressed GCN5 to reduce the levels of succinylated cccDNA, indicating that IFNs act upstream to control GCN5 function and repress cccDNA transcription, the specific mechanism of which remains to be elucidated. Taken together, IFNs epigenetically silence cccDNA function through the recruitment of epigenetic factor complexes that add repressive PTMs or remove activating PTMs so that cccDNA enters a transcriptionally repressed chromatin structural state. Importantly, IFNs can also do so by inducing ISGs that bring about both the removal of activating PTMs and the addition of suppressive PTMs. For example, IFI16 reduces cccDNA transcription activity[91] by recruiting histone deacetylase 1 (HDAC1) and Sirtuin 1 (SIRT1) to increase repressive H3K27me3 PTMs on cccDNA, and concurrently impairs the recruitment of p300/CBP to prevent the addition of activating PTMs on cccDNA.

Interestingly, cccDNA also carries the ISRE cis-element, and this is critical for establishing IFN-mediated epigenetic changes on cccDNA. The HBV ISRE is located at the enhancer I/X promoter region[92,93], and is recognized by the ISGF3 complex and the ISGs IRF1 and IRF7. In transcriptionally active cccDNA, the HBV ISRE is bound by phosphorylated and unphosphorylated STAT1 and STAT2 transcription factors to active cccDNA (Figure 2). IFN treatment induces redistribution of the STAT proteins from the HBV ISRE towards the IFN signalling pathway, resulting in antiviral effects against HBV cccDNA by the upregulation of cccDNA-targeting ISGs. One of these ISGs is IRF9, which binds directly to the HBV ISRE element to suppress cccDNA transcription[94]. When the HBV ISRE is mutated, loss of IRF9 binding was shown to abrogate IFN-induced suppression of cccDNA transcription. This is clinically significant, as mutations in the HBV ISRE affects CHB patient response to IFN treatment[95] to render IFN treatment less effective. In addition, the HBV ISRE sequence is HBV genotype dependent, thus its sequence-dependent functionality partially accounts for differences in patient responder rates between carriers of HBV genotypes B and C[96]. Another ISG, ISG20, also directly inhibits transcription from cccDNA by direct binding to the enhancer II/CP region[97]. Higher ISG20 expression level also correlates to better response to IFN-α treatment in CHB patients[98] and viral clearance in HBV-infected chimpanzees[99]. With the withdrawal of activating transcription factors from cccDNA and binding of specific transcription repressors induced by IFN treatment, cccDNA transcription is further suppressed by epigenetic modifications of histones such as histone hypoacetylation which occurs through the recruitment of the HDAC1 and SIRT1, and hypermethylated by the PRC2 complex. Thus, in addition to epigenetic silencing of cccDNA, IFNs also suppress cccDNA transcription activity by generating IFN-induced transcription repressors specific to cccDNA.

Inhibition of cccDNA synthesis

IFNs are also known to directly inhibit cccDNA synthesis. The antiviral ISG myxovirus resistance protein 2 has been shown to reduce cccDNA formation when overexpressed[100]. Its specific knockdown abrogates the loss of cccDNA induced by IFN-α, providing confirmation for its role in IFN-α induced reduction in cccDNA levels. It has been proposed that this occurs through inhibiting cccDNA synthesis from rcDNA, as well as from downregulated HBV transcripts.

HBV DISRUPTION OF IFN SIGNALLING

HBV is not without its defences. It generates multiple factors that dampen the IFN signalling pathway. Inter-individual variability in the balance of these against antiviral IFN-mediated mechanisms could explain why CHB patients do not respond equally well to IFN-treatment. By understanding how HBV proteins antagonize IFN action, therapeutic approaches that interrupt the anti-HBV functions of IFNs can be developed to improve the response and efficacy of IFN treatments.

HBV disrupts the IFN signalling cascade at various steps. HBx[21] and HBe[20] downregulate the expression of IFN receptors, IFNAR1 and IL10RB, disrupting the initiation of type I and type III IFN signalling (Figure 3). HBe and HBx achieve this by upregulating SOCS2 and SOCS3 respectively, suppressing the IFN signalling cascade and inhibiting TYK2 activation (Figure 1). As SOCS2 also prevents STAT1 phosphorylation, STAT1 is prevented from entering the nucleus to induce ISG expression. Since SOCS2 and SOCS3 are the most direct upstream inhibitors of the IFN signaling pathway, their increased expression significantly attenuates IFN-dependent transcription of ISGs to compromise the therapeutic efficacy of IFNs. It was found that overexpression of HBe alone leads to 6-fold reduction in phosphorylated STAT1 nuclear translocation, hence downregulating protein kinase R (PKR) and oligo-adenylate synthetase (OAS) gene expression by 50%[20]. Similarly, HBx overexpression alone doubled SOCS3 expression and increased PP2A expression by 5-fold, significantly reducing PKR and OAS expression by 3-fold[101]. It is thus clear to see, that the combined effects of expressing HBx and HBe in infected cells, especially in cells with high viral titre and virological markers, will compromise the clinical efficacy of direct IFN therapy. Other HBV proteins act further down the IFN signalling cascade to prevent STAT1 nuclear translocation, further disrupting ISG expression. HBV pol/RT inhibits PKC-δ, preventing STAT1 phosphorylation and competitively binding to importin[102,103]. HBV pol/RT also significantly reduces the induction of IRF9 critical for the generation of ISGs through formation of the ISGF3 complex. p22 also binds importin[104] to prevent STAT1 nuclear translocation. In the nucleus, HBx and HBs upregulate PP2A, which inhibits PRMT1 hence reduces STAT1 methylation, enabling PIAS1 to sequester phosphorylated STAT1 away from its DNA-binding elements *in vitro* and in liver tissues from HBV infected patients[105]. As HBV proteins specifically target multiple regulatory elements of IFN signalling to block ISG expression, the expression of anti-cccDNA and antiviral ISGs would therefore be significantly reduced in the presence of HBV, rendering the effort to eliminate HBV by direct IFN administration futile. Therefore, to circumvent HBV mechanisms that antagonize IFN signalling, therapies aimed at targeting HBV molecules and components of the IFN-signalling pathway would greatly enhance the efficacy of IFN treatment for the elimination and suppression of cccDNA and HBV.

THERAPEUTIC AGENTS AND STRATEGIES AGAINST cccDNA

IFNs may eliminate and suppress cccDNA formation for a therapeutic cure of HBV infections, but IFN therapy has not been particularly efficacious due to the adverse effects from pleiotropic off-target effects and the existence of multiple HBV mechanisms that antagonize the IFN signalling pathway and downstream effector functions. As these greatly limit the clinical use of IFNs for treating HBV infections, alternative approaches that specifically enhance the anti-HBV effects of IFNs and suppress the antagonistic effects of HBV proteins can be developed to achieve cccDNA elimination and suppression without adverse effects from IFN signalling. Multiple classes of drugs that are in development or have been developed, that act on such targets may have the beneficial effect by downregulating HBV cccDNA (Figure 3). They act by modulating the functions of IFN signalling pathway components or HBV proteins that antagonize IFN signalling and its anti-cccDNA effector functions. Of note, as HBV disruption of IFN response is multi-factorial, combination therapy may be necessary to achieve cccDNA elimination for a true HBV cure.

Inducers of APOBECs

Perhaps the most obvious strategy to eliminate cccDNA is to enhance and sustain the expression of APOBECs which actively result in cccDNA degradation. While APOBEC mimics are not yet available, APOBEC expression can be induced through multiple pathways including activation of IFN signalling using other IFN sub-types such as IFN-γ and IFN-λ, and alternative pathways involving TNF-α and LTβR activation.

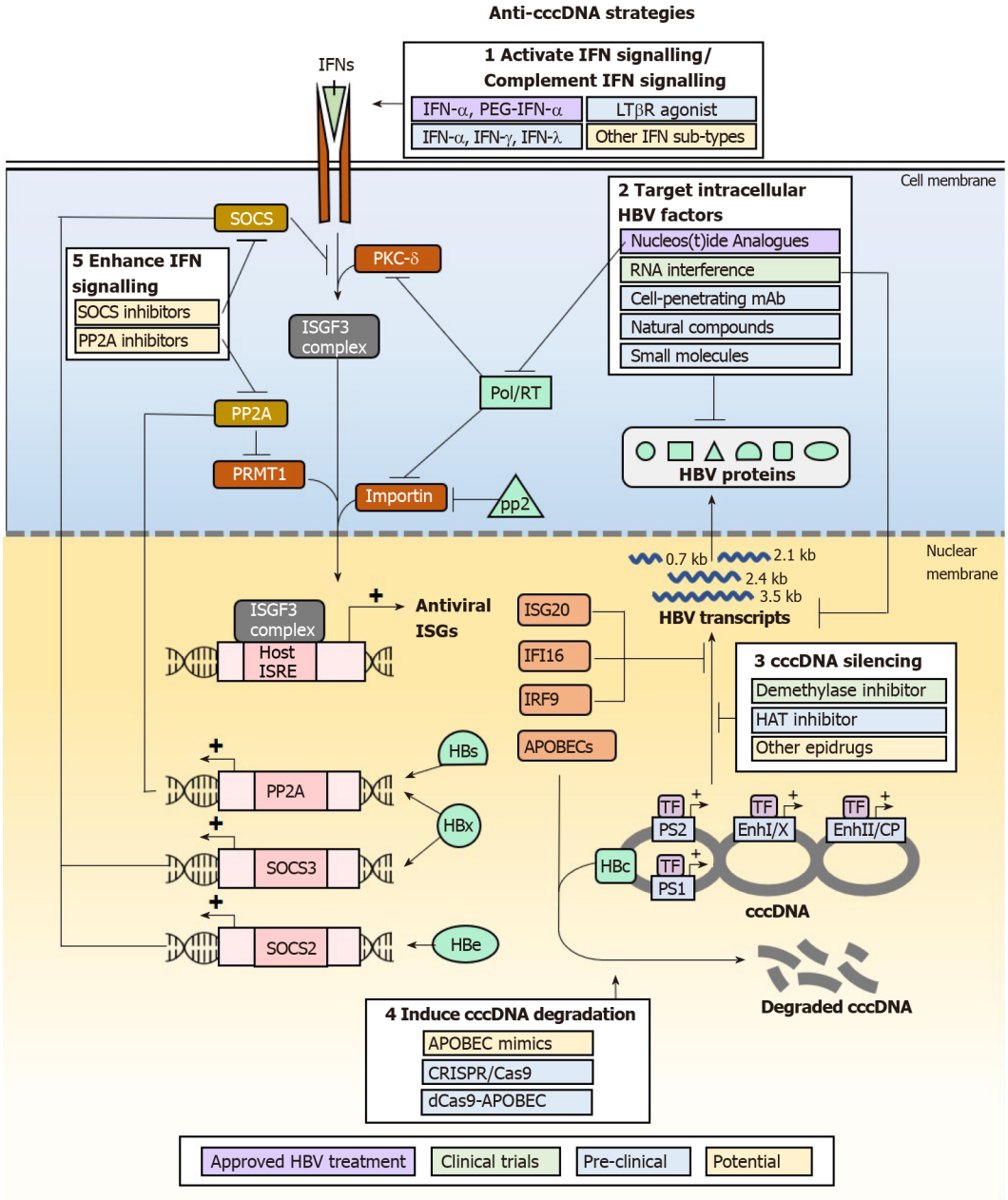


Figure 3 Anti-covalently closed circular DNA strategies and antagonistic hepatitis B virus proteins that modulate the interferon signalling pathway. Hepatitis B virus (HBV) has developed multiple mechanisms that target different parts of the intracellular interferon (IFN) signalling pathway to avert elimination by IFN treatment. Therapeutic agents that counter HBV anti-IFN signalling activities or enhance the strength of host cell IFN signalling may be beneficial to increase the efficiency of IFN treatment for the elimination or silencing of covalently closed circular DNA. HBV: Hepatitis B virus; IFN: Interferon; PEG: Pegylated form; SOCS: Suppressor of cytokine signalling; PKC-δ: Protein kinase C-delta; IRF: Interferon regulatory factor; JAK: Activate janus kinase; STAT: Signal transducers and activators of transcription; Pol/RT: Polymerase/reverse transcriptase; TNF: Tumour necrosis factor; ISGs: Interferon-stimulated genes; ISRE: Interferon-stimulated response elements; cccDNA: Covalently closed circular DNA.

IFN-λ3 was found to be specifically upregulated in patients treated with adefovir or tenofovir, and further shown in cell culture models to be effective in reducing HBs by inducing ISGs[106]. Studies in HepaRG differentiated hepatocytes also show that IFN-β, IFN-λ1 and IFN-λ2 induce longer-lasting APOBEC expression than IFN-α2, and are just as efficient in mediating cccDNA degradation[107]. IFN-γ and TNF-α have also been shown to upregulate APOBEC expression. In particular, the utility of IFN-γ in the treatment of HBV infections can be explored as it is currently used clinically for the treatment of hepatic fibrosis[108]. An LTβR agonist antibody has already been developed and was shown to be safe in mice at lower dosing requirements than clinical IFN-α. This could be further developed and tested in clinical settings for efficacy and safety in the treatment of HBV infections.

The IFN response may also be strengthened by reducing the action of negative regulators of the IFN signalling pathway, primarily by acting on SOCS2 or SOCS3 and

PP2A. This directly inhibits the degradation of IFN signaling, allowing ISGF3 and GAF complexes to form hence carry out transcription of ISGs. Thiazolidinediones such as pioglitazone and rosiglitazone are known to reduce the expression of SOCS proteins[109], and consequently would prevent the loss of IFN signalling transduction hence upregulate APOBEC expression in the presence of appropriate IFNs. They are currently used in the treatment of type II diabetes, thus may be readily re-purposed for the treatment of HBV infections to reduce cccDNA levels. The first-in-class small molecule inhibitor of PP2A, LB-100, would theoretically achieve similar effects of inducing APOBEC expression. It was found to be well-tolerated in patients with solid tumours in phase I clinical trials[110].

Epidrugs

As cccDNA function is highly dependent on its epigenetic PTM status, "epidrugs" are also being investigated for their efficacy in silencing cccDNA function. HAT inhibitor C646, which is widely used in cancer studies, has been shown to reduce H3K27ac and H3K122ac thus silence cccDNA for reduced HBV transcription[10]. The pro-drug GS-5801 (Gilead), which specifically inhibits KDM5 (lysine demethylase 5) has been shown to suppress cccDNA transcription by removing H3K4me3[111]. Given the importance of other epigenetic modifiers such as GCN5 and p300/CBP for cccDNA transcriptional activation, and SIRT1, HDAC1 for transcriptional repression and silencing, other unexplored epidrugs that inhibit the transcription activation group or activate the transcription repression and silencing group may also be tested for efficacy in directly silencing cccDNA. While epidrugs directly silence cccDNA *in vitro*, their greatest challenge in utility as an anti-HBV therapeutic depends on their ability to specifically target cccDNA in infected cells while sparing host genome to avoid carcinogenesis and toxicity[112,113]. In addition, due to compromised functionality of HBV-infected livers, many drugs that have safe profiles in the treatment of non-liver diseases are contraindicated in CHB. For example, the Food and Drug Administration-approved DNMT inhibitor, 5-azacytidine commonly used in the treatment of hematologic malignancies is contraindicated in patients with advanced liver cancer to control HBV infection due to hepatotoxicity[114]. Thus, epidrugs need to be carefully evaluated for safety and suitability in the treatment of HBV infections.

Targeted degradation of HBV products

To avoid drug-induced toxicity, approaches that directly target HBV products (cccDNA, RNA and proteins) are being developed. One such approach is to directly degrade cccDNA using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats)/CRISPR-associated protein 9) DNA editing machinery, where guide RNA-directed gene editing of cccDNA specifically results in its erroneous repair by non-homologous end-joining which cleaves and/or mutates cccDNA. This has been shown to effectively reduce cccDNA copies and is followed by concomitant clearance of HBs, HBc and HBe expression in mice with humanized liver[115,116] and multiple human liver cell lines[117,118]. While the cccDNA-targeting system is being developed and improved for clinical use, a major concern for this strategy is the generation of off-target double-strand breaks in the host genome that may facilitate HBV genomic integration to cause liver cancer. A suggested solution for this is to tether impaired Cas9 to APOBECs so that cccDNA may be mutated by base-changes instead of strand-breaks[119], highlighting again the importance of APOBECs in the role of targeted cccDNA degradation.

Another approach that has potentially fewer off-target effects is to specifically destroy HBV proteins that disrupt the IFN signalling pathway. Cell-penetrating monoclonal antibodies (mAb) have been developed for such purpose. For example, the genetically engineered antibody 9D11-Tat, formed by fusing a cell-penetrating peptide on the C-terminus of the heavy chain of a mAb specific to HBx, can target HBx for proteosomal degradation[120]. Alternatively, the transcripts for HBV proteins may be targeted for degradation by small interfering RNA (siRNA) duplexes[121]. siRNA therapeutics are already in clinical trials and currently assessed for efficacy and safety. siRNA candidates ARC-520, JNJ-3989 (Arrowhead Pharmaceuticals), and GSK3389404 (Ionis/GlaxoSmithKline)[122-124] have displayed promising efficacy in clearing viral transcripts to reduce HBs and HBV DNA production. Small molecules may also achieve the same effect by reducing HBV transcripts. Of note, the small molecule RG7834 was found to be even more potent than using the current clinical therapeutic agent entecavir in reducing HBs levels in cell culture model and HBV-infected human liver chimeric uPA-SCID mice[125,126]. With increased interest in natural compounds and their potential antiviral functions, curcumin was shown to reduce the expression of HBe and HBs[127] which antagonizes the IFN signalling cascade. Another natural

compound, Dicoumarol, can enhance HBx degradation. It inhibits NQO1 to destabilize its interaction with HBx, rendering it susceptible to the action of proteasomes[128]. With such promising new candidate drugs for tackling HBV, combination therapy that couples IFN treatment with these HBV protein-targeting drugs have been proposed to be hopeful for achieving cccDNA elimination without giving HBV the chance to evade the IFN response.

Clinical updates on the use of IFNs

Neither NAs nor IFNs alone can achieve effective HBV elimination in majority of HBV carriers. However, their mechanisms of action complement one another to potentially achieve viral elimination. NAs are effective in suppressing viral titre in most patients, allowing IFNs to effectively mount a cellular immune response in a less immunotolerant environment when HBV titre is decreased, and concurrently allow IFN-mediated intracellular antiviral mechanisms to effectively act against HBV and its cccDNA with less antagonistic effects from decreased HBV titre. As such, combination therapy is increasingly explored, with many experimenting the types of combinations that can be administered (Table 3). Combinations explored include NA monotherapy followed by IFN monotherapy or vice versa, periods of monotherapy followed by periods of IFN and NA co-administration, or co-therapy followed by monotherapy. Interestingly, the efficacies of combination therapy differ greatly. When NA monotherapy is switched to IFN monotherapy, higher rates of HBe and HBs seroconversion are observed together with lower relapse rates. Simultaneous administration of NAs and IFNs for more than 24 wk, followed by sustained NA treatment gives very high HBe seroconversion rate of 50%, accompanied by the loss of HBs expression in 16% patients. This is a remarkable feat considering the loss of HBs is usually less than 5% with NA or IFN monotherapy (Table 1). In contrast, multiple reports show that simultaneous administration of NAs and IFN yield conflicting results, with many studies showing little benefit from adding IFNs into the regime of NA treatment[129,130]. Further large-scale clinical studies are needed to ascertain the differences in these findings.

CONCLUSION

It is widely accepted that elimination or silencing of cccDNA is necessary to cure HBV infections so that no active template remains for HBV to reactivate. It is possible for IFNs to achieve this through induction of ISGs, primarily APOBECs that specifically target cccDNA for degradation. IFN signalling also results in the recruitment of epigenetic modifiers that render cccDNA in a transcriptionally repressed and silent state. However, HBV prevents these by inhibiting IFN signalling through upregulating negative regulators of IFN signal transduction such as SOCS2, and sequestration of factors that transduce IFN signalling. Thus, to achieve an anti-HBV response that targets cccDNA specifically, drugs that enhance and sustain the anti-cccDNA effects of IFN signalling and drugs that target HBV products that disrupt these IFN-mediated mechanisms need to be developed. Fortunately, many of the anti-cccDNA mechanisms are not unique to HBV infection. Drugs known to modulate these IFN-mediated pathways can be re-purposed and tested for efficacy in suppressing HBV replication and cccDNA levels. These include LTβR agonists and other cytokines that serve as alternative pathways to strengthen IFN-mediated APOBEC expression, inhibitors in the treatment of type II diabetes that act on SOCS proteins to prevent dampening of IFN signalling transduction, and epidrugs that render cccDNA transcriptionally silent. These drugs may be used together with current HBV therapies, new anti-HBV drugs in the pipelines such as cell-penetrating antibodies, cccDNA-targeting CRISPR/Cas9 systems and even natural compounds that directly target HBV proteins that disrupt IFN signalling for degradation. Hopefully, the success of these novel strategies would materialize for the eradication of HBV.

Table 3 Updates on combination therapy

Ref.	Treatment		% loss of HBV markers				% seroconvert		% normal ALT	% HBV reactivation
	Type	Schedule	HBs	HBe	rcDNA	cccDNA	α-HBs	α-HBe		
Hagiwara *et al*[160]	Sim	Combi: 48 wk	4	60	62	85	-	60	77	38
Zhang *et al*[63]	Sim	Combi: 48 wk	9	-	58	-	-	30	73	-
	Mono	IFN: 48 wk	6	-	31	-	-	25	56	-
	Add	IFN: 12 wk; +Combi: 36 wk; +NA: 12 wk	16	-	72	-	-	50	78	-
	Add	IFN: 24 wk; +Combi: 24 wk; +NA: 24 wk	9	-	69	-	-	31	78	-
Huang *et al*[48]	Swi	NA: ≥ 2 yr +IFN: 60 wk	33	91	n.a.	-	26	65	n.a.	-
	Mono	NA: ≥ 2 yr	0	38	n.a.	-	0	22	n.a.	-
Zhou *et al*[49]	Swi	NA: ≥ 4 yr +IFN: 48 wk	36	n.a.	n.a.	-	27	n.a.	-	25
	Mono	NA: ≥ 5 yr	4	n.a.	n.a.	-	0	n.a.	-	58

HBV: Hepatitis B virus; rcDNA: Relaxed circular DNA; cccDNA: Covalently closed circular DNA; ALT: Alanine aminotransferase; NA: Nucleoside/nucleotide analogue; IFN: Interferon; n.a.: Not applicable; Sim: Simultaneous therapy; Add: Add-on therapy; Mono: Monotherapy; Swi: Switch therapy.

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MINIREVIEWS

Artificial intelligence-assisted endoscopic detection of esophageal neoplasia in early stage: The next step?

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Abstract

Esophageal cancer (EC) is a common malignant tumor of the digestive tract and originates from the epithelium of the esophageal mucosa. It has been confirmed that early EC lesions can be cured by endoscopic therapy, and the curative effect is equivalent to that of surgical operation. Upper gastrointestinal endoscopy is still the gold standard for EC diagnosis. The accuracy of endoscopic examination results largely depends on the professional level of the examiner. Artificial intelligence (AI) has been applied in the screening of early EC and has shown advantages; notably, it is more accurate than less-experienced endoscopists. This paper reviews the application of AI in the field of endoscopic detection of early EC, including squamous cell carcinoma and adenocarcinoma, and describes the relevant progress. Although up to now most of the studies evaluating the clinical application of AI in early EC endoscopic detection are focused on still images, AI-assisted real-time detection based on live-stream video may be the next step.

Key Words: Early esophageal cancer; Artificial intelligence; Endoscopy; Diagnosis; Trend

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Core Tip: Esophageal cancer (EC) is one of the most common malignant tumors of the digestive tract. Early EC lesions can be cured by endoscopic therapy, and the curative effect is equivalent to that of surgical operation. Upper gastrointestinal endoscopy is still the gold standard for EC diagnosis, while the accuracy of endoscopy depends in part on professional experience. Artificial intelligence applied in the screening of early EC has been shown to be a good assistant for those less-experienced endoscopists. This manuscript reviews the state of the art of artificial intelligence applications in clinical

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early EC detection by endoscopy for those who will be interested in this field.

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INTRODUCTION

Esophageal cancer (EC) is one of the most common malignant tumors of the digestive tract and originates from the epithelium of the esophageal mucosa[1]. In 2018, the global incidence of EC was ranked seventh among malignant tumors (6.3/100000), and the mortality rate ranked sixth (5.5/100000)[2]. There are significant differences in the incidence and patterns of EC among different countries and regions, with the highest incidence in East Asia, which is twice the world average (12.2/100000). The main pathological type is esophageal squamous cell carcinoma (ESCC), while esophageal adenocarcinoma (EAC) is the main pathological type in relatively low-incidence areas such as Europe and America. Experiences in Japan and South Korea have confirmed that early EC lesions can be cured by endoscopic therapy. The curative effect is equivalent to that of surgical operation, and the 5-year survival rate of patients can reach 95%[3]. Therefore, early detection of EC and timely endoscopic treatment are the only ways to reduce EC mortality.

Upper gastrointestinal endoscopy is still the gold standard for EC diagnosis. After years of research and practice, the current upper gastrointestinal endoscopy diagnosis technology has made considerable progress. In terms of diagnostic methods, the original single ordinary white light imaging (WLI) has gradually developed into a variety of technologies such as pigment endoscopy, confocal laser endomicroscopy (CLE), electronic staining, magnifying endoscopy (ME), and autofluorescence imaging (AFI) endoscopy[4]. Among them, narrow band imaging (NBI) and blue laser can improve the sensitivity (SEN) of early EC diagnosis to more than 90% compared with ordinary white light endoscopy. CLE, also known as optical biopsy, can be comparable to pathological slices. The diagnostic specificity (SPE) of AFI is 50%, and the SEN is 100%[5]. At present, NBI, blue laser, *etc.* have been widely used in clinical practice, but clinical applications such as CLE and AFI are not universal.

British and American gastroenterologists have developed a series of guidelines for the screening and surveillance of Barrett's esophagus (BE) and EAC[6,7]. In China, gastroenterologists recommend endoscopic screening of key populations to improve the diagnosis rate of early EC. Endoscopic iodine staining and indicative biopsy screening programs implemented in high-risk areas in China can effectively reduce the incidence and mortality of esophageal cancer[8]. It is recommended that 40 years of age is the starting age for screening for EC, and screening should be terminated when the age of 75 or life expectancy is less than 5 years. Early EC and intraepithelial neoplasia (or dysplasia) are the main screening targets[9].

As we all know, the accuracy (ACC) of endoscopic examination results largely depends on the professional level of the examiner. Studies have shown that the use of proton pump inhibitors, less experienced endoscopists (< 5 years and < 1000 cases of endoscopy), and smaller lesions are significantly related to missed diagnosis of EC[10]. A great amount of needs in EC-related endoscopic screening increase the burden of clinical work, and the increase in staff fatigue may in turn affect the efficiency and ACC of the examination. How to solve the problem of the drastic increase in endoscopy workload has plagued clinical work managers.

Artificial intelligence (AI), machine learning (ML), and deep learning (DL) have relatively specific meanings, but they are usually widely used to refer to any modern processing method related to big data. AI is not "new". When Turing first designed his test, the phrase was mainly reserved for a technology that could broadly imitate human intelligence. Today, AI is widely and universally used to refer to any kind of ML program. At the most basic level, ML refers to any type of computer program that can "learn" on its own without having to be explicitly programmed by humans. ML includes supervised learning, unsupervised learning, and semi-supervised learning, which combines supervised and unsupervised approaches. In supervised learning,

algorithms generate answers based on known and labeled data sets. Classification and regression algorithms, such as random forests and support vector machines (SVM), are commonly used in supervised learning. In unsupervised ML, algorithms generate answers to unknown and unlabeled data.

DL is a form of ML that can use supervised or unsupervised algorithms, or both. Through the hierarchical learning process to extract high-level and complex abstractions as data representations, DL models produce results faster than standard ML methods. Convolutional neural network (CNN) can be composed of many layers of models. Each layer takes input from the previous layer, processes it, and then outputs it to the next layer in a daisy chain.

The emergence of AI has gradually emerged in clinical work, and a large number of verifications have been obtained in medical imaging, especially the screening and diagnosis of lung cancer[11], and the analysis of pathological characteristics[12]. Some AI technologies have also been applied in the screening of early EC and have shown their advantages. This article will review the application status of AI in the screening of early EC under endoscopy and discuss its future development trends.

AI IN ENDOSCOPIC DETECTION OF ESCC

In 2002, experts of digestive endoscopy summarized the Paris classification of early digestive tract lesions[13]. Among them, the endoscopic morphology of superficial ESCCs roughly divided into three categories: Protruded, flat, and depressed types. However, in squamous epithelium, the microvascular pattern of intrapapillary capillary loops (IPCLs) is the only reliable indicator of tissue atypia[14]. In diagnostic criteria of the Japanese Esophageal Society (JES) classification, the microvascular irregularity is evaluated for the presence or absence of each of the following morphological factors: Weaving (*i.e.* tortuosity), dilatation, irregular caliber, and different shape (*i.e.* various shapes). Currently, clinical endoscopists generally use the AB classification proposed by JES. If microvessels have three or fewer factors, they are classified as type A; if they have four factors, they are classified as type B[15].

At present, NBI-ME has become the first choice clinical method for the diagnosis of early ESCC. With the help of NBI-ME, endoscopists can clearly observe the morphology of the diseased IPCL and guide the selection of targeted biopsy and clinical treatment strategies. However, the ACC of NBI-ME in diagnosing early ESCC is limited by the experience and level of endoscopists. The diagnostic ACC of experienced experts is as high as 90%, while for inexperienced or non-expert endoscopists, the diagnostic ACC is only 78.6%[16]. AI may help those inexperienced endoscopists to analyze medical images intelligently, detect and/or classify lesions, and improve the ACC of diagnosis by using variant algorithms[17] (Table 1).

ESCC diagnosis

In 2015, Shin *et al*[18] developed a class 2 Linear classification algorithm to identify squamous high-grade dysplasia (HGD) or ESCC by using nuclear-related features of high-resolution microendoscope (HRM) images. The area under the curve (AUC), SEN, and SPE of the test and validation data sets were 0.95, 87%, and 97% and 0.93, 84%, and 95%, respectively. In order to reduce equipment costs, Quang *et al*[19] developed a small tablet-interfaced HRM with real-time algorithms. The algorithm can automatically identify ESCC, and its AUC, SEN, and SPE were 0.937, 95%, and 91%, respectively. It was believed to be especially beneficial in lower-resource settings for operators with less experience interpreting HRM images.

In 2016, Liu *et al*[20] designed an algorithm called Joint Diagonal Principal Component Analysis (JDPCA) for detection of EC. In their research, a novel image feature extraction method was established through combining the algorithm of ML based on JDPCA and conventional feature extraction algorithm without learning. Then a computer-aided method was proposed to identify the endoscopic images obtained from conventional gastroscopy and wireless capsule endoscopy containing lesions. The algorithm can correctly detect 90.75% EC, and the AUC reached 0.9471 as a result. Although ESCC is the main pathological type in China, the specific classification of EC was not mentioned in the study.

Horie *et al*[21] first tried to use DL to diagnose ESCC through a large number of endoscopic images in 2019. The CNN took 27 s to analyze 1118 test images captured using WLI and NBI and correctly detected EC cases with a SEN of 98%. It could distinguish superficial EC from advanced cancer with ACC of 98%. Subsequently, Cai *et al*[22] proposed a novel computer-aided diagnosis (CAD) system using a deep neural

Table 1 Artificial intelligence application for esophageal squamous cell cancer

AI Application	Study design	Data category	Type of Images	AI architecture	Training dataset	Validation Method or dataset	AUC	SEN	SPE	ACC	PPV	NPV	Compared with experts	Ref.
Diagnosis	Retrospective	Still image	HRM	2-class LDA	104 sites	167 sites	0.93	84%	95%	NA	NA	NA	NA	Shin *et al*[18], 2016
Diagnosis	Prospective	Still image	HRM	Fully automated algorithm	104 sites	167 sites	0.937	95%	91%	NA	NA	NA	NA	Quang *et al*[19], 2016
Detection	Retrospective	Still image	WCE	JDPCA + CCV	400 images	10-fold-CV	0.9471	93.33%	89.20%	90.75%	NA	NA	NA	Liu *et al*[20], 2016
Diagnosis	Retrospective	Still image	WLI/NBI	CNN-SSD	8428 images	Caffe DL framework/1118 images	NA	81% (WLI)/89% (NBI) (per-patient) 72% (WLI)/86% (NBI) (per-image)	79%	99%	39%	95%	NA	Horie *et al*[21], 2019
Detection	Retrospective	Still image	WLI	DNN-CAD	2428 images	187 images	0.9637	97.8%	85.4%	91.4%	86.4%	97.6%	Superior	Cai *et al*[22], 2019
Diagnosis	Retrospective	Still image	WLI/NBI/BLI	CNN-SSD	22562 images	Caffe DL framework/727 images	NA	100% (Non-ME + NBI/BLI) 90% (Non-ME + WLI) 98% (ME)	63% (Non-ME + NBI/BLI) 76% (Non-ME + WLI) 56% (ME)	77% (Non-ME + NBI/BLI) 81% (Non-ME + WLI) 77% (ME)	NA	NA	Equivalent	Ohmori *et al*[23], 2020
Diagnosis	Retrospective	Still image	ME-NBI	FCN-CAD	1383 lesions	3-fold-CV	NA	87% (lesion level)	84.1 (lesion level)	89.2 (lesion level) 93.0 (pixel level)	NA	NA	Equivalent	Zhao *et al*[24], 2019
Diagnosis	Retrospective	Still image	ME-NBI	CNN	7046 images	5-fold-CV	NA	89.3%	98%	93.7%	NA	NA	NA	Everson *et al*[25], 2019
Diagnosis	Retrospective	Still image	ECS	CNN-GoogLeNet	4715 images	Caffe DL framework/1520 images	0.85	92.6%	89.3%	90.9%	NA	NA	NA	Kumagai *et al*[27], 2019
Invasion depth measurement	Retrospective	Still image	WLI/NBI/BLI	CNN-SSD	14338 images	Caffe DL framework/914 images	NA	90.1%	95.8%	91.0%	99.2%	63.9%	Equivalent	Nakagawa *et al*[28], 2019
Invasion depth measurement	Retrospective	Still image	WLI/NBI	CNN-SDD-GoogLeNet	1751 images	Caffe DL framework/291 images	NA	84.1%	73.3%	80.9%	NA	NA	Superior	Tokai *et al*[29], 2020
Diagnosis	Retrospective	Still	NBI	CAD-SegNet	6473	6671 images/80	0.989	98.04% (per-	95.03% (per-	NA	NA	NA	NA	Guo *et al*[30],

		image/Real-time video			images	videos		image) 91.5%(per-frame)	image) 99.9%(per-frame)					2020
Diagnosis	Retrospective/ Prospective	Still image/Real-time image	WLI	GRAIDS/DeepLab V.3+	4091 images	3323 images	NA	NA	NA	NA	NA	NA	Equivalent	Luo *et al*[31], 2020
Detection/Invasion depth measurement	Retrospective	Still image/Real-time video	NBI/BLI	CNN-SSD	17274 images	5277 images/144 videos	NA	91.1%	51.5%	63.9%	46.1%	92.7%	Superior	Fukuda *et al*[32], 2020
Invasion depth measurement	Retrospective	Still image/video images	WLI/NBI/BLI	CNN-SSD	23977 images	PyTorch DL framewor/102 video images	NA	50% (non-ME) 70.8%(ME)	98.7% (non-ME) 94.9%(ME)	87.3% (non-ME) 89.2%(ME)	92.3% (non-ME) 81.0%(ME)	86.5% (non-ME) 91.4%(ME)	Superior	Shimamoto *et al*[33], 2020

Although no detailed information about the classification of esophageal cancer (EC) was found in the research of Liu *et al*[20] and Luo *et al*[31], I prefer to summarize the data in this table because most EC patients in China suffer from esophageal squamous cell carcinoma. AI: Artificial intelligence; AUC: Area under the curve; SEN: Sensitivity; SPE: Specificity; ACC: Accuracy; PPV: Positive predictive value; NPV: Negative predictive value; HRM: High-resolution microendoscopy; 2-class LDA: Two-class linear discriminant analysis; NA: Not available; WCE: Wireless capsule endoscopy; JDPCA: Joint diagonalization principal component analysis; CCV: Color coherence vector; n-fold-CV: n-fold cross-validation; WLI: White light imaging; NBI: Narrow-band imaging; CNN: Convolutional neural network; SSD: Single-Shot Multibox Detector; DNN: Deep neural network; DL: Deep Learning; BLI: Blue Laser Imaging; ME: Magnifying endoscopy; FCN-CAD: Full convolutional network computer-aided detection; ECS: Endocytoscopic system; GRAIDS: Gastrointestinal Artificial Intelligence Diagnostic System.

network (DNN). Two thousand four hundred twenty-eight (1332 abnormal, 1096 normal) esophagoscopic images from 746 patients were collected to set up a novel DNN-CAD system in two centers, and a validation dataset containing 187 images from 52 patients was prepared. Only standard WLIs were used to train the model. The DNN-CAD model can detect 91.4% of early ESCC, which is higher than that of advanced endoscopists.

Ohmori *et al*[23] evaluated ME and non-ME images [including WLI and NBI/Blue Laser Imaging (BLI)] using Single-Shot Multibox Detector (SSD) based CNN to identify ESCC. The ACC of ME, non-ME+WLI, and non-ME+NBI/BLI was high SEN and moderate SPE, respectively, 77%, 81%, and 77%. It was proved in the study that no significant difference existed in diagnostic performance between AI and experienced endoscopists.

Endocytoscopic diagnosis

Zhao *et al*[24] conducted another CAD model to evaluate the feasibility of automated classification of IPCLs to improve the detection of ESCC. ME-NBI images were collected, and a double-labeled full convolutional network was developed for image segmentation. In the research, it was shown that the diagnostic ACC of senior observers was much higher than that of mid-level and junior observers. When this model distinguished the lesions A, B1, and B2 IPCL, the diagnostic ACC rates of the lesion and pixel level reached 89.2% and 93%, respectively, similar to the seniors. Specifically, the model was more sensitive to type A IPCL than clinicians (71.5% *vs* 28.2%-64.9%), which can avoid unnecessary radical treatment. A total of 7046

sequential HD ME-NBI images from 17 patients were used to train a CNN in another study conducted by Everson *et al*[25]. IPCL patterns were also classified according to the JES classification. This CNN differentiated abnormal from normal IPCL patterns with 93.7% ACC.

The endocytoscopic system (ECS) provides magnified endoscopy, which can be stained with methylene blue to observe surface epithelial cells[26,27]. The optical magnification of ECS is 500 ×, which can be increased to 900 × by using the digital magnification in the video processor[26]. Kumagai *et al*[27] used a CNN-based AI system based on GoogLeNet and trained using 4715 ECS images of the esophagus (1141 malignant and 3574 non-malignant images) to diagnose ESCC using ECS images. An independent set of 1520 images, collected from 55 consecutive patients (27 ESCCs and 28 benign esophageal lesions) were tested. The AI correctly diagnosed 25 of the 27 ESCC with an overall SEN of 92.6%, SPE of 89.3%, and an overall ACC of 90.9%.

Depth of invasion diagnosis of ESCC

The study conducted by Nakagawa *et al*[28] did not determine the IPCL pattern but aimed to predict the depth of cancer invasion by using a DL system based on the CNN-SSD architecture. For all, non-ME and ME images, the system's ability to distinguish correctly pathologic mucosal/submucosal microinvasive (EP/SM1) cancers from submucosal deep invasive (SM2/3) cancers was 91%, 92.9%, and 89.7%, respectively, with comparable performance to experienced endoscopists.

In Tokai *et al*[29] research, AI's ability to measure the depth of ESCC invasion was demonstrated. They collected 1751 ESCC training images obtained by WLI and NBI from the Japan Cancer Institute Hospital and developed a CNN-SSD diagnosis system. The magnified images obtained *via* ME were excluded. Subsequently, 291 test images from 55 patients confirmed by pathological findings were used to compare the AI system with 13 board-certified endoscopists. The AI system detected 95.5% of the ESCC in the test images in 10 s and correctly estimated the ESCC infiltration depth in 6 s, with a SEN of 84.1% and an ACC of 80.9%. The ACC score of this system exceeded 12 of 13 endoscopists, and its AUC was greater than the AUC of all endoscopists. It was indicated that the AI system could potentially be used for ESCC diagnosis and measuring depth of invasion.

Real-time diagnosis of ESCC

Guo *et al*[30] developed a CAD system based on SegNet architecture that can perform real-time automatic diagnosis of precancerous lesions and early ESCC under non-ME and ME. Data from four medical centers in three countries including China, the United States, and India were collected and used for CAD model training. Four sets of data from early ESCC and noncancerous endoscopy including still images and real-time videos were used to verify their effects. The SEN and SPE of the system were 98.04% and 95.03%, respectively, for still images. In 27 non-magnifying videos, the per-frame and the per-lesion SEN were 60.8% and 100%, respectively. In 20 magnifying videos, the per-frame and the per-lesion SEN were 96.1% and 100%, respectively. Unaltered full-range normal esophagus videos included 33 videos (per-frame SPE 99.9%, per-case SPE 90.9%).

In another multi-center, case-control, diagnostic study, Luo *et al*[31] developed and validated the Gastrointestinal Artificial Intelligence Diagnostic System (GRAIDS) for the diagnosis of upper gastrointestinal cancers through analysis of imaging data from clinical endoscopies. Six hospitals of different levels with varying experience in the endoscopic diagnosis of upper gastrointestinal cancer in China participated in the study. The GRAIDS algorithm was based on the concept of DeepLab's V3+ [released by Google (Mountain View, CA, United States) in 2018]. In total, 1036496 endoscopic images from 84424 individuals were used to develop and test GRAIDS. GRAIDS achieved a diagnostic SEN similar to that of expert endoscopists {0.942 [95% confidence interval (CI): 0.924-0.957] *vs* 0.945 (0.927-0.959); $P = 0.692$}. The SEN was higher than that of qualified endoscopists [0.858 (0.832-0.880), $P < 0.0001$] and trainees [0.722 (0.691-0.752), $P < 0.0001$]. In this big data study, the number of EC in the training set and verification set reached about 27% of the total, but the proportion of early EC and the detection effect were not explained. More detailed data on early EC are to be expected.

The latest research results on real-time detection of early EC are from Fukuda *et al*[32]. They used 23746 images of 1544 cases of superficial ESCC confirmed by pathology and 4587 images of 458 cases of non-cancerous and normal tissues to construct a deep CNN-SSD model. NBI or BLI technology was used to collect 5-9 s video clips of 144 patients as a verification data set. The SEN, SPE, and ACC for the AI were 86%, 89%, and 88%, respectively, and 74%, 76%, and 75% for the experts,

respectively. This is an important study that paves the way for the development of better models to detect EC lesions in a real-time clinical environment, but randomized prospective clinical trials are needed for verification.

Another research team from Japan developed an AI system to calculate ESCC invasion depth[33]. In total, 23977 images, including WLI and NBI/BLI images of pathologically proven ESCC from endoscopic videos and still images, were obtained as a learning dataset. An independent validation dataset of 102 video images of ESCC was taken in research. Two types of videos, non-ME with WLI and ME with NBI/BLI of 4-12s, were included. The CNN model was compared with 14 experts in endoscopic field. ACC, SEN, and SPE performance for AI were superior to those for experts in both types of videos. The AI model was concluded to be effective in real-time measurement of ESCC invasion depth.

AI IN ENDOSCOPIC DETECTION OF BE AND EAC

BE is the precursor of EAC. The prevalence of EAC is rising rapidly, and the prognosis of advanced cases is poor. However, early detection of cases can be promptly treated with endoscopic treatment, which has a chance of cure. Due to the difficulty in detecting early neoplasms under endoscopy, early lesions are often missed[34]. This is because early Barrett tumors are usually flat, with only subtle changes in the color and texture of the mucosa. These changes can be visible in the HD-WLI performed by experts. However, due to the low progression rate of tumor formation (< 1% per patient year), many general-level endoscopists rarely encounter the early formation of Barrett's tumor, so that they are not familiar with its endoscopic morphology and cannot recognize the lesions[35]. In order to improve EAC screening and increase the SEN and speed of the examination, AI-assisted endoscopy has important value. In addition, it can also provide convenience for endoscopists facing challenges and anxiety, so as not to miss early lesions (Table 2).

EAC diagnosis

In 2013, van der Sommen *et al*[36] first proposed a SVM algorithm for detecting early cancer in the EAC using HD endoscopic images, color histograms ,and simple statistical data of Gabor features, with a classification ACC of 95.9% and an AUC of 0.992. Later, they found that the system detected 36 from 38 lesions with a recall of 0.95 and a precision of 0.75 through clinical validation. In 2016, van der Sommen *et al*[37] tested a computer algorithm (automated image recognition system) that employed specific texture, color filters, and ML for detecting early neoplastic lesions on BE based on specific imaging details. All images were of high perceptual quality. The algorithm was developed and tested using 100 endoscopic images of 44 BE patients. Early tumor lesions were identified in the per-image analysis, with a SEN and SPE of 0.83. At the patient level, the SEN and SPE of the system were 0.86 and 0.87, respectively.

Swager *et al*[38] conducted the first study based on volume laser endoscopy (VLE) images with direct histological correlation in 2017, which developed a clinically inspiring computer algorithm for BE neoplasia[39]. VLE is an advanced imaging system that can scan the esophagus wall up to 3 mm deep with near-microscopic resolution. A total of 60 VLE images from a high-quality *ex vivo* VLE histological correlation database were used in the study, including 30 nondysplastic BE and 30 HGD/early EAC images. The algorithm of the feature "layering and signal decay statistics" showed good performance in detecting BE neoplasia (AUC = 0.95), and thus it might possible to assist endoscopists in detecting early neoplasia on VLE. van der Sommen *et al*[40] also compared the performance of the CAD methods with the classification ACC of two VLE experts, with a maximum AUC in the range of 0.90-0.93 for the ML method *vs* an AUC of 0.81 for the medical experts.

Horie *et al*[21] proved that the diagnosis ACC for EAC of the CNN was 90% (19/21). Four lesions of EAC were missed, which may be because educational images for EAC were not enough. Only eight were used in the study. In another study about CNN, four methods including Regional-based CNN (R-CNN), Fast R-CNN, Faster R-CNN, and SSD were adapted to detect abnormal regions in the esophagus HD-WLE images automatically[41]. A total of 100 images from 39 patients that have been manually annotated as ground truth by five experienced clinicians were tested. The SSD outperformed other methods achieving SEN of 0.96, SPE of 0.92, and F-measure of 0.94. Hashimoto *et al*[42] conducted a pilot study on the endoscopic detection of early esophageal neoplasia on BE using a CNN system. They pre-trained the system on ImageNet, then received training on 1853 images, and finally tested it with 458 images.

Table 2 Artificial intelligence application for esophageal adenocarcinoma

AI Application	Study design	Data category	Type of Images	AI architecture	Training dataset	Validation Method or dataset	AUC	SEN	SPE	ACC	PPV	NPV	Compared with experts	Ref.
Detection	Retrospective	Still image	WLI	CAD-SVM	64 images	LOOCV	NA	95%	NA	75%	NA	NA	NA	van der Sommen *et al* [36], 2014
Detection	Retrospective	Still image	WLI	CAD-SVM	100 Images	LOOCV	NA	83% (per-image) 86% (per-patient)	83% (per-image) 87% (per-patient)	NA	NA	NA	Inferior	van der Sommen *et al* [37], 2016
Detection	Retrospective	Still image	VLE	CAD	60 images	LOOCV	0.95	90%	93%	NA	NA	NA	Superior	Swager *et al* [38], 2017
Detection	Retrospective	Still image	VLE	CAD	60 images	LOOCV	0.90-0.93	NA	NA	NA	NA	NA	Superior	van der Sommen *et al* [40], 2018
Diagnosis	Retrospective	Still image	WLI/NBI	CNN	8 patients	Caffe DL framework	NA	88% (WLI)/88% (NBI) (per-patient) 69% (WLI)/71% (NBI) (per-image)	NA	90%	NA	NA	NA	Horie *et al* [21], 2019
Detection	Retrospective	Still image	WLE	CNN-SSD	100 images/39patients	20% patients/5-fold-CV/LOOCV	NA	96%	92%	NA	NA	NA	NA	Ghatwary *et al*[41], 2019
Detection	Retrospective	Still image	WLI/NBI	CNN-Inception-ResNet-v2	1832 images	458 images	NA	96.4%	94.2%	95.4%	NA	NA	NA	Hashimoto *et al*[42], 2019
Detection	Retrospective	Still image	WLI	CAD	60 images	LOOCV	0.92	95%	85%	91.7%	NA	NA	NA	de Groof *et al*[43], 2019
Detection	Retrospective	Still image	WLI	CAD-ResNet-UNet	1544 images	4-fold-CV (internal validation)/160 images (external validation)	NA	87.6% (internal validation) 92.5% (external validation)	88.6% (internal validation) 82.5% (external validation)	88.2% (internal validation) 87.5% (external validation)	NA	NA	NA	de Groof *et al*[44], 2019
Diagnosis	Retrospective	Still image	WLI/NBI	CAD-ResNet	248 images	LOOCV	NA	97% (WLI)/94% (NBI) (Augsburg data)92% (MICCAI)	88% (WLI)/80% (NBI) (Augsburg data)100% (MICCAI)	NA	NA	NA	NA	Ebigbo *et al*[45], 2019
Diagnosis	Retrospective	Random images from real-time	WLI	CAD-ResNet-/DeepLab V.3+	129 images	36 images (real time)	NA	83.7%	100%	89.9%	NA	NA	NA	Ebigbo *et al*[49], 2020

		video												
Surveillance	Prospective	Real-time image	WLI/NBI/VLE	IRIS	NA	Real-time image	NA	NA	NA	NA	NA	NA	NA	Trindade *et al*[50], 2019
Detection	Prospective	Live endoscopic procedure	Live endoscopic procedure	CAD-ResNet/U-Net	1544 images	48 levels/144 images/20 live endoscopic procedure	NA	90.9% (per level) 75.8% (per image) 90% (per patient)	89.2% (per level) 86.5% (per image) 90% (per patient)	89.6% (per level) 84.0% (per image) 90% (per patient)	NA	NA	NA	de Groof *et al*[51], 2020

AI: Artificial intelligence; AUC: Area under the curve; SEN: Sensitivity; SPE: Specificity; ACC: Accuracy; PPV: Positive predictive value; NPV: Negative predictive value; WLI: White light imaging; CAD: Computer-aided diagnosis; SVM: Support vector machines; LOOCV: Leave-one-out cross-validation; NA: Not available; VLE: Volume laser endoscopy; NBI: Narrow-band imaging; CNN: Convolutional neural network; DL: Deep learning; SSD: Single-Shot Multibox Detector; n-fold-CV: n-fold cross-validation; MICCAI: Medical Image Computing and Computer Assisted-Intervention; IRIS: Intelligent real-time image segmentation.

The size of the tumor lesions in the image data they selected was between 3 mm and 20 mm, and most of the lesions were not significant, so that endoscopists with less training were likely to miss the diagnosis. The CNN accurately detected 95.4% of early lesions, including 96.4% of SEN and 94.2% of SPE, and the running speed of the algorithm was allowed for real-time implementation.

Progress on CAD development was made by de Groof *et al*[43] for BE detection. All WLIs recorded in 40 cases of neoplastic Barrett lesions and 20 cases of non-dysplastic BE were collected through WLE in full HD format (1280 × 1024 pixels). Experts delineated the images of neoplasm. The CAD system had received training in color and texture features. The ACC, SEN, and SPE for detection were 92%, 95%, and 85%, respectively, which were evaluated by leave-one-out cross-validation. In the randomized controlled trial (No: NTR7072.) reported in 2019, de Groof *et al*[44] developed a hybrid ResNet-UNet model CAD system using five independent endoscopy data sets. Compared with general endoscopists, the ACC of the CAD system was 88% *vs* 73%, the SEN was 93% *vs* 72%, and the SPE was 83% *vs* 74%. This report details the first externally validated DL-CAD system for early BE neoplasia.

In the study of Ebigbo *et al*[45], two databases [Augsburg data and the 'Medical Image Computing and Computer Assisted-Intervention' (MICCAI) data] were used to train and test a CAD system on the basis of a CNN with a residual net (ResNet) architecture. Based on still images, the diagnosis of EAC by CAD-DL reached SEN/SPE of 97%/88% (Augsburg data) and 92%/100% (MICCAI data) for WLIs and 94%/80% for NBIs (Augsburg data), respectively.

Depth of invasion diagnosis of EAC

Several studies questioned whether surgery should always be used to treat EAC with deep submucosal invasion. However, it is accepted that the depth of invasion is at least one of the biggest risk factors for metastasis[46]. In the searching item related to the word "depth of invasion/ infiltration of EAC", no article published was found. Fortunately, research on AI's infiltration depth measurement of gastric cancer and

colon cancer has been published, which may be worth learning for research of invasion depth of EAC. In the study of Zhu *et al*[47], a total of 790 WLIs served as a development dataset and another 203 images as a test dataset, and a CNN-CAD system, developed from ResNet50, was used to determine the invasion depth of gastric cancer. At a threshold value of 0.5, the SEN was 76.47%, SPE was 95.56%, and the overall ACC was 89.16%. The AI system achieved significantly higher ACC than human endoscopists. Scholars from Japan analyzed endocytoscopic images by using CAD-SVM, and the algorithm obtained after extracting 5543 images from 238 lesions can diagnose invasive colorectal cancer in 200 test images with an ACC of 94.1%[48].

Real time diagnosis of EAC

Ebigbo *et al*[49] developed a real-time DL system based on deep CNN and ResNet architecture with the latest encoder/decoder network DeepLab V.3+. The endoscopy was performed by an expert endoscopist. The system captured random images from real-time camera livestream and provided global prediction (classification) and dense prediction (segmentation), which can accurately distinguish between normal BE and early EAC. The ACC of the AI system for 14 cases of early EAC was 89.9%. The AI-based CAD was indicated to improve the quality of BE assessment, especially for non-expert endoscopists.

In 2019, Trindade *et al*[50] reported an AI software termed intelligent real-time image segmentation for endoscopic surveillance of BE. The intelligent real-time image segmentation identified three established VLE features previously associated with histologic dysplasia and displayed them using different color schemes superimposed over the VLE image. A multi-center randomized controlled trial (NCT03814824) is underway to validate further the AI system. In another study, de Groof *et al*[51] evaluated the preliminary diagnostic ACC of a CAD system in detecting Barrett's neoplasm during live endoscopic procedures. The test dataset composed of the live endoscopy procedure of 10 patients with nondysplastic Barrett's esophagus and 10 patients with confirmed Barrett's neoplasm. WLIs at every 2 cm level of the Barrett's segment were obtained and analyzed. The ACC, SEN, and SPE of the CAD system were 90%, 91%, and 89%, respectively. This result revealed that the CAD system was ready to be tested in larger multi-center trials.

LIMITATION OF AI APPLICATION IN EARLY EC DETECTION

AI is becoming rapidly integrated into modern gastroenterology with the expansion of diagnostic images and treatments. Among the three common tumor diseases of the digestive tract, esophageal cancer, gastric cancer, and colon cancer, the value of AI has been fully demonstrated. Multiple AI algorithms have been developed to run real-time during gastroscopy and colonoscopy for cancerous detection, diagnosis, or invasion depth measurement[48,52,53]. The application of AI in the field of gastric cancer and colon cancer seems to be earlier and more comprehensive than that in the field of esophageal cancer.

It can be seen from the above review that the clinical application of AI in early EC is still in its infancy. The vast majority of studies are retrospective studies, and only two clinical trials have been reported, one of which is still in research progress. The above-mentioned research mainly focused on AI algorithms, training and verification and compared with the detection ACC, SEN, and SPE of different levels of endoscopists. There has not been a large-scale clinical application of AI, whether for surveillance or detection. It is foreseeable that the difficulties in applying AI to clinical practice are manifold, and there are still many obstacles before large-scale implementation.

First of all, the current AI applications mainly focus on files such as images, pictures, and videos. The establishment of an AI model requires a sufficiently large training set labeled by experts for AI learning. This training set requires the participation of a number of expert-level staff, and experts are obviously scarce resources, which makes it difficult for the quantity and quality of the training set to meet sufficient demand. As natural persons, experts are also likely to make mistakes. This may lead to incorrect diagnostic information in the training set. Secondly, a large amount of medical imaging data, especially endoscopy data related to EC, need to be obtained during manual examination. The quality of the data depends not only on the level of the endoscopist but also on the function of the endoscopic instrument. Not all endoscopists can complete a qualified endoscopy, and not all endoscopy equipment can provide the best images. Most medical units can only use their own data, which can be considered a selection bias. AI must be applied to external multi-centers for

validation to explore its ACC. At present, only a multi-center study from China had conducted external verification[31]. Third, the subject of AI use in early EC detection is currently mainly focused on retrospective research. It is difficult to use AI technology for prospective clinical research, especially blinded randomized controlled trials, although this type of research is the most reliable "gold standard" in medical science. To conduct such a trial, it requires medical ethics approval. Fourth, the current generation of AI is numerous but proprietary. Costs including equipment and person training may become a limiting factor for widespread implementation. Fifth, the acceptance and approval of AI by governmental and professional organizations are needed. For each country, any legal issues related to the use of AI in endoscopes must be resolved before widespread implementation.

FUTURE OF AI APPLICATION IN EARLY EC DETECTION

Papers on AI in the diagnosis of early EC are gradually increasing, but except for a few, they basically discuss AI algorithms and clinical validation. The training data and verification data used are also still images. In the study on EAC , the authors also used still images for real-time analysis during endoscopy[49], which may be useful for evaluating EAC and other situations because the area of BE is limited. It is more useful to use real-time video for detection, because the detection can be performed immediately without the need to capture pictures frequently. In order to investigate whether AI is useful for preventing missed cancerous diagnosis, validation videos should not be limited to slow observation videos but include fast videos. However, this high accuracy comes from high-resolution videos, and it is not easy to provide accurate and high-quality images in videos containing low-resolution. As a guarantee of computing speed, the improvement of graphics processing unit and other hardware needs to be synchronized.

Existing papers discuss the still images of the esophageal surface and the depth of invasion, indicating that endoscopic equipment can not only improve the image quality but also become three-dimensional. When combined with other sensing technologies, it is eager to perform three-dimensional mapping of esophageal lesions through an endoscope. If AI technology is combined with three-dimensional endoscopy, what will happen in the future? There is no ending.

The AI system is expected to become a qualified assistant, but not a substitute, for inexperienced endoscopists to get similar judgment capabilities as experts. In order to establish an AI training database applicable to the whole world, it is necessary to establish a gold standard for AI diagnosis. This requires endoscopy experts from different countries and regions to label accurately and supplement the database.

Even though AI is helpful for diagnosis during endoscopy, it is temporarily difficult for AI to perform endoscopy operations on behalf of humans because the shape of the digestive tract varies from person to person. Therefore, for endoscopists, efforts to improve the technology used to operate endoscopes will continue to be indispensable.

CONCLUSION

This review summarizes the current status of research and development of AI in endoscopes related to early EC detection and the application prospects of AI. The use of AI-based endoscopes will enable detection accuracy of early EC and improve prognosis. As the diagnosis ability and accuracy of early EC vary greatly among endoscopists, AI may have better diagnostic capabilities and will help endoscopists to reduce missed diagnosis of early EC in the near future. Maybe only a step is needed to move for AI application in such a clinical field, but the step needs efforts from multiple fields including medicine, AI, optics, legislation, ethics, *etc*. We might as well look forward to this step as soon as possible.

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MINIREVIEWS

COVID-19 and the gastrointestinal tract: Source of infection or merely a target of the inflammatory process following SARS-CoV-2 infection?

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Abstract

Gastrointestinal (GI) symptoms have been described in a conspicuous percentage of coronavirus disease 2019 (COVID-19) patients. This clinical evidence is supported by the detection of viral RNA in stool, which also supports the hypothesis of a possible fecal-oral transmission route. The involvement of GI tract in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is corroborated by the theoretical assumption that angiotensin converting enzyme 2, which is a SARS-CoV-2 target receptor, is present along the GI tract. Studies have pointed out that gut dysbiosis may occur in COVID-19 patients, with a possible correlation with disease severity and with complications such as multisystem inflammatory syndrome in children. However, the question to be addressed is whether dysbiosis is a consequence or a contributing cause of SARS-CoV-2 infection. In such a scenario, pharmacological therapies aimed at decreasing GI permeability may be beneficial for COVID-19 patients. Considering the possibility of a fecal-oral transmission route, water and environmental sanitation play a

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crucial role for COVID-19 containment, especially in developing countries.

Key Words: COVID-19; SARS-CoV-2; Gastrointestinal symptoms; Gut microbiome; Dysbiosis; Zonulin; Fecal-oral transmission

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Core Tip: Coronavirus disease 2019 (COVID-19) patients may suffer from gastrointestinal symptoms that are associated with gastrointestinal dysbiosis. Even though the exact role of gut microbiome perturbation as a either a cause or a consequence of the disease is still to be elucidated, pharmacological interventions aimed at containing intestinal permeability may be of support in COVID-19 patients.

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INTRODUCTION

In December 2019, in Wuhan, one of the largest cities in the Chinese province of Hubei, a new coronavirus appeared that was responsible for a pneumonia epidemic that was named by the World Health Organization (WHO) on February 11, 2020 as coronavirus disease 2019 (COVID-19), and declared as a dangerous threat to public health and an international health emergency[1,2]. Indeed, COVID-19 infections rapidly spread and was declared a pandemic by the WHO one month later on March 11, 2020. In a short time, the pandemic involved all continents, and at the time of writing this review, accounts for more than 100 million confirmed cases worldwide, also becoming a serious threat to the global economy[3,4].

At the beginning of January 2020, the new coronavirus responsible for this pandemic was named "severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)" by the International Committee on Taxonomy of Viruses[5]. It is a single-stranded RNA virus with positive polarity and is closely related to the B-line of beta-coronaviruses, which are typically responsible for severe and lethal respiratory syndromes[2,6]. Phylogenetic analysis showed that the viral sequence of this new coronavirus has a strong similarity (89%) with two other coronaviruses similar to SARS and deriving from bats, namely bat-SL-CoVZC45 (GenBank access n. MG772933.1) and bat-SL-CoVZXC21 (GenBank access n. MG772934.1). Similarities were also found with SARS-CoV, the virus that caused the SARS pandemic in 2002/2003, and the Middle East Respiratory Syndrome (MERS-CoV) pandemic of 2012, with which SARS-CoV-2, shares sequence homologies (79% with SARS-CoV and 50% with MERS-CoV)[7-9] as well as zoonotic transmission and several clinical features.

MECHANISM OF INFECTION

Studies carried out so far suggest that the human angiotensin converting enzyme 2 (ACE2) is the main target receptor for the virus to gain access into human host cells. Binding occurs between the ACE2 binding domain located at the C-terminal end of the viral spike protein and the ACE2 target receptor located on the surface of the host cell membrane[10]. After binding to ACE2 receptors on human cells[11], the SARS-CoV-2 spike protein is cleaved by two mucosa-specific serine proteases, namely TMPRSS2 and TMPRSS4, to facilitate viral cellular infection[12].

Tissues with the highest expression of ACE2, and therefore a high sensitivity to infection are present in both the respiratory and the gastrointestinal (GI) tracts. The receptor is present in the pulmonary epithelium, in type 2 pneumocytes that are present in the alveoli where the exchange between oxygen and carbon dioxide takes

place, in the nose, mouth, stratified epithelial cells in the upper esophagus, and absorbent enterocytes of the ileum and colon. Other receptors that are known to allow entry of SARS-CoV-2 are DC-SIGN (CD209), CD147 and L-SIGN (CD209L)[13-15]. Transmission of the virus can take place through direct contact with respiratory droplets from infected subjects or indirectly from contaminated objects and surfaces[16].

CLINICAL SYMPTOMS

Clinical manifestations associated with COVID-19 infection range from complete absence of symptoms (asymptomatic subjects) to serious and even fatal infections. In most cases, the disease is mild to moderate, so that symptoms can be managed safely at home without the need for hospitalization. The main target of the infection is the respiratory tract, and therefore, the clinical manifestations associated with the disease are mostly respiratory. The most common symptoms are similar to those of seasonal flu and include fever, dry cough, dyspnea, sore throat, headache, muscle aches, and asthenia. Severe respiratory distress, chest pain, pneumonia, and pulmonary insufficiency can also be found in severe cases[17-19]. In SARS-CoV-2 pneumonia, lung computed tomography scans shows bilateral, subpleural ground-glass opacity lesions[20-22]. In addition to pulmonary clinical manifestations, heart damage can also occur in infected patients. Shi *et al*[23] in a study conducted in Wuhan on 400 patients, found that about one-fifth experienced heart disease as a risk factor for worse outcome. Heart damage essentially consists of sudden and severe myocardial inflammation leading to heart failure and arrhythmias[24]. For this reason, hypertensive patients, and in general all individuals with a positive history of cardiovascular disease, are more at risk of experiencing a poor prognosis than other patients[25]. SARS-CoV-2 can also damage the central nervous system, with symptoms that range from mild (anosmia, dysgeusia) to severe (viral encephalitis). Moreover, COVID-19 infection, like other pulmonary infectious diseases, may represent a risk factor for acute cerebrovascular events. The observed D-dimer elevation in COVID-19 patients may indicate additional risk of cerebrovascular events[26]. Kidney and liver damage have also been reported. Proteinuria, hematuria, increased aspartate transaminase (AST, also known as aspartate aminotransferase or glutamic oxaloacetic transaminase), alanine aminotransferase (ALT, also known as alanine transaminase or glutamic-pyruvic transaminase), and bilirubin have been found in about half of infected patients[27,28].

The virulence of microorganisms determines the damage to host cells before the microorganisms are cleared by host immunity[29]. Yet, the virulence of the infective microorganism does not fully account for the virulence of the disease, the majority of which results from inflammation produced by the immune response[29]. That has been demonstrated for many infective diseases and is particularly true for SARS-CoV-2. Most viral and bacterial infections in humans are self-limiting[29,30], and infecting microorganisms become commensal if they can coexist with the human host. The SARS-CoV-2 virus infection is no different[31], and that could explain why most COVID-19 cases are asymptomatic or mild.

COVID-19 AND THE GI TRACT

Although, as reported, SARS-CoV-2 infection mainly affects the respiratory system[32], leading to breathing difficulties, dry cough, and nasal congestion to respiratory failure, this novel coronavirus can be found in the GI tract as well[33]. Furthermore, SARS-CoV-2 RNA has been isolated from stools. Viral nucleocapsid protein can be observed in duodenal and rectal glandular epithelial cells by laser scanning confocal microscopy. The available results provide evidence of the activity of this virus in the GI tract[34].

GI SYMPTOMATOLOGY

GI symptoms are present in approximately 50% of COVID-19 patients, consisting mainly of diarrhea, nausea, vomiting, and abdominal discomfort[35]. Moreover, several studies have shown that SARS-CoV-2 is able to interact with ACE2 receptors on ileal enterocytes and colon epithelial cells, so that a trophism for the GI tract has been hypothesized as well[27,36]. Further evidence supporting the possibility that both the

small and large intestines may be susceptible to SARS-CoV-2 infection, was the isolation of viral RNA from the GI epithelium and staining of the viral nucleocapsid protein in these cells[34]. In addition, viral RNA was also isolated in stool samples from COVID-19 patients, and it was presumed that the viral components originated from infected enterocytes[34,36]. Considering the overall evidence, several reports have expressed concerns of possible fecal-oral transmission of SARS-CoV-2[37,38]. Nevertheless, the findings were not unexpected as other well-known coronaviruses, including those causing the MERS and SARS epidemics, have shown to infect enterocytes, leading to several GI symptoms[39].

Several reports have focused on the GI symptomatology in COVID-19 patients, underlining clinical outcomes that varied widely in terms of frequency. Nine of those studies that included a total of 4177 patients found that the incidence of GI symptoms varied from 4.9% to 61.3%. The most frequent symptoms were diarrhea (8.4%) and lack of appetite (7.4%); 4.1% of patients complained about nausea-related disorders, while vomiting was reported in 2.4% and abdominal pain in 1.7% of the cases[40-48]. Interestingly, the frequency of diarrhea, nausea, and abdominal pain in COVID-19 patients was shown to be higher among women than men[49]. Finally, Yang *et al*[50] examined 50 COVID-19 patients by dividing them in two groups by the presence of either initial pulmonary or initial GI symptoms. They found that patients with GI manifestations as initial symptoms had more severe clinical outcomes, leading to longer hospitalization.

As stated above, and similar to SARS-CoV-1, SARS-CoV-2 infection is mediated by ACE2, which allows the virus to enter the cells and replicate by taking advantage of the host synthesis apparatus[51]. A recent study has shown that the binding affinity of the spike protein of SARS-CoV-2 is about 10-20 times higher than that of SARS-CoV-1[52]. ACE2 receptors are widely expressed in the epithelia of both the respiratory and GI tracts. A recent multiomic *in vitro* study has shown that expression of these membrane enzymes is higher in intestinal cell lines than lung cell lines[36].

The pathophysiology of GI symptoms is not entirely clear, but it seems that they originate as a result of several phenomena. ACE2 performs an essential function in the intestine by regulating amino acid homeostasis and microbiome balance[53]. It is possible that the binding of SARS-CoV-2 to these membrane receptors reduces their availability, giving rise to an alteration of physiological function that induces a dysbiosis that leads to diarrhea, one of the most frequent symptoms reported during SARS-CoV2 infection[54]. The development of a cytokine storm could be indeed the cause of damage to the GI tract. Autopsies of SARS patients found increased expression of proinflammatory cytokines in ACE2-expressing cells compared with those that do not express this enzyme on their membranes[55]. It is fair to think that this happens following SARS-CoV-2 infection. Inflammation could also stimulate the gut microbiota to release molecules that increase the inflammatory state of the intestine and subsequently spread from the intestine into the circulatory system to cause systemic damage with more severe consequences than the viral infection itself[56]. In addition to locally occurring phenomena, the gut-lung axis seems to have a pivotal role in the development of GI symptoms as well. This axis represents a link between two organs that share a common mucous immune system[57]. Its influence has been hypothesized as a result of studies that have described COVID-19 patients with GI symptoms but without SARS-CoV-2 RNA-positive stool samples[54].

The impact of GI comorbidities on the development of symptoms in COVID-19 patients is yet to be defined. While the relationship between disorders such as diabetes, cardiovascular diseases, and hypertension and COVID-19-related severity was proven by several studies[58]. There are not yet sufficient studies to demonstrate a causal link between the presence of previous GI conditions and the onset of COVID-19-related symptomatology.

Indeed, existing studies do not provide indisputable results. A study carried out in China found that pre-existing GI disorders represent a third comorbidity in COVID-19 patients[59]. Another study carried out in Wuhan reported a relatively high percentage of COVID-19 patients with GI disorders[60]. Conversely, other studies carried out in the United States[61] and in Italy[62] have not found a significant correlation between SARS-CoV-2 infection and the presence of inflammatory bowel disease. On the contrary, Papa *et al*[63] observed a better clinical outcome in patients presenting with digestive comorbidities, as presumably they do not hinder the immune response against SARS-CoV-2. However, the authors point out that the number of cases observed was small[63].

FECAL-ORAL TRANSMISSION OF SARS-COV-2

A potential fecal-oral transmission route of SARS-CoV-2, in addition to respiratory droplets, person-to-person contact, or indirect infection following contact with contaminated surfaces, has been proposed by several authors[37,38,64,65]. The presence of common GI symptoms in the COVID-19 patients mentioned above is evidence that the GI tract is involved in SARS-CoV-2 infection.

Viral RNA has been detected in esophagus, stomach, duodenum, and rectum tissue from patients with severe SARS-CoV-2 infections[66]. Notably, the first COVID-19 patient in the United States tested positive for SARS-CoV-2 in loose stool specimens by real-time reverse transcription polymerase chain reaction[67]. Several studies report detection of viral RNA in stool samples in adults[34,68-72] and children[73-75] diagnosed with COVID-19. Interestingly, two studies confirmed that SARS-CoV-2 was detectable in fecal samples even after negative testing of respiratory swabs[70,72], and in patients who did not present GI symptoms[66,69,71]. In addition to SARS-CoV-2 RNA detection in stool samples, positive staining of viral nucleocapsid protein in gastric, duodenal, and rectal epithelia might demonstrate the infection of GI glandular epithelial cells[34]. Wang *et al*[68] found a high content of viral RNA in fecal specimens from COVID-19 patients, and electron microscopy confirmed the presence of living viral particles in those samples. Wölfel *et al*[69] could not isolate living SARS-CoV-2 from stool samples despite the presence of high concentrations of viral RNA, but did detect cells in a few stools that contained subgenomic mRNA, which suggests active replication of the virus in the intestinal tract. The presence of viral subgenomic mRNA indicates actively infected cells because such mRNA is transcribed only in infected cells and is not found packaged into virions[69]. As in adults, SARS-CoV-2 in children might persist in the GI tract longer than in the respiratory system. Actually, a high percentage of children diagnosed of COVID-19 tested positive on rectal or anal swabs, and some were positive after nasopharyngeal or throat swabs became negative[73-75].

Other evidence that supports an alternative fecal-oral transmission of COVID-19 is the expression of SARS-CoV-2 entry-genes in the cells of intestinal tissues. Mucosal cells of the lower intestine can be infected by other coronaviruses, resulting in diarrhea and other enteric symptoms. In this case, SARS-CoV-2 could be carried by saliva and secretions into the digestive tract, where it would infect the ACE2-expressing enterocytes. It has been confirmed that SARS-CoV-2 can infect enterocytes present in human small intestine organoid models, which supports virus replication and a GI route of infection[76]. However, another study demonstrated that human GI secretions were able to inactivate SARS-CoV-2, which suggests that although virus infection and pathogenesis is enhanced in the intestine, it is not clear if it would be a primary site of infection and possible route of transmission[77]. An alternative route of transmission that acts together with the respiratory route might explain the rapid spread of disease.

GUT MICROBIOME ROLE IN COVID-19 SEVERITY

COVID-19 symptomatology is strictly related to both a physiological and an aberrant activation of immune system, leading in very severe cases to a cytokine storm[78]. Based on this premise, it is not surprising that the gut microbiome, which is closely linked to the immune status of the host, may play a role in COVID-19 severity. A gut microbiome imbalance has been associated in COVID-19 patients with elevated concentrations of inflammatory cytokines, and blood markers, including C-reactive protein (CRP), lactate dehydrogenase, and aspartate aminotransferase[79]. Yeoh *et al*[79] examined blood and fecal samples from 100 young (mean age of about 36 years) confirmed positive COVID-19 patients in two Hong Kong hospitals. They also analyzed samples from patients for up to 30 days after clearance of SARS-CoV-2. All patients had slight or moderate symptomatology, with only 5% with severe and 3% with critical conditions. Compared with a group of healthy adult controls, COVID-19 patients had higher fecal populations of *Ruminococcus gnavus*, *Ruminococcus torques*, and *Bacteroides dorei* species. This imbalance seemed not to depend on the pharmacological therapy administered. The same investigators reported a stratification of gut microbiota composition associated with disease severity in hospitalized COVID-19 patients. Cytokine (IL-10 and tumor necrosis factor-), enzyme (AST, -glutamyl transferase, lactate dehydrogenase), CRP, N-terminal proB-type natriuretic peptide levels, and erythrocyte sedimentation rate were previously reported as associated with increased COVID-19-related symptomatology[80-82]. Those parameters were also significantly associated with microbiota composition in COVID-19 patients.

Remarkably, the levels of those molecules increased in parallel with changes in the microbiota composition, representing more severe disease states[79]. Overall, the results suggest that the gut microbiota composition might be linked to the extent of the immune response to COVID-19 and subsequent tissue damage, thus playing a role in regulating disease severity. The persistence of gut microbiota dysbiosis even 30 days after clearance of SARS-CoV-2 might also be linked to the persistence of COVID-19 related symptoms, such as fatigue, dyspnea, and joint pain.

Moreover, Yu[83] suggested that nutritional disorders impair immunity, causing hyperinflammation and leading to an overload of cytokines in COVID-19 patients. Autophagy induced by restrictive eating could be an efficient strategy to prevent COVID-19-related symptomatology. Indeed, it inhibits overgrowth of the microbiota and partially reduces excessive nutrition for replication. Autophagy also attenuates inflammation. On that basis, Yu[83] recommended restoration of good nutritional status together with restriction of food intake, especially in older subjects, to reduce the risk of developing severe symptomatology if infected by COVID-19.

On the other hand, as inflammation can be considered as a physiological response of the immune system to damaged tissues[84], it is a protective reaction to remove the injurious stimuli and to initiate the healing of damaged tissues[84]. Nutrition excess slows tissue healing, indeed nutrition derived from the degradation of the damaged tissue coupled with excess nutrition intake will be mostly turned into lipid intermediates and deposited in nonadipose tissue, lipotoxicity and further tissue damage. Thus, in a state of overnutrition, inflammation can become chronic. In this regard, undernutrition might be beneficial in fighting viral infection, as anorexia nervosa patients seem to be free of common viral infections[85,86].

ROLE OF THE GI TRACT IN COVID-19 COMPLICATIONS

Multisystem inflammatory syndrome in children (MIS-C) is defined by the Centers for Disease Control and Prevention[87] as a severe illness requiring hospitalization that occurs in individuals < 21 years of age with evidence of current or recent SARS-CoV-2 infection, or recent exposure to an individual with COVID-19. Children with MIS-C have fever, laboratory evidence of inflammation, and multiorgan involvement without an alternative plausible diagnosis. Ninety-two percent of children with MIS-C report GI symptoms[88] and 80% develop cardiac pathology. MIS-C is characterized by a superantigen-mediated hyperinflammatory response with a unique T-cell profile and multiple autoantigens[89-91]. It has been recently shown that the spike protein of SARS-CoV-2 contains sequence and structural motifs similar to a *Staphylococcal Enterotoxin B* superantigen[92], and superantigen-induced skewing of the T-cell repertoire in MIS-C patients with expansion of the T-cell receptor beta variable gene correlates with the cytokine storm and hyperinflammation seen in MIS-C[91]. Inflamed expansion of antibodies for multiple non-COVID-19 pathogens, including common coronaviruses, influenza and respiratory syncytial virus[93] plus numerous self-antigens[89,90] also correlates with the severe cardiac complications of MIS-C. Immune complexes stimulate monocytes/macrophages, eliciting phagocytosis, macrophage activation and cytokine storm[94,95] (Figure 1). The instigating factor for MIS-C has not been elucidated, but prominent GI symptoms suggest an intestinal source. The upper or lower respiratory tract are unlikely sources of antigens triggering this hyperinflammatory response, given the relative lack of respiratory symptoms in MIS-C, low/undetectable viral load in respiratory secretions[93], and the 2-4 wk delay between initial infection/exposure and development of MIS-C. Rather, ongoing antigen exposure is much more likely to be from the GI tract as has been shown in Kawasaki disease (KD), a similar illness with a hyperinflammatory profile leading to fever, rash, elevated inflammatory markers resulting in vasculitis, coronary aneurysms, and cardiac pathology. In KD, increased intestinal permeability secondary to upregulation of zonulin, a molecule that regulates paracellular molecule trafficking, has been associated with increased circulating immunoglobulin A and cardiac complications in children with KD[96].

PHARMACOLOGICAL IMPLICATIONS OF FECAL-ORAL TRANSMISSION

A study by Yeoh *et al*[79] was not designed to determine whether dysbiosis is the consequence of patient immune response rather than a contributing cause in disease onset and severity. Nevertheless, the reported association seems to be sufficiently

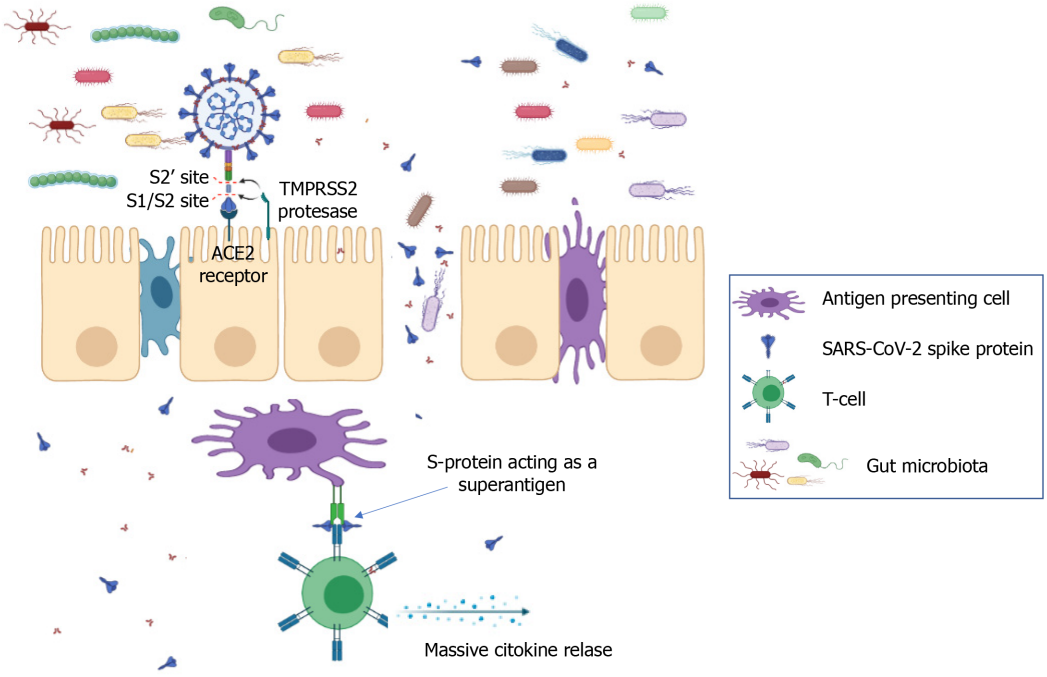


Figure 1 Gut dysbiosis induced by the immune response to severe acute respiratory syndrome coronavirus 2 in the gastrointestinal tract increases gut permeability, which in turn increases spike protein trafficking. Spike protein was reported to act as a superantigen that links the T-cell receptor and major histocompatibility complex II on antigen-presenting cells. ACE2: Angiotensin converting enzyme 2; S-protein: Spike protein; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

robust to support the benefit of pharmacological intervention aimed at decreasing gut permeability for COVID-19 GI symptoms management, and potentially to reduce the risk of its complications, including MIS-C. Such a strategy could both reduce GI tract inflammation and help accelerate naturally occurring dysbiosis correction. Larazotide acetate, a synthetic octapeptide designed as a specific zonulin receptor antagonist, was successfully and safely used to modulate gut permeability and the related inflammation[97,98]. Di Micco *et al*[99] recently proposed an additional role for larazotide in COVID-19 patients. Based on an in-silico evaluation, they reported the ability of larazotide to inhibit the N3 protease of SARS-CoV-2. As larazotide was specifically designed as an oral drug able to reach the lower GI tract, it might be useful both for its direct anti-SARS-CoV-2 effect and to prevent antigen trafficking of SARS-CoV-2-derived molecules, including spike protein from the gut lumen to the bloodstream, which could trigger a cytokine storm leading to MIS-C.

CONCLUSION

In summary, whether digestive symptoms are a direct response to SARS-CoV-2 infection of the GI tract, or a secondary outcome of COVID-19, is still to be completely understood. The presence of viral RNA in the GI tract might partially explain GI symptoms even if it does not mean that there is infection in the intestine. In either case, robust evidence supports GI tract involvement in COVID-19 as well as a potential fecal-oral transmission route. The GI seems to be not only a reservoir of SARS-CoV-2, but also an active battlefield in which virus, innate and adaptive immune system responses, epithelial cells, and gut microbiota play critical roles. The interventions for managing GI-related issues should be twofold. From a patient perspective, pharmacological treatment to reduce the severity of GI symptoms and support a quick re-establishment of eubiosis should be evaluated. Moreover, interventions to ensure water sanitation should be added to the current policies for COVID-19 pandemic control. Considering that many people worldwide lack access to clean drinking water and good sanitation, the possible containment of COVID-19 through water treatment should be carefully taken into account to reduce the actual and future magnitude of the pandemic.

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ORIGINAL ARTICLE

Basic Study

Lipotoxic hepatocyte-derived exosomal miR-1297 promotes hepatic stellate cell activation through the PTEN signaling pathway in metabolic-associated fatty liver disease

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Abstract

BACKGROUND

Exosomes play an important role in metabolic-associated fatty liver disease (MAFLD), but the mechanism by which exosomes participate in MAFLD still remain unclear.

AIM

To figure out the function of lipotoxic exosomal miR-1297 in MAFLD.

METHODS

MicroRNA sequencing was used to detect differentially expressed miRNAs (DE-miR) in lipotoxic exosomes derived from primary hepatocytes. Bioinformatic tools were applied to analyze the target genes and pathways regulated by the DE-miRs. Quantitative real-time PCR (qPCR) was conducted for the verification of DE-miRs. qPCR, western blot, immunofluorescence staining and ethynyl-20-deoxyuridine assay were used to evaluate the function of lipotoxic exosomal miR-1297 on hepatic stellate cells (LX2 cells). A luciferase reporter experiment was performed to confirm the relationship of miR-1297 and its target gene *PTEN*.

RESULTS

MicroRNA sequencing revealed that there were 61 exosomal DE-miRs ($P < 0.05$) with a fold-change > 2 from palmitic acid treated primary hepatocytes compared with the vehicle control group. miR-1297 was the most highly upregulated according to the microRNA sequencing. Bioinformatic tools showed a variety of target genes and pathways regulated by these DE-miRs were related to liver fibrosis. miR-1297 was overexpressed in exosomes derived from lipotoxic hepatocytes by qPCR. Fibrosis promoting genes (*α-SMA*, *PCNA*) were altered in LX2 cells after miR-1297 overexpression or miR-1297-rich lipotoxic exosome incubation *via* qPCR and western blot analysis. Immunofluorescence staining and

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ethynyl-20-deoxyuridine staining demonstrated that the activation and proliferation of LX2 cells were also promoted after the above treatment. *PTEN* was found to be the target gene of miR-1297 and knocking down *PTEN* contributed to the activation and proliferation of LX2 cells *via* modulating the PI3K/AKT signaling pathway.

CONCLUSION

miR-1297 was overexpressed in exosomes derived from lipotoxic hepatocytes. The lipotoxic hepatocyte-derived exosomal miR-1297 could promote the activation and proliferation of hepatic stellate cells through the PTEN/PI3K/AKT signaling pathway, accelerating the progression of MAFLD.

Key Words: Metabolic-associated fatty liver disease; miRNA-1297; Exosome; Hepatic stellate cell; PTEN; Liver fibrosis

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Core Tip: In this study, the expression of miR-1297 was increased in exosomes derived from primary hepatocytes. Exosomal miR-1297 from lipotoxic LO2 (a hepatocyte cell line) could promote LX2 (a hepatic stellate cell line) activation and proliferation by regulating the PTEN/PI3K/ATK pathway. Currently, the study between exosomes and metabolic-associated fatty liver disease is very limited. This is the first time that exosomal miR-1297 from lipotoxic hepatocytes was confirmed to accelerate liver fibrosis, and the new mechanism may become a promising treatment target for metabolic-associated fatty liver disease.

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INTRODUCTION

Metabolic-associated fatty liver disease (MAFLD), which was formerly called nonalcoholic fatty liver disease, is highly prevalent worldwide and affects approximately 25% of the global adult population[1]. Recently, a consensus of international experts proposed the terminology change from nonalcoholic fatty liver disease to MAFLD in order to better demonstrated the etiological features of this disease[2]. The incidence of MAFLD has been rising rapidly in recent years, and in China the incidence of MAFLD is 15%-30%[3]. MAFLD is characterized by fatty degeneration of the liver due to different metabolic conditions, such as obesity and type 2 diabetes, leading to fatty deposition in the liver[4]. Lipid deposition will increase the oxidative stress of cells in the liver, damage normal cell function and finally lead to liver fibrosis[5]. Hepatic stellate cell (HSC) activation was regarded as the core part of all kinds of liver fibrosis. The abnormal activation and proliferation of HSCs will cause them to produce more cellular extracellular matrix, destroy the normal structure of liver lobules and eventually result in cirrhosis[6]. However, the exploration of the specific pathogenesis underlying MAFLD is still in the preliminary stages.

Exosomes are circular or cup-shaped lipid bilayer vesicles with diameters of 30-150 nm, which are secreted by the host cells and transfer a variety of compounds, including miRNAs, mRNAs, lncRNAs, DNA and proteins[7]. Exosomes derived from lipotoxic hepatocytes could accelerate the activation of HSCs and lead to fibrosis[8]. However, to date, studies on the exact mechanism of exosomal miRNA and MAFLD are still very limited. In this study, we perform exosomal miRNA profiling from lipotoxic hepatocytes and further determine the potential mechanism by which exosomal miRNAs participate in the progression of MAFLD.

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MATERIALS AND METHODS

Cell culture

Hepatocytes (HCs), including primary HCs (PHCs), a normal human HC cell line (LO2) and a human HSC cell line (LX2) were used in this study. PHCs from male wild-type mice were isolated by pronase perfusion and cultured as reported previously[9]. LO2 cells and LX2 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, United States) supplemented with 100 mg/mL streptomycin (Gibco), 100 IU/mL penicillin (Gibco) and 10% fetal bovine serum (Gibco). PHCs were used to obtain exosomes for microRNA sequencing (miRNA seq) and quantitative real-time PCR (qPCR) verification. LO2 cells were used to obtain exosomes for cell incubation, and LX2 cells were used for other experiments. These procedures for PHC isolation were approved by the Ethical Committee of the Shanghai General Hospital.

Human sample collection

There were 20 MAFLD patients who shared similar demographic features and personal histories enrolled in this study. Among the 20 MAFLD patients with similar liver fibrosis severity, 10 patients were diagnosed as mild fatty liver while the other 10 patients were diagnosed as severe fatty liver depending on the hepatic steatosis. FibroScan (Echosens, Paris, France) analysis was applied to assess the controlled attenuation parameter and liver stiffness measurement of these patients according to the 2017 ASSLD guidelines[10]. The liver stiffness measurement value of these patients showed no significant difference. Patients with controlled attenuation parameter values from 265 dB/m to 295 dB/m were regarded as mild fatty liver, while patients with controlled attenuation parameter values over 295 dB/m were defined as severe fatty liver. Serum exosome samples from these patients were collected for miRNA qPCR. The study was approved by the Ethical Committee of the Shanghai General Hospital, and written informed consent was provided by all patients. The clinical features of the patients enrolled in this study are shown in Supplementary Table 1.

Exosome isolation and incubation

Exosomes from PHCs, LO2 cells and human serum samples were isolated using an ultracentrifugation method that was reported in a previous study[11]. To obtain the lipotoxic exosomes, we treated PHCs and LO2 cells with 200 μmol/L palmitic acid (PA, Sigma-Aldrich, St. Louis, MO, United States) or 5% BSA (Sigma-Aldrich) (diluted in phosphate buffered saline, Sigma-Aldrich) as the vehicle control for 24 h. Then, exosomes were isolated from the cell culture medium. For experiments related to exosome incubation, LX2 cells were treated with 50 μg/mL LO2 exosomes for 48 h.

Transmission electron microscopy observation of exosomes

To observe the exosome morphology, transmission electron microscopy (JEOL, Tokyo, Japan) was used. The experimental procedures were performed with assistance from Shanghai Bohao Biotechnology Co., Ltd. (Shanghai, China).

Nanoparticle tracking analysis for exosomes

To analyze the particle diameter distribution, nanoparticle tracking analysis was performed using a NanoSight NS300 system (Malvern Instruments, Worcestershire, United Kingdom) as in a previous study[12].

Exosome uptake experiment

For the exosome uptake experiment, 50 μg/mL LO2-derived exosomes (LO2-exosomes) diluted in phosphate buffered saline were labelled using the red fluorescent dye PKH26 Kit (Sigma-Aldrich) After incubating LX2 with PKH26 labelled LO2-exosomes for 6 h, a fluorescence microscope (Leica, Wetzlar, Germany) was used to observe the exosomes that were absorbed by LX2 cells.

miRNA high-throughput sequencing and data analysis

The miRNA seq experimental procedures were performed with the assistance of Bohao Biotechnology Co., Ltd. (Shanghai, China). The miRNA library was constructed using a TruSeq miRNA Library Prep Kit (Illumina, San Diego, CA, United States) following the standard procedures of the manufacturer's instructions.

Low-quality reads and adapters were filtered out from the raw reads to obtain clean reads. The clean reads were aligned against the miRBase database, and the expressions of miRNAs in each sample were determined. Differentially-expressed miRNAs were

screened using edgeR, under the following criteria: |log2 (fold-change)| ≥ 1 and $P < 0.05$. Heatmaps of gene expression were generated with MeV software (MeV Ltd., Stockport, United Kingdom).

Target gene bioinformatic analysis

Target gene prediction was performed for the significant differentially expressed miRNAs (DE-miRs). GO and pathway enrichment analyses were performed for the target genes using the Gene Ontology Database and Kyoto Encyclopedia of Genes and Genomes.

Cell transfection

For cell transfection, negative control mimics (mi-NC) and miR-1297 mimics (mi-miR) were used to overexpress miR-1297. Small interfering RNA for the negative control (si-NC) and small interfering RNA for PTEN were used to knock down PTEN. Cell transfection was conducted using Lipofectamine 2000 Regent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. All of the above vectors were purchased from GenePharma Co., Ltd. (Shanghai, China).

Luciferase reporter assay

The luciferase reporter assay experiment was conducted using the pGL3-basic luciferase vector (Promega, Madison, WI, United States). The 3'-UTR sequence of PTEN predicted to interact with miR-1297, together with a corresponding mutated sequence within the predicted target sites, were synthesized and inserted into the luciferase reporter (Promega, Madison, WI, United States) to generate PTEN wild-type or PTEN mutant (primers shown in Supplementary Table 2). The pGL3-basic luciferase vector containing PTEN wild-type or PTEN mutant were cotransfected with miR-1297 mimics or negative control mimics using Lipofectamine2000 (Invitrogen). After transfection, cells were incubated in suitable conditions for 24 h. The Luciferase Reporter Assay Kit (Promega) was used to assess the relative luciferase activity in accordance with the manufacturer's instructions. Firefly luciferase values were normalized to those of Renilla luciferase.

qPCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen). qPCR was performed using Hieff qPCR SYBR Green Master Mix (Applied Biosystems, Foster City, CA, United States) and Hieff First Strand cDNA Synthesis Super Mix (Applied Biosystems). β-actin was used as the internal reference to measure the mRNA expression of specific genes. U6 was used to normalize the expression of specific miRNA. Primers used in this research were fully listed in Supplementary Table 2.

Western blot analysis

These included antibodies were used for western blotting analysis: anti-TSG101 (1:1000, Santa Cruz Biotechnology, Santa Cruz CA, United States), anti-CD63 (1:1000, Abcam, Cambridge, MA, United States), anti-PCNA (1:500, Abcam), anti-α-SMA (1:1000, Abcam), anti-PTEN (1:1000, Abcam), anti-TGFβ1 (1:1000, Abcam), anti-SMAD3 (1:1000, Abcam), anti-p-SMAD3 (1:1000, Abcam) and anti-GAPDH (1:2000, Abcam). HRP-conjugated IgG (1:5000, Abcam) was used as the secondary antibody.

Immunofluorescence staining

Antibodies against α-SMA (1:100, Abcam) and 4' 6-diamidino-2-phenylindole (Abcam) were used for immunofluorescence staining. Alexa Fluor 594 AffiniPure goat anti-rabbit IgG (H+L) (Santa Cruz Biotechnology) was used as the secondary antibody. The laser microscope (Leica, Wetzlar, Germany) was used to capture the representative images.

Ethynyl-20-deoxyuridine staining

The proliferation of HSCs was detected by an ethynyl-20-deoxyuridine staining (EdU) cell lighting kit (RiboBio, Shanghai, China). The cell nuclei were stained using Hoechst (Beyotime, Shanghai, China). The laser microscope (Leica, Wetzlar, Germany) was used to capture the representative images.

Statistical analysis

The SPSS software (version 22.0; SPSS, Inc., Chicago, IL, United States) and GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, United States) were applied to

analyze all the data presented in this study. Data are expressed as the mean ± SD for at least three independent experiments for each group. Continuous variables showing a normal distribution were analyzed by an independent-samples *t*-test or one-way ANOVA. A P value < 0.05 was considered statistically significant.

RESULTS

Identification of exosomes derived from PHCs

Many studies have clarified that exosomes can participate in a variety of metabolic diseases, but the function of exosomes in MAFLD remains unclear. First, the morphology of exosomes derived from PA-treated (Exo-PA) PHCs or vehicle-treated (Exo-CN) PHCs was observed by transmission electron microscopy. Exosomes were small, quasi-circular vesicles with an intact membrane structure (Figure 1A). Then, exosome size and particle concentration were assessed *via* nanoparticle tracking analysis. Exosomes with a diameter of 50-150 nm accounted for 87.50% (Exo-PA) and 81.70% (Exo-CN) of all vesicles, which met the standard of high-quality exosomes (Figure 1B). In addition, the concentration of Exo-PA was significantly higher than that of Exo-CN (P < 0.05, Figure 1C). Finally, the exosomal markers CD63 and TSG101 could be observed in both Exo-PA and Exo-CN (Figure 1D). These results indicated that the method we used could obtain high-quality exosomes for follow-up research.

miRNA seq profiling and bioinformatic analysis of lipotoxic HC-secreted exosomes

To explore the differentially abundant exosomal miRNAs of lipotoxic HCs, we detected the miRNA expression profiles in Exo-PA and Exo-CN using miRNA seq. According to the results, 61 miRNAs were differentially expressed (P < 0.05) with a fold change > 2 (defined as DE-miR) in the Exo-PA group compared with the Exo-CN group. Among the 61 miRNAs, 20 were upregulated, and 41 were downregulated (Supplementary Table 3). In addition, three upregulated miRNAs had a fold change > 5 (hsa-miR-1297, hsa-miR-708-5p, hsa-miR-671-5p), while two downregulated miRNAs had a fold change > 5 (hsa-miR-718, hsa-miR-193a-3p). miR-1297 was the most highly upregulated miRNA (a 7.81-fold change), and miR-718 was the most highly downregulated miRNA (a 6.62-fold change) in the Exo-PA group. Heatmaps and the volcano map of gene expression are shown based on the expression of these DE-miRs (Figure 2A and 2B).

GO functional enrichment analysis was performed for the DE-miRs to explore the target gene function. The results revealed that these DE-miRs were associated with ten cell components, ten molecular functions and ten biological processes. Among these functions, seven functions were related to ubiquitin activity, which plays an important role in liver fibrosis. The results of the level 2 GO term enrichment analysis are presented in Figure 2C.

According to the Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis, there were 20 significantly enriched pathways. Based on a literature review, four pathways, the AMPK, PI3K-AKT, Hippo and Ras pathways, were highly correlated with liver fibrosis (Figure 2D).

Based on the miRNA seq results, 61 DE-miRs were found to be abnormally expressed in the Exo-PA group compared to the control group. miR-1297 was the top upregulated miRNA among these DE-miRs. Furthermore, seven gene functions and four pathways regulated by these DE-miRs were found to be highly correlated with fibrosis.

Expression levels of exosomal miR-1297 from lipotoxic HCs were significantly upregulated

To verify the results of miRNA seq, we measured exosomal DE-miRs with a fold change > 3 in cell culture samples and human serum samples. For cell culture samples, Exo-PA and Exo-CN were used. For human serum samples, exosomes derived from the serum of patients with severe fatty liver or mild fatty liver were selected in order to clarify the actual change of miR-1297 during the pathogenesis of MAFLD.

In cell culture samples, miR-1297 was the top upregulated candidate with a 5.83-fold change in the Exo-PA group compared to the Exo-CN group, while miR-718 was also the top downregulated miRNA with a 3.62-fold change, which was consistent with the results of miRNA seq. The detailed expression levels for the DE-miRs are shown in Figure 3A.

In human serum exosomal samples from exosomes derived from the serum of

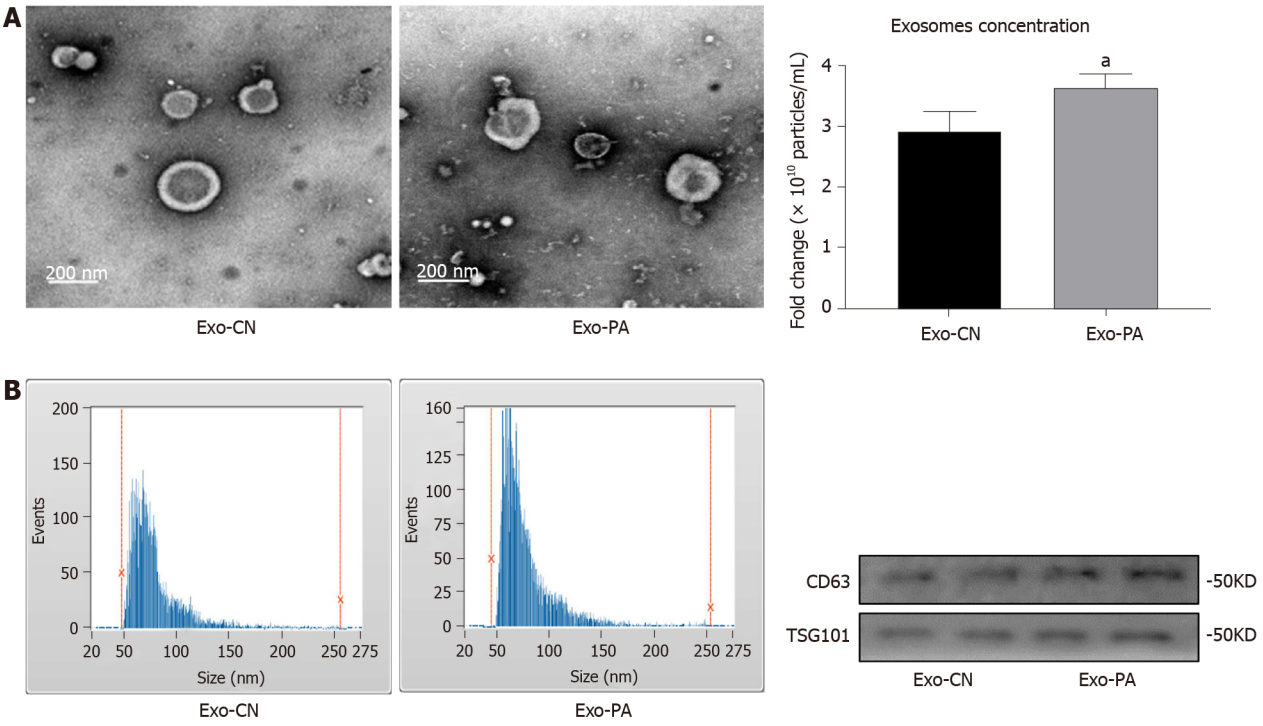


Figure 1 Identification of exosomes derived from primary hepatocytes. A: Exosomes derived from primary hepatocytes (Exo-PHC) were visualized by electronic microscopy (× 30000); B: Nanoparticle tracking analysis for Exo-PHC; C: The concentration of Exo-PHC was measured. Exosomes derived from vehicle control (Exo-CN) treated PHC: 2.92 ± 0.32 × 10^{10} particles/mL *vs* exosomes derived from palmitic acid (Exo-PA) treated PHC: 3.62 ± 0.22 × 10^{10} particles/mL; D: Exosomal markers CD63 and TSG101 were detected by western blot in Exo-PHC. Statistical significance, $^{a}P < 0.05$.

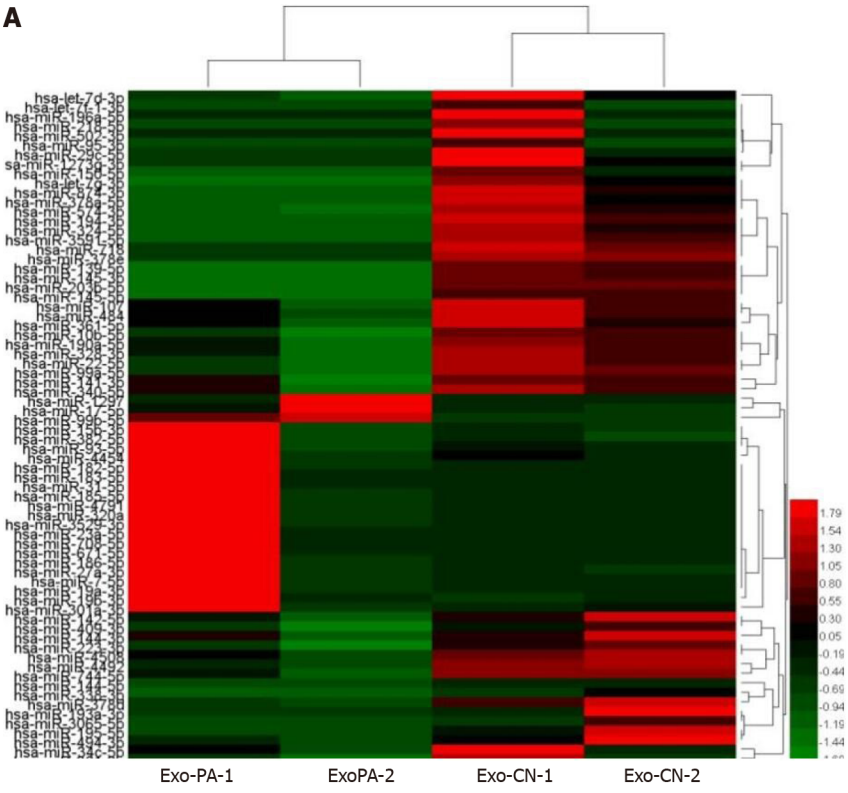
patients with mild fatty liver, miR-1297 had the greatest upregulation (a 3.32-fold change) compared to exosomes derived from the serum of patients with severe fatty liver among all the DE-miRs, while miR-193a was the top downregulated miRNA with a 2.96-fold change. The detailed expression levels for the DE-miRs are shown in Figure 3B.

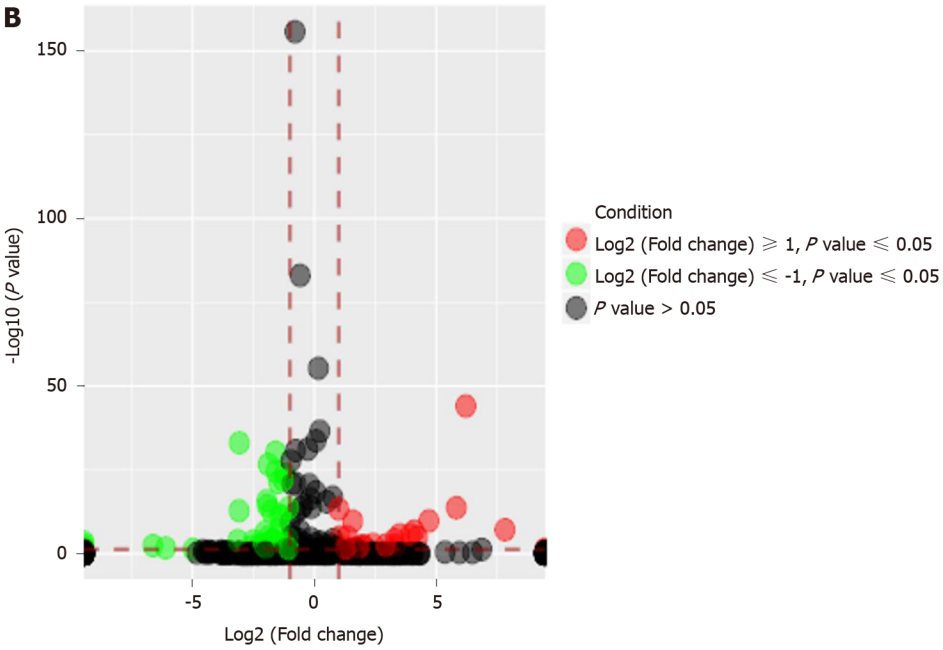
Although the results of cell culture samples did not completely parallel the results of human serum samples, miR-1297 showed a higher expression level in both groups. Therefore, miR-1297 was finally selected for follow-up research. The above findings suggested that the expression levels of exosomal miR-1297 from lipotoxic HCs were significantly upregulated.

miR-1297 accelerated HSC activation and proliferation

Currently, there is no research related to miR-1297 and liver fibrosis. To explore the effect of miR-1297 on HSCs, we first transfected the hepatic stellate cell line LX2 with miR-1297 mimics (mi-miR) or miRNA negative control mimics (mi-NC). After transfection, the expression was upregulated approximately 1400 times in the mi-miR LX2 cells (Figure 4A). Because α-SMA is an activation marker of HSCs, the expression of α-SMA could reflect collagen production when HSCs are activated. After miR-1297 overexpression in LX2, the mRNA and protein levels of α-SMA were increased (Figure 4B and 4C). In addition, immunofluorescence analysis further revealed that the relative red fluorescence intensity of α-SMA (red fluorescence) was markedly increased in the mi-miR LX2 cells compared to the mi-NC cells (mi-miR: 1.87 ± 0.15 *vs* mi-NC: 1.00 ± 0.22, Figure 4D). During the process of liver fibrosis, the activation and proliferation of HSCs play a central role. PCNA is a proliferation marker of HSCs. The mRNA and protein levels of PCNA were found to be significantly elevated in the mi-miR LX2 cells compared to the mi-NC LX2 cells (Figure 4B and 4C). In addition, an EdU assay was conducted to evaluate the proliferation of HSCs. As shown in Figure 4E, 32.10% of the mi-miR LX2 cells incorporated EdU, while only 24.15% of the mi-NC LX2 cells were EdU positive, which revealed that miR-1297 could promote the proliferation of LX2 cells as well.

These results proved that miR-1297 could accelerate HSC activation and proliferation.


A
Exo-PA-1
ExoPA-2
Exo-CN-1
Exo-CN-2
1.79
1.54
1.30
1.05
0.80
0.55
0.30
0.05
-0.19
-0.44
-0.69
-0.94
-1.19
-1.44


B
150
100
50
0
-Log10 (P value)
-5
0
5
Log2 (Fold change)
Condition
Log2 (Fold change) ⩾ 1, P value ⩽ 0.05
Log2 (Fold change) ⩽ -1, P value ⩽ 0.05
P value > 0.05

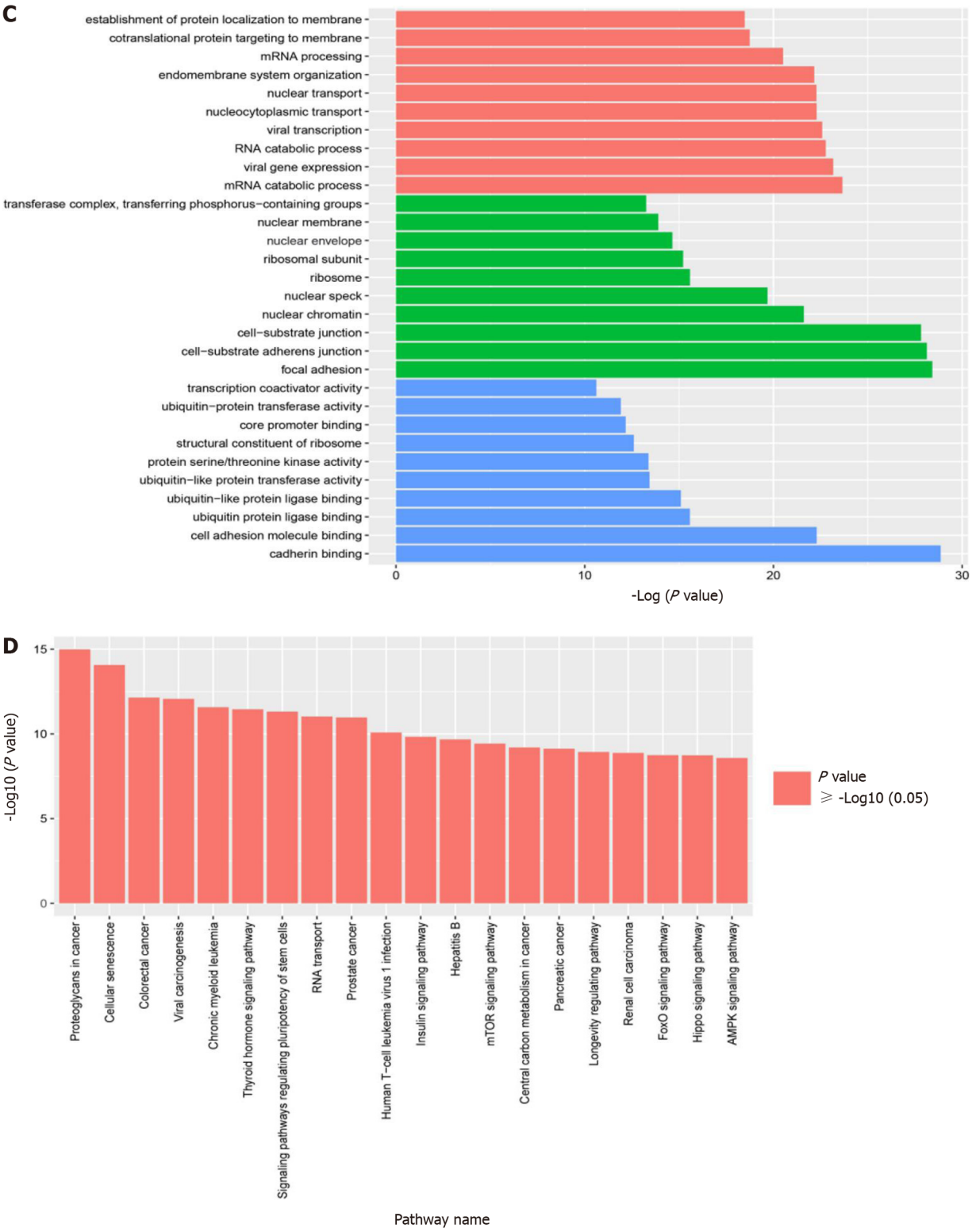


Figure 2 MicroRNA sequencing profiling and bioinformatic analysis. A: Heatmap of differentially-expressed microRNAs (DE-miRs) in the microRNA sequencing; B: Volcano map of DE-miR expression in the miRNA sequencing; C: GO function analysis for target genes regulated by DE-miRs; D: Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of target genes for the DE-miR. Exo-PA-1/Exo-PA-2: Group 1/2 of exosomes derived from palmitic acid treated primary hepatocytes; Exo-CN-1/Exo-CN-2: Group 1/2 of exosomes derived from vehicle control treated primary hepatocytes.

miR-1297 exerted its function by regulating the PTEN/PI3K/AKT pathway in HSCs

As miR-1297 was found to promote HSC activation and proliferation in LX2 cells, we tried to find the target gene it may regulate to exert its function. In the TargetScan database (http://www.targetscan.org/, Figure 5A), a total of three fibrosis-related genes, *COL10A1*, *PTEN* and *TRAF3*, were selected. A previous study suggested that PTEN was found to be the target gene of miR-1297 in human cervical carcinoma

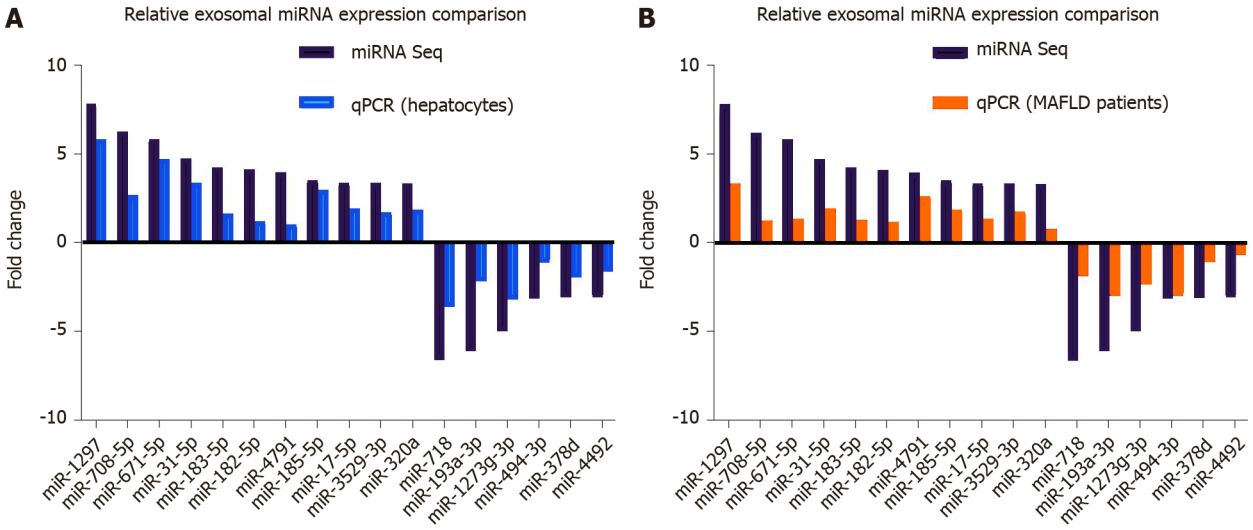


Figure 3 Quantitative real-time PCR analysis verification for differentially expressed microRNAs in exosomes derived from primary hepatocytes and patient serum samples of metabolic-associated fatty liver disease. A: Quantitative real-time PCR was performed on exosomes derived from vehicle control treated primary hepatocyte and palmitic acid treated primary hepatocyte. The fold change ratio of microRNA (miRNA) in the Exo-PA group was compared to the result of differentially expressed miRNAs in the miRNA sequencing (miRNA seq). Detailed data of individual miRNA expression could be seen Supplementary Table 2; B: Quantitative real-time (q)PCR was performed between mild and severe fatty liver human serum exosome samples. The fold change ratio of miRNA in severe fatty liver were compared to the result of the differentially expressed miRNAs of the miRNA sequencing. Detailed data of individual miRNA expression could be seen Supplementary Table 2. MAFLD: Metabolic-associated fatty liver disease.

cells[13], so a luciferase reporter assay was conducted to determine the relationship of miR-1297 and PTEN in LX2 cells. In the PTEN wild-type group, the relative luciferase activity in the mi-miR LX2 cells was decreased compared with that in the mi-NC LX2 cells (mi-NC: 1.00 ± 0.14 *vs* mi-miR: 0.56 ± 0.12) (Figure 5B). However, in the PTEN mutant group, there were no obvious luciferase activity changes between the mi-miR and mi-NC LX2 cells (Figure 5B), which confirmed that PTEN was the target gene of miR-1297 in LX2 cells. Then, the mRNA and protein levels of PTEN were also found to be significantly inhibited after miR-1297 mimic transfection, which indicated that miR-1297 could negatively regulate the expression of PTEN (Figure 5C and 5D).

Previous studies have shown that PTEN could negatively regulate the downstream PI3K/AKT pathway and accelerate liver fibrosis[14-16]. Therefore, PTEN was knocked down to evaluate its function in HSCs. The protein expression of PI3K and p-AKT (Figure 5D and 5E) was markedly elevated in both the miR-1297 and small interfering RNA for PTEN LX2 cells compared to their control cells, while α-SMA and PCNA were also significantly upregulated (Figure 5E), which revealed that PTEN could negatively regulate the PI3K/AKT pathway and HSC activation. These findings suggested that miR-1297 could modulate the PTEN/PI3K/AKT pathway, accelerating the activation and proliferation of HSCs.

Lipotoxic HC-secreted exosomes could transfer exosomal miR-1297 to HSCs and contribute to HSC activation through the PTEN/PI3K/AKT pathway

Given the observed effects, miR-1297 was found to promote the activation and proliferation of LX2. To further determine the function of lipotoxic HC-secreted exosomes (HC-Exos) on HSCs, we incubated LX2 cells with exosomes from PA-treated LO2 cells or exosomes from CN-treated LO2 cells. Through the exosome uptake experiment, both Exo-PA and Exo-CN were observed to be engulfed by LX2 cells, suggesting that HC-Exos were successfully taken up by the recipient HSCs (Figure 6A).

miR-1297 was overexpressed after Exo-PA treatment (Exo-PA: 3.95 ± 0.18 *vs* Exo-CN: 1.00 ± 0.11), while the mRNA and protein levels of PTEN were obviously decreased (Figure 6B and 6D). In addition, the expression of PI3K and p-AKT was significantly increased in the Exo-PA-treated LX2 cells, indicating that miR-1297 modulated the PTEN/PI3K/AKT pathway after lipotoxic HC-Exo treatment (Figure 6D).

In addition, the expression of α-SMA was found to be significantly upregulated in the Exo-PA LX2 group (Figure 6C and 6D). Immunofluorescence analysis revealed a remarkable overexpression of α-SMA (red fluorescence) in the Exo-PA-treated LX2

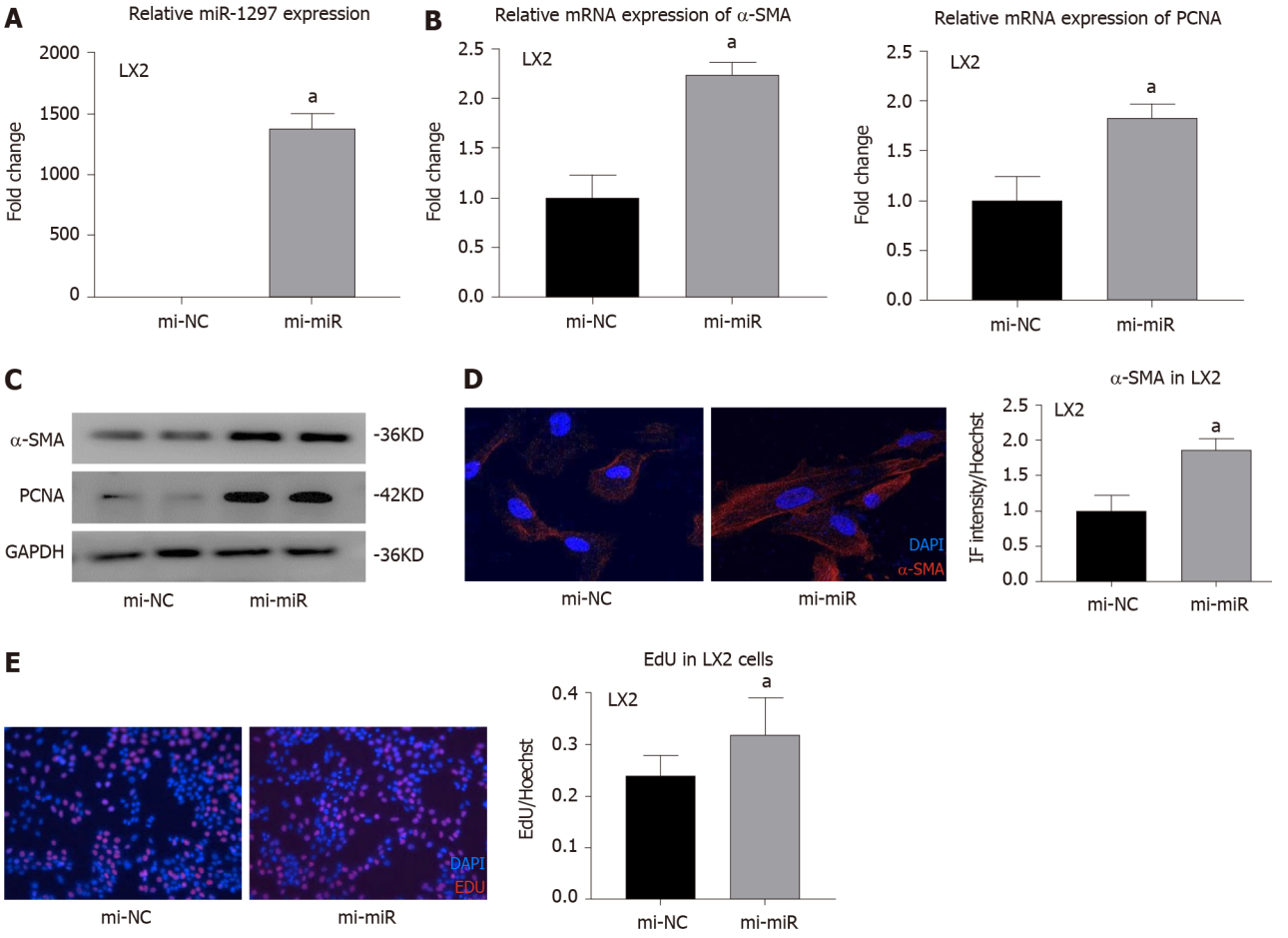


Figure 4 MicroR-1297 promoted hepatic stellate cell activation and proliferation by targeting the PTEN signaling pathway. A: The transfection efficiency of 50 nM miR-1297 mimics in LX2 cells was assessed by quantitative real-time PCR. microRNA mimics of negative controls (mi-NC): 1.00 ± 0.12 *vs* miR-1297 mimics (mi-miR): 1380.25 ± 121.16; B: Relative mRNA expression of *α-SMA* (the activation marker of hepatic stellate cells) and PCNA (the proliferation marker of hepatic stellate cells) were assessed by quantitative real-time PCR. α-SMA: mi-NC: 1.00 ± 0.23 *vs* mi-miR: 2.24 ± 0.12; PCNA: mi-NC: 1.00 ± 0.25 *vs* mi-miR: 1.83 ± 0.14; C: Relative protein expression of α-SMA, PCNA, PTEN, PI3K, AKT and p-AKT were assessed by western blot after treatment of 50 nmol/L miR-1297 mimics for 48 h; D: Immunofluorescence staining of α-SMA was performed to evaluate the activation of LX2 cells after 50 nmol/L miR-1297 mimics for 48 h. mi-NC: 1.00 ± 0.22 *vs* mi-miR: 1.87 ± 0.15, Bar = 200 μm; E: Ethynyl-20-deoxyuridine test was performed to evaluate the proliferation of LX2 cells after 50 nmol/L miR-1297 mimics for 48 h. mi-NC: 0.24 ± 0.04 *vs* mi-miR: 0.32 ± 0.07, Bar = 50 μm. Statistical significance, $^{a}P < 0.05$. DAPI: 4'6-Diamidino-2-phenylindole; EdU: Ethynyl-20-deoxyuridine.

cells (Exo-PA: 1.68 ± 0.21 *vs* Exo-CN: 1.00 ± 0.16) (Figure 6E). The mRNA and protein levels of PCNA were markedly elevated in the Exo-PA group as well (Figure 6C and 6D). The proportion of EdU-incorporated LX2 cells was dramatically elevated (Exo-PA: 0.41 ± 0.06 *vs* Exo-CN: 0.21 ± 0.04) (Figure 6F).

Taken together, these results demonstrated that lipotoxic HC-Exos could transfer exosomal miR-1297 to recipient HSCs, thus promoting HSC activation and proliferation by modulating the PTEN/PI3K/AKT pathway.

DISCUSSION

MAFLD is highly prevalent worldwide and may become the leading cause of end-stage liver disease in the coming decades[17]. Because the majority of MAFLD patients do not show any symptoms, MAFLD may further evolve into liver fibrosis or even hepatocellular carcinoma[18]. Recently, an increasing number of studies focused on the relationship between exosomes and MAFLD-related fibrosis[12], but the exact mechanisms by which exosomes participate in fibrosis remain unclear. In our present research, we observed that the exosomal miR-1297 expression was significantly upregulated in lipotoxic HCs. Furthermore, a novel mechanism through which exosomal miR-1297 mediates cellular communication between HCs and HSCs, subsequently contributing to HSC activation *via* the PTEN/PI3K/ATK pathway, was confirmed.

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 41-47 of PTEN 3' UTR hsa-miR-1297	5' …ACACCAUGAAAAUAAACUUGAAU… 3' GUGGACUUAAUGAACUU	7mer-A1	-0.24	96	-0.24	6.860	0.73

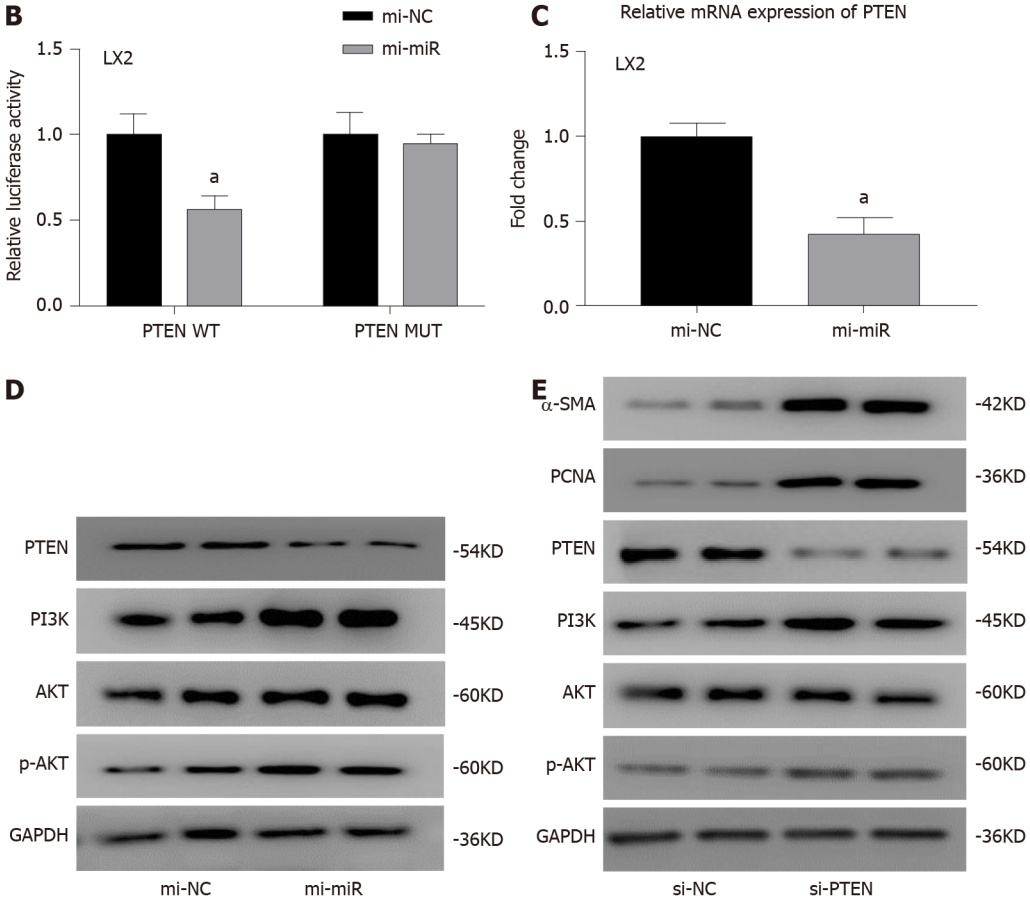
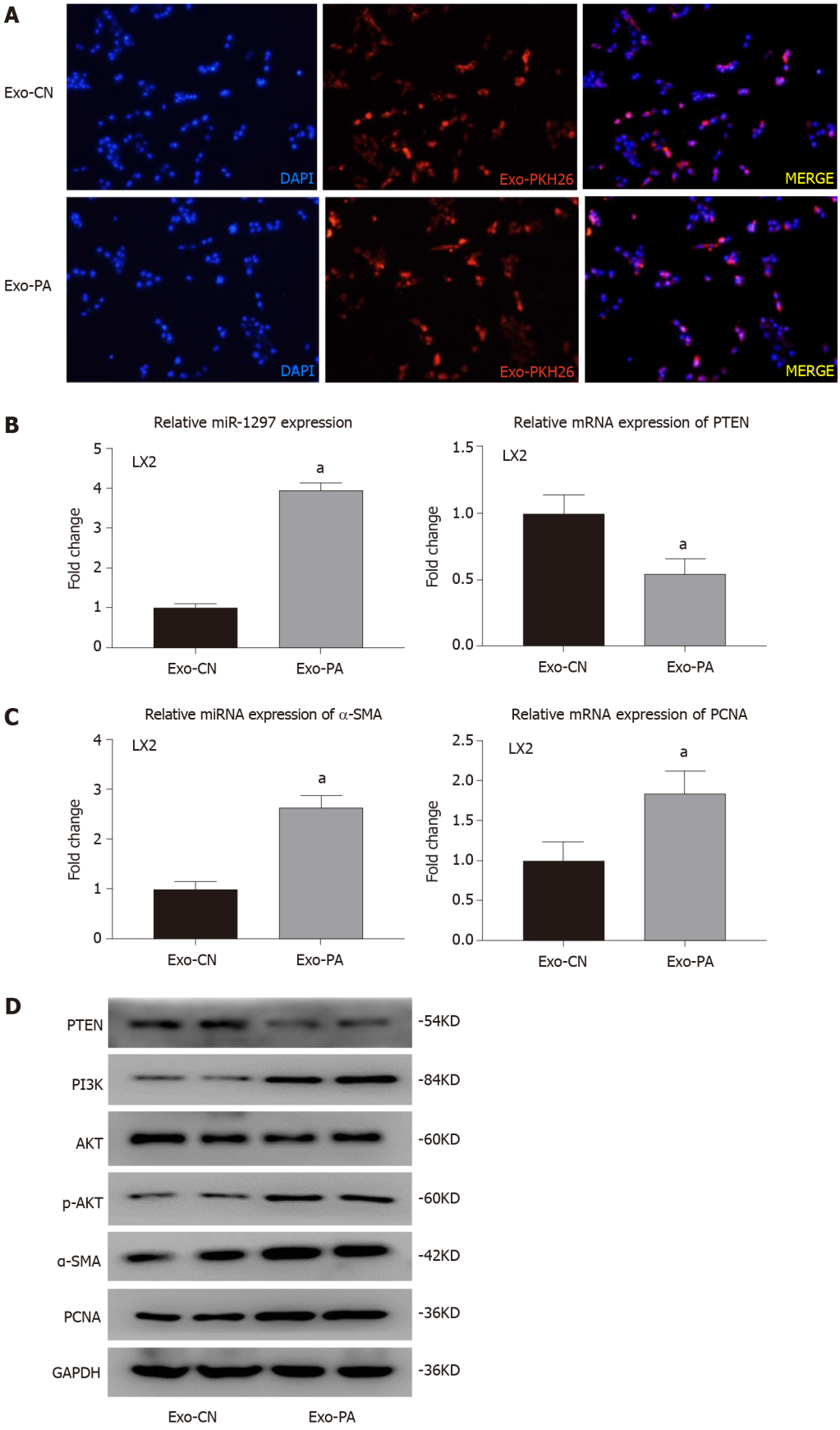


Figure 5 MicroR-1297 promoted hepatic stellate cell activation and proliferation by targeting the PTEN signaling pathway. A: Binding site of PTEN mRNA with microRNA (miR)-1297 from TargetScan database; B: LX2 cells were cotransfected with luciferase reporter constructs containing a wild-type or mutant 3'-UTR of PTEN and 50 nmol/L miR-1297 mimics for 24 h. Then relative luciferase activity was assessed. PTEN wild-type (PTEN WT) microRNA mimics of negative controls: 1.00 ± 0.12 *vs* miR-1297 mimics: 0.56 ± 0.08; PTEN mutant (PTEN MUT) microRNA mimics of negative controls (mi-NC): 1.00 ± 0.13 *vs* miR-1297 mimics (mi-miR): 0.95 ± 0.05; C: Relative mRNA expression of *PTEN* were assessed by quantitative real-time PCR after 50 nmol/L miR-1297 mimics for 48 h. microRNA mimics of negative controls: 1.00 ± 0.08 *vs* miR-1297 mimics: 0.43 ± 0.09; D: Relative protein expression of PTEN, PI3K, AKT and p-AKT were assessed by western blot after 50 nmol/L miR-1297 mimics for 48 h; E: Relative protein expression of α-SMA, PCNA, PTEN, PI3K, AK and p-AKT were assessed by western blot after 100 nmol/L small interfering RNA for PTEN (si-PTEN) transfection for 48 h. Statistical significance: $^{a}P < 0.05$. si-NC: Small interfering RNA for negative controls.

Exosomes have been identified as important mediators for cell-cell communication by transferring various biological components, such as miRNAs, lncRNAs and proteins[19]. The evidence that exosomes played an important role in the process of liver fibrosis, acting as a means of communication among different hepatic cells, have been confirmed by multiple studies[20]. Exosomal miR-223 from natural killer cells and exosomal miR-181-5p from mesenchymal stem cells were found to inhibit HSC activation by modulating autophagy[21,22]. Lipopolysaccharide-activated THP-1 macrophages could release exosomal miR-103-3p to induce the activation of HSCs[23]. Exosomes derived from human bone marrow mesenchymal stem cells could alleviate liver fibrosis through the Wnt/β-catenin pathway[24]. However, research on HC-HSC crosstalk is very limited. To determine the effect of lipotoxic HC-Exos on HSCs, we performed exosomal miRNA seq for an MAFLD HC-Exo model in our research. A total of 61 miRNAs were found to be abnormally expressed in the lipotoxic HC-Exo group compared to the control group, and seven gene functions and four pathways regulated by these DE-miRs were found to be highly correlated with fibrosis.

Then miR-1297 was found to be the most highly upregulated miRNA in both


A
Exo-CN
DAPI
Exo-PKH26
MERGE
Exo-PA
DAPI
Exo-PKH26
MERGE
B
Relative miR-1297 expression
LX2
a
Fold change
Exo-CN
Exo-PA
Relative mRNA expression of PTEN
LX2
a
Fold change
Exo-CN
Exo-PA
C
Relative miRNA expression of α-SMA
LX2
a
Fold change
Exo-CN
Exo-PA
Relative mRNA expression of PCNA
LX2
a
Fold change
Exo-CN
Exo-PA
D
PTEN
PI3K
AKT
p-AKT
α-SMA
PCNA
GAPDH
-54KD
-84KD
-60KD
-60KD
-42KD
-36KD
-36KD
Exo-CN
Exo-PA

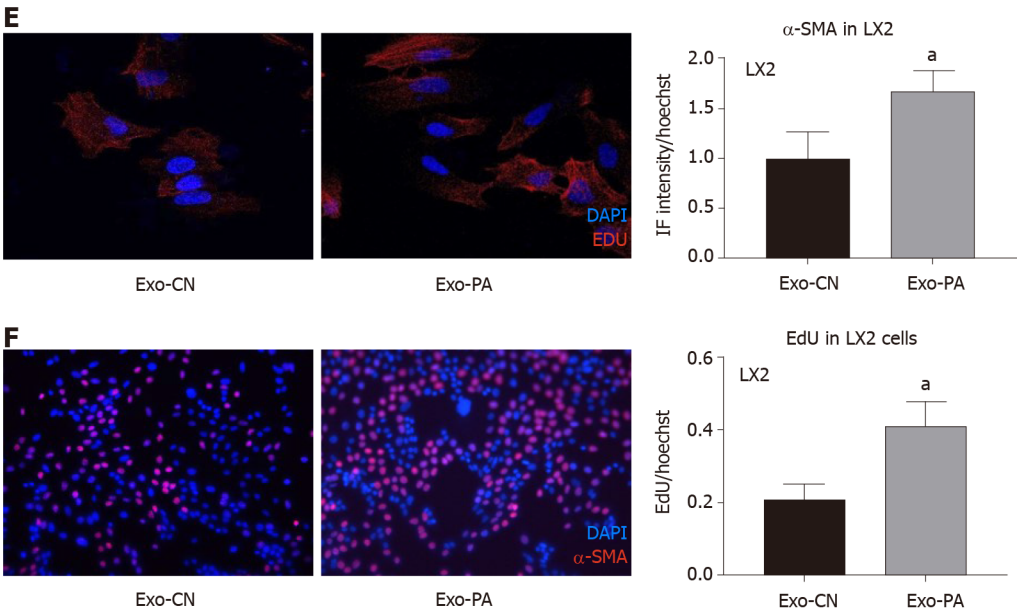


Figure 6 Lipotoxic hepatocyte-secreted exosomes could transfer exosomal microRNA-1297 to hepatic stellate cells and contribute to hepatic stellate cell activation through the PTEN/PI3K/AKT pathway. A: The PKH-26 stained exosomes were absorbed by LX2 cells observed by a fluorescence microscope; B: The relative microRNA expression of miR-1297 and mRNA expression of *PTEN* were assessed by quantitative real-time PCR after exosomes derived from vehicle control or palmitic acid (Exo-CN or Exo-PA) treatment for 48 h. miR-1297 mimics of negative controls (mi-NC): 1.00 ± 0.12 *vs* miR1297 mimics (mi-miR): 3.95 ± 0.18, PTEN mi-NC: 1.00 ± 0.13 *vs* mi-miR: 0.54 ± 0.12; C: The relative mRNA expression of *α-SMA* and *PCNA* were assessed by quantitative real-time PCR after Exo-CN or Exo-PA treatment for 48 h. α-SMA mi-NC 1.00 ± 0.14 *vs* mi-miR 2.63 ± 0.23, PCNA mi-NC 1.00 ± 0.23 *vs* mi-miR 1.85 ± 0.23; D: Protein levels of α-SMA, PCNA, PTEN, PI3K, AKT and p-AKT in LX2 cells were assessed by western blot after Exo-CN or Exo-PA treatment for 48 h; E: Immunofluorescence staining was performed to evaluate the activation of LX2 cells after Exo-CN or Exo-PA treatment for 48 h; mi-NC: 1.00 ± 0.26 *vs* mi-miR: 1.68 ± 0.21; F: Ethynyl-20-deoxyuridine (EdU) staining was performed to evaluate the proliferation of LX2 cells after Exo-CN or Exo-PA treatment for 48 h, mi-NC: 0.21 ± 0.04 *vs* mi-miR: 0.41 ± 0.07. Statistical significance: $^{a}P < 0.05$. DAPI: 4′6-Diamidino-2-phenylindole; Exo-CN: Exosomes from control vehicle; Exo-PA: Exosomes from palmitic acid.

lipotoxic PHC-derived exosomes and severe fatty liver patient serum exosomes compared to their control groups *via* qPCR verification. Cellular exosomes were isolated from cell culture media and could represent an MAFLD cell model progression. We found miR-1297 was significantly upregulated in lipotoxic PHC-derived exosomes. However, sometimes the experimental results were not parallel with the actual disease progression. Therefore, we measured the expression of miR-1297 in serum exosome samples of MAFLD patients. Exosomes derived from serum usually originated from different organs and could reveal a rather complicated but more real condition during the disease progression. In serum exosome samples we also found miR-1297 was markedly overexpressed in severe MAFLD patients. Both results revealed that miR-1297 was obviously upregulated during MAFLD progression, and miR-1297 was finally chosen for the follow-up study.

Previous studies have confirmed the role of miR-1297 in liver diseases. miR-1297 promoted the progression of liver cancer by inhibiting the expression of RB1[25]. Liu *et al*[26,27] found that miR-1297 could negatively regulate hepatocellular carcinoma progression by modulating EZH2 and HMGA2. But currently, the role of miR-1297 has not been reported in liver fibrosis. To determine the function of miR-1297 in liver fibrosis, the human hepatic stellate cell line LX2 was treated with miR-1297 mimics and miR-1297-rich lipotoxic exosomes in our research. Upregulation of miR-1297 was found to suppress the expression of its target gene *PTEN* and contribute to the activation and proliferation of LX2 cells.

PTEN is a well-known regulatory gene to suppress tumors and fibrosis, and many studies have demonstrated that it can prevent the development of various fibrotic-related diseases, such as renal, lung and liver fibrosis[28-30]. The loss of PTEN induced by hypoxia inducible factor-1 promoted HSC activation by regulating the P65 signaling pathway[30]. miR-1273g-3p modulated the activation and apoptosis of HSCs by directly targeting PTEN in HCV-related liver fibrosis[31]. In addition, PTEN inhibited macrophage activation by regulating the PI3K/Akt/STAT6 signaling pathway in hepatic Kupffer cells and alleviated liver fibrosis[32]. In the present study, *PTEN* was confirmed to be the target gene of miR-1297. High expression levels of miR-1297 induced by miR-1297 mimics and miR-1297-rich lipotoxic HC-Exos could

negatively regulate PTEN in LX2, inhibiting its function in preventing liver fibrosis. Therefore, the expression of fibrosis-related genes (*α-SMA* and *PCNA*) was found to be increased after miR-1297 overexpression, miR-1297-rich lipotoxic HC-Exo incubation or *PTEN* knockdown, which was hypothesized to explain why miR-1297 could promote HSC activation.

The progression of liver fibrosis is a complex physiological process, including the regulation of multiple signaling pathways, such as the TGFβ1/SMAD, MAPK and PI3K/ATK pathways[33]. The PI3K/ATK pathway is a classic pathway regulated by PTEN that participates in a variety of biological functions, including cell growth, proliferation and differentiation[34]. It has been reported that PTEN could hydrolyze PI3K protein, thereby phosphorylating AKT and stimulating fibroblast accumulation and myofibroblast differentiation[35,36]. Our results demonstrated that the low expression of PTEN induced by miR-1297 or small interfering RNA could promote the protein expression of PI3K and p-AKT, thus elevating the expression of fibrosis-related genes (*α-SMA* and *PCNA*), which may explain the downstream molecular mechanism by which miR-1297 regulates PTEN and activates HSCs.

CONCLUSION

Taken together, our research is the first to clarify that exosomal miR-1297 originating from PA-treated HCs could promote the activation and proliferation of HSCs by targeting the PTEN/PI3K/AKT pathway and accelerate the progression of liver fibrosis. This molecule may become a new target for the treatment of MAFLD in the future.

ARTICLE HIGHLIGHTS

Research background

Accumulating evidence has revealed that exosomes play an important role in the progression of metabolic-associated fatty liver disease, but the exact mechanism remains unclear.

Research motivation

Previous studies demonstrated that exosomes from lipotoxic hepatic cells could contribute to hepatic stellate cell (HSC) activation, but the specific cellular crosstalk interaction was not fully illustrated. We aimed to figure out if exosomal microRNA could participate in this procedure.

Research objectives

To determine the role of exosomal miR-1297 in the metabolic-associated fatty liver disease cell model.

Research methods

miRNA sequencing was conducted to screen differentially-expressed microRNAs. *In vitro* experiments like quantitative real-time PCR analysis, western blot, immunofluorescence and ethynyl-20-deoxyuridine staining were performed to explore the function of exosomal miR-1297 on HSC activation and proliferation.

Research results

miR-1297 was obviously overexpressed in exosomes derived from lipotoxic hepatocytes. The lipotoxic hepatocyte-derived exosomal miR-1297 could promote the activation and proliferation of HSCs through the PTEN/PI3K/AKT signaling pathway.

Research conclusions

The lipotoxic hepatocyte-derived exosomal miR-1297 could accelerate the progression of metabolic-associated fatty liver disease in a cell model.

Research perspectives

This study provided some new evidence on the crosstalk between hepatocyte-secreted exosomes and HSC. miR-1297 may become a potential target for the treatment of

metabolic-associated fatty liver disease.

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ORIGINAL ARTICLE

Basic Study

Cyanidin 3-glucoside modulated cell cycle progression in liver precancerous lesion, *in vivo* study

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Author contributions: Matboli M and Hasanin AH conceived the presented idea, developed the theory; Matboli M performed the statistics and the molecular assay; Hasanin AH verified the analytical methods; Hussein R and Saleh LA carried out the experimental animal work, performed the statistical analysis, writing the manuscript; El-Nakeep S developed the theory and writing the manuscript; Ellackany R has shared in the revision of the manuscript; Habib EK performed the histopathological examination and writing the comments; all authors discussed the results and contributed to the final manuscript revision.

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Abstract

BACKGROUND

Cyanidin-3-O-glucoside (cyan) exhibits antioxidant and anticancer properties. The cell cycle proteins and antimitotic drugs might be promising therapeutic targets in hepatocellular carcinoma.

AIM

To investigate the effect of cyan administration on cell cycle in hepatic precancerous lesion (PCL) induced by diethylnitrosamine/2-acetylaminofluorene (DEN/2-AAF) in Wistar rats.

METHODS

In vivo, DEN/2-AAF-induced hepatic PCL, rats were treated with three doses of cyan (10, 15, and 20 mg/kg/d, for four consecutive days *per* week for 16 wk). Blood and liver tissue samples were collected for measurement of the followings; alpha fetoprotein (AFP) liver function and RNA panel differential expression was evaluated *via* real time polymerase chain reaction. Histopathological examination of liver sections stained with H&E and immunohistochemical study using

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glutathione S-transferase placental (GSTP) and proliferating cell nuclear antigen (PCNA) antibodies were assessed.

RESULTS

Cyan administration mitigated the effect of DEN/2-AFF induced PCL, decreased AFP levels, and improved liver function. Remarkably, treatment with cyan dose dependently decreased the long non-coding RNA MALAT1 and tubulin gamma 1 mRNA expressions and increased the levels of miR-125b, all of which are involved in cell cycle and mitotic spindle assembly. Of note, cyan decreased GSTP foci percent area and PCNA positively stained nuclei.

CONCLUSION

Our results indicated that cyan could be used as a potential therapeutic agent to inhibit liver carcinogenesis in rat model *via* modulation of cell cycle.

Key Words: Hepatocellular carcinoma therapy; Hepatocellular-carcinoma growth; Hepatocellular-carcinoma model; Hepatocellular-carcinoma size

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Core Tip: Several regulatory RNA networks are important in regulation of liver cell cycle progression and are linked to the pathogenesis of hepatocellular carcinoma. Identification of critical steps involved in hepatocarcinogenesis are likely to lead to the development of new therapeutics that will inhibit tumor proliferation alongside with verification of promising drug targets biomarkers that may lead to improved patient survival. Cyanidin-3-O-glucoside (cyan) has a significant antioxidant and anticancer activities. Cyan could be used as potential agent to inhibit liver carcinogenesis in rat model *via* modulation of cell cycle. Cyan dose dependently decreased the long non-coding RNA-MALAT1 and tubulin 1 mRNA expressions and increased the hsa-miR-125b expression which participate in cell cycle and mitotic spindle assembly.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the commonest primary malignancy of the liver. Its main risk factors include hepatitis B, hepatitis C, and non-alcoholic steatohepatitis. HCC is associated with high mortality and morbidity. Its incidence has increased from 1.4 to 6.2 *per* 100000 cases *per* year within the last 30 years[1]. HCC burden in Egypt is high due to the high prevalence of HCV, where liver cancers account for 11.75% of all gastrointestinal cancers and 1.68% of all malignancies. In addition, over 70% of all liver malignancies in Egypt are HCC[2].

To date, HCC is holding its place as an unresolved dilemma; its resistance to systemic chemotherapy and radiation makes the cure approachable only in very early stages according to Milan criteria, that is, where the tumor size is < 5 cm (when only one lesion is present) or < 3 cm (when 2-3 lesions are present)[3].

Genes involved in cell cycle control are usually mutated in presence of cancer. Dysregulated mitosis results in genomic instability, which leads to tumor aggressiveness[4]. Microtubules are a key component of mitotic spindles, and thus, a crucial part of the process of mitosis. They form an important target in cancer chemotherapy, inducing arrest of mitosis and cell death. On the other hand, while the overexpression of γ-tubulin (γ-TUBG) showed improved survival among small cell lung cancer patients[5], the overexpression of γ-TUBG is found to be characteristic feature of thyroid and breast cancers[6]. Such variation may arise due to differences in gene transcription and check point regulations in different cancer tissues[7].

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Cyanidin-3-glucoside (cyan) is a potential chemotherapeutic and chemo-protective agent. Through its antioxidant activity, it scavenges radical oxygen species, which decreases the overall number of tumorous cells (malignant and benign) in *in vivo* studies[8]. Cyan has been implicated in some beneficial health activities[9], including reducing age-associated oxidative stress[10], improving cognitive brain function, and exhibiting anti-diabetic[11], anti-inflammation[12], anti-atherogenic[13], and anti-obesity activities[14]. Previously, it has been reported that cyan mediates caspase-3 cleavage with DNA fragmentation. Cyan has also been associated with induction of autophagy, a key element involved in cancer elimination, *via* induction of autophagy-related gene 5 and microtubule-associated protein 1 light chain 3-II[15]. Cyan has drawn increasing attention because of its potential anti-cancer properties. Cyan may offer a novel avenue for treating HCC. a potential antiproliferative effect on HepG2 cells[16].

The present study was conducted to evaluate the effect of cyan in three different doses on cell cycle progression and mitotic assembly disorder in hepatic precancerous lesion (PCL) *via* assessment of levels of long non-coding RNA (lncRNA) MALAT1, miR-125b, and tubulin 1. We also conducted histopathological and immunohistochemical examination using H&E staining assessment of levels of glutathione S-transferase placental (GSTP) and proliferating cell nuclear antigen (PCNA) antibodies in male Wistar rat model of diethylnitrosamine/2-acetylamino-fluorene (DEN/2-AAF)-induced hepatic PCL.

MATERIALS AND METHODS

Chemicals and drugs

DEN was obtained as 1 g solution in serum bottle diluted in 0.9% NaCl. 2-AAF was obtained as white powder, while cyan was obtained as black powder. Both 2-AAF and cyan were dissolved in 0.9% NaCl. All chemicals were purchased from Sigma-Aldrich, Cairo, Egypt.

Experimental animals

All animal procedures were approved by the Institutional Animal Ethics Committee of Ain Shams University, Faculty of Medicine. Thirty male Wistar rats weighing 200-250 g were purchased from Nile Pharmaceuticals Company (Cairo, Egypt). Animals were housed in an animal room with temperature 25 ± 2 °C and 12 h light/dark cycle controls. An adaptation period of 1 wk was allowed before initiation of the experimental protocol.

Experimental procedures

Induction of hepatic PCL: DEN was injected intraperitoneally (*i.p.*) at a dose of 100 mg/kg once weekly for three consecutive weeks. One week after the last DEN injection, a single dose (300 mg/kg) of 2-AAF was injected.

Animal groups: Rats were divided into five groups (6 rats/each group), including: Naïve group: Rats were injected with a vehicle (0.9% NaCl), PCL group: Rats were injected with DEN and 2-AAF, and three cyan groups (cyan-10, cyan-15, and cyan-20 groups): Rats were injected with DEN and 2-AAF, and orally administered through gastric gavage with cyan in doses of 10, 15, and 20 mg/kg respectively, for four consecutive days *per* week for 16 wk[17].

Assessment of the effects of treatment

Biochemical and molecular studies: After scarifying the animals, blood was withdrawn from each rat from the retro orbital vein and incubated for about half an hour for clotting. Then, the blood sample was centrifuged for 20 min at 5000 RPM to get serum samples. Livers were dissected. The right lobe of the livers was removed, cut into longitudinal sections 2-4 mm in thickness and kept in 10% formalin for histopathological and immunohistochemical examination. The other lobe was kept frozen at -80 °C for molecular analyses. The samples were analyzed for the following parameters: (1) alpha fetoprotein (AFP) levels: AFP level was analyzed using quantitative sandwich rat AFP ELISA kit purchased from MyBiosource Inc. (San Diego, United States) with a sensitivity range of 0.625-20 ng/mL; (2) serum alanine aminotransferase (ALT) levels: The serum ALT was measured according to method described by using kits purchased from Diamond Diagnostic (Cairo, Egypt). ALT catalyzes the transfer of amino groups from specific amino acids to ketoglutaric acid, yielding glutamic acid and oxaloacetic or pyruvic acid, respectively. Ketoacids were

then determined calorimetrically after their reaction with 2, 4- dinitrophenylhydrazine; and (3) determination of serum total bilirubin and direct bilirubin: Alkaline methanolysis of bilirubin followed by chloroform extraction of bilirubin methyl esters, and later, separation of these esters by chromatography and spectrophotometric determination at 430 nm.

Determination of serum albumin

Quantitative determination of serum albumin level was quantified using rat albumin ELISA kit. The absorbance at 450 nm is a measure of the concentration of albumin in the test sample.

Bioinformatics-based selection of cell cycle-microtubule assembly- and HCC-specific RNA panel

The RNA panel was chosen in three steps: (1) *Tubulin, gamma 1* (*TUBG1*) was retrieved as a crucial player in microtubule formation and progression of the cell cycle through gene atlas expression database (available at https://www.ebi.ac.uk/gxa/home) alongside with verification of the chosen gene from protein Atlas database (available at https://www.proteinatlas.org/), followed by analysis of *TUBG1* gene ontology to ensure that it is linked to *G2-M* transition and mitotic spindle assembly, which is closely linked to cancer development through gene card database (available at https://www.genecards.org/); (2) we selected miR-125b-1-3p targeting *TUBG1* mRNA though) miRWALK database available at http://mirwalk.umm.uni-heidelberg.de/), followed by pathway enrichment, which revealed that miR-125b-1-3p is linked to MAP kinase and ubiquitin-mediated proteolysis (strongly correlated with mitotic assembly and cell cycle progression) through (Diana database available at http://diana.imis.athena-innovation.gr/DianaTools/index.php); and (3) lncRNA metastasis-associated lung adenocarcinoma transcript 1, lncRNA MALAT1, was obtained as a lncRNA specific to HCC and targeting miR-125b-1-3p using noncode database available at http://www.noncode.org/ and European Bioinformatics institute database available at https://www.ebi.ac.uk.

Extraction of total RNA, including lncRNA and miRNA from liver tissue

Total RNA was extracted from liver tissues using miRNeasy Mini kit (Cat No. 217004, Qiagen, United States) according to manufacturer's protocol. The extracted RNA concentration and integrity were assessed using [(NanoDrop Technologies/Thermo Scientific, Wilmington, DE, United States)], with RNA purities were 1.8-2. The extracted total RNA was reverse transcribed into cDNA using a miScript II RT kit (Cat No. 218160, 218161, Qiagen, United States) on thermal cycler (Bio-Rad; Hercules, CA, United States), according to the manufacturer's protocol.

Real time-polymerase chain reaction quantification of the RNA panel in liver tissues

The relative expression of TUBG1 mRNA and lncRNA-MALAT1 in liver tissue were measured using a Quantitect SYBR Green Master Mix kit and an RT2 SYBR Green ROX real time-polymerase chain reaction (qPCR) Mastermix (Qiagen), respectively, using a 7500 qPCR Systems (Applied Biosystems, Foster City, CA, United States) detection system) and specific primers (Accession: NM_001128148, NM_003234, NR_002819, and ENST00000534336, respectively) supplied by Qiagen. GAPDH (Accession NM_002046.7) was used as a housekeeping gene. MiR-3163 expression in liver tissue was quantified by PCR using a miScript SYBR Green kit (Qiagen), a miScript universal primer, and a miRNA-specific forward primer (mir-125b-1-3p miScript Primer Assay) (Accession: MIMAT0004592 (5'-ACGGGUUAGG CUCUUGGGAGCU). All steps followed the manufacturer's suggested protocol, and SNORD68 was used as an internal control. Ct values more than 36 were considered as negative. The specificities of the amplicons for the SYBR Green−based PCR amplification were affirmed by the melting curves. The $2^{-\Delta\Delta Ct}$ technique was used to measure the relative expression of the HCC-specific RNA panel.

Histopathological and immunohistochemical studies

Liver sections were cut at 5-μm thickness and stained with H&E. Immunohistochemistry S-P method was used to detect GSTP and PCNA levels. Briefly, the protocol was as follows: (1) the tissues were treated with endogenous peroxidase blocking solution at room temperature for 10 min, and then, incubated in normal nonimmune serum at room temperature for 10 min; (2) mouse anti GSTP or PCNA antibody was added to adjacent tissue sections respectively and incubated overnight at 4 °C; (3) biotin-conjugated second antibody was added to the sections and incubated at

room temperature for 10 min; and (4) S-P complex was added at room temperature for 10 min, and then, 2,4-diaminobutyric acid was used for the color reaction. The tissue sections were washed with Poly (butylene succinate) (PBS) (0.01 mol/L, pH 7.4) between each step. Positive and negative controls were simultaneously used to ensure specificity and reliability of the staining process. A positive section was taken as positive control. In negative control, PBS was used to replace the first antibody. GSTP-positivity was indicated by brown coloration of the cytoplasm. Morphometric analysis of the foci percent area was carried out using Leica Q win V.3 software after capturing the images using a Leica DM2500 microscope (Leica, Wetzlar, Germany). The PCNA labeling indices are represented as the expression of positively-stained nuclei (10 fields/slide at 400 ×) as shown below: +, positive expression found in 1-3 fields; ++, positive expression found in 4-6 fields; and +++, positive expression found in 7-10 fields. Rabbit monoclonal PCNA and GSTP antibodies were purchased from Abcam (San Francisco, United States).

Statistical analysis

All results were expressed as mean ± SD. Statistical analysis was carried out using GraphPad Prism version 6.01. Unpaired t-test was done to compare between naïve and PCL groups. One-way ANOVA, followed by Tukey's test, was carried out to compare between all groups. P values < 0.05 were considered statistically significant.

RESULTS

Effect on AFP levels and liver function tests

As shown in Figure 1, following induction of PCL, rats exhibited a significant increase in AFP levels. AFP levels were significantly decreased in cyan-10, cyan-15, and cyan-20 groups compared to PCL group. There was no significant difference between the AFP levels of the three cyan groups. Liver function tests were affected after receiving DEN/2-AAF in which rats exhibited a significant increase in levels of ALT and total and direct bilirubin, and a significant decrease in the serum albumin (Figure 2). As shown in Figure 2A, rats received cyan. in different doses exhibited a significant decrease in serum ALT compared to PCL group. Cyan-15 group showed significant decrease in serum ALT compared to cyan-10 group. Moreover, cyan-20 group showed significant decrease in serum ALT compared to cyan-10 group. In As depicted in Figure 2B, compared to PCL group, the rats in the three cyan groups exhibited a significant decrease in serum total bilirubin. Furthermore, cyan-20 group showed significant decrease in serum total bilirubin compared to cyan-10 group. Figure 2C: Serum direct bilirubin was significantly decreased in the three cyan groups compared to the PCL group. Moreover, cyan-20 group showed significant decrease in serum direct bilirubin compared to cyan-10 group. Administration of cyan in doses 5, 10 and 20 mg/kg/d produced a significant increase in serum albumin compared to PCL group. However, there was no significant difference between the serum albumin levels of the three cyan groups (Figure 2D).

Effect on expression of lncRNA MALAT1, miR-125b, and TUBG1 (G2/M transition of mitotic cell cycle) in the liver tissue

As shown in Figure 3, induction of PCL resulted in significant increase of lncRNA MALAT a significant decrease in RQ miR-125b and significant increase in RQ TUBG1 expressions in comparison to naïve group. RQ of lncRNA MALAT1 in the liver tissue was significantly decreased in cyan -10,15 and cyan-20 groups compared to PCL group. Furthermore, cyan-20 group showed significant decrease in RQ of lncRNA MALAT1 in the liver tissue compared to cyan-10 group (Figure 3A). On the contrary, RQ of miR-125b in the liver tissue was significantly increased in cyan -10, cyan-15 and cyan-20 groups compared to PCL group. In addition, cyan-20 group showed significant increase in RQ of miR-125b in the liver tissue compared to cyan-10 group (Figure 3B). Furthermore, RQ of TUBG1 in the liver tissue was significantly decreased in cyan-10, cyan-15 and cyan-20 groups compared to PCL group. Also, cyan-20 group showed significant decrease in RQ of TUBG1 in the liver tissue compared to cyan-10 group (Figure 3C).

Histopathological and immunohistochemical studies

Light microscopic examination of H&E-stained sections of naïve rats' liver showed normal hepatic architecture, cords of hepatocytes radiating from central vein, portal

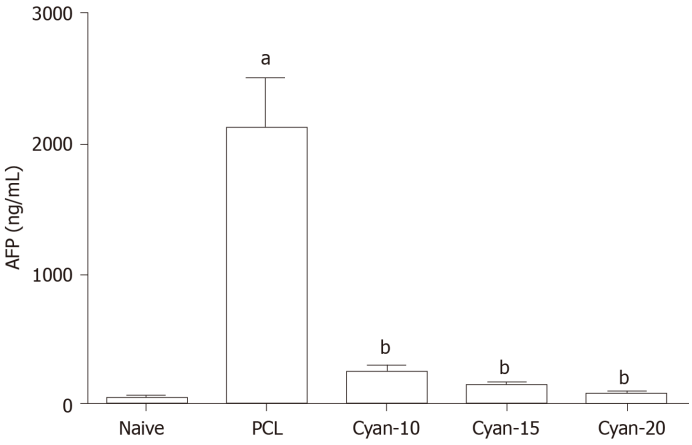


Figure 1 Effect of cyanidin 3-glucoside at different doses (10, 15 and 20 mg/kg/d) on alpha fetoprotein in the serum of rats. Values are mean ± SEM; number of animals = 6 rats/each group. [a]*P* < 0.05 significant differences compared to naïve group, unpaired t test; [b]*P* < 0.05 significant differences compared to precancerous lesion group. PCL: Precancerous lesion; Cyan: Cyanidin-3-O-glucoside; AFP: Alpha fetoprotein.

triads present in between, polygonal hepatocytes with centrally rounded vesicular nuclei, and hepatic sinusoid in between (Figure 4A and B). Liver sections obtained from rats received DEN/2-AAF (Figure 4C-E) showed disruption of normal hepatic lobular architecture along with presence of large, discriminated dysplastic nodules compressing the surrounding liver tissue, with high-grade dysplastic cells displaying increased nuclear: Cytoplasmatic ratio (Figure 4E), nuclear hyperchromatosis, and basophilic cytoplasm. Liver sections of rats treated with different doses of cyanidin (10, 15, and 20 mg/kg) respectively, showed small and less discriminated dysplastic nodules (Figure 4F-H).

Immunohistochemical examination with GSTP antibody appeared as large hyperplastic nodules were demonstrated by the brown color. Figure 5A and B shows the liver sections of naïve group, while Figure 5C shows large-positively stained GSTP foci demonstrated in PCL group. Liver sections from rats of all cyan-treated groups exhibited small GSTP-positive small hepatic foci of different sizes scattered in-between negatively-stained hepatic parenchyma (Figure 5D-F). The percent area of GSTP foci was significantly increased in the PCL group compared to naïve group, while all cyan-treated groups showed significant decrease in the GSTP percent area. Furthermore, cyan-15 and -20 showed significant decrease in the GSTP percent area over cyan-10 (Table 1). PCNA immunohistochemical analysis showed an elevated expression of PCNA in the group that received DEN/2-AAF as compared to naïve group (Figure 6A and B). Liver sections obtained from rats received cyan. in its three doses showed decrease in PCNA-positively stained nuclei (Figure 6C-E and Table 1).

DISCUSSION

Altered expression of mitotic spindle assembly genes has been detected in many cancers. For example, *MAD2* gene levels are downregulated in breast carcinoma[18], while mitotic checkpoint serine/*BUB1* gene expression is dysregulated in colorectal carcinoma[19]. The impairment of spindle assembly genes are frequently detected in HCC[20,21]. Moreover, inhibition of the mitotic assembly genes has been found to be lethal to cancer cells, and has promising therapeutic effect in cancer treatment[22,23].

In this study, we used several public microarray databases for the construction of simple genetic-epigenetic network specific to HCC and linked to cell cycle and mitotic spindle formation. We assessed the antiproliferative effect of cyan in HCC animal model *via* modulation of lncRNA-MALAT1-miR-125b-TUBG1 mRNA axis expression.

Microtubules are involved both in maintenance of cell shape and motility and in mitotic spindle formation[24]. γ-TUBG is a major player that supports microtubule nucleation. γ-TUBG acts as a binding site for the α/β-tubulin dimer and it has two *γ-TUBG* genes, TUBG1 and TUBG2 in mammals only[25]. The TUBG levels in the centrosome change during cell cycle progression and usually increases at the start of mitosis. At the end of mitosis, TUBG levels are decreased to interphase levels[26]. Overexpression and altered compartmentalization of γ-TUBG may lead to carcinogenesis[27-29]. Interestingly, γ-TUBG associates interact with tumor suppressor

Table 1 Expression rate of hepatocytes positive for glutathione S-transferase placental and proliferating cell nuclear antigen were calculated as number of positive field expression in 10 fields *per* rat liver tissue

	Naive	PCL	Cyan-10	Cyan-15	Cyan-20
GSTP +ve foci % area	0.09 ± 0.07	47.2 ± 12.4[a]	33.4 ± 11.4[b]	8.95 ± 4.29[b,c]	3.83 ± 2.45[b,c]
PCNA		+++	++	+	+

Values are mean ± SD; number of animals = 6 rats/each group. Examined field; 10 fields/liver section.
[a]$P < 0.05$ significant differences compared to naïve group.
[b]$P < 0.05$ significant differences compared to precancerous lesion group.
[c]$P < 0.05$ significant compared to cyanidin-3-O-glucoside-10, One-way ANOVA followed by Tukey's test. +: Positive expression found in 1-3 fields; ++: Positive expression found in 4-6 fields; +++: Positive expression found in 7-10 field; PCL: Precancerous lesion; Cyan: Cyanidin-3-O-glucoside; GSTP: Glutathione S-transferase placental; PCNA: Proliferating cell nuclear antigen.

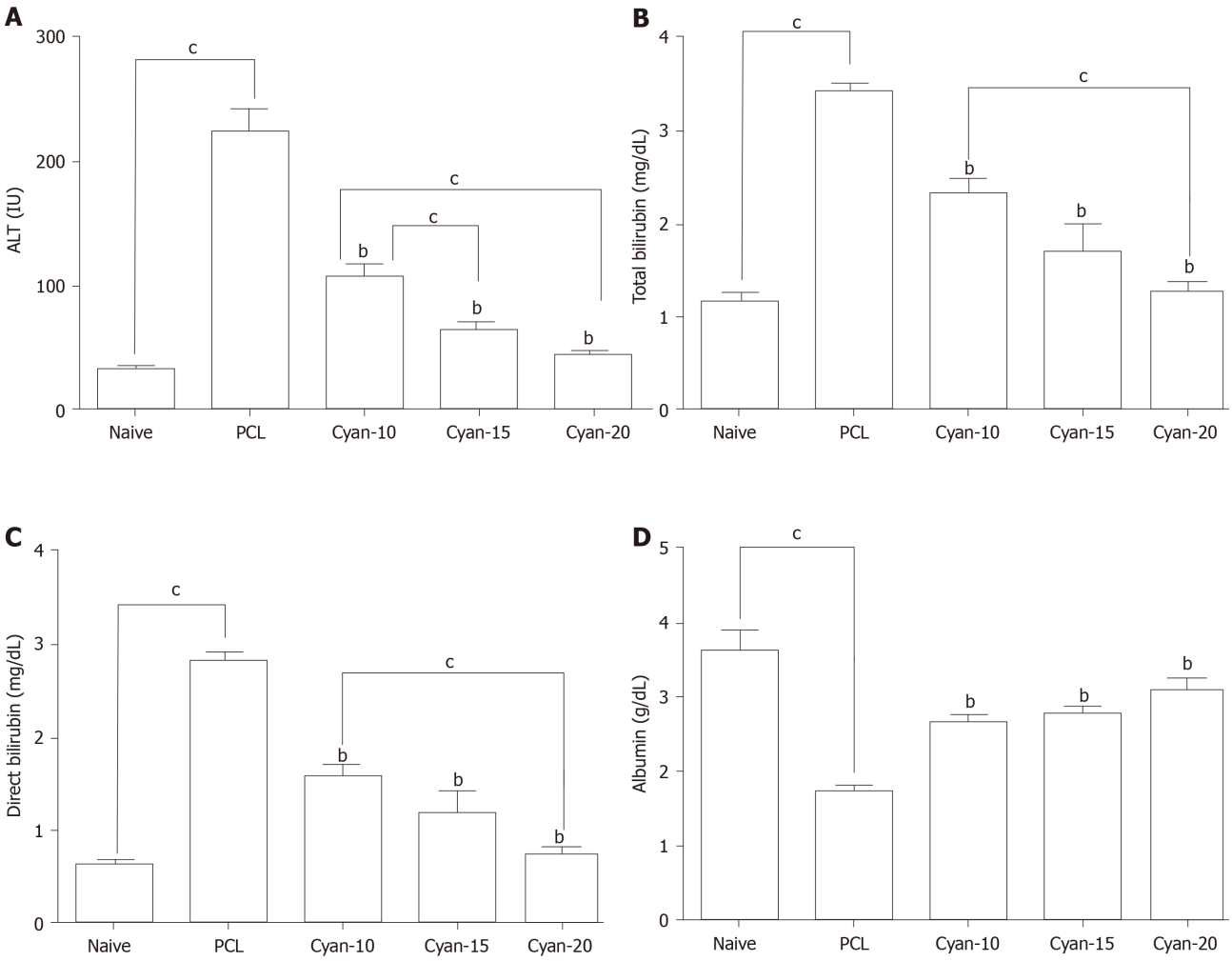


Figure 2 Effect of cyanidin 3-glucoside at different doses (10, 15 and 20 mg/kg/d) in rats. A: Alanine aminotransferase; B: Total bilirubin; C: Direct bilirubin; D: Serum albumin. Values are mean ± SEM; number of animals = 6 rats/each group. [b]$P < 0.05$ significant differences compared to precancerous lesion group; [c]$P < 0.05$ significant differences between the 2 selected groups, One-way ANOVA followed by Tukey's test. PCL: Precancerous lesion; Cyan: Cyanidin-3-O-glucoside; ALT: Alanine aminotransferase.

protein C53 in mammalian interphase cells of different cellular origins[30]. Interestingly, γ-TUBG interacts with E2F transcription factors, resulting in upregulation of E2F target genes PCNA and CDKB1[31] and glutathione S transferase activity[32]. On the other hand, the mRNA and protein levels of TUBA1C are upregulated in HCC. Clinically, it affects the survival of patients and the level of metastatic burden along with portal vein thrombosis. In addition, it activates cellular proliferation and migration and is a potential therapeutic target for HCC[33]. Recent studies have shed light on the efficacy of microtubule-binding agents in the treatment of HCC, especially

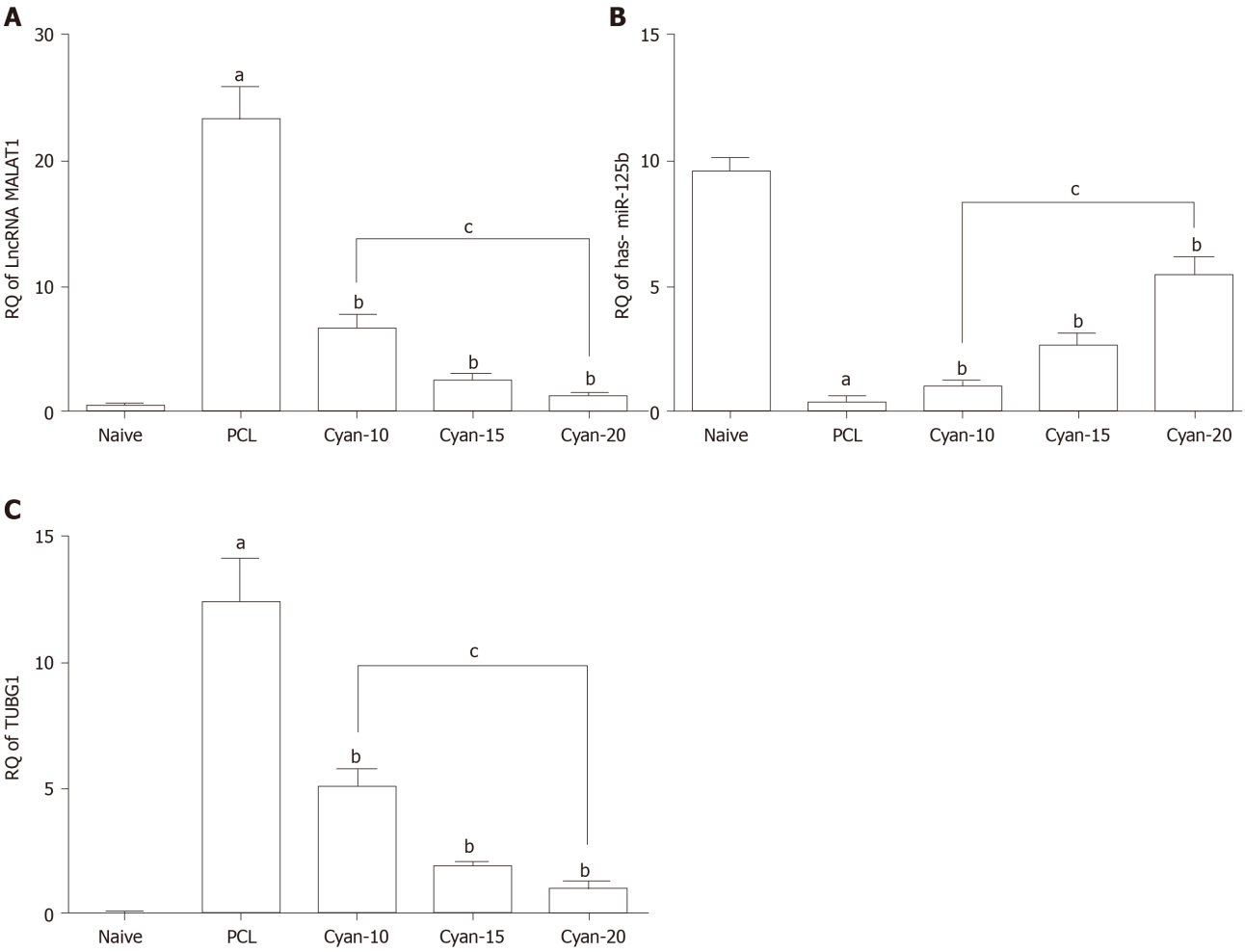


Figure 3 Effect on expression of of long non-coding RNA, MALAT1, miR-125b and tubulin 1 (G2/M transition of mitotic cell cycle) in the liver tissue. A: RQ of long non-coding RNA MALAT1; B: RQ miR-125b; C: RQ TUBGI . Values are mean ± SEM; number of animals = 6 rats/each group. $^{a}P < 0.05$ significant differences compared to naïve group; $^{b}P < 0.05$ significant differences compared to precancerous lesion group; $^{c}P < 0.05$ significant differences between the 2 selected groups, One-way ANOVA followed by Tukey's test. PCL: Precancerous lesion; Cyan: Cyanidin-3-O-glucoside; TUBG: Tubulin gamma 1; lncRNA: Long non-coding RNA.

in combination with mammalian target of rapamycin inhibitors[34].

One of the few miRNAs that regulate Spindle Assembly Checkpoints (SAC) is miR-125b. It suppresses the expression of Mad1 (a core SAC protein) that is responsible for inhibiting entry into anaphase till metaphase defects are corrected[35]. Importantly, miRNA-125b has been found to be associated with colorectal cancer[36], multiple myeloma[37], and breast cancer[38]. Song *et al*[39] demonstrated that the expression of miR-125b in hepatocytes was protective and reflected better survival in HCC tissue samples due to restoration of SIRT6 expression[39]. miR-125b-5p inhibits HCC proliferation by targeting TXNRD1, the terminal step of biosynthesis of nucleotides[40].

Deregulation of lncRNAs has been shown to be crucial in cancer progression, especially HCC[41,42]. Hung *et al*[43] demonstrated the involvement of lncRNAs in regulation of the expression of cell cycle-related genes, p53 gene regulatory pathway, and apoptosis[43,44]. The lncRNA MALAT1 is overexpressed in several solid tumors and is linked with tumor recurrence[45-47]. MALAT1 coordinate RNA polymerase II transcription, pre-mRNA splicing, and mRNA export[48]. Thus, this lncRNA holds great potential in influencing the local concentration of a specific splicing factor during specific stages of the cell cycle. Depletion of MALAT1 in cancer cells inhibits tumorigenicity[49]. MALAT1 regulates E2F1 transcription factor activity, highlighting its pro-proliferative function[50]. MALAT1 is upregulated in many cancer types, including breast cancer[51], cervical cancer, lung cancer[52], and hepatocarcinoma[53-55]. In a previous study, hypoxia enhanced MALAT1 expression, leading to increase in proliferating and invading activity of Hep3B cells due to negative interaction with miR200a[56]. Huang and his group explored the role of specificity protein 1/3 in regulation of MALAT1 expression in HCC cells[57].

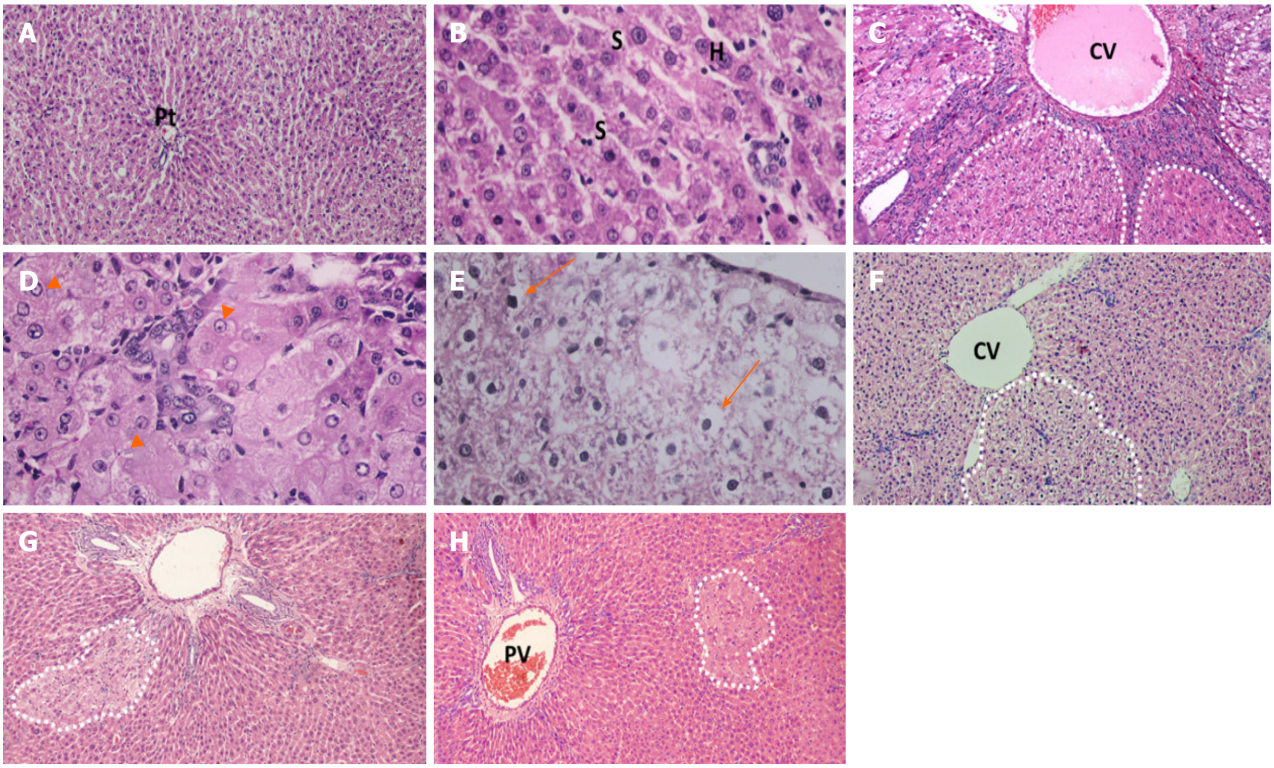


Figure 4 Photomicrographs of liver sections stained with H&E staining. A and B: Naïve group liver sections showed normal hepatic architecture, cords of hepatocytes radiating from central vein and portal triads present in-between and polygonal hepatocytes with central rounded vesicular nuclei, and hepatic sinusoid in-between; C-E: Liver sections of rats received diethylnitrosamine/2-acetylaminofluorene (DEN/2-AAF) showed larger, discriminated dysplastic nodules (doted shapes) compressing the surrounding liver tissue with disruption of normal hepatic lobular architecture; D: Liver sections of rats received DEN/2-AAF showed eosinophilic foci of cellular alteration consisting of enlarged hepatocytes with increased acidophilic staining and vaculated nuclei (arrow head); E: Liver sections of rats received DEN/2-AAF showed foci of Clear cell formed of hepatocytes showing variable degrees of cytoplasmic vacuolations and ballooning with pyknotic nuclei (arrow); F-H: Liver sections of rats treated with different doses of cyanidin (10, 15, 20 mg/kg) respectively, showing small and less discriminated dysplastic nodules (doted shapes). (Magnification: A, C, F, G, H × 1000; B, D, E × 400). CV: Central vein; PV: Portal vein; Pt: Portal triads; S: Sinusoid; H: Hepatocytes.

Cyanidin or anthocyanin (a polyphenolic pigment found in plants) confers several pharmacological benefits, including anticancer properties[58-62]. Cyan mediates cytotoxicity against human monocytic leukemia cells *via* G2/M phase arrest and induction of apoptosis[63]. Tsai *et al*[64] evaluated anthocyanins as promising anticancer or chemopreventive agents to induce G2/M arrest in HAs-induced leukemia cell cycle arrest *via* ATM-Chk1/2-Cdc25C axis modulation[64]. They also found that anthocyanin inhibits the proliferation of HCC cells[65,66].

In our study, the effect of cyan on cancerous tissue was dose-dependent (10, 15 and 20 mg/kg/d), that is, increasing its effect with increasing the concentration of the drug. This was proved also, with the correlated significant decrease in the percent area of GSTP foci, PCNA expression which are the target of TUBG1 mRNA and the molecular epigenetic markers associated with HCC (lncRNA MALAT1 and miR-125b-1-3p) in the liver tissue of rats in our study (Figure 7). Thus, we can hypothesize that administration of cyan in PCL may result in downregulation of lncRNA MALAT1 with subsequent release of free miR-125b-1-3p that binds to TUBG1 mRNA and downregulate its expression[65]. The changes in the epigenetic-genetic network affect their target genes PCNA and GSTP.

Lastly, the cyan-mediated modulation of cell cycle and mitotic spindle assembly that regulate HCC proliferation might affect the capability of the HCC cells to overcome oxidative stress[66,67]. Our data confers the rationale for designing antimitotic approach combining the conventional cytotoxic drugs with phytochemical extracts to inhibit cell cycle progression in cancer. More *in vitro* functional studies are required to explore the functional mechanism of the chosen RNA panel and validate their role as drug target biomarkers.

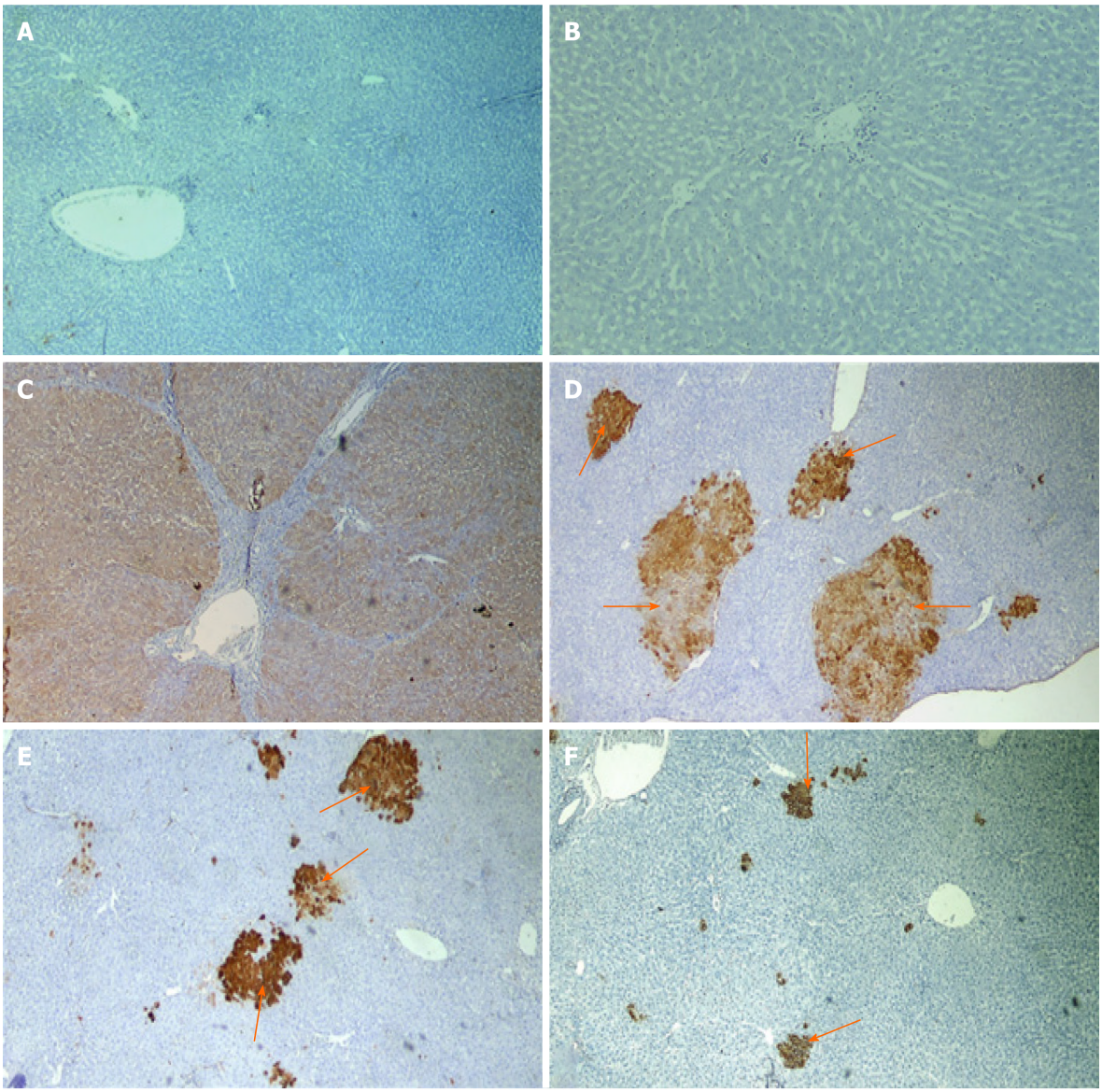


Figure 5 Photomicrographs of liver sections of rats immunohistochemical stained with glutathione S-transferase placental antibody. A and B: Naive group; C: Precancerous lesion group showing multiple glutathione S-transferase placental (GSTP)-positive large hepatic nodules (brown stained nodules) occupying most of section; D-F: Liver sections of rats treated with different doses of cyanidin (10, 15, 20 mg/kg) showing GSTP positive small hepatic foci (brown stained cells = arrow) of different size scattered in-between negatively stained hepatic parenchyma. (Magnification: × 40)

CONCLUSION

Cyanidin is a natural molecule that holds great potential in unraveling the mystery of cytotoxic pharmacy in the future. It can be used to devise novel antimitotic drugs that can target cell cycle in HCC. Our results also indicated great potential of the TUBG1 mRNA- miR-125b-lncRNA MALAT1 and -1-3p axis as potential drug target biomarker for further investigations.

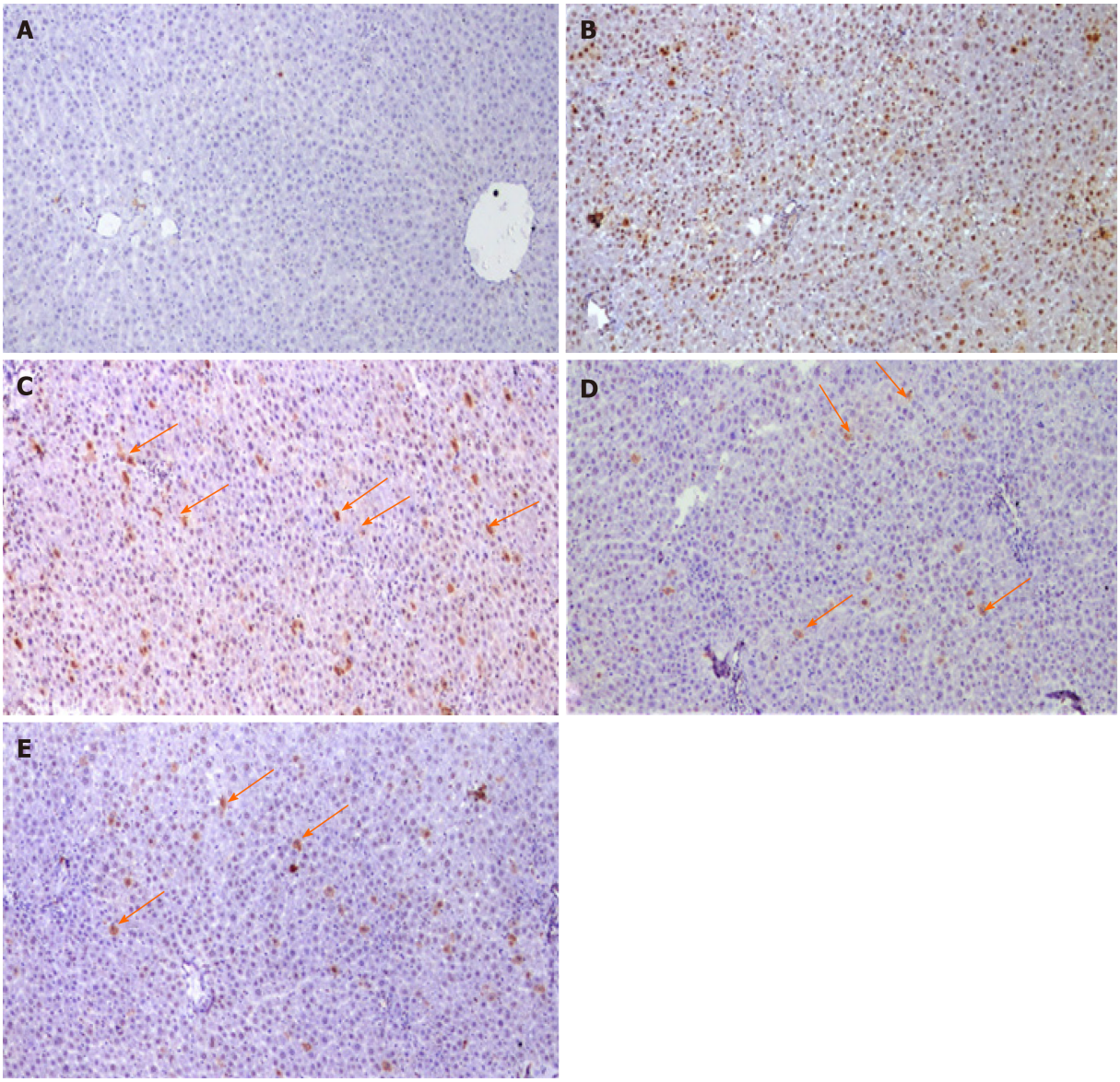


Figure 6 Photomicrographs of liver sections Immunohistochemical stained with proliferating cell nuclear antigen. A: Negative reaction of control group; B: Diethylnitrosamine + 100 mg 2-acetylaminofluorene group showing positive stained nuclei scattered all over the field; C-E: Liver sections of rats treated with different doses of cyamidine (10, 15, 20 mg/kg) respectively, show few positive hepatocytes sporadically distributed over the field (arrow). (Magnification × 100).

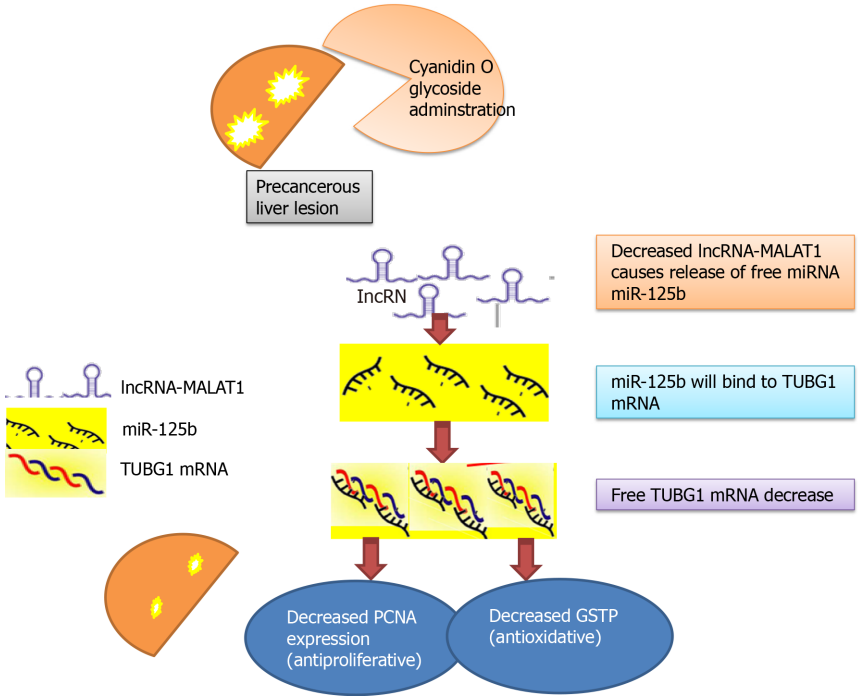


Figure 7 Concept map of study design. GSTP: Glutathione S-transferase placental; PCNA: Proliferating cell nuclear antigen; TUBG: Tubulin; lncRNA: Long non-coding RNA.

ARTICLE HIGHLIGHTS

Research background

Several regulatory RNA networks are important in regulation of liver cell cycle progression.

Research motivation

Cyanidin-3-glucoside (cyan) is a potential chemotherapeutic and chemo-protective agent.

Research objectives

The present study aimed to investigate the effect of cyan administration on cell cycle in hepatic precancerous lesion induced by diethylnitrosamine/2-acetylaminofluorene in Wistar rats.

Research methods

We used bioinformatic analysis followed by experimental validation.

Research results

Cyan dose dependently decreased the long non-coding RNA-MALAT1 and tubulin gamma 1 mRNA expressions and increased the hsa-miR-125b expression which participate in cell cycle and mitotic spindle assembly. Cyan administration decreased alpha-fetoprotein and improved liver function. Cyan decreased glutathione S-transferase placental foci percent area and proliferating cell nuclear antigen positively stained nuclei.

Research conclusions

Cyanidin may offer a natural molecule to unravel the mystery a cytotoxic pharmacy for the future.

Research perspectives

Further larger *in vitro* and *in vivo* studies are needed to elucidate the mechanism of cyanidin cytotoxicity in hepatocellular carcinoma.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Stapled transperineal repair for low- and mid-level rectovaginal fistulas: A 5-year experience and comparison with sutured repair

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Abstract

BACKGROUND

Currently, rectovaginal fistula (RVF) continues to be a surgical challenge worldwide, with a relatively low healing rate. Unclosed intermittent suture and poor suture materials may be the main reasons for this.

AIM

To evaluate the efficacy and safety of stapled transperineal repair in treating RVF.

METHODS

This was a retrospective cohort study conducted in the Coloproctology Department of The Sixth Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). Adult patients presenting with RVF who were surgically managed by perineal repair between May 2015 and May 2020 were included. Among the 82 total patients, 37 underwent repair with direct suturing and 45 underwent repair with stapling. Patient demographic data, Wexner faecal incontinence score, and operative data were analyzed. Recurrence rate and associated risk factors were assessed.

RESULTS

The direct suture and stapled repair groups showed similar clinical characteristics for aetiology, surgical history, fistula features, and perioperative Wexner score.

University, Guangzhou, China (No. E2018012).

Informed consent statement: Patients underwent sutured or stapled repair surgery after each agreed to treatment by written consent.

Conflict-of-interest statement: The authors declare having no conflicts of interest related to this study or its publication.

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The stapled repair group did not show superior results over the suture repair group in regard to operative time, blood loss, and hospital stay. However, the stapled repair group showed better postoperative Wexner score (1.04 ± 1.89 *vs* 2.73 ± 3.75, $P = 0.021$), less intercourse pain (1/45 *vs* 17/37, $P = 0.045$), and lower recurrence rate (6/45 *vs* 17/37, $P = 0.001$). There was no protective effect from previous repair history, smaller diameter of fistula (< 0.5 cm), better control of defecation (Wexner < 10), or stapled repair. Direct suture repair and preoperative high Wexner score (> 10) were risk factors for fistula recurrence. Furthermore, stapled repair gave better efficacy in treating complex RVFs (*i.e.*, multiple transperineal repair history, mid-level fistula position, and poor control of defecation).

CONCLUSION

Stapled transperineal repair is advantageous for management of RVF, providing a high primary healing rate and low recurrence rate.

Key Words: Rectovaginal fistula; Surgical repair; Transperineal approach; Stapled technique; Recurrence

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Core Tip: This retrospective cohort study evaluated efficacy and safety of the novel usage of stapling in repairing rectovaginal fistula (RVF). The overall recurrence risk of patients treated by staple repair was significantly lower than that of patients who underwent the conventional direct suture transperineal repair, especially for cases of complex RVFs, including multiple repair history, ≥ 10 preoperative Wexner score, or mid-level RVF status. According to the 5-year follow-up experience, stapled repair appears to be a promising surgical option for treating RVF, with high efficacy and safety.

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INTRODUCTION

Rectovaginal fistula (RVF) continues to be a surgical challenge worldwide, presenting with a variety of symptoms, which include the passage of air and/or stool from the vagina or development of urinary and/or vaginal sepsis. The aetiology of RVF is mixed, but it most commonly results from obstetric trauma (with 3rd or 4th degree perineal lacerations), perianal Crohn's disease[1], radiation damage or malignancy, and complication of anorectal or gynaecological surgery[2]. Each among the range of RVF-related symptoms exerts a significant negative impact on the patient's quality of life and may substantially limit social interaction and even independence[3].

The vast majority of RVF cases require surgical treatment; despite a variety of surgical approaches being in use, primary healing rates in many series remain variable and low[4-8]; moreover, the risk of a poor longer term outcome and the need for more than one surgical repair remain appreciable[9,10]. Rupture of the incisions in the rectum and perineal diaphragm is a major cause of recurrence, with the incision dehiscence largely owing to the high local pressure exerted on the incision itself[9]. In conventional repair, the intermittent suture method often results in inadequate tight closure of the incision. The intervals of stitches are vulnerable to be pressed open by the rapid increase of local pressure when defecation. In addition, the silk thread suture is not reliable to withstand local high pressure, as it cracks easily. Therefore, looking for stronger suture materials and adopting a continuous suture to stabilize the incision may effectively reduce the risk of incision fracture. The stapled cartridge used in stapler operation is both continuous and close in its closure arrangement, facilitating

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achievement of tight closure in our experience.

Given the range of surgical approaches, both the surgeon and the patient may be left unsatisfied with the result and with the occasional consequences of stoma, diminished sphincter function, poor perineal healing, and dyspareunia. It is accepted that outcomes for RVF repairs are influenced by many factors, including the primary aetiology, the type and times of prior repair, the use of a temporary diverting stoma, the time interval between recurrence of symptoms and a subsequent repair, the underlying integrity of the sphincter and the perineal body, any prior history of irradiation, and patient comorbidity[11-13]. Patients with Crohn's disease represent a particular group with uniformly worse results, wherein a combination of medical and surgical treatment by a healthcare management team is required[14-16].

Patients may be classified into anovaginal and RVFs, with sub-categorization of the RVF cases into low *vs* high and simple *vs* complex[17]. The location of the fistula defines the operative approach (*i.e.*, anal, perineal, or vaginal). Low RVF is typically located through or distal to the anal sphincter complex, with communication to the vaginal introitus above the dentate line. High RVF has its vaginal opening near the cervix, with a mid-level RVF defined as situated at a point between that of a low and a high case. Complicated cases are more likely to require some form of interposition graft and/or faecal diversion. Simple fistulas are typically small in size (< 2.5 cm in diameter), more distally located, and have either a traumatic or cryptoglandular origin. In contrast, complex fistulas include cases with an inflammatory bowel disease origin, relation to cancer or radiation treatment, and a recurrent RVF following an unsuccessful prior repair. The wide range of options for RVF repair reflect these varying aetiologies and the armamentarium available for recurrent cases. Beyond primary repair, these options selectively include a mucosal advancement flap (with or without sphincteroplasty), muscle or soft-tissue interposition (Martius grafting, graciloplasty, and biologic mesh interposition), fibrin glue, fistula plugs, ligation of the intersphincteric fistula tract (known as LIFT) procedure, and faecal diversion[18].

Our group has previously reported the safety of the novel use of a stapled transperineal repair by means of the Echelon Flex 60 Endopath (Ethicon Endo-Surgery Inc., Cincinnati, OH, United States) over a medium-term follow-up, as determined in a pilot series of obstetric-related non-Crohn's disease cases, showing a high success rate[19]. We have now assessed a retrospective cohort of patients presenting with low- and mid-level RVF, comparing outcomes and the probability of recurrence between those undergoing either a direct sutured or stapled fistula closure.

MATERIALS AND METHODS

Study design

Ethical permission for conduct of the study was provided by the Ethics Committee of The Sixth Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). Patients presenting with an RVF who were surgically managed by perineal repair between May 2015 and May 2020 were included in the analysis. Patients were identified from a prospectively maintained database, crosschecked with operating theatre records of The Sixth Affiliated Hospital of Sun Yat-sen University (a university-affiliated 1383-bed tertiary referral centre, with 800 dedicated beds for coloproctology).

For comparison, patients undergoing standard direct suture repair were classified as Group 1 and those who underwent stapled transperineal repair were included in Group 2. In the event of a repeat repair, a minimum waiting period of 6 mo posto-perative from the previous repair was needed. All patients underwent a consultant clinical examination, colonoscopy, anorectal manometry (Manoscan™360; Sierra Scientific Instruments, Los Angeles, CA, United States), and magnetic resonance imaging (Optima MR360, 1.5 T; GE Healthcare, Waukesha, WI, United States) of the anal sphincter[6].

Patients with only a low-level or mid-level RVF (as defined) were included in the analysis. The exclusion criteria were < 18 years of age, underlying inflammatory bowel disease or diagnosed Crohn's disease, malignant cases of RVF, and cases where the RVF was associated with active perianal sepsis and/or an undrained perianal abscess (Figure 1).

Surgical procedure

Our group has previously described the stapled RVF repair[19]. Briefly, all patients underwent a preoperative rectal and vaginal lavage with a Betadine wash. One hour prior to surgery, ciprofloxacin and/or metronidazole was administered intravenously.

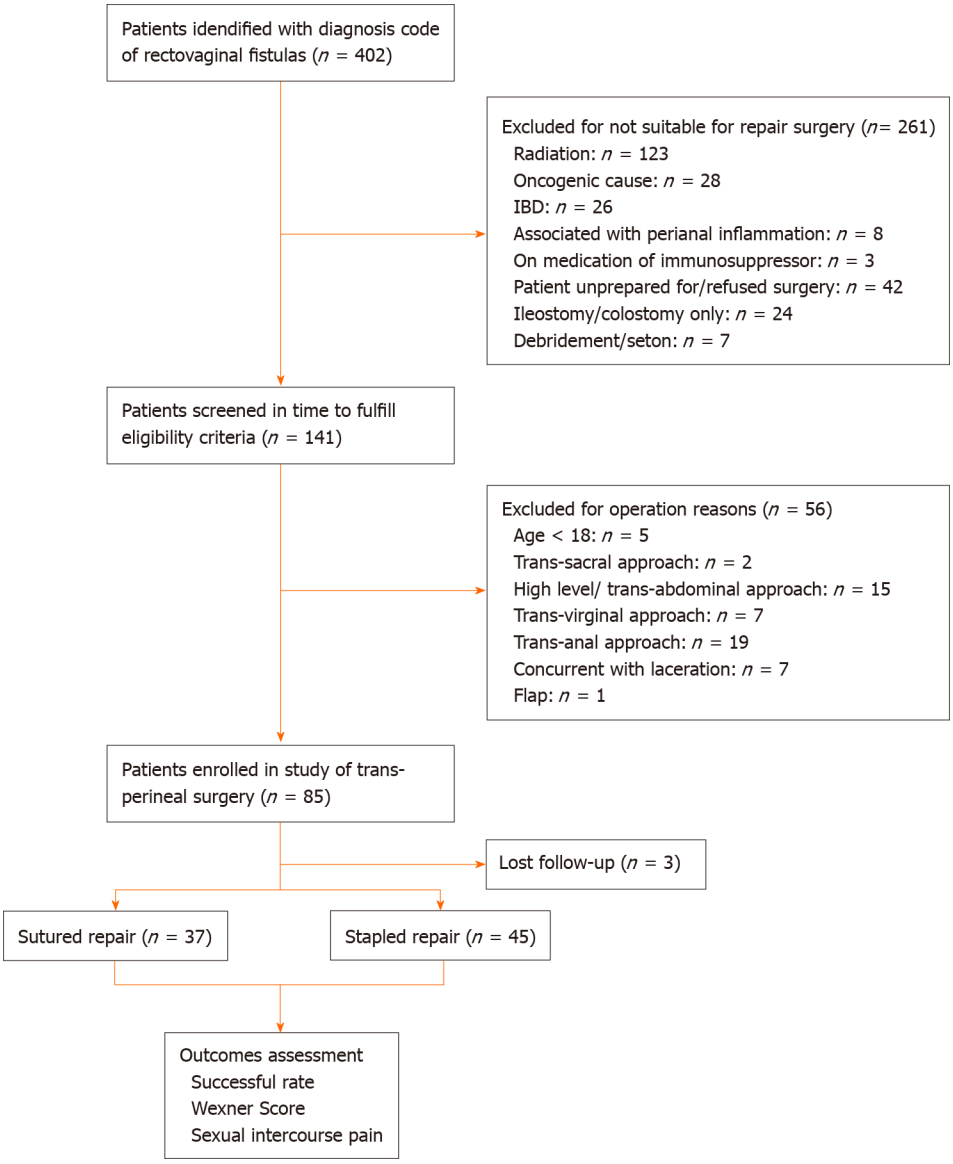


Figure 1 Participant enrolment and follow-up. A total number of 402 rectovaginal fistula patients were treated at The Sixth Affiliated Hospital of Sun Yat-sen University between May 2015 and May 2020. Among them, 85 were treated by stapled or sutured transperineal repair in our hospital. Apart from three patients who were lost to follow-up, 82 patients were included in this study. The clinical data for each were collected and analysed retrospectively. A total of 37 patients underwent conventional direct sutured transperineal repair and were classified as Group 1, and 45 patients who underwent stapled repairs were included in Group 2. Basic characteristics, perioperative details, and surgical outcomes were evaluated. IBD: Inflammatory bowel disease.

Following induction of the anaesthesia, a urinary catheter was inserted with the patient in the lithotomy position. A Lonestar retractor (Saisheng Medical Technology Co., Ltd, Changzhou, Jiangsu Province, China) was used for exposure, with insertion of a No. 6 Fr Nelaton tube into the fistula, so as to identify the fistula during the dissection. A "U-shaped" incision was then made in the region, mid-way between the vagina and the rectum, with dissection of the rectovaginal septum and injection in the dissection area of a 1:200000 epinephrine-saline solution in order to facilitate a bloodless dissection. Slight traction on the Nelaton tube assisted in identification of the fistula from surrounding normal tissues.

For those patients in Group 1, the fistula was sharply severed with a scalpel, after which the rectum and vagina were closed separately with interrupted 2/0 or 3/0 Vicryl (Ethicon Endo-Surgery Inc.) sutures. No specific attempt was made to reduce the suture line tension or to interpose tissue to support the rectovaginal septum. In Group 2 patients, the Echelon Flex 60 Endopath (Ethicon Endo-Surgery Inc.) stapler was used for fistula closure by means of a stapled cartridge (3.5 mm staple height, 1.5 mm when closed). The staples left on the vaginal wall side were removed, after which the defect in the vaginal wall was closed by direct suture. After clear identification of the edges of the external anal sphincter, those cases where an external anal sphincter defect had been demonstrated by preoperative imaging underwent an attendant anal

sphincteroplasty. Equally, if there was any tension on the suture line, a levatorplasty was also performed using a continuous absorbable monofilament barbed Stratafix (Ethicon Endo-Surgery Inc.) suture. The skin was closed over a suction drain, with a gauze roll pack inserted in the vagina. Both antibiotics were continued for 72 h postoperatively, with removal of the gauze roll in the ward after 48 h and removal of the drain usually till the drainage less than 5 mL during a 48-h period.

Assessment parameters and outcome

Patient demographic data collected included age, fistula aetiology, fistula duration, the measured distance of the lower edge of the fistula from the anal and vaginal margins, and the fistula diameter. The Wexner faecal incontinence score was determined before surgery[20]. Operative data collected included the operative time (interval between the beginning of the operation and commencement of application of the dressing) and the degree of intraoperative blood loss (based on the number of gauze pads used). Postoperative complications (*e.g.*, wound infection, wound dehiscence, and anal canal stenosis) were recorded, along with the use of a diverting stoma and the length of hospital stay (referred to as LOHS). Patients were routinely followed with clinical examination and performance of Wexner scoring at 1 mo after surgery, as well as with recording of any reported dyspareunia in sexually active patients. Recurrence of a fistula was specifically assessed in patients who reported vaginal flatus or faecal discharge undergoing repeat imaging.

Statistical analysis

Statistical analyses were performed using the SPSS statistical software package (version 26.0; IBM Corp., Armonk, NY, United States). All statistical methods used in this study were evaluated by an expert in Biomedical Statistics (Department of Medical Statistics, Sun Yat-sen University). Student's *t*-test was used for comparisons where data were normally distributed and with Wilcoxon's rank sum test for non-normally distributed data. Categorical data were assessed by either a chi-squared test or a Fisher's exact test, where appropriate. Groups 1 and 2 were compared using the log rank test, with significant variables on univariate analysis inserted into a multivariate Cox regression model. Risk factors for fistula recurrence were identified with graphic construction of a projected risk using the Kaplan-Meier method[21]. *P* values < 0.05 were considered statistically significant.

RESULTS

A total of 82 patients with mid- and low-level RVF were included in the analysis, with 37 patients in Group 1 (direct sutured repair) and 45 patients in Group 2 (stapled repair). The overall mean age was 37.82 ± 12.63 years, with division of the groups into those < 60 and ≥ 60 years of age. Given the exclusion criteria, the commonest aetiologies of RVF in the cohort included post-obstetric (n = 39; 47.56%), congenital-related (n = 18; 21.95%), post-anorectal surgical (n = 24; 29.27%), and other (traumatic, n = 1; 1.22%) causes. The overall mean duration of disease was 110.38 ± 141.4 mo, with the fistula opening located a mean distance of 2.26 ± 1.33 cm from the anal margin and 1.76 ± 1.22 cm from the vaginal introitus. The mean fistula width was 0.69 ± 0.46 cm, with overall 0.48 ± 0.83 prior attempts at RVF repair. The mean preoperative Wexner score for the entire cohort was 6.77 ± 3.45, with sub-categorization of the groups by < 10 or ≥ 10 on the scale. The mean follow-up period was 13.7 mo (range: 1.0-54.7 mo).

Table 1 shows the clinical characteristics of the patients. The etiological distribution of patients was quite different between the two groups, but the majority of cases were secondary to surgery, particularly conventional surgery. Other parameters showed no statistical difference between the two groups. Table 2 shows the intraoperative factors, where there was no statistical difference noted between Group 1 and Group 2 cases in operative time, intraoperative blood loss, or LOHS. Compared with Group 1, the postoperative Wexner score was significantly lower in Group 2. Otherwise, the Wexner score was largely improved within both of the two groups after surgery. There was no difference in the incidence of postoperative dyspareunia, with one patient from each group complaining of new-onset painful intercourse. There were 17 recurrent cases in Group 1 and significantly less in Group 2 (n = 6; P = 0.001).

As shown in Table 3, univariate analysis of factors affecting recurrence showed significance for previous repair history, fistula diameter, surgical technique, and preoperative Wexner score, with a non-significant effect noted for obstetrical fistulas, occurrence secondary to surgery, and existing intestinal stoma. In multivariate

Table 1 Clinical characteristics of the patient groups

Variable	Group 1	Group 2	Test value	*P* value
	n = 37	*n* = 45		
Age[1], yr, *n* (%)			χ^2 = 2.139	0.235
< 60	32 (86.49)	43 (95.56)		
≥ 60	5 (13.51)	2 (4.44)		
Aetiology[1], *n* (%)			χ^2 = 8.601	0.025
Congenital	5 (13.51)	13 (28.89)		
Obstetrical	15 (40.54)	24 (53.33)		
Secondary to surgery	16 (43.24)	8 (17.78)		
Other (traumatic aetiology)	1 (2.7)	0 (0)		
Fistula duration time[2], mo	91.1 ± 128.38	126.24 ± 150.85	*U* = 695	0.199
Previous repair history[1], *n* (%)			χ^2 = 1.311	0.515
0	26 (70.27)	31 (68.89)		
1	8 (21.62)	7 (15.56)		
≥ 2	3 (8.11)	7 (15.56)		
Fistula location[1], *n* (%)			χ^2 = 1.072	0.351
Low-level	27 (72.97)	28 (62.22)		
Mid-level	10 (27.03)	17 (37.78)		
Distance from anal margin[2], cm	2.16 ± 1.53	2.34 ± 1.16	*U* = 750.5	0.443
Distance from vaginal introitus[2], cm	1.65 ± 1.32	1.84 ± 1.13	*U* = 692.5	0.188
Diameter of fistula[2], cm	0.78 ± 0.47	0.62 ± 0.43	*U* = 639.5	0.069
Stoma[1], *n* (%)	5 (13.51)	7 (15.56)	χ^2 = 0.068	1
Preoperative Wexner score[2]	7.03 ± 3.45	6.69 ± 3.48	*t* = 0.614	0.541

[1]Categorical data presented as number and percentage of patients in each group.
[2]Measurement data presented as mean ± SD. Group 1: Direct sutured rectovaginal fistula patients; Group 2: Stapled rectovaginal fistula patients. χ^2: Chi-squared testing; *U*: Mann-Whitney *U* testing.

Table 2 Operative and postoperative parameters—comparison between groups

Variable	Group 1	Group 2	Test value	*P* value
	n = 37	*n* = 45		
Operative time[1], min	74.84 ± 29.24	84.47 ± 33.46	*U* = 705	0.233
Blood loss[1], mL	24.38 ± 17.05	23.89 ± 20.42	*U* = 779.5	0.613
Hospital stay[1], d	14.19 ± 5.44	14.04 ± 4.54	*t* = 0.131	0.896
Postoperative Wexner score[1]	2.73 ± 3.75	1.04 ± 1.89	*U* = 610	0.021
Recurrence[2], *n* (%)	17 (45.95)	6 (13.33)	χ^2 = 10.701	0.001

[1]Measurement data presented as mean ± standard variance.
[2]Categorical data presented as number and percentage of patients in each group. Group 1: Direct sutured rectovaginal fistula patients; Group 2: Stapled rectovaginal fistula patients. χ^2: Chi-squared testing; *U*: Mann-Whitney *U* testing.

analysis (Table 4), both the surgical approach (P = 0.005) and preoperative Wexner score (P = 0.025) remained as significant independent predictive variables for fistula recurrence, but previous repair history, existing intestinal stoma, and fistula diameter showed no significant impact on recurrence with no confidence interval and hazard ratio.

Table 3 Univariate analysis of risk factors for fistula recurrence

Variable	Recurrent RVF	Non-recurrent RVF	*P* value
Age, yr, *n* (%)			0.655
< 60	22 (95.65)	55 (93.33)	
≥ 60	1 (4.35)	4 (6.78)	
Aetiology, *n* (%)			0.203
Congenital	6 (26.09)	12 (20.34)	
Obstetrical	7 (30.43)	32 (54.24)	
Secondary to surgery	10 (43.48)	14 (23.73)	
Other	0 (0)	1 (1.69)	
Fistula duration time, mo, *n* (%)			0.289
< 24	14 (60.87)	29 (49.15)	
≥ 24	9 (39.13)	30 (50.85)	
Previous repair history, *n* (%)			0.037
0	12 (52.13)	45 (76.27)	
≥ 1	11 (47.83)	14 (23.73)	
Fistula location, *n* (%)			0.804
Low-level	15 (65.22)	40 (67.8)	
Mid-level	8 (34.78)	19 (32.2)	
Diameter of fistula, cm, *n* (%)			0.039
< 0.5	3 (13.04)	22 (37.29)	
≥ 0.5	20 (86.96)	37 (62.71)	
Stoma, *n* (%)			0.068
Yes	17 (73.91)	53 (89.83)	
No	6 (26.09)	6 (10.17)	
Surgical approach, *n* (%)			0.002
Conventional suture repair	17 (73.91)	20 (33.9)	
Stapled repair	6 (26.09)	39 (66.1)	
Preoperative wexner score, *n* (%)			0.031
< 10	15 (65.22)	51 (86.44)	
≥ 10	8 (34.78)	8 (13.56)	

RVF: Rectovaginal fistula.

Kaplan-Meier curves generated for the postoperative recurrence-free period are shown in Figure 2 and demonstrate a significantly higher risk of recurrence if a sutured rather than a stapled repair was performed and if the preoperative Wexner score in the cohort was ≥ 10. The effect of the preoperative Wexner score was particularly evident in the first 1 mo following surgery (Figure 2B). Figure 3 shows a generated Kaplan-Meier curve comparing the effects of surgical repair (sutured *vs* stapled) with the preoperative Wexner score, demonstrating an overall significant effect of an interaction of the mode of surgery with the Wexner score recorded 1 mo after surgery ($P = 0.031$). In this regard, for those patients with a preoperative Wexner score of < 10, the recurrence rate for conventional sutured repair was 38.71% (12/31) but only 8.57% (3/35) for those managed by stapled repair (Figure 3B). For those patients presenting with a preoperative Wexner score of ≥ 10, a higher recurrence rate was predicted for patients undergoing sutured repair compared with stapled repair (83.33% *vs* 30%, respectively) (Figure 3C).

Despite the fact that failed prior repair history and fistula location were not found to

Table 4 Multivariate analysis of risk factors for fistula recurrence

Variable	Multivariate analysis	*P* value
	HR (95%CI)	
Previous repair history ≥ 1	-	0.053
Stoma	-	0.159
Diameter of fistula ≥ 0.5 cm	-	0.369
Conventional suture repair	3.838 (1.502-9.807)	0.005
Preoperative Wexner score ≥ 10	2.696 (1.131-6.427)	0.025

CI: Confidence interval; HR: Hazard ratio.

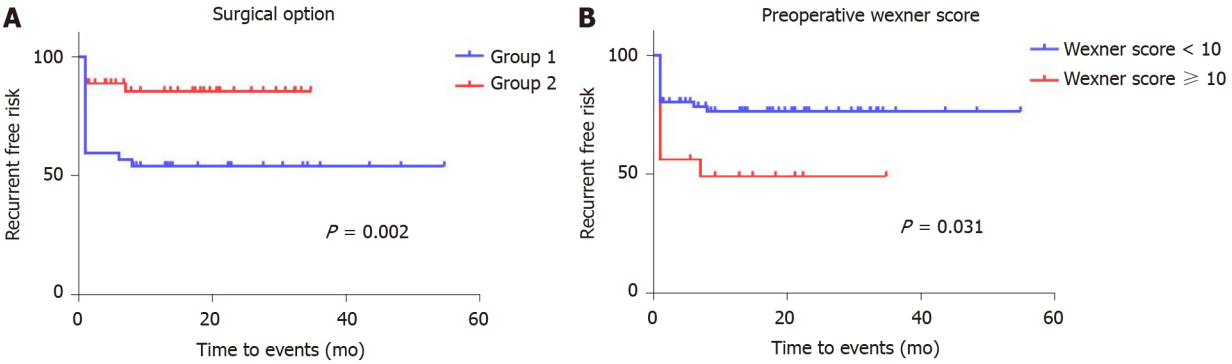


Figure 2 Kaplan-Meier analysis for the risk of rectovaginal fistula recurrence — effect of surgical type and preoperative Wexner score. A: Sutured *vs* stapled repair; B: Preoperative Wexner score categorized as < 10 or ≥ 10.

be significant for recurrence in the Cox proportional hazards model, we redrew the Kaplan-Meier curves with separation of different groups to develop the latent advantages of the stapled technique. The effect of prior repairs significantly impacted those cases undergoing multiple sutured repairs (Figure 4). There was no difference noted in the recurrence rate between Groups 1 and 2, if there was a single prior RVF repair experience [4/8 (50%) *vs* 2/7 (28.57%), respectively; $P = 0.466$], whereas the risk of recurrence in patients who had not undergone a prior repair was higher in Group 1 than in Group 2 [10/26 (38.46%) *vs* 2/31 (6.45%), respectively; $P = 0.005$] (Figure 4B and C). Meanwhile, the stapled technique largely resolved the risk of recurrence in patients who had undergone multiple prior repairs [3/3 (100%) *vs* 2/7 (28.57%), respectively; $P = 0.016$] (Figure 4D). Furthermore, the recurrent risk of sutured repair cases was significantly higher than that of stapled repair cases among both the low-level subgroup [11/27 (40.74%) *vs* 4/28 (14.29%), respectively; $P = 0.04$] and the mid-level subgroup [6/10 (60%) *vs* 2/17 (11.76%), respectively; $P = 0.009$] (Figure 5).

DISCUSSION

This retrospective comparative study showed that the stapled closure of RVF is safe and efficient, with a significantly lower fistula recurrence rate. Multivariate analysis showed the surgical approach and the preoperative Wexner incontinence score to be independent predictors of postoperative success, with predicted recurrence risk greatest in the first postoperative year. There was also an interactive effect on recurrence risk between high-grade preoperative incontinence and the use of a stapled repair.

Our group had recently reported on the safety of the Echelon Flex 60 Endopath stapler, as determined in a pilot study of 7 RVF cases, showing its relative ease of use and low morbidity[19]. As far as we are aware, our latest study (presented herein) is the first to compare stapled RVF closure with a direct sutured repair. Outside of separated local rectal and vaginal sutures, given the high rate of RVF recurrence over time, alternative techniques have included the use of augmentations, such as the Martius

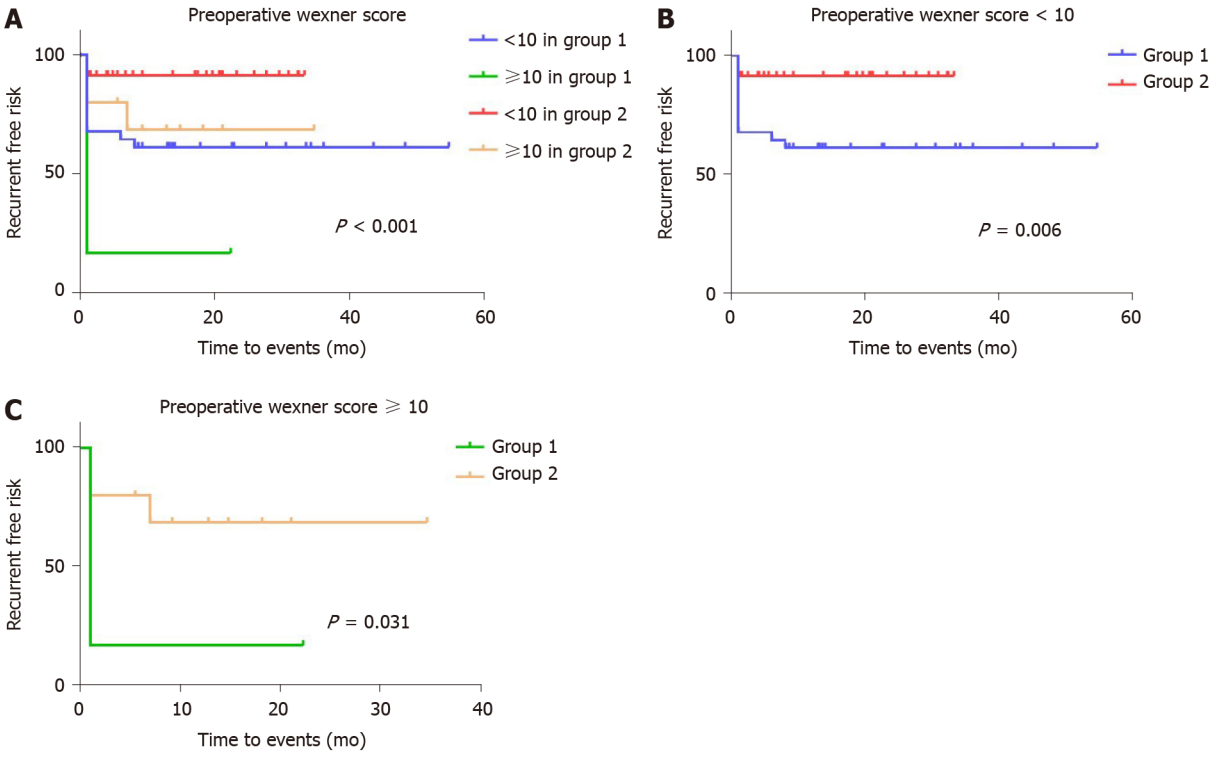


Figure 3 Kaplan-Meier curves assessing recurrence-free risk combining the mode of surgery (sutured *vs* stapled repair) with the preoperative Wexner score. A: Preoperative Wexner score categorized as < 10 or ≥ 10 in each group; B: Patients with preoperative Wexner score < 10 in each group; C: Patients with preoperative Wexner score ≥ 10 in each group.

labial fat pad replacement[22,23], interposition graciloplasty[24,25], ometoplasty or fat instillation[26,27], and the insertion of absorbable biocompatible mesh[28]. Despite these newer augmented techniques, the surgical management of RVF remains a major challenge, with recurrence rates ranging widely in the literature (from 0% to 80%)[4,7,10]. The principal clinical features associated with recurrence after an initial repair include the number of prior repairs[11], the fistula aetiology, and a history of cigarette smoking[4]; there has been no demonstrable effect of age, body mass index, presence of comorbidities, or steroid use[29].

Our present study showed advantage for the stapled fistula closure with the groups assessed (well matched for the number of multiple previous fistula repairs). In this regard, Lowry *et al*[11] showed a reduction in success rate (from 88% down to 55%) between the first repair and those patients who had undergone two prior failed attempts. Since there is a correlation between the number of surgical attempts and the ultimate rate of failure, future investigations need to prospectively assess the stapled RVF closure in cases with higher risk of recurrence. Otherwise, the recurrence rate of each aetiology in Group 1 was higher than that in Group 2, especially in secondary-to-surgery patients [9/16 (56.25%) *vs* 1/8 (12.5%), respectively]. Given that secondary-to-surgery patients had a history of more than or equal to one repair operation, this finding also indicates that patients with multiple operations are not optimally suitable for another sutured repair. The relative high healing rate in mid-level fistulas also suggests stapled repair as a potential surgical option for treating complex RVF. Data concerning the value of a protective stoma in RVF repair remain controversial[30]. Our stoma use in both cohorts was comparatively low, with the two groups being well matched. Outside of more complicated cases or those excluded from our analysis[13], diversion of stomas is more likely to be used in RVF cases with coincident sepsis or after multiple failed repairs.

It is important to recognize that our results are biased towards lower and simpler cases, wherein most specialist interventions should have a moderately high success rate. In this regard, our study merely confirmed the feasibility of the stapled procedure, with a very low attendant perioperative morbidity. Our results may also depend upon factors which lie outside of the particular repair method. In this respect, Lo *et al*[7], in a combined 12-mo follow-up study of patients from Taiwan, Malaysia, and the Philippines, showed a worse overall outcome after simple local RVF repair when the women were older and of lower socioeconomic status or when their fistula

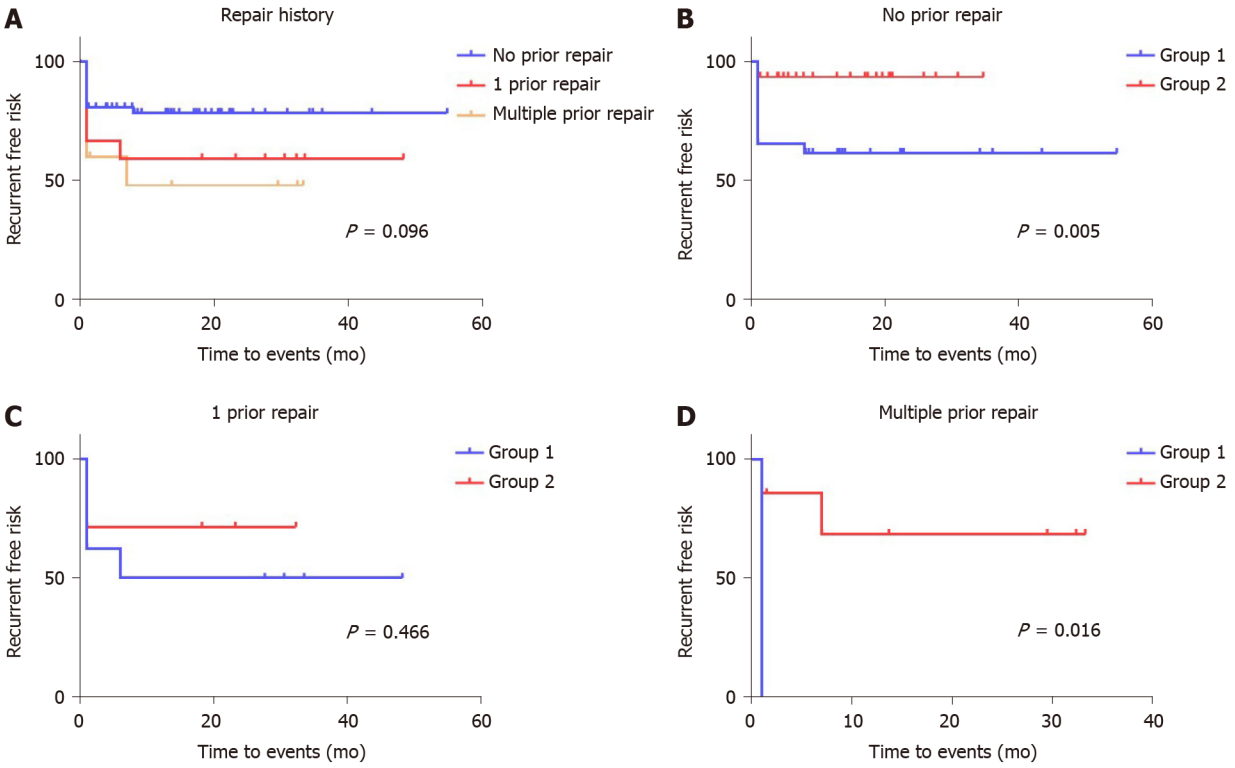


Figure 4 Kaplan-Meier curves of risk of recurrence and the effect of prior rectovaginal fistula repair history, according to the mode of surgery (sutured *vs* stapled repair). A: Transperineal repair categorized as no prior repair, single, and multiple prior repair(s) in each group; B: Patients who had no prior repair history in each group; C: Patients with a single prior repair in each group; D: Patients who had more than one prior repair in each group.

resulted from non-obstetric-related causes. In our practice, the majority of our cases were post-obstetric, even though RVF is an uncommon sequel of vaginal delivery[31]. The success of surgery depends upon the integrity of the local tissues, the absence of active inflammation and infection, the amount of scar tissue available, and the degree of attenuation of the perineal body. We postulate that failure after local repair is consequent to tension within the soft-tissues, an effect which may be diminished with the use of a stapled closure.

Given the differential forces across a high-pressure rectum to a comparatively low-pressure vaginal circuit[32], spontaneous healing of an RVF is rare[33]. We consider it appropriate to perform a coincident sphincteroplasty and levatorplasty in cases with demonstrable sphincter defects, where there has been a prior third or fourth degree perineal laceration. This is an added advantage of the transperineal repair (however performed) that readily permits an anterior levatorplasty, which acts as a bulwark against the high-pressure rectal side of the repair. This is consistent with results from other studies that have used different types of repair; for example, Tsang *et al*[12] showed that with advancement mucosal anoplasty, there was a substantial improvement in success for those cases presenting with preoperative incontinence and a visible external anal sphincter defect if combined with both a sphincteroplasty and a levatorplasty. Besides, the additional separation towards the fistula helps alleviate local tension. In our experience, the plane in the rectovaginal septum should be expanded sufficiently around the entire fistula. It represents the cornerstone for tension-free cutting closure of the fistula when the stapler is applied. Second, the levator ani muscle should be exposed bilaterally for consequent continuous suturing. This facilitates a better reinforcement of levatorplasty and closure of the fistula, to withstand future tension.

For the different local RVF repair method in the present study, preoperative incontinence in the stapled group appeared to be a predictor of a better outcome, whereby the interposition of well-vascularised tissue between the rectum and the vagina can lead to improved healing. In our opinion, a preoperative Wexner score exceeding 10 strongly indicates a dysfunctional anal sphincter. Therefore, the surgeon should dissociate enough space to reconcile the use of the stapler and sphincter repair or levatorplasty. It seems that having adequate space exposed in order to place the stapler and perform levatorplasty or sphincteroplasty during the stapled repair process in patients with a high Wexner score will greatly alleviate the local pressure on

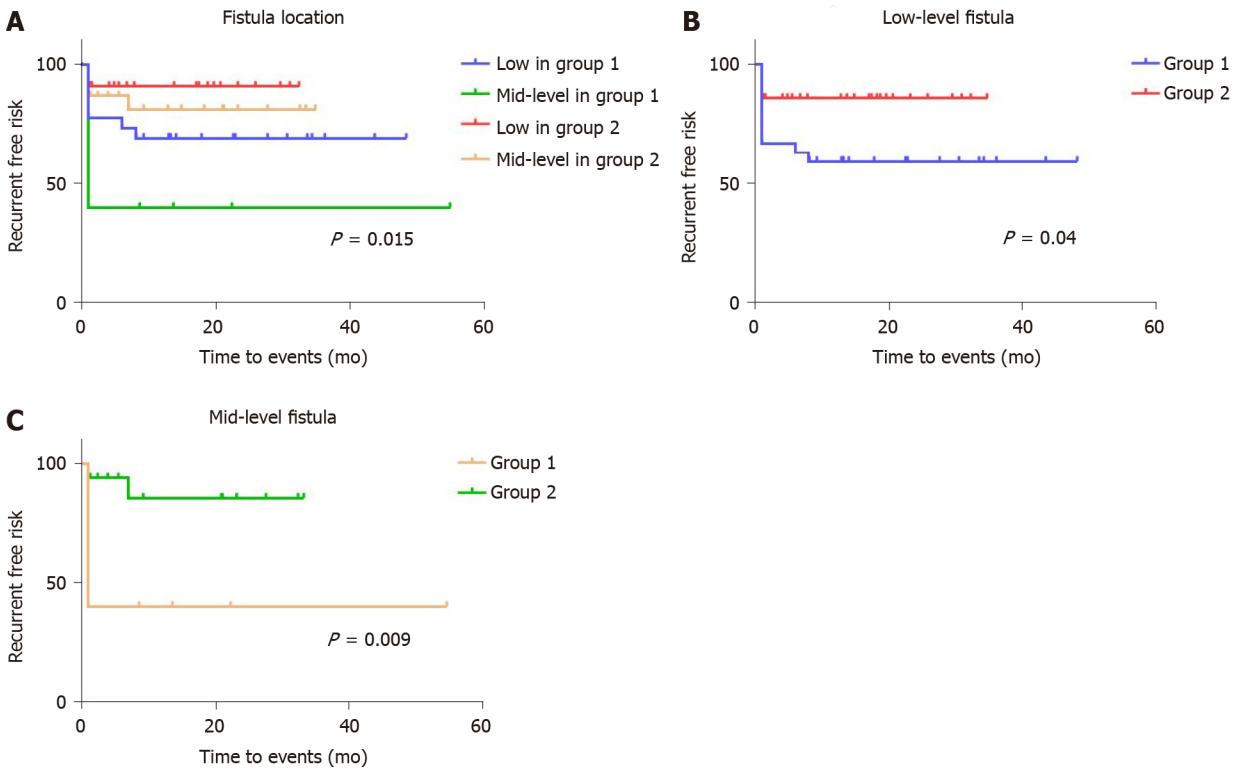


Figure 5 Kaplan-Meier curves of risk of recurrence and the effect of height of the fistula, according to the mode of surgery (sutured *vs* stapled repair). A: Fistula location categorized as low- and mid-level in each group; B: Patients with low-level fistula in each group; C: Patients with mid-level fistula in each group.

the incision, which may have contributed to the lower recurrence rate.

Our study has several limitations. The retrospective design with focused consideration of low- and mid-level fistulas introduces biases towards particular types of surgery in less complicated cases. In addition, our median follow-up was relatively short, with the expectation of later recurrent fistulas in both groups over time. The overall low incidence of inflammatory bowel disease in China can also influence the outcome of RVF repair, limiting the ability to translate our experience to other countries. Further, in China, patients with congenital RVF quite commonly wait to present to an adult Coloproctology Unit until after marrying age, to request repair. Another potential limitation of our study lies in the fact that, although Wexner scoring was performed preoperatively, the true prevalence and severity of incontinence may be greater since some symptoms can be inaccurately attributed to the fistula rather than to pre-existing incontinence. This emphasizes the importance of a thorough physical examination and preoperative sphincter imaging for successful functional outcome and patient satisfaction following treatment, reflecting not only fistula healing but also continence improvement.

Our study has shown the advantage of management of non-inflammatory RVF using a stapled repair over a direct suture, with reduced recurrence rates over a short- to medium-term follow-up.

CONCLUSION

In conclusion, the stapled transperineal repair technique shows better efficacy and acceptable safety in surgical treatment of low- and mid-level RVFs.

ARTICLE HIGHLIGHTS

Research background

Currently, rectovaginal fistula (RVF) continues to be a surgical challenge worldwide, on account of low primary healing rates and uncertainty regarding secondary repair.

Research motivation

Based on findings from a preliminary pilot study of the safety of stapled transperineal repair on low- and mid-level RVF, we designed a retrospective study to compare outcomes and recurrence rates between sutured and stapled transperineal repair.

Research objectives

Patient demographic data, Wexner faecal incontinence score, and operative data were analyzed. Recurrence rate and associated risk factors were specifically assessed.

Research methods

This was a retrospective cohort study conducted on patients from the Coloproctology Department of The Sixth Affiliated Hospital of Sun Yat-sen University. In total, 82 adult patients presenting with RVF who were surgically managed by perineal repair between May 2015 and May 2020 were included. Among them, 37 patients were repaired with direct suture and 45 patients with stapler.

Research results

The two treatment groups shared similar clinical characteristics, such as aetiology, surgical history, fistula features, and Wexner score. The stapled repair group did not show superior results over the sutured repair group in regard to operative time, blood loss, and length of hospital stay. However, the patients in the stapled repair group showed a better postoperative Wexner score (1.04 ± 1.89 *vs* 2.73 ± 3.75, $P = 0.021$), less intercourse pain (2.22% *vs* 2.7%, $P = 0.045$) and, most important, lower recurrence rate (13.33% *vs* 45.95%, $P = 0.001$). No previous repair history, smaller diameter of fistula (Wexner < 0.5 cm), better control of defecation (Wexner < 10), and stapled repair showed protective effects on healing. Direct suture repair and preoperative high Wexner score (≥ 10) were further demonstrated to be risk factors for fistula recurrence.

Research conclusions

Stapled transperineal repair shows an advantage for management of non-inflammatory, low- and mid-level, or even with prior failure of repair of RVF, with high primary healing rate and low recurrence rates.

Research perspectives

Our retrospective analysis of only low- and mid-level fistulas introduces biases towards particular types of surgery in less complicated cases. In addition, our median follow-up was relatively short, with the expectation of later recurrent fistulas in both groups over time. The long-term efficacy of stapled repair needs further prospective, randomized controlled trials to fully understand and capitalise on its advantages in clinic.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Impact of preoperative antibiotics and other variables on integrated microbiome-host transcriptomic data generated from colorectal cancer resections

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Abstract

BACKGROUND

Integrative multi-omic approaches have been increasingly applied to discovery and functional studies of complex human diseases. Short-term preoperative antibiotics have been adopted to reduce site infections in colorectal cancer (CRC)

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Institutional review board statement: This study was approved by the Stony Brook University Institutional Review Board (approval No. 163184).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Data sharing statement: All de-multiplexed, paired-end 16S rRNA gene sequence files and RNA-sequence data along with associated metadata were deposited into the Gene Expression Omnibus, which is a public repository that archives and freely distributes comprehensive sets of microarray, next-generation sequencing, and other forms of high-throughput functional genomic data submitted by the scientific community data base under project accession number GSE165255. All the tissues analyzed were collected from patients that gave informed consent for sharing results of the analysis of their anonymized tissues on public databases, where the risk of identification is very low. The statistical code used in this study is available from the corresponding author at ellen.li@stonybrookmedicine.edu.

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resections. We hypothesize that the antibiotics will impact analysis of multi-omic datasets generated from resection samples to investigate biological CRC risk factors.

AIM

To assess the impact of preoperative antibiotics and other variables on integrated microbiome and human transcriptomic data generated from archived CRC resection samples.

METHODS

Genomic DNA (gDNA) and RNA were extracted from prospectively collected 51 pairs of frozen sporadic CRC tumor and adjacent non-tumor mucosal samples from 50 CRC patients archived at a single medical center from 2010-2020. The 16S rRNA gene sequencing (V3V4 region, paired end, 300 bp) and confirmatory quantitative polymerase chain reaction (qPCR) assays were conducted on gDNA. RNA sequencing (IPE, 125 bp) was performed on parallel tumor and non-tumor RNA samples with RNA Integrity Numbers scores ≥ 6.

RESULTS

PERMANOVA detected significant effects of tumor *vs* nontumor histology (P = 0.002) and antibiotics (P = 0.001) on microbial β-diversity, but CRC tumor location (left *vs* right), diabetes mellitus *vs* not diabetic and Black/African Ancestry (AA) *vs* not Black/AA, did not reach significance. Linear mixed models detected significant tumor *vs* nontumor histology*antibiotics interaction terms for 14 genus level taxa. QPCR confirmed increased *Fusobacterium* abundance in tumor *vs* nontumor groups, and detected significantly reduced bacterial load in the (+)antibiotics group. Principal coordinate analysis of the transcriptomic data showed a clear separation between tumor and nontumor samples. Differentially expressed genes obtained from separate analyses of tumor and nontumor samples, are presented for the antibiotics, CRC location, diabetes and Black/AA race groups.

CONCLUSION

Recent adoption of additional preoperative antibiotics as standard of care, has a measurable impact on -omics analysis of resected specimens. This study still confirmed increased *Fusobacterium nucleatum* in tumor.

Key Words: Colorectal cancer; Antibiotics; African Continental Ancestry Group; Diabetes mellitus; 16S rRNA gene; RNA-sequencing

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Core Tip: This pilot study explored the effect of five variables [tumor histology, preoperative antibiotics, laterality of colorectal cancer (CRC) location, diabetes mellitus, Black/African Ancestry (AA) race] on analysis of microbiome and host transcriptome among archived frozen CRC resection samples. The introduction of short-term preoperative antibiotics as standard of care has a measurable effect on the analysis. Despite the small sample size and variable exposure to preoperative antibiotics, it was still possible to use the data for discovery studies. Fusobacterium abundance was increased in tumor *vs* nontumor regions. Expression of VBP1 was decreased in expression in both Black/AA tumor and nontumor samples.

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INTRODUCTION

Imbalances in mucosal associated microbiota (dysbiosis) have been reported in human colorectal cancer (CRC)[1]. The increased risk of CRC in patients with inflammatory bowel diseases and in animal models of intestinal inflammation support the concept that bacteria promoting colonic inflammation may be proto-oncogenic[2]. According to the driver and passenger hypothesis, there are key pathogens that can drive tumorigenesis and support other bacteria as passengers to proliferate and exacerbate disease in sporadic CRC[3]. Similarly, the keystone hypothesis states that certain low abundance pathogens can promote inflammation by altering a normal microbiota into a dysbiotic one[4]. There are certain pathogens that are associated with CRC including increased abundances of *Peptostreptococcus, Bacteroides fragilis, Fusobacterium nucleatum* (*F. nucleatum*), and *Escherichia coli* and decreased abundances of *Clostridium, Bifidobacterium, Faecalibacterium* and *Roseburia*[1-6]. The potential mechanisms at which these particular bacteria may affect the adenoma-carcinoma sequence, include gene expression alternations, promotion of chronic inflammation and release of carcinogenic metabolites[1-6]. The approach of collecting and integrating multi-omic host and microbiome data has been increasingly applied to discovery and functional studies of human gastrointestinal diseases[7,8]. In an ongoing multi-omic analysis of archived frozen mucosal samples of tumor and adjacent nontumor regions of CRC surgical resection specimens (2010-2020), we discovered that a major change was made in the preoperative antibiotic protocol as of January 2017. Prior to that time, the standard of care was to administer only intravenous antibiotics within 30 min of incision, and only a few CRC resection patients were placed on short-term oral antibiotics within a month of the surgery, for various clinical indications. However, after this time, the standard operating protocol for preoperative antibiotics was to prescribe outpatient oral neomycin and metronidazole 24 h in advance of the procedure, in order to reduce surgical site infections[9-11]. The use of antibiotics can shift the microbiome depending on the dosage and duration of the antibiotic exposure. Several studies have shown that tumorigenesis and tumor growth can be attenuated with different antibiotic cocktails and timing of antibiotic exposure with duration of inflammation. On the other hand, early exposure to antibiotics increased risk of CRC and interfered with chemotherapy efficacies due to microbial dysbiosis[5]. With these conflicting findings and this change in protocol at our institution, it allowed us to examine how differential use of antibiotics, along with other clinical/demographic factors influences integrative, multi-omic analyses of CRC.

The five following variables were included in the analysis of the microbiome and host transcriptome datasets generated in this pilot study: (1) Tumor histology (tumor *vs* nontumor); (2) Preoperative antibiotics (yes/no); (3) Laterality of CRC location (left *vs* right); (4) Diabetes mellitus (yes/no); and (5) Black/African Ancestry (AA) race (yes/no). The rationale for comparing tumor *vs* nontumor histology is based on multiple previous reports of differences between tumor and nontumor regions of CRC resection specimens[1,12,13]. Since antibiotics clearly have an effect on the human microbiome[14], it was crucial to assess the impact of the change in antibiotic protocol on the metagenomic and transcriptomic data, before making the commitment to expand the pilot study by analyzing more CRC resection samples. Because molecular differences have been previously reported right sided and left sided CRCs[15], laterality of CRC location was included in the analysis.

The original targets of this pilot discovery and functional study were two biological CRC risk factors: (1) Diabetes mellitus; and (2) Black/AA race. Diabetes mellitus has been associated with increased CRC risk and poorer survival[16,17], and has been implicated in CRC pathogenesis[18]. Several studies have described alterations in the fecal microbiome of patients with diabetes mellitus compared to those without diabetes mellitus in the absence of a CRC diagnosis[19-21]. Transcriptomic profiling of CRC resection tumor and adjacent nontumor samples suggested evidence of diabetic "field cancerization" of the nontumor region[22].

CRC incidence and mortality has been persistently higher in Black/AA compared to all other races in the United States[23,24]. Paredes *et al*[25] recently reported that transcriptomic profiling of Black/AA compared to White/European Ancestry (EA) revealed reduced immune related gene expression in tumor samples and plasma cytokine levels collected from Black/AA CRC patients. We report here the results of this pilot study to investigate the effects of the tumor histology, preoperative antibiotics, laterality of CRC location, diabetes mellitus status and Black/AA race on microbiome and human transcriptomic data of CRC patients.

MATERIALS AND METHODS

Patient samples

Sequencing analysis of 51 pairs of tumor and nontumor frozen mucosal samples collected from 50 treatment-naïve sporadic CRC subjects (excluding samples collected after neoadjuvant therapy), archived in the Stony Brook Cancer Center Tissue Analytics Shared Resource, was approved by the Stony Brook Institutional Review Board (IRB, IRB2019-00682) and by the Cold Spring Harbor Laboratory IRB (IRB 1467912). All the archived samples were collected prospectively from CRC patients scheduled between 2010-2020 for surgical resection for clinical care, who had given their informed consent for banking of surgical remnant for possible genomic sequencing, collection of longitudinal clinical metadata and access to clinical formalin-fixed paraffin embedded (FFPE) tissues for possible genomic sequencing under a protocol approved by the Stony Brook University IRB (No. 163184). CRC samples collected from individuals diagnosed with hereditary CRC syndromes or inflammatory bowel diseases and individuals who received neoadjuvant treatment, were excluded from this analysis. The clinical metadata included age at the time of tissue collection, self-identified race and ethnicity, family history of a first degree relative with CRC, tumor location (right, cecum-transverse colon; left, splenic flexure-rectum), pathologic cancer stage, smoking status (current, past, never), body mass index, diabetes mellitus status, preoperative antibiotics within a month of surgery (yes/no), cancer stage (0, 1, 2, 3, 4). Participants who enrolled in the study after 2017 were automatically assumed to be exposed to preoperative antibiotics as the new protocol prescribes oral neomycin (1 g × 3 doses) and metronidazole (500 mg × 3 doses) to the patient 24 h prior to surgery. DNA and RNA were extracted from the archived frozen tissues using the Qiagen Allprep DNA/RNA/miRNA kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA and RNA were extracted from 5 μm sections of FFPE tumor and non-tumor samples using the Qiagen Allprep DNA/RNA FFPE (Qiagen, Hilden, Germany) kits according to the manufacturer' protocol except that xylene was used to de-paraffinize the FFPE samples.

16S rRNA gene sequencing

The 16S rRNA libraries were constructed by generating broad-range polymerase chain reaction (PCR) amplicons using barcoded primers that target the V3V4 variable region of the 16S rRNA gene: Primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), as previously described from 51 paired tumor and nontumor DNA samples[22]. Illumina paired-end sequencing was performed on the Miseq platform with versions v2.4 of the Miseq Control Software and of MiSeq Reporter, using a 600-cycle version 3 reagent kit. Illumina Miseq paired-end reads were aligned to human reference genome hg19 with bowtie2 and matching sequences were discarded as previously described[25]. All de-multiplexed, paired-end 16S rRNA gene sequence files along with associated metadata were deposited into the Gene Expression Omnibus under project accession number GSE165255. The paired end reads were assembled, aligned and classified using the SINA/SILVA classifier as previously described[26-28]. Operational taxonomic units (OTUs) were produced by binning sequences with identical taxonomic assignments.

Taqman quantitative PCR analysis of mucosal genomic DNA

Real time Taqman PCR analyses were conducted using established specific primers and probe for the *F. nucleatum* nusG gene[29,30] and broad range primers and probe bacterial 16S rRNA gene[31] shown in Table 1. The Taqman primers and probe for the human solute carrier family 21 or *SLCO2A1* also termed prostaglandin cotransporter or *PGT* gene[29], a single copy autosomal gene located on human chromosome 3, were from Taqman assay ID Hs00194554_m1 (Thermo Fisher Scientific, Waltham, MA, United States). The probes and primers were custom ordered as two separate Taqman assay kits (Thermo Fisher Scientific, Waltham, MA, United States). The first kit combined the *F. nucleatum* NUS gene assay (FAM probe) with the total bacterial 16S rRNA gene assay (VIC probe). The second kit combined the *F. nucleatum* nus gene (FAM probe) with the human *SLCO2A1* gene assay (VIC probe). The PCR assays were carried out in triplicate with 80 hg of sample DNA in 20 μL in each well. The thermal conditions were 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. *F. nucleatum* subsp. *nucleatum* Knorr ATCC 25586 genomic DNA (gDNA) purchased from ATCC (Manassas, VA, United States) and a gene block was synthesized using the predicted PCR amplicon from the *F. nucleatum* subsp. *animalis 4_8* genome reference

Table 1 Specific Taqman polymerase chain reaction primer and probe sequences for the *Fusobacterium nucleatum* nusG gene and broad range Taqman primer and probe sequences for the bacterial 16S rRNA gene

Target gene	Sequence
F. nucleatum nusG gene forward primer[29]	5′-CAACCATTACTTTAACTCTACCATGTTCA-3′
F. nucleatum nusG gene reverse primer[29]	5′-GTTGACTTTACAGAAGGAGATTATGTAAAAATC-3′
F. nucleatum nusG gene probe[30]	5′-TCAGCAACTTGTCCTTCTTGATCTTTAAATGAACC-3′
Broad range 16S rRNA gene forward primer[31]	5′-TCCTACGGGAGGCAGCAGT-3′
Broad range 16S rRNA gene reverse primer[31]	5′- GGACTACCAGGGTATCTAATCCTGTT-3′
Broad range 16S rRNA gene probe[31]	5′-CGTATTACCGCGGCTGCTGGCAC-3′

F. nucleatum: *Fusobacterium nucleatum*.

sequence for the Human Microbiome Project (Assembly: GCA_000400875, Integrated DNA Technologies, Coralville, IA, United States) were used as positive controls. The 80 hg of DNA template was used in each 20 μL reaction and with either *F. nucleatum* (FAM probe)/*SLCO2A1* (VIC probe) or the *F. nucleatum* (FAM probe) broad-range 16S rRNA gene (VIC probe) in triplicate.

RNA sequence analysis

RNA-sequencing was conducted on a subset of tumor and nontumor RNA samples with RIN scores ≥ 6 at the CSHL Cancer Center Sequencing Technologies and Analysis Shared Resource and unpaired tumor RNA samples at the New York Genome Center. At both sequencing centers, the RNA sequence libraries were prepared with the Kapa RNA kit with RiboErase (Roche Sequencing and Life Science Kapa Biosystems, Wilmington, MA, United States) according to the manufacturer's protocol and Illumina 125 bp PE sequencing was conducted on HiSeq2500 instruments with a targeted depth of 20 million. The sequences were aligned using Salmon in Patro *et al*[32]. The sequences were deposited in NCBI's Gene Expression Omnibus database with accession number GSE165255.

Statistical analysis

Statistical analysis was performed utilizing the Biostatistics and Bioinformatics Shared Resource at the Stony Brook University Cancer Center. Patient demographics were compared between the subset of samples analyzed and the total set of samples archived in the Stony Brook Cancer Center Tissue Analytics Shared Resource (2010-2020) using either Wilcoxon rank sum test for continuous variables, and chi-square test using GraphPad Prism. Alpha diversity indices [*e.g.*, Sobs, Shannon complexity (H), Shannon Evenness (H/Hmax)] were calculated on samples yielding a total OTU count inferred through 1000 replicate resamplings using Explicet[33]. Linear mixed models were used to compare alpha-diversity (Sobs, ShannonH, ShannonE) between different groups of histology, antibiotics, location, diabetes and Black/AA race. Log-transformation was applied if the normality assumption was not satisfied. Compound symmetry (CS) covariance structure was utilized to model the correlation among measurements from the same patient. β-diversity was assessed by calculating distance matrices based on the Morisita-Horn dissimilarity index and visualized by principal coordinate analysis (PCoA). A PERMANOVA analysis[34] was performed to assess the statistical significance of differences in β-diversity with the following five variables: (1) Tumor histology (tumor/nontumor); (2) Pre-operative antibiotics (yes/no); (3) Anatomic location (left/right); (4) Diabetes mellitus (yes/no); and (5) Black/AA race (yes/no). These analyses were conducted using the vegan 2.56 package of R 3.6.1, and the default setting for number of permutations (n = 999). To compare relative abundance of each OTU between tumor *vs* nontumor, generalized linear mixed model (GLMM) or generalized estimating equation (GEE) models were used by using the actual counts of each OTU as the outcomes, which were assumed to follow a negative binomial distribution[26]. CS covariance structure was utilized to model the correlation among measurements from the same patient. The log overall count for each individual in each group was considered as an offset in order to normalize for differences in sequencing depth of coverage. When models had a fitting issue because of excessive 0 counts, all sample's counts+1 were used as the outcome and log (overall count+1) was used as the offset. The two-way interaction term between tumor histology

(tumor/nontumor) and preoperative antibiotics (yes/no) was first added in each model to estimate the difference between tumor *vs* normal within patients with or without pre-operative antibiotics. For OTUs that had model fitting difficulty and OTUs with non-significant interaction, the two-way interaction term was removed and the aforementioned models were fit again. Univariate linear mixed models were used to estimate the coefficient of correlation and the *P* values for the log transformed relative abundances of selected OTUs. The *P* values were based on the *t*-test for GLMM and the *Z*-test for GEE. The Bonferroni adjusted *P* values < 0.05 were considered as statistically significant. These analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, United States).

Differentially expressed genes (DEGs) ($\log_2$ fold change ≥ 1, adjusted *P* value < 0.05 were identified using DESeq2[35] for the five groups: (1) Tumor histology (tumor *vs* nontumor); (2) Preoperative antibiotics within a month of the surgery (yes/no); (3) Anatomic location of the CRC and adjacent nontumor region (left *vs* right); (4) Diabetes mellitus status (yes/no); and (5) Black/AA self-declared race (yes/no). For the latter four variables, the tumor and nontumor samples were analyzed separately. The DEG lists were also deposited in NCBI's Gene Expression Omnibus database with accession number GSE165255.

RESULTS

Patient characteristics of samples analyzed

The characteristics of the patients for the subset of samples analyzed compared to that of the total collection of archived sporadic CRC samples (2010-2020) are shown in Table 2. Since the samples in the subset were originally selected to provide Black/AA samples and "matched" White/EA samples for another transcriptomic profiling performed at a separate sequencing facility[25], there was significantly higher representation of Black/AA samples in this pilot study. Left-sided CRCs were also more highly represented in the subset compared to the overall collection. Because most of the subset samples were collected prior to January 2017, (when the protocol adding preoperative oral antibiotic prophylaxis was first introduced), there was a lower representation of samples collected from patients who had preoperative antibiotic treatment compared to the total sample collection. Of the 18 diabetic patients, only one had type 1 diabetes mellitus and the remaining 17 patients were diagnosed with type 2 diabetes mellitus.

Colonic mucosal microbiome analysis

We generated 5706758 16S rRNA gene sequences from 51 pairs of CRC tumor and nontumor samples. After excluding sequence libraries with total counts < 1000, the remaining 89 sequence libraries (41 tumor, 48 nontumor, 26 preoperative antibiotics, 63 no preoperative antibiotics, 55 left, 34 right, 32 diabetic, 57 not diabetic, 21 Black/AA, 68 not Black/AA) had an average sequencing depth of 64752 (minimum 1032; maximum 248838). As shown in Table 3, no significant differences were detected in any of the three alpha diversity indices between the following five groups: (1) Preoperative antibiotics (yes/no); (2) Tumor *vs* nontumor histology; (3) Anatomic location of sample (left *vs* right); (4) Diabetes mellitus status (yes/no), and (5) Black/AA race *vs* not Black/AA race. Exploratory PERMANOVA using the Morasita Horn index for β-diversity was performed with the same five groups (see Table 4). Tumor histology and preoperative antibiotics demonstrated significant *F* statistics, but anatomic location, diabetes and Black/AA race did not reach significance. The PCoA comparisons using these indices for these five groups are displayed in Figure 1.

Because PERMANOVA identified tumor histology and preoperative antibiotics as significant, fitted linear mixed models were used to measure the effect of preoperative antibiotics and tumor histology along with first order preoperative antibiotics*tumor histology interactions on the relative abundances of individual genus-level taxa. For this analysis, we included those OTUs with average relative abundances greater than 0.001% and that were observed in at least 25% of the samples. Because of model convergence issues, 4 OTUs (*Firmicutes/Eubacterium, Firmicutes/Acidaminococcus, Proteobacteria/Neisseriaceae, Proteobacteria/Desulfovibrio)* were also excluded because of failure of their models to converge. Of the 106 OTUs included in the relative abundance regression analysis, 14 exhibited significant first order tumor histology*preoperative antibiotics interaction terms (see Table 5). The estimated differences in tumor *vs* nontumor samples were analyzed separately for those 14 OTUs depending on whether the patients were prescribed additional preoperative

Table 2 Comparison of patient characteristics between the subset of sporadic colorectal cancer samples analyzed with that of the total collection of archived samples in the Stony Brook Cancer Center Tissue Analytics Shared Resource (2010-2020)

	Subset analyzed, *n* = 51	Total collection, *n* = 190	*P* value
Age (yr) ± IQR	62 ± 20	65 ± 20	0.4329
Male sex (%)	30 (58.8)	115 (60.5)	0.8254
Race (%)			0.0295
White	38 (74.5)	163 (85.8)	
Black	11 (21.6)	14 (7.4)	
Asian	1 (2.0)	6 (3.2)	
Other	1 (1.9)	7 (3.7)	
Hispanic ethnicity (%)	6 (11.8)	22 (11.6)	0.9707
Family history 1st degree relative (%)	7 (13.7)	34 (17.9)	0.4817
BMI (kg/m^2)	27.8 ± 8	28.2 ± 8	0.5683
Diabetes mellitus (%)	18 (35.3)	55 (28.9)	0.4853
Smoking (%)			0.1103
Current	7 (13.7)	10 (5.3)	
Past	21 (41.2)	88 (46.3)	
Never	23 (45.1)	92 (48.4)	
Anatomic location (%)			0.0210
Left	31 (60.8)	81 (42.6)	
Right	20 (39.2)	109 (57.4)	
Cancer stage (%)			0.4578
Stage 0	1 (2.0)	6 (3.2)	
Stage 1	9 (17.6)	27 (14.2)	
Stage 2	14 (27.4)	63 (33.2)	
Stage 3	25 (49.0)	75 (39.5)	
Stage 4	2 (3.9)	19 (15.8)	
Preoperative antibiotics (%)	16 (31.4)	91 (47.9)	0.0350

IQR: Inter-quartile range; BMI: Body mass index.

antibiotics. One of 14 OTUs, *Fusobacteria/Leptotrichia,* was associated with increased relative abundance in tumor *vs* nontumor, regardless of preoperative antibiotic exposure (adjusted $P < 0.0001$ after Bonferroni correction = unadjusted P value*28 with antibiotics, and adjusted $P = 0.01$ after Bonferroni correction with no antibiotics). Of the 92 OTUs without significant first order preoperative antibiotic*tumor histology interaction terms, 19 OTUs, including *Fusobacterium* spp. demonstrated significant differences in tumor *vs* nontumor samples with adjusted $P < 0.05$ after Bonferroni correction (see Table 6).

Taqman quantitative PCR confirmation of increased F. nucleatum relative abundance in tumor vs non-tumor samples

*F. nucleatum (*both subspecies *nucleatum* and subspecies *animalis)* were enumerated in tissues specimens by quantitative PCR (qPCR) of the nusG gene, while total bacterial load was assayed by broad-range 16S rRNA gene qPCR. The human *SLCOA2* gene was assayed in parallel in order to normalize results. QPCR assays were performed on 37 paired tumor/nontumor mucosal gDNA samples, (10 pairs, +preoperative antibiotics; 27 pairs, -preoperative antibiotics). DNA samples were depleted in 7 tumor samples and 1 nontumor samples of the 51 paired tumor/nontumor samples and were therefore excluded from the analysis. *F. nucleatum* nusG was undetectable

Table 3 Estimated differences in alpha diversity indices between the following groups

Variable	Sobs		ShannonH		ShannonE	
	Estimate (95%CI)	*P* value	Estimate (95%CI)	*P* value	Estimate (95%CI)	*P* value
Tumor *vs* nontumor	-1.34 (-4.04, 1.35)	0.32	-0.05 (-0.25, 0.15)	0.61	-0.004 (-0.04, 0.03)	0.77
Pre-op antibiotics *vs* none	2.93 (-3.51, 9.37)	0.36	0.27 (-0.12, 0.66)	0.17	0.04 (-0.02, 0.09)	0.20
Left *vs* right	2.83 (-2.44, 8.10)	0.29	-0.10 (-0.43, 0.23)	0.53	-0.03 (-0.08, 0.02)	0.26
Diabetic *vs* nondiabetic	3.73 (-2.30, 9.75)	0.22	0.26 (-0.11, 0.62)	0.16	0.03 (-0.02, 0.09)	0.22
Black/AA race *vs* not Black/AA race	2.70 (-4.43, 9.83)	0.45	0.09 (-0.34, 0.52)	0.69	0.01 (-0.06, 0.07)	0.80

Linear mixed models were used to estimate differences. The *P* values are based on *t*-tests from the linear mixed models. CI: Confidence interval; AA: African Ancestry.

Table 4 PERMANOVA results for the following groups

Variable	*P* value
Tumor *vs* nontumor	0.002
Pre-operative antibiotics *vs* no preoperative antibiotics	0.001
Left *vs* right anatomic location	0.367
Diabetes mellitus *vs* not diabetic	0.061
Black/AA race *vs* not Black/AA race	0.258

AA: African Ancestry.

Table 5 Operational taxonomic units (genus level) with significant tumor histology*pre-operative antibiotics interaction terms

OTU (genus level)
Actinobacteria/Gardnerella
Actinobacteria/Eggerthella
*Firmicutes/Bacilli/*unspecified
Firmicutes/Staphylococcus
Firmicutes/Parvimonas
Firmicutes/Anaerostipes
Firmicutes/Howardella
Firmicutes/Solobacterium
*Fusobacteria/Fusobacteriales/*unspecified
Fusobacteria/Leptotrichia
Proteobacteria/Sutterella
Proteobacteria/Ralstonia
Proteobacteria/Enterobacter
Proteobacteria/Haemophilus

OTU: Operational taxonomic unit.

(undetermined) in the qPCR assays for at least one of the remaining 7 paired tumor and normal samples, and were also excluded from analysis. Both the *F. nucleatum* subspecies *nucleatum* and subspecies *animalis* were detected under these assay conditions. The mean threshold cycles (Ct) for the *F. nucleatum* nusG, broad-range 16S

Table 6 The operational taxonomic units (genus level) without significant tumor histology*preoperative antibiotics interaction terms, with significant (*P* < 0.05 after Bonferroni correction) estimated tumor/nontumor ratios of relative abundance and 95% confidence intervals

OTU	Ratio (95%CI)	*P* value
Increased in tumor		
*Firmicutes/Carnobacteriaceae/*unspecified	22.21 (7.57, 65.21)	< 0.0001
Firmicutes/ Peptoniphilus	2.13 (1.45, 3.13)	0.0042
Firmicutes/Catenibacterium	3.89 (1.79, 8.46)	0.0206
Firmicutes/Dialister	2.05 (1.48, 2.83)	0.0005
Fusobacteria/Fusobacterium	5.30 (2.82, 9.97)	< 0.0001
Proteobacteria/Campylobacter	7.40 (3.30, 16.62)	< 0.0001
Proteobacteria/Citrobacter	3.51 (2.12, 5.81)	< 0.0001
Decreased in tumor		
*Actinobacteria/Coriobacteriaceae/*unspecified	0.45 (0.32, 0.64)	0.0003
*Bacteroidetes/*unspecified	0.49 (0.32, 0.74)	0.0240
Bacteroidetes/S24-7	0.50 (0.36, 0.70)	0.0016
Cyanobacteria/Chloroplast	0.44 (0.31, 0.64)	0.0021
*Firmicutes/Lachnospiraceae/*unspecified	0.76 (0.68, 0.84)	< 0.0001
Firmicutes/Marvinbryantia	0.39 (0.24, 0.65)	0.0113
Firmicutes/vadinBB60	0.50 (0.36, 0.70)	0.0052
Firmicutes/Turicibacter	0.44 (0.30, 0.66)	0.0074
Firmicutes/Allobaculum	0.43 (0.29, 0.62)	0.0002
*Proteobacteria/Rickettsiales/*mitochondria	0.32 (0.19, 0.53)	0.0003
Tenericutes/Anaeroplasma	0.42 (0.28, 0.62)	0.0004
Verrucomicrobia/Akkermansia	0.58 (0.42, 0.80)	0.0350

OTU: Operational taxonomic unit; CI: Confidence interval.

rRNA and human *SLCOA2* assays are shown in Figure 2A-C . Pairwise comparisons of the mean tumor *F. nucleatum* nusG gene Ct ± SD with nontumor samples (32.2 ± 4.8 *vs* 34.0 ± 4.2, $P = 0.0125$), confirmed that there was a higher abundance of *F. nucleatum* in tumor samples compared with their paired adjacent nontumor samples. The mean 16S rRNA gene Ct was significantly higher in the (+)antibiotics group compared with the (-)antibiotics group (24.8 ± 3.7 *vs* 22.3 ± 3.4, $P = 0.0085$, missing 1 tumor (+)antibiotics and 1 tumor (-)antibiotics Ct value). This suggests that the preoperative antibiotics significantly reduced mucosal associated bacteria load. The difference in the mean *F. nucleatum* Ct between the (+)antibiotics group and the (-)antibiotics group (34.1 ± 3.7 *vs* 32.7 ± 4.8, $P = 0.24$) did not reach significance. The difference in the mean human *SLCOA2* Ct between the (+)antibiotics group and the (-)antibiotics group also did not reach significance (34.5 ± 0.8 *vs* 34.4 ± 2.3, $P = 0.74$, missing 2 nontumor (-)antibiotics Ct values).

In this current study the detection rates for the detection of *F. nucleatum* nusG gene were 84% and 93% respectively for the frozen tumor and nontumor samples. Of note, the Taqman probe used in the current study differed from the probe used in the previous FFPE studies, in that the probe in the current study did not overlap the reverse primer. The mean Ct values for each of the three qPCR assays performed on paired FFPE tumor/nontumor samples were compared with the Ct values obtained on the parallel pairs of frozen tumor/nontumor samples collected from the same two CRC patients (see Table 7). The broad range 16S rRNA gene Ct values were significantly higher in the FFPE samples compared to the paired frozen mucosal samples (30.6 ± 1.6 *vs* 22.7 ± 1.7, $P = 0.0034$, $n = 4$), despite using 80 hg of gDNA in all assays. The differences in the mean *F. nucleatum* nusG Ct values did not reach

Table 7 Comparison of the mean quantitative polymerase chain reaction mean threshold cycles ± SD between genomic DNA extracted from paired frozen and formalin-fixed paraffin embedded samples

	F. nucleatum nusG		16S rRNA		Human SLCO2A1	
	Frozen	FFPE	Frozen	FFPE	Frozen	FFPE
Tumor A	25.9 ± 1.2	35.4 ± 0.1	21.8 ± 0.1	31.4 ± 0.1	32.6 ± 2.5	39.3 ± 0.6
Nontumor A	37.2 ± 0.6	39.9/ND	22.2 ± 0.1	31.7 ± 0.3	34.8 ± 0.5	ND
Tumor B	26.0 ± 1.0	27.5 ± 1.0	21.6 ± 0.2	28.3 ± 0.1	33.2 ± 1.0	32.5 ± 1.3
Nontumor B	31.5 ± 1.4	35.1 ± 0.7	25.2 ± 0.1	31.2 ± 0.6	34.6 ± 0.9	38.2 ± 0.1

F. nucleatum: *Fusobacterium nucleatum*; FFPE: Formalin-fixed paraffin embedded; ND: Not detected.

significance, but one of the FFPE samples bordered on being not detectable (ND). The differences in the mean human *SLCOA2* Ct values did not reach significance although one of the FFPE samples was ND.

Effect of tumor histology and preoperative antibiotics on human RNA-sequencing data

Since significant effects of tumor histology and preoperative antibiotics were noted in the microbiome analysis, we were particularly interested in the potential effect of preoperative antibiotics on the human transcriptomic profiles generated in parallel. RNA-seq data was obtained from CSHL for 33 paired tumor/nontumor RNA samples and 4 unpaired tumor RNA samples at CSHL. RNA-sequencing was completed on an additional 11 unpaired tumor RNA samples at the NYGC sequencing center. PCoA demonstrated a clear separation between the tumor and nontumor transcriptomic profiles (see Figure 3). Out of a total of 31052 genes, 1235 DEGs were decreased in tumor (n = 48) *vs* nontumor (n = 33, Log_2 fold change ≤ -1, adjusted P value < 0.05) and 1229 DEGs were increased.

No clear separation was observed on PCoA for the remaining four variables: Preoperative antibiotics (see Figure 3), left *vs* right CRC location (not displayed), diabetic *vs* not diabetic (not displayed), or Black/AA race *vs* not Black/AA race (not displayed). DESeq2 analyses were conducted on the tumor samples separately from the nontumor samples for these four variables. For the (+)antibiotics (n = 15) *vs* the (-)antibiotics (n = 33) group of tumor samples, 30 DEGs were decreased and 188 DEGs were increased. For the (+)antibiotics (n = 13) *vs* (-)antibiotics (n = 20) nontumor samples, no DEGs were decreased and 32 DEGs were increased. Six upregulated DEGs were common with the same polarities in both tumor and nontumor (+)antibiotics/(-)antibiotics DEG lists (see Table 8).

For the left (n = 30) *vs* right (n = 18) CRC location groups of tumor samples, 12 DEGs were decreased and 7 DEGs were increased, and for the left (n = 18) *vs* right (n = 15) CRC location groups of nontumor samples, 72 DEGs were decreased and 36 DEGs were increased. However, no DEGs were common to both tumor and nontumor left *vs* right DEG lists. For the diabetic (n = 18) *vs* nondiabetic (n = 30) groups of tumor samples, 52 DEGs and 9 DEGs were increased, and for the diabetic (n = 12) *vs* nondiabetic (n = 21) groups of nontumor samples, 2 DEGs were decreased and 6 DEGs were increased. No DEGs were common to both tumor and nontumor diabetic/nondiabetic DEG lists.

In the analysis of the Black/AA (n = 11) *vs* not Black/AA (n = 37) groups of tumor samples, 5 DEGs were decreased and 11 DEGs were increased in Black/AA samples. For the Black/AA (n = 10) *vs* not Black/AA (n = 33) groups of nontumor samples, 42 DEGs were decreased and 5 DEGs were increased in Black/AA samples. Three DEGs were common to both tumor and nontumor Black/AA *vs* not Black/AA groups (see Table 9).

DISCUSSION

The results of this pilot study illustrate the need for thorough curation of the clinical metadata linked to archived gastrointestinal resection specimens for discovery and functional studies on CRC pathophysiology. Even after prospectively interviewing

Table 8 Differentially expressed genes, (+)antibiotics *vs* (-)antibiotics, common to both tumor and nontumor samples

Gene (increased)	Symbol	Tumor log_2 fold change	Tumor adjusted *P* value	Nontumor log_2 fold change	Nontumor adjusted *P* value
ENSG00000023902	*PLEKHO1*	1.127	0.0461	1.694	0.0130
ENSG00000253304	*TMEM200B*	1.326	0.0445	1.719	0.0481
ENSG00000187608	*ISG15*	1.534	0.0301	2.202	0.0102
ENSG00000185745	*IFIT1*	1.708	0.0243	2.245	0.0176
ENSG00000109705	*NKX3-2*	2.082	0.0476	2.859	0.0264
ENSG00000145936	*KCNMB1*	2.011	0.0480	2.874	0.0176

Table 9 Differentially expressed genes, Black/African Ancestry *vs* not Black/African Ancestry, common to both tumor and nontumor samples

Gene	Symbol	Tumor log_2 fold change	Tumor adjusted *P* value	Nontumor log_2 fold change	Nontumor adjusted *P* value
Decreased					
ENSG00000155959	*VBP1*	-8.217	5.26×10^{-10}	-8.318	5.26×10^{-10}
ENSG00000102109	*PCSK1N*	-5.965	0.0050	-7.175	0.0004
Increased					
ENSG00000225972	*MTND1P23*	4.0913	5.67×10^{-7}	3.801	0.0001

research subjects for antibiotic exposure prior to surgical resection and carefully reviewing the electronic medical record for prescribed medications, it was difficult to assess this potentially important confounding variable. Further discussion with the colorectal surgery service revealed that a major change in pre-operative antibiotics was established as standard of care as of January 2017 to reduce site infections. This change in protocol was made, because there was accumulating evidence that the addition of short term perioperative oral antibiotics reduced intra-abdominal surgical site infections[9-11]. The reduced mucosal associated bacterial abundance based on our 16S rRNA broad range PCR assays, may contribute to the reduced incidence of postoperative intra-abdominal infections. Our results indicate that there is a measurable effect not only on the host mucosal-associated microbial communities but also on the host colonic mucosal transcriptomic profiles. Reviewing the literature, while some studies clearly excluded CRC subjects who had received additional preoperative antibiotics[13], it is not clear in a number of published reports, whether exposure to preoperative antibiotics was taken into consideration in the analysis. Though the effect of the antibiotics is transient, and the baseline microbiome is shown to recover within 1.5 mo with the most potent antibiotics[36], the impact of preoperative antibiotics has a measurable effect on the mucosal transcriptomic profiles generated from the resection specimens.

A major limitation of this pilot study is the small number of subjects who were analyzed and potential selection bias with respect to the five variables included in the analyses. We detected significant differences in microbial β-diversity between tumor *vs* nontumor histology groups and (+)antibiotics *vs* (-)antibiotics. However, failure to detect differences with respect to CRC location, diabetes or Black/AA race could be because this study was underpowered to detect a significant effect of these three variables. Despite heterogeneity in pre-operative antibiotic protocols, qPCR analysis confirmed previous reports showing that *F. nucleatum* abundance was increased in tumor compared to paired nontumor samples[29,37-41]. Lower detection rates of *F. nucleatum* nusG gene by PCR were previously reported on archived FFPE CRC tissues, ranging from 13%-45%[37,38] while our study reports 84% and 93% detection rates for tumor and non-tumor fresh frozen regions respectively. Furthermore, *F. nucleatum* has been detected in advanced adenomas but not in early adenomas, which are precursor stages along the adenoma-carcinoma sequence[39,41]. *F. nucleatum* abundance has been associated with poor prognosis and resistance to chemotherapy[37,41,42]. In xenograft models of human CRC, antibiotic treatment for three weeks reduced *Fusobacterium* load, cancer cell proliferation and overall tumor growth, however treatment for only

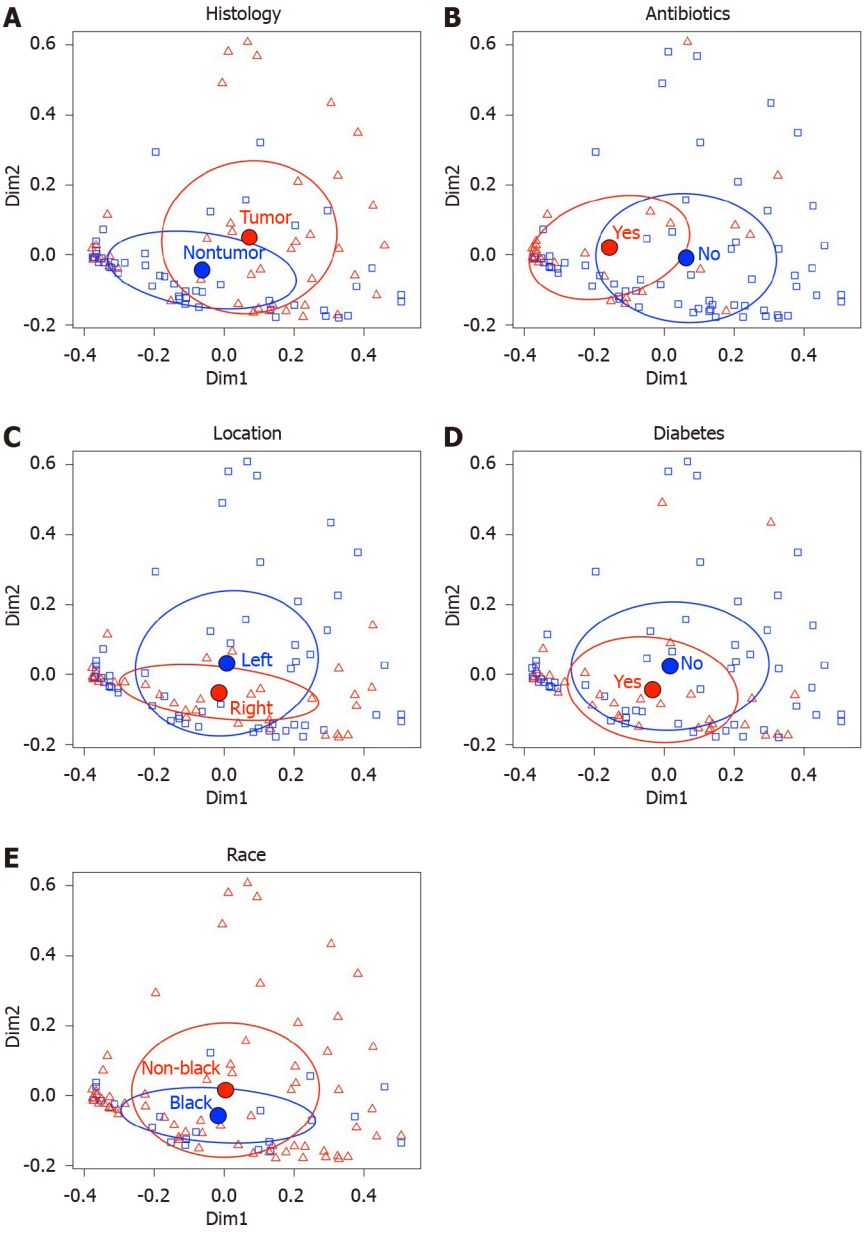


Figure 1 Principal coordinate analysis plots for Morita Horn dissimilarity indices. A: Preoperative antibiotic treatment (yes/no); B: Tumor *vs* nontumor histology; C: Anatomic location of sample (left *vs* right); D: Diabetes mellitus status (yes/no); E: Black/African Ancestry race (yes/no). The filled circles and surrounding ovals indicate the mean ± SD centroid for each group.

24 h had no significant effect[38]. There has been one report that *F. nucleatum* abundance is higher in colonic effluents collected from Black/AA male veterans compared to White/EA male veterans undergoing screening colonoscopy[43].

Despite the small sample size and variable exposure to preoperative antibiotics, it was still possible to use the data for discovery studies. Expression of the von Hippel-Landau binding protein 1 or *VBP1* in Black/AA CRC tumors (n = 64) was observed to be significantly lower compared to White/EA CRC tumors (n = 284, P = 0.026) in The Cancer Genome Atlas (TCGA) database and in a recently published transcriptomic profiling study[21]. The polarity observed for differences in proprotein convertase subtilisin/kexin type 1 inhibitor or *PCSK1N* Black/AA *vs* White/EA CRC tumors in the TCGA database however, was opposite to what was observed in this pilot study. The *MTND1P23* gene was not present in the TCGA database. We are particularly intrigued by the results indicating reduced *VBP1* expression in Black/AA tumor *vs* not Black/AA tumor and Black/AA nontumor *vs* not Black/AA nontumor, because of a recent report that *VBP1* suppresses HIF-1α-induced epithelial□mesenchymal transition *in vitro* and tumor metastasis *in vivo*[44].

Given that the new preoperative antibiotic protocol adopted at this medical center is 24 h of oral antibiotics in addition to current IV antibiotics administered within 30 min

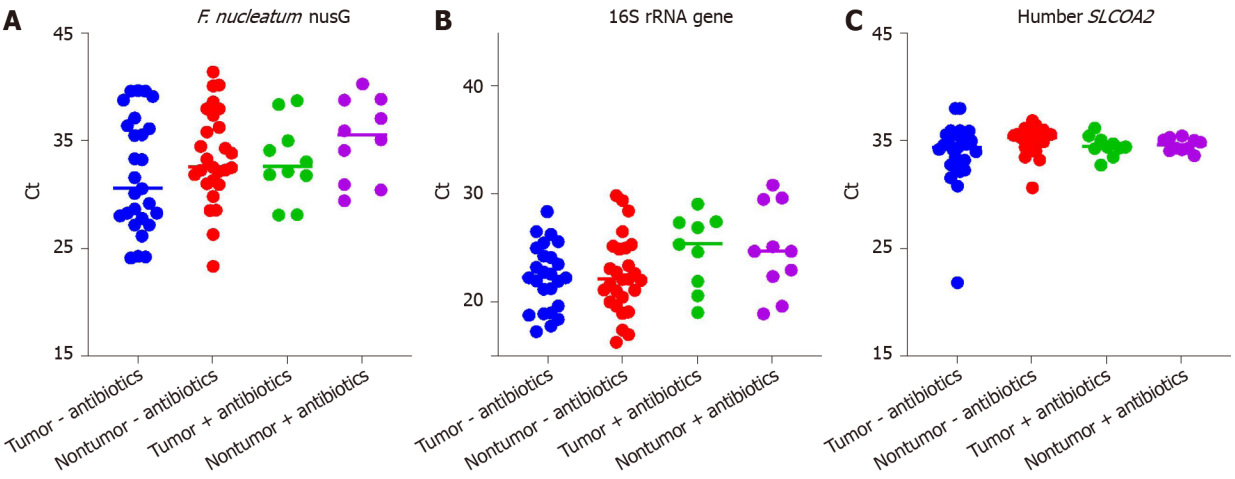


Figure 2 Scatter plots of threshold cycle values. A: *Fusobacterium nucleatum* nusG gene; B: Broad range 16S rRNA gene; C: Human *SLCOA2* gene quantitative polymerase chain reaction assays. *F. nucleatum*: *Fusobacterium nucleatum*.

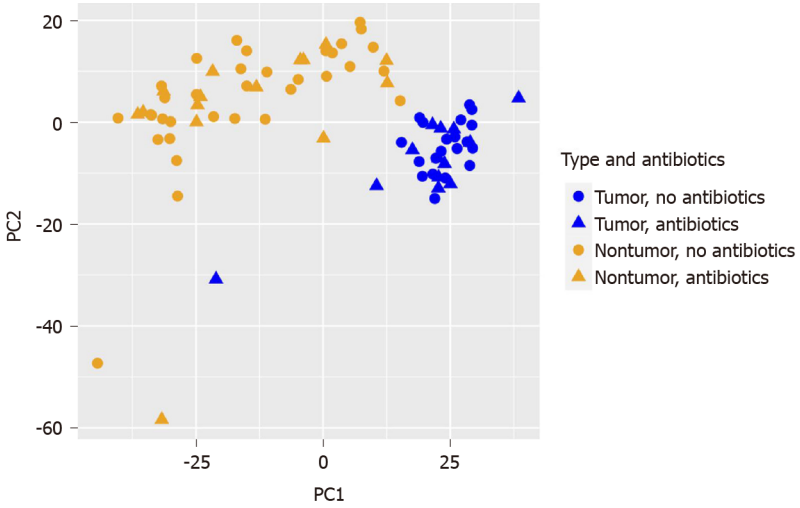


Figure 3 Principal coordinate analysis of human colorectal cancer RNA-seq data with respect to tumor histology (tumor *vs* nontumor) and preoperative antibiotics (yes/no).

of incision for colorectal resections, a relevant question is how to further pursue the findings of this initial discovery and functional study. Going forward, probably the best solution is to perform targeted PCR assays on archived FFPE CRC and advanced adenoma tissues collected prior to the change in preoperative antibiotics protocol in order to sufficiently power the analysis of evaluating the effect of laterality, diabetes status and race on microbiome and transcriptomic data of CRC patients. In particular, utilizing the targeted PCR to detect *Fusobacterium*, it can possibly be used as a clinical prognostic biomarker for diabetic CRC patients. We also plan to shift our prospective collection of colonic tissues away from surgical resections to prospective collection of colonoscopic biopsy samples.

CONCLUSION

The recent addition of preoperative oral antibiotics 24 h to the standard administration of IV antibiotics within 30 min of incision has a measurable effect on colonic mucosal gene expression in addition to its effect on the amount and composition of mucosal associated bacteria in the resected specimen. Despite heterogeneity in the preoperative antibiotics in this study cohort, increased abundance of *F. nucleatum*, was observed in tumor *vs* nontumor regions of the resected specimen. This study identified the *VBP1* gene, which may suppress CRC metastasis, as having decreased RNA expression in

both tumor and nontumor regions of the resected specimen collected from Black/AA subjects.

ARTICLE HIGHLIGHTS

Research background

Imbalances in mucosal associated microbiota (dysbiosis) have been reported in human colorectal cancer (CRC). There are certain pathogens that are associated with CRC including increased abundances of *Peptostreptococcus, Bacteroides fragilis, Fusobacterium nucleatum,* and *Escherichia coli* and decreased abundances of *Clostridium, Bifidobacterium, Faecalibacterium* and *Roseburia.* The approach of collecting and integrating multi-omic host and microbiome data has been increasingly applied to discovery and functional studies of human gastrointestinal disease.

Research motivation

A major change was made in the preoperative antibiotic protocol at this hospital as of January 2017. Prior to that time, the standard of care was to administer only intravenous antibiotics within 30 min of incision, and only a few CRC resection patients were placed on short-term oral antibiotics within a month of the surgery, for various clinical indications. However, after this time, the standard operating protocol for preoperative antibiotics was to prescribe outpatient oral neomycin and metronidazole 24 h in advance of the procedure, in order to reduce surgical site infections. The use of antibiotics can shift the microbiome depending on the dosage and duration of the antibiotic exposure. Several studies have shown that tumorigenesis and tumor growth can be attenuated with different antibiotic cocktails and timing of antibiotic exposure with duration of inflammation. On the other hand, early exposure to antibiotics increased risk of CRC and interfered with chemotherapy efficacies due to microbial dysbiosis. With these conflicting findings and this change in protocol at our institution, it allowed us to examine how differential use of antibiotics, along with other clinical/demographic factors influences integrative, multi-omic analyses of CRC.

Research objectives

To examine the effect of the five following variables were included in the analysis of the microbiome and host transcriptome datasets generated in this pilot study: (1) Tumor histology (tumor *vs* nontumor); (2) Preoperative antibiotics (yes/no); (3) Laterality of CRC location (left *vs* right); (4) Diabetes mellitus (yes/no); and (5) Black/African Ancestry (AA) race (yes/no).

Research methods

Genomic DNA (gDNA) and RNA were extracted from prospectively collected 51 pairs of frozen sporadic CRC tumor and adjacent non-tumor mucosal samples from 50 CRC patients archived at a single medical center from 2010-2020. 16S rRNA gene sequencing (V3V4 region, paired end, 300 bp) and confirmatory quantitative polymerase chain reaction (PCR) assays were conducted on gDNA. RNA sequencing (IPE, 125 bp) was performed on parallel tumor and non-tumor RNA samples with RNA Integrity Numbers scores ≥ 6.

Research results

Exploratory PERMANOVA using the Morasita Horn index for β-diversity was performed with each of the five groups. Tumor *vs* nontumor histology (P = 0.002) and preoperative antibiotics (P = 0.001) demonstrated significant F statistics, but anatomic location, diabetes and Black/AA race did not reach significance. Differences in α-diversity did not reach significance between the five groups. Fourteen taxa at the genus level exhibited significant tumor*preoperative antibiotic interactions. Of the taxa without tumor*preoperative antibiotic interactions, 7 taxa were significantly increased in tumor *vs* nontumor, including *Fusobacterium*, and 11 taxa were significantly decreased. The increase in *Fusobacterium nucleatum* (*F. nucleatum*) abundance was confirmed by Taqman PCR assays. Additional preoperative antibiotics significantly reduced mucosa-associated total bacterial abundance, which may contribute to reduction of intra-abdominal surgical site infections. Analysis of a subset of parallel formalin-fixed paraffin embedded (FFPE) samples retained polarity of the observed trends but impaired signal strength. Principal coordinate analysis of the

transcriptomic data showed a clear separation from tumor and nontumor samples. Consequently, differentially expressed genes were analyzed separately for the other four variables separately in tumor and nontumor samples. Differentially expressed genes common to the tumor and nontumor groups were identified for additional preoperative antibiotics and Black/AA race. The *VBP1* gene, which may suppress CRC metastasis, exhibits reduced expression in Black/AA subjects compared to not Black/AA subjects.

Research conclusions

The recent addition of preoperative oral antibiotics 24 h to the standard administration of IV antibiotics within 30 min of incision has a measurable effect on colonic mucosal gene expression in addition to its effect on the amount and composition of mucosal associated bacteria in the resected specimen. Despite heterogeneity in the preoperative antibiotics in this study cohort, increased abundance of *F. nucleatum,* was observed in tumor *vs* nontumor regions of the resected specimen. This study identified the VBP1 gene, which may suppress CRC metastasis, as having decreased RNA expression in both tumor and nontumor regions of the resected specimen collected from Black/AA subjects.

Research perspectives

Given that the addition of preoperative antibiotics to the standard administration of IV antibiotics within 30 min of incision has a measurable effect on colonic mucosal gene expression, in addition to its effect on the amount and composition of mucosa-associated bacteria in the resected specimen, is being widely adopted as standard of care, we plan to perform targeted PCR assays on archived CRC FFPE tissues to confirm our results collected prior to when the change in protocol was adopted (January 2017). Because patients undergoing colonoscopy are not routinely prescribed antibiotics before the procedure, we also plan to shift prospective collection of colonic neoplastic and normal tissues away from surgical resections to prospective collection of research colonoscopic biopsy samples.

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ORIGINAL ARTICLE

Retrospective Study

Apolipoprotein E variants correlate with the clinical presentation of paediatric inflammatory bowel disease: A cross-sectional study

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Abstract

BACKGROUND

It has been suggested that apolipoprotein E (*APOE*) polymorphisms are associated with the risk of developing inflammatory bowel disease (IBD) and the early age of disease onset. However, there are no reports regarding the relationship with clinical characteristics and disease severity.

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AIM

To summarise that APOE polymorphisms are associated with the risk of developing IBD and the early age of disease onset.

METHODS

In total, 406 patients aged 3-18 with IBD (192 had ulcerative colitis and 214 had Crohn's disease) were genotyped using the TaqMan hydrolysis probe assay. Clinical expression was described at diagnosis and the worst flare by disease activity scales, albumin and C-reactive protein levels, localisation and behaviour (Paris classification). Systemic steroid intake with the total number of courses, immunosuppressive, biological, and surgical treatment with the time and age of the first intervention were determined. The total number of exacerbation-caused hospitalisations, the number of days spent in hospital due to exacerbation, the number of relapses, and severe relapses were also estimated.

RESULTS

Ulcerative colitis patients with the *APOEε4* allele had lower C-reactive protein values at diagnosis (P = 0.0435) and the worst flare (P = 0.0013) compared to patients with the *APOEε2* allele and genotype *APOEε3/ε3*. Crohn's disease patients with the *APOEε2* allele scored lower on the Pediatric Crohn's Disease Activity Index at diagnosis (P = 0.0204). IBD patients with *APOEε2* allele spent fewer days in the hospital due to relapse (P = 0.0440).

CONCLUSION

APOE polymorphisms are associated with the risk of developing IBD and the clinical expression of IBD. However, the clinical relevance of the differences identified is rather modest.

Key Words: Apolipoprotein E polymorphism; Crohn's disease; Ulcerative colitis; Immunosuppression; Surgery; Disease severity

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Core Tip: Apolipoprotein E polymorphisms are associated with the risk of developing inflammatory bowel disease and seem to be associated with the disease expression and treatment. However, the clinical relevance of the differences is relatively modest.

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INTRODUCTION

Heritability and disease risk can only be partly explained by genetic factors alone[1-4]. Inflammatory bowel disease (IBD) has a strong genetic makeup. To date, 240 risk gene loci have been associated with the disease[1]. Several genetic variations are linked to specific IBD phenotypes. For instance, *NOD2*, *IRGM*, *ATG16L1*, and *NCF4/NCF2* are related to segmental, structuring, or early-onset disease[5-10]. Genetic testing for these and other variants may prove useful in predicting the disease course for future clinical use.

One of the well-known genetic determinants of some diseases other than IBD is apolipoprotein E (*APOE*), most commonly known for its role in Alzheimer's disease[11]. Although first recognised for its role in lipoprotein metabolism, APOE is involved in several biological processes not directly related to lipid transport function[12]. Importantly, APOE is a key player in immunoregulation[13-15] and

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associated with autoimmune disorders such as multiple sclerosis, rheumatoid arthritis, and psoriasis[16-18]. It has been reported that APOE has several immune-related functions such as suppressing T-cell proliferation[19-21], possibly by downregulating DNA synthesis and reducing phospholipid turnover in T cells[22-24], neutrophil activation[25], and modulation of macrophage assisted[26-28] antigen presentation[14,15].

APOE is a polymorphic protein present in three major isoforms that differ only by two single amino acid substitutions, APOEε4 (arg112, arg158), APOEε3 (cys112, arg158), and APOEε2 (cys112, cys158). The amino acid replacement causes profound functional changes at the cellular and molecular level as well as in the immune system. APOE suppresses the production of proinflammatory cytokines such as tumour necrosis factor-α in microglia in an isoform-dependent manner (ε2 > ε3 > ε4)[29]. In turn, inflammatory cytokines can promote APOE synthesis and release or downregulate the production of APOE in different tissues[30,31]. However, interactions between APOE and cytokines are occasionally conflicting, highlighting the complex roles of APOE and cytokines in various disorders[15].

In IBD, inflammation alters lipid, apolipoprotein, and lipoprotein profiles in subjects with active disease[32,33] and patients with limited response to infliximab[34]. A previous study from Saudi Arabia showed that the genetic distribution of *APOE* polymorphisms in IBD seems to be altered compared to healthy subjects[35]. The study also suggested that the *ε4* allele increased the risk of IBD and was associated with an early onset of the disease. Similarly, *APOEε4* has been associated with severity in another immunologic disorder: rheumatoid arthritis[16]. For these reasons, this study aimed to investigate the relationship between *APOE* variants with disease severity in IBD.

MATERIALS AND METHODS

Patients

Patients recruited to the study belonged to the Polish Paediatric Crohn's and Colitis cohort and involved 406 paediatric IBD patients: 214 with Crohn's disease (CD; 86 females, 128 males) and 192 with ulcerative colitis (UC; 87 females, 105 males) (Table 1). Patients were recruited in the course of hospital treatment or during scheduled visits at outpatient clinics (Department of Pediatric Gastroenterology and Metabolic Diseases, Poznań University of Medical Sciences; The Department of Gastroenterology, Hepatology, Feeding Disorders and Paediatrics; The Children's Memorial Health Institute, Warsaw; Department of Pediatric Gastroenterology and Nutrition, Medical University of Warsaw; Department and Clinic of Pediatrics, Gastroenterology and Nutrition, Wroclaw Medical University; Department of Pediatrics, Faculty of Medical Sciences in Zabrze, Medical University of Silesia, Katowice; Department of Pediatrics, Faculty of Medical Sciences, Medical University of Silesia in Katowice and Department of Pediatric Endoscopy and Gastrointestinal Function Testing, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Bydgoszcz, Poland). The diagnosis of IBD was confirmed by experienced gastroenterologists using standard diagnostic criteria[36,37]. The inclusion criteria were a diagnosis of CD or UC and aged 3-18. Patients in life-threatening, severe general condition were excluded from the study. The study obtained the approval of the Bioethical Committee at Poznań University of Medical Sciences (960/15 with the associated amendments).

Disease severity evaluation

Disease activity was assessed using appropriate scales at diagnosis and the worst flare [Pediatric Ulcerative Colitis Activity Index and Pediatric Crohn's Disease Activity Index (PCDAI)][38], which was defined by the highest Pediatric Ulcerative Colitis Activity Index and PCDAI scales in their medical history. Albumin (g/dL) and C-reactive protein (CRP; mg/L) concentrations at diagnosis and the worst flare were obtained from medical records (CRP reference range 0-5 mg/L). The treatment domain included data regarding systemic steroid intake with the total number of courses, immunosuppressive treatment with the time and age of the first intake, biological therapy with time and age of first admission, and operative treatment with time and age of first surgery. The localisation and behaviour of the disease were defined by the Paris Classification at the diagnosis and worst flare[39]. Most CD patients presented with an ileocolonic location and nonstricturing behaviour of the disease (Supplementary Table 1), while most UC patients presented with pancolitis and were never severe (S0: > 65 on the Pediatric Ulcerative Colitis Activity Index scale;

Table 1 Demographic and clinical expression of Crohn's disease and ulcerative colitis

Variables median (IQR) or *n* (%)	*n*	Crohn's disease	Ulcerative colitis	*P* value
Age in yr				
At inclusion	397	15.18 (13.32-17.05)	15.11 (11.70-16.75)	0.044
At diagnosis	404	12.58 (10.02-14.32)	12.14 (7.89-14.94)	0.365
At worst flare	355	13.63 (11.54-15.85)	13.76 (10.13-15.84)	0.244
Duration of the disease (yr)	390	2.23 (0.82-4.25)	1.88 (0.36-3.77)	0.239
Female	173	86 (40.2)	87 (45.3)	0.297
Nutritional status				
Weight at diagnosis in kg	387	38.0 (27.0-49.8)	40.0 (27.8-53.9)	0.490
Weight at diagnosis, z score	383	-0.82 [(-1.39)-(-0.04)]	-0.51 [(-1.12)-0.22]	0.003
Height at diagnosis in cm	382	151.0 (137.0-164.5)	152.0 (130.5-168.3)	0.718
Height at diagnosis, z score	378	-0.37 [(-1.29)-0.47]	0.06 [(-0.67)-0.81]	0.001
Body mass index at diagnosis in kg/m^2	382	16.6 (14.5-18.4)	17.4 (15.5-19.3)	0.019
Body mass index at diagnosis, z score	378	-0.79 [(-1.47)-(-0.04)]	-0.49 [(-1.00)-0.16]	0.006
Albumin level at diagnosis in g/dL	345	3.90 (3.51-4.25)	4.10 (3.70-4.40)	< 0.003
Parameter of inflammation				
CRP at diagnosis in mg/L[1]	386	12.94 (2.10-29.25)	2.24 (0.50-10.80)	< 0.001
CRP at worst flare in mg/L	347	13.95 (3.03-32.43)	2.70 (0.63-13.44)	< 0.001
Disease activity scales				
PCDAI/PUCAI at diagnosis	190/166	32 (23-48)	45 (28-60)	
PCDAI/PUCAI at worst flare	170/155	40 (30-53)	50 (35-65)	
Treatment				
Systemic steroids[2]	406	115 (53.7)	138 (71.9)	< 0.001
Immunosuppressive treatment[3]	405	168 (78.5)	112 (58.6)	< 0.001
Biological therapy[4]	406	107 (50.0)	49 (25.5)	< 0.001
Operative treatment[5]	406	29 (13.6)	4 (2.1)	< 0.001

[1]C-reactive protein reference range 0-5 mg/L.
[2]Systemic steroid therapy included: methylprednisolone, prednisone, hydrocortisone.
[3]Immunosuppressive and anti-inflammatory agents included: Azathioprine, methotrexate, mercaptopurine, cyclosporine, mycophenolate mofetil, tacrolimus, sulfasalazine.
[4]Biological agents included: infliximab, adalimumab, golimumab, vedolizumab.
[5]Only surgery related to inflammatory bowel disease-specific problems (*e.g.*, colectomy, resection, fistula, perforation, abscess) was included. CRP: C-reactive protein; IBD: Inflammatory bowel disease; IQR: Interquartile range; PCDAI: Pediatric Crohn's Disease Activity Index; PUCAI: Pediatric Ulcerative Colitis Activity Index.

Supplementary Table 2). Based on medical records, the total number of exacerbation hospitalisations, the number of days spent in hospital due to exacerbation, the number of relapses, and severe relapses from diagnosis were estimated and calculated per year of the disease duration. The associated extraintestinal symptoms and concomitant diseases were collected from the medical history.

Genotyping

DNA was isolated from whole blood using the Blood Mini (A and A Biotechnology). A hydrolysis probe assay (TaqMan assay) was used with the following probes, C_904973_10 and C_3084793_20, to genotype patients (Life Technologies Corp. Carlsbad. California, United States). The genotyping was performed on the CFX-96 thermocycler system with allele discrimination plots provided by CFX Manager Software (Bio-Rad, Hercules, CA, United States).

Statistical analysis

Differences in categorical variables were compared with two-tailed Fisher's exact test. Differences in continuous variables were evaluated by the Mann Whitney *U* test and Kruskal-Wallis test. Post hoc comparisons were performed with Dunn's test, and the significance level for the time-to-treatment analysis was evaluated by Gehan's test. The explanatory factor analysis was used to analyse the underlying factors in the questionnaire. The significance level was set at $P < 0.05$, and statistical analyses were performed using Statistica 13.1 software (StatSoft Inc, Tulsa, OK, United States), JASP 0.10.2 (University of Amsterdam, Amsterdam, the Netherlands), and G*Power (Dusseldorf University, Germany). Comparisons between groups with less than ten patients were not included.

RESULTS

Genotyping

The most prevalent genotype in UC and CD was *APOEε3/ε3* (Table 2). No differences in the distribution of alleles and genotypes between UC and CD were documented.

The distribution of the *APOE* genotypes was compared to previous studies in the Polish population (Supplementary Table 3). Pooling available data[40-42] to obtain a similar sample size (n = 425) showed a significantly lower frequency of *APOEε3/ε3* genotype in IBD patients compared to controls (62.3% *vs* 71.5%; P = 0.0051; odds ratio = 0.66; 95% confidence interval: 0.49-0.88) and simultaneously higher frequency of *APOEε3/ε4* genotype (21.7% *vs* 15.1%; P = 0.0153; odds ratio = 1.56; 95% confidence interval: 1.09-2.23) with no difference in other genotypes or for the *APOEε3* allele (P = 0.8625). However, in the study of Bojar *et al*[43] (postmenopausal women; n = 402), the distribution of *APOEε3/ε3* genotype was similar to the present study (62.9% *vs* 62.3%; P = 0.8555; odds ratio = 0.97; 95% confidence interval: 0.73-1.30).

UC patients with *APOEε3ε3* had higher CRP values, and the *APOEε2/ε3* genotype were predisposed to left-sided colitis (E2) at diagnosis (Table 3). Concomitant diseases in CD patients occurred at different frequencies in major *APOE* genotypes, and children with *APOEε2ε3* genotype had significantly lower PCDAI scores at diagnosis than patients with the remaining genotypes (Table 4). UC patients with *t*he *APOEε4* allele had significantly lower CRP levels than the patients with *APOEε3ε3* genotype and *APOEε2*-positive, both at diagnosis and at the worst flare (Table 5). There were also differences in age at first biological treatment. Additionally, *APOEε2*-positive patients with IBD spent significantly fewer days in the hospital due to relapse per year of disease duration than *APOEε4*-positive patients and with the *APOEε3/ε3* genotype (Table 5). Patients with CD and *APOEε3ε3* genotype had lower values of standardised body height at diagnosis (Table 5). No difference was observed in the frequency of systemic steroids, immunosuppressive, and biological treatment between *APOE* genotypes in UC and CD patients. Supplementary Table 4 shows the results for the whole group of IBD patients.

DISCUSSION

The present study investigated the relationship between *APOE* genotype and disease severity in IBD, suggesting that the *APOE* genotype might be associated with some indices of disease course such as CRP and albumin levels at the worst flare, age at surgery and numbers of hospitalisation days. UC patients with the *APOEε4* allele had the lowest values of CRP, both at diagnosis and the worst flare. The median age at first biological therapy in UC was lowest in patients with the *APOEε4* allele, whereas left-side colitis was more frequent among patients with the *APOEε2* allele. In CD patients, the *APOEε4* allele was associated with higher albumin at worst flare and higher standardised body height at diagnosis. Moreover, patients with the *APOEε2* allele scored lower on the PCDAI. This study is the largest to show the genetic distribution of *APOE* polymorphisms in IBD to date.

APOE is known to be associated with inflammation indicators[13]. The findings of the present study confirm this relationship as the CRP levels differed between *APOE* genotypes. Patients with the *APOEε4* allele and *APOEε3ε4* genotype had lower CRP values at diagnosis and the worst flare, while patients with the *APOEε3ε3* genotype had higher levels of CRP at the worst flare. These results are similar to those obtained in healthy adults, which showed that subjects with *APOEε3ε3* had the highest plasma

Table 2 Apolipoprotein E genotype and allele distribution compared between ulcerative colitis and Crohn's disease

Genotype/allele	UC, *n* = 192	CD, *n* = 214	*P* value, two-tailed Fisher exact	Odds ratio (95%CI)
ε3/ε3	118	135	0.7590	0.93 (0.62-1.40)
ε3/ε4	47	41	0.2278	1.37 (0.85-2.20)
ε2/ε3	18	35	0.0397	0.53 (0.29-0.97)
ε3+	183	211	0.0757	0.29 (0.08-1.08)
ε4+	54	43	0.0629	1.56 (0.98-2.46)
ε2+	24	37	0.2108	0.68 (0.39-1.19)

CD: Crohn's disease; CI: Confidence interval; UC: Ulcerative colitis.

levels of CRP and individuals with *APOEε4ε4* and *APOEε2ε4* had the lowest levels[13]. A similar pattern has also been observed in other diseases such as coronary artery disease[43-46]. März *et al*[47] proved that in coronary artery disease, both white cell count and fibrinogen were not related to the *APOE* genotype, suggesting that the underlying mechanism is not associated with inflammation[46] but rather to the mevalonate/cholesterol synthetic pathway, which may be downregulated in patients with *APOEε4* in response to altered lipoprotein metabolism and hepatic uptake[46]. In another study, the *APOEε4* allele was also associated with lower CRP but not white blood cell count[47]. Further mechanistic studies are needed to explain the link.

Our study is the first to report that in CD patients, the *APOEε4* allele is associated with higher median levels of albumins at the worst flare. Albumin level is negatively correlated with the extent of the inflammatory response, which is caused by a hypercatabolic state and a decrease of albumin synthesis in the liver[48]. Tumour necrosis factor-α inhibits albumin expression causing hypoalbuminemia[48] , a state associated with IBD activity, unresponsiveness to treatment, and increased risk of colectomy in UC. Patients with hypoalbuminemia had a higher likelihood of having more than two courses of corticosteroids, thiopurine, or anti-tumour necrosis factor treatment[49]. In CD, albumin levels were reported as a marker of postoperative complications[50] and active clinical disease[51]. Low albumin level together with high CRP may correlate with an increased inflammatory response[52]. In the study of Sayar *et al*[53], the area under the curve values for severe UC were 0.883 for albumin levels (cut-off 3.6 g/dL) and 0.941 for CRP/albumin ratio (cut-off 0.6)[52]. Given these data, the results of our study may suggest that the *APOEε4* allele is associated with a milder disease course of CD. The association of the *APOEε2* allele with lower PCDAI scores and fewer days of hospitalisation due to relapse might suggest a protective role of this allele on disease severity. However, this relationship is more complicated as we found that the *APOEε2* allele is also associated with a younger age at first surgery. This finding should be verified, preferably in a group of adult patients with a longer disease course and higher surgery rates.

The biology of APOE in IBD has not been fully elucidated, but recent studies have shown that the *APOE* transcript is overexpressed in paediatric IBD patients[53]. Studies in colonic epithelial cells in a mouse model showed that the apoE-mimetic peptide (COG112) inhibited the inflammatory response to *Citrobacter rodentium*[54], a bacterium known to cause colitis in mice[55]. The authors suggested this occurred by preventing the activation of nuclear factor κB[54]. Therefore, further mechanistic studies of APOE action are warranted.

A previous study on *APOE* in IBD in a group with a different genetic background (Saudi Arabia) did not focus on disease severity. Therefore, any comparisons are difficult[35]. In that study, the *APOEε4* allele was associated with the risk of developing IBD and early onset, whereas our study did not identify significant differences between *APOE* genotypes and age at diagnosis. The frequencies of *APOEε3ε3* genotype were lower in IBD patients in comparison to controls, which is consistent with the above-mentioned report[35].

The present study involved a large multicentre paediatric cohort, including a comprehensive clinical description, which allowed a detailed genotype-phenotype analysis. However, defining the global severity of the disease course remains challenging, especially in diseases with such a differentiated clinical presentation. The major limitation of this study is related to the retrospective character of the data collection regarding diagnosis and the worst flare. Need for surgery, which is one of

Table 3 Clinical characteristics in patients with ulcerative colitis depending on major apolipoprotein E genotypes

Variables median (IQR) or *n* (%)	*n*	ε2/ε3	ε3/ε3	ε3/ε4	*P* value
Age in yr					
At inclusion	184	15.7 (12.5-16.9)	15.3 (11.9-16.9)	14.3 (11.5-16.3)	0.2464
At diagnosis	191	11.4 (7.9-14.6)	12.4 (7.9-15.0)	12.4 (8.2-14.9)	0.9070
At worst flare	171	14.6 (9.9-16.4)	13.7 (10.4-16.0)	13.7 (10.0-15.7)	0.7255
Duration of the disease in yr	179	3.0 (1.4-6.2)	1.9 (0.4-3.5)	1.2 (0.0-3.5)	0.0868
Nutritional status					
Weight at diagnosis in kg	180	40.0 (28.8-59.5)	39.0 (27.8-54.0)	43.8 (29.5-53.4)	0.9704
Weight at diagnosis, z score	179	-0.20 [(-0.86)-0.43]	-0.5 [(-1.1)-0.1]	-0.24 [(-0.95)-0.63]	0.3037
Height at diagnosis in cm	175	146.5 (129.0-169.0)	153.0 (131.0-168.5)	156.0 (131.5-169.0)	0.9175
Height at diagnosis, z score	174	0.12 [(-0.62)-0.75]	0.09 [(-0.69)-0.79]	0.22 [(-0.44)-1.06]	0.5823
Body mass index at diagnosis in kg/m^2	175	17.61 (16.02-19.74)	17.0 (15.4-19.1)	17.9 (15.4-20.3)	0.5121
Body mass index at diagnosis, z score	174	-0.11 [(-0.70)-0.29)]	-0.56 [(-0.99)-0.11]	-0.30 [(-1.12)-0.56]	0.2293
Weight at worst flare in kg	164	46.1 (31.6-62.0)	46.2 (31.9-55.6)	50.0 (28.0-61.0)	0.9600
Weight at worst flare, z score	161	-0.33 [(-1.00)-0.56]	-0.58 [(-0.95)-0.16]	-0.52 [(-0.90)-0.40]	0.6559
Height at worst flare in cm	162	162.5 (138.5-173.5)	159.0 (140.9-171.0)	160.0 (135.0-172.0)	0.9688
Height at worst flare, z score	161	0.11 [(-0.72)-1.16]	-0.09 [(-0.62)-0.78]	0.06 [(-0.62)-0.89]	0.8376
Body mass index at worst flare in kg/m^2	160	18.20 (16.47-19.74)	17.36 (15.75-19.71)	17.93 (15.89-20.96)	0.6013
Body mass index at worst flare, z score	159	-0.22 [(-1.16)-0.14]	-0.68 [(-1.10)-0.16]	-0.43 [(-1.12)-0.63]	0.6789
Albumin level					
At diagnosis in g/dL	159	4.2 (4.0-4.6)	4.1 (3.7-4.4)	4.1 (3.6-4.4)	0.2569
At worst flare in g/ dL	148	4.3 (4.0-4.7)	4.1 (3.6-4.4)	4.2 (4.0-4.4)	0.3488
Parameter of inflammation					
CRP at diagnosis in mg/L	178	3.8 (0.7-6.6)	2.5 (0.7-12.2)	1.1 (0.2-8.0)	0.0515
CRP at worst flare in mg/L	162	2.1 (1.1-23.3)	3.7 (1.1-19.0)	0.8 (0.3-2.9)	0.0012
Disease activity scales					
PUCAI at diagnosis	166	40 (18-55)	45 (30-60)	50 (25-60)	0.5144
PUCAI at worst flare	155	48 (20-65)	55 (40-65)	50 (30-65)	0.3766
Disease localisation and behaviour					
E1 at diagnosis	19/192	3 (16.7)	10 (8.5)	6 (12.8)	0.4694
E2 at diagnosis	33/192	8 (44.4)	16 (13.6)	9 (19.1)	0.0063
E3 at diagnosis	28/192	1 (5.6)	18 (15.3)	9 (19.1)	0.3953
E4 at diagnosis	83/192	5 (27.8)	60 (50.8)	18 (38.3)	0.0990
S0 at diagnosis	110/192	13 (72.2)	69 (58.5)	28 (59.6)	0.5383
S1 at diagnosis	37/192	3 (16.7)	23 (19.5)	11 (23.4)	0.7885
E1 at worst flare	9/192	1 (5.6)	4 (3.4)	4 (8.5)	0.3863
E2 at worst flare	27/192	3 (16.7)	18 (15.3)	6 (12.8)	0.8943
E3 at worst flare	23/192	3 (16.7)	16 (13.6)	4 (8.5)	0.5814
E4 at worst flare	75/192	7 (38.9)	50 (42.4)	18 (38.3)	0.8750
S0 at worst flare	83/192	9 (50.0)	52 (44.1)	22 (46.8)	0.8713
S1 at worst flare	49/192	5 (27.8)	34 (28.8)	10 (21.3)	0.6114
Treatment					

Systemic steroids[1]	192	11 (61.1)	92 (78.0)	29 (61.7)	0.0599
Number of courses of steroid treatment	190	1 (0-2)	1 (1-2)	1 (0-2)	0.0672
Immunosuppressive treatment[2]	191	9 (50.0)	74 (63.2)	25 (53.2)	0.3451
Number of immunosuppressants	191	1 (0-1)	1 (0-1)	1 (0-1)	0.2572
Time-to-first dose of immunosuppressive treatment in mo	109	3.0 (2.0-17.0)	4.0 (0.0-10.0)	2.8 (0.0-8.0)	0.4356
Age at first intake of immunosuppressive treatment in yr	109	14.7 (10.4-16.1)	12.3 (7.8-14.1)	11.0 (7.3-15.5)	0.2381
Biological therapy[3]	192	4 (22.2)	29 (24.8)	13 (27.7)	0.8781
Total number of biologics	192	0 (0-0)	0 (0-0)	0 (0-1)	0.8164
Time-to-first dose of biological treatment in mo	48	19.9 (12.8-50.3)	16.4 (9.1-28.1)	10.8 (4.0-27.7)	0.3152
Age at first biological treatment	49	15.7 (14.7-15.9)	11.5 (7.9-14.6)	10.7 (4.5-15.5)	0.0852
Operative treatment[4]	192	0 (0.0)	3 (2.5)	1 (2.1)	0.7893
Age at first surgery in yr	6	7.7 (5.9-9.6)	14.8 (6.8-17.1)	13.0 (10.4-15.6)	0.2969
Time-to-first surgery in mo	4		16.7 (5.0-28.7)	19.1 (0.9-37.4)	1.0000
Hospitalisations, if duration ≥ 1 yr					
Hospitalisations for relapse, per 1 yr of the disease	98	0.3 (0.3-0.8)	0.6 (0.3-1.6)	0.9 (0.5-1.3)	0.2518
Days of hospitalisation for relapse, per 1 yr of the disease	98	2.5 (0.6-4.5)	4.8 (1.8-9.3)	7.3 (3.8-8.7)	0.1362
Relapses from diagnosis, per 1 yr of the disease	98	0.3 (0.1-0.8)	0.6 (0.3-1.2)	0.8 (0.3-1.3)	0.3491
Severe relapses from diagnosis, per 1 yr of the disease	100	0.0 (0.0-0.3)	0.1 (0.0-0.6)	0.2 (0.0-0.4)	0.7150
Concomitant diseases[5]	192	9 (50.0)	41 (34.7)	15 (31.9)	0.3781
Extraintestinal manifestations	192	3 (16.7)	23 (19.5)	10 (21.3)	0.9131

[1]Systemic steroid therapy included: methylprednisolone, prednisone, hydrocortisone.
[2]Immunosuppressive and anti-inflammatory agents included: azathioprine, methotrexate, mercaptopurine, cyclosporine, mycophenolate mofetil, tacrolimus, sulfasalazine.
[3]Biological agents included: Infliximab, adalimumab, golimumab, vedolizumab.
[4]Only surgery related to inflammatory bowel disease-specific problems (*e.g.*, colectomy, resection, fistula, perforation, abscess) was included.
[5]*e.g.*, celiac disease, bronchial asthma, obesity, gastroesophageal reflux disease, epilepsy, hypothyroidism. CRP: C-reactive protein; IBD: Inflammatory bowel disease; IQR: Interquartile range; PUCAI: Pediatric Ulcerative Colitis Activity Index.

the most crucial measures of disease course, would require longer follow-up in order to describe disease severity. Although we did not include a control group, *APOE* polymorphisms in healthy subjects have been studied in the Polish population[40-42,56], which allowed us to estimate whether there was any frequency distribution difference.

CONCLUSION

APOE polymorphisms are associated with the risk of developing IBD and seem to be associated with the clinical expression of the disease and applied treatment (with inflammatory markers and nutritional status, disease activity and localisation, hospitalisations). However, the clinical relevance of the differences identified is relatively modest.

Table 4 Clinical characteristics in patients with Crohn's disease depending on major apolipoprotein E genotypes

Variables median (IQR) or *n* (%)	*n*	ε2/ε3	ε3/ε3	ε3/ε4	*P* value
Age in yr					
At inclusion	213	15.5 (13.2-16.8)	15.2 (13.3-17.2)	15.2 (13.4-16.2)	0.8055
At diagnosis	213	11.8 (10.1-14.6)	12.7 (9.9-14.5)	12.6 (10.0-13.9)	0.8796
At worst flare	184	13.3 (11.6-15.2)	13.6 (11.3-15.8)	14.3 (12.8-15.9)	0.5121
Duration of the disease in yr	211	2.8 (0.6-5.4)	2.0 (0.8-4.0)	2.3 (0.8-4.1)	0.7843
Nutritional status					
Weight at diagnosis in kg	207	38.3 (27.6-48.0)	37.3 (25.3-49.5)	38.4 (28.3-57.6)	0.5360
Weight at diagnosis, z score	204	-0.53 [(-1.02)-(-0.02)]	-0.91 [(-1.46)-(-0.12)]	-0.73 [(-1.34)-0.38]	0.2062
Height at diagnosis in cm	207	148.3 (141.0-164.0)	151.5 (134.0-164.0)	151.3 (141.0-170.0)	0.6757
Height at diagnosis, z score	204	-0.17 [(-0.85)-0.51]	-0.47 [(-1.43)-0.32]	0.05 [(-1.10)-0.96]	0.0617
Body mass index at diagnosis in kg/m^2	207	16.73 (14.28-18.42)	16.59 (14.41-18.22)	16.40 (14.78-20.78)	0.8397
Body mass index at diagnosis, z score	204	-0.72 [(-1.33)-(-0.16)]	-0.79 [(-1.53)-(-0.08)]	-0.88 [(-1.29)-0.49]	0.7878
Weight at worst flare in kg	181	41.8 (34.8-50.3)	41.9 (29.6-52.6)	46.8 (36.2-58.9)	0.2294
Weight at worst flare, z score	178	-0.67 [(-1.16)-0.10]	-1.14 [(-1.64)-(-0.25)]	-0.60 [(-1.22)-0.02]	0.0756
Height at worst flare in cm	183	153.0 (148.5-166.0)	158.0 (141.5-167.0)	162.0 (148.5-171.5)	0.3088
Height at worst flare, z score	180	-0.15 [(-1.09)-0.61]	-0.52 [(-1.41)-0.21]	-0.24 [(-1.10)-0.43]	0.1234
Body mass index at worst flare in kg/m^2	181	17.29 (15.53-18.60)	16.89 (14.87-19.03)	17.09 (15.56-21.74)	0.4172
Body mass index at worst flare, z score	178	-0.87 [(-1.38)-0.01]	-1.03 [(-1.55)-(-0.19)]	-0.53 [(-1.46)-0.49]	0.3913
Albumin level					
At diagnosis in g/dL	186	3.9 (3.7-4.3)	3.8 (3.4-4.2)	3.9 (3.4-4.3)	0.5796
At worst flare in g/dL	179	3.9 (3.8-4.3)	3.9 (3.4-4.1)	3.9 (3.6-4.3)	0.0611
Parameter of inflammation					
CRP at diagnosis in mg/L	208	13.8 (0.8-40.0)	13.0 (2.1-29.6)	12.0 (3.4-24.9)	0.8818
CRP at worst flare in mg/L	185	18.3 (1.7-31.5)	14.0 (3.3-38.5)	13.6 (3.2-26.8)	0.7672
Disease activity scales					
PCDAI at diagnosis	190	25 (20-35)	35 (25-50)	30 (25-43)	0.0282
PCDAI at worst flare	170	35 (23-50)	45 (30-53)	38 (30-53)	0.1898
Disease localisation and behaviour					
L1 at diagnosis	53/213	9 (25.7)	35 (26.1)	8 (19.5)	0.6852
L2 at diagnosis	40/213	9 (25.7)	19 (14.2)	11 (26.8)	0.0935
L3 at diagnosis	99/213	13 (37.1)	67 (50.0)	16 (39.0)	0.2507
L4a at diagnosis	23/213	4 (11.4)	14 (10.4)	4 (9.8)	0.9721
L4b at diagnosis	8/213	1 (2.9)	7 (5.2)	0 (0.0)	0.2950
B1 at diagnosis	146/213	24 (68.6)	89 (66.4)	33 (80.5)	0.2287
B2 at diagnosis	15/213	3 (8.6)	11 (8.2)	1 (2.4)	0.4263
B3 at diagnosis	19/213	3 (8.6)	15 (11.2)	1 (2.4)	0.2304
B2B3 at diagnosis	4/213	1 (2.9)	3 (2.2)	0 (0.0)	0.5927
G0 at diagnosis	145/213	24 (68.6)	92 (68.7)	29 (70.7)	0.9667
G1 at diagnosis	33/213	3 (8.6)	24 (17.9)	6 (14.6)	0.3921
P at diagnosis	19/213	0 (0.0)	16 (11.9)	3 (7.3)	0.0824
L1 at worst flare	40/213	5 (14.3)	26 (19.4)	9 (22.0)	0.6873

L2 at worst flare	27/213	7 (20.0)	14 (10.4)	6 (14.6)	0.3007
L3 at worst flare	92/213	10 (28.6)	66 (49.3)	16 (39.0)	0.0708
L4a at worst flare	18/213	3 (8.6)	12 (9.0)	3 (7.3)	0.9477
L4b at worst flare	9/213	1 (2.9)	5 (3.7)	3 (7.3)	0.5507
B1 at worst flare	114/213	17 (48.6)	74 (55.2)	23 (56.1)	0.7549
B2 at worst flare	19/213	2 (5.7)	12 (9.0)	5 (12.2)	0.6165
B3 at worst flare	21/213	1 (2.9)	16 (11.9)	4 (9.8)	0.2798
B2B3 at worst flare	5/213	1 (2.9)	4 (9.8)	0 (0.0)	0.5367
G0 at worst flare	121/213	18 (51.4)	79 (59.0)	24 (58.5)	0.7184
G1 at worst flare	34/213	2 (5.7)	24 (17.9)	8 (19.5)	0.1776
P at worst flare	20/213	0 (0.0)	17 (12.7)	3 (7.3)	0.0649
Treatment					
Systemic steroids[1]	214	19 (34.3)	73 (54.1)	21 (51.2)	0.9455
Number of courses of steroid treatment	212	1 (0-2)	1 (0-2)	1 (0-1)	0.5535
Immunosuppressive treatment[2]	214	25 (71.4)	110 (81.5)	31 (75.6)	0.3756
Number of immunosuppressants	214	1 (0-1)	1 (1-1)	1 (1-1)	0.2632
Time-to-first dose of immunosuppressive treatment in mo	166	1.3 (0.0-13.0)	2.0 (0.0-7.0)	1.0 (0.0-9.6)	0.8866
Age at first intake of immunosuppressive treatment in yr	166	12.9 (10.3-13.9)	13.0 (10.7-14.9)	12.7 (9.6-14.3)	0.6668
Biological therapy[3]	214	15 (42.9)	73 (54.1)	18 (43.9)	0.3303
Total number of biologics	214	0 (0-1)	1 (0-1)	0 (0-1)	0.2243
Time-to-first dose of biological treatment in mo	102	17.8 (6.3-44.0)	12.6 (5.6-25.9)	13.3 (6.1-26.7)	0.6313
Age at first biological treatment	102	13.8 (12.7-14.8)	13.6 (11.3-15.3)	14.0 (10.7-15.6)	0.8880
Operative treatment[4]	214	2 (5.7)	19 (14.1)	8 (19.5)	0.2158
Age at first surgery in yr	30	11.3 (9.4-13.1)	14.5 (12.5-16.5)	14.9 (14.0-15.7)	0.1698
Time-to-first surgery in mo	26	12.0	19.4 (0.0-41.1)	25.1 (7.9-43.5)	0.7807
Hospitalisations, if duration ≥ 1 yr					
Hospitalisations for relapse, per 1 yr of the disease	133	0.4 (0.2-0.7)	0.5 (0.3-0.8)	0.4 (0.2-1.3)	0.6615
Days of hospitalisation for relapse, per 1 yr of the disease	132	2.7 (0.7-5.6)	4.7 (1.6-7.5)	4.0 (1.1-7.6)	0.4001
Relapses from diagnosis, per 1 yr of the disease	132	0.4 (0.2-0.9)	0.5 (0.2-0.9)	0.4 (0.2-1.4)	0.8664
Severe relapses from diagnosis, per 1 yr of the disease	129	0.0 (0.0-0.3)	0.2 (0.0-0.5)	0.2 (0.0-0.5)	0.1996
Concomitant diseases[5]	214	16 (45.7)	40 (29.6)	8 (19.5)	0.0446
Extraintestinal manifestations	214	7 (20.0)	34 (25.2)	11 (26.8)	0.7660

[1]Systemic steroid therapy included: methylprednisolone, prednisone, hydrocortisone.
[2]Immunosuppressive and anti-inflammatory agents included: azathioprine, methotrexate, mercaptopurine, cyclosporine, mycophenolate mofetil, tacrolimus, sulfasalazine.
[3]Biological agents included: infliximab, adalimumab, golimumab, vedolizumab.
[4]Only surgery related to inflammatory bowel disease-specific problems (*e.g.* colectomy, resection, fistula, perforation, abscess) was included.
[5]*e.g.*, celiac disease, bronchial asthma, obesity, gastroesophageal reflux disease, epilepsy, hypothyroidism. CRP: C-reactive protein; IQR: Interquartile range; PCDAI: Pediatric Crohn's Disease Activity Index.

Table 5 Summary of relevant findings depending on apolipoprotein E genotypes and alleles

Variables median (IQR) or *n* (%)	*n*	*ε3/ε3*	*APOEε2*-positive	*APOEε4*-positive	*P* value
IBD					
Albumin level at worst flare in g/dL	327	3.9 (3.4-4.3)	4.0 (3.9-4.5)	4.1 (3.8-4.4)	0.0176[a]
CRP at worst flare in mg/L	347	7.7 (1.9-31.3)	4.3 (1.1-28.3)	3.2 (0.5-16.7)	0.0146[b]
Age at first surgery in yr	36	14.5 (11.7-16.7)	9.5 (7.7-11.4)	14.9 (14.0-15.6)	0.0378
Days of hospitalisation for relapse, per 1 yr of the disease	230	4.7 (1.6-8.3)	2.2 (0.7-4.8)	6.1 (1.7-8.7)	0.0440[c]
CD					
Albumin level at worst flare in g/dL	327	3.9 (3.4-4.1)	3.9 (3.8-4.4)	4.4 (3.6-4.3)	0.0363
PCDAI at diagnosis	190	35 (25-50)	25 (20-35)	30 (25-45)	0.0204[c]
Height at diagnosis, z score	378	-0.47 [(-1.43)-0.32]	-0.16 [(-0.85)-0.61]	0.00 [(-1.10)-0.96]	0.0482
UC					
CRP at diagnosis in mg/L	386	2.5 (0.7-12.2)	3.8 (0.8-7.3)	1.1 (0.2-8.2)	0.0435
CRP at worst flare in mg/L	347	3.7 (1.1-19.0)	2.1 (1.8-7.3)	0.9 (0.3-3.6)	0.0013
Age at first biological treatment	151	11.5 (7.9-14.6)	15.7 (15.3-15.7)	10.7 (4.8-15.5)	0.0432
E2 at diagnosis	192	16 (13.6)	8 (40.0)	9 (18.0)	0.0160

[a]Post hoc APOEε3ε3 *vs* APOEε2-positive $P = 0.0383$ (Bonferroni and Holm); APOEε3ε3 *vs* APOEε4-positive $P = 0.0417$ (Bonferroni) and $P = 0.0383$ (Holm).
[b]Post hoc APOEε3ε3 *vs* APOEε4-positive $P = 0.0056$ (Bonferroni and Holm).
[c]APOEε3ε3 *vs* APOEε2-positive $P = 0.0534$ (Bonferroni) and $P = 0.0356$ (Holm), APOEε2-positive *vs* APOEε4-positive $P = 0.0216$ (Bonferroni and Holm).
CD: Crohn's disease; CRP: C-reactive protein; IBD: Inflammatory bowel disease; PCDAI: Pediatric Crohn's Disease Activity Index; UC: Ulcerative colitis.

ARTICLE HIGHLIGHTS

Research background

Apolipoprotein E (*APOE*) polymorphisms were previously reported to be linked with the risk of developing inflammatory bowel diseases (IBD).

Research motivation

No data on the relationship between *APOE* polymorphisms and disease severity are available.

Research objectives

This study aimed to investigate the link between *APOE* variants and disease severity in IBD.

Research methods

The TaqMan hydrolysis probe assay was used to genotype 406 patients with IBD (192 had ulcerative colitis and 214 had Crohn's disease). Clinical expression involved disease activity scales, albumin and C-reactive protein levels, disease localisation and behaviour, and treatment with the time and age of the first intervention. The number of hospitalisations and days spent in hospital due to exacerbation as well as the number of relapses and severe relapses were also estimated.

Research results

Ulcerative colitis patients with the *APOEε4* allele had the lowest C-reactive protein values both at diagnosis ($P = 0.0435$) and the worst flare ($P = 0.0013$) compared to patients with the *APOEε2* allele and genotype *APOEε3/ε3*. Crohn's disease patients with the *APOEε2* allele scored lower on the Pediatric Crohn's Disease Activity Index at diagnosis ($P = 0.0204$). All IBD patients with the *APOEε2* allele spent fewer days in the hospital due to relapse ($P = 0.0440$).

Research conclusions

The *APOE* genotype seems to be associated with some indices of disease course such

as inflammatory markers, disease activity, and applied treatment. However, the clinical significance of the differences identified remains modest.

Research perspectives

Further mechanistic studies of APOE action in IBD are warranted.

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ORIGINAL ARTICLE

Observational Study

Exploration of nucleos(t)ide analogs cessation in chronic hepatitis B patients with hepatitis B e antigen loss

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Abstract

BACKGROUND

Nucleos(t)ide analogs (NAs) cessation in chronic hepatitis B (CHB) patients remains a matter of debate in clinical practice. Current guidelines recommend that patients with hepatitis B e antigen (HBeAg) seroconversion discontinue NAs after relatively long-term consolidation therapy. However, many patients fail to achieve HBeAg seroconversion after the long-term loss of HBeAg, even if hepatitis B surface antigen (HBsAg) loss occurs. It remains unclear whether NAs can be discontinued in this subset of patients.

AIM

To investigate the outcomes and factors associated with HBeAg-positive CHB patients with HBeAg loss (without hepatitis B e antibody) after cessation of NAs.

METHODS

We studied patients who discontinued NAs after achieving HBeAg loss. The Cox proportional hazards model was used to identify predictors for virological relapse after cessation of NAs. The cut-off value of the consolidation period was confirmed using receiver operating characteristic curves; we confirmed the cut-off value of HBsAg according to a previous study. The log-rank test was used to

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compare cumulative relapse rates among groups. We also studied patients with CHB who achieved HBeAg seroconversion and compared their cumulative relapse rates. Propensity score matching analysis (PSM) was used to balance baseline characteristics between the groups.

RESULTS

We included 83 patients with HBeAg loss. The mean age of these patients was 32.1 ± 9.5 years, and the majority was male (67.5%). Thirty-eight patients relapsed, and the cumulative relapse rate at months 3, 6, 12, 24, 36, 60, 120, and 180 were 22.9%, 36.1%, 41.0%, 43.5%, 45.0%, 45.0%, 45.0%, and 52.8%, respectively. Twenty-six (68.4%) patients relapsed in the first 3 mo after NAs cessation, and 35 patients (92.1%) relapsed in the first year after NAs cessation. Consolidation period (≥ 24 mo *vs* < 24 mo) (HR 0.506, $P = 0.043$) and HBsAg at cessation (≥ 100 IU/mL *vs* < 100 IU/mL) (HR 14.869, $P = 0.008$) were significant predictors in multivariate Cox regression. In the PSM cohort, which included 144 patients, there were lower cumulative relapse rates in patients with HBeAg seroconversion ($P = 0.036$).

CONCLUSION

HBeAg-positive CHB patients with HBeAg loss may be able to discontinue NAs therapy after long-term consolidation, especially in patients with HBsAg at cessation < 100 IU/mL. Careful monitoring, especially in the early stages after cessation, may ensure a favorable outcome.

Key Words: Chronic hepatitis B; Hepatitis B e antigen; Nucleos(t)ide analogs; Cessation

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Core Tip: A considerable proportion of patients fail to achieve hepatitis B e antigen (HBeAg) seroconversion after the long-term loss of HBeAg, even if hepatitis B surface antigen loss occurs. It remains unclear whether nucleos(t)ide analogs (NAs) can be discontinued in this subset of patients. The current study included patients who discontinued NAs after achieving HBeAg loss (without hepatitis B e antibody) for long periods since 2001. It was concluded that these patients may be able to discontinue NAs therapy after long-term consolidation. HBsAg at cessation < 100 IU/mL predicts sustained virological response after NAs cessation. Careful monitoring, especially in the early stages after cessation, may ensure a favorable outcome.

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INTRODUCTION

Nucleos(t)ide analogues (NAs) are primary therapeutic agents for hepatitis B virus (HBV) infection; they significantly improved outcomes in patients with chronic hepatitis B (CHB)[1-3]. However, taking these medicines for life presents a substantial obstacle to compliance among patients with CHB. Therefore, NAs withdrawal is being studied[4-6].

Hepatitis B e antigen (HBeAg) has been proved to be associated with several immunomodulatory functions in HBeAg-positive patients with CHB, including replication of HBV and responses of the host to the virus[7,8]. Current guidelines recommend that patients with HBeAg seroconversion discontinue NAs after relatively long-term consolidation therapy[1-3]. Although only a small number of patients achieved a loss of hepatitis B surface antigen (HBsAg), our previous study showed acceptable cumulative relapse rates for HBeAg-positive patients who achieved HBeAg seroconversion[5].

A considerable proportion of patients fail to achieve HBeAg seroconversion after

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the long-term loss of HBeAg, even if HBsAg loss occurs[9,10]. It remains unclear whether NAs can be discontinued in this subset of patients[1-3,11,12]. In an outcome study of patients with CHB and HBeAg loss after NAs cessation, 17 of 25 patients maintained sustained virological response (SVR)[10]. Another study also included CHB patients with HBeAg loss and the off-treatment virologic responses remained poor[13]. However, these studies were not explicitly designed for this subpopulation; furthermore, the sample sizes were small, and the consolidation periods were short[10,13]. Therefore, the persuasiveness of the conclusions was limited.

Some patients in our previous cohort[5,14] discontinued NAs after achieving HBeAg loss for long periods because of personal decisions and were not included in our previous analyses, while some maintained SVR. Therefore, the purpose of the present study was to determine the outcomes and related factors in patients with HBeAg-positive CHB with HBeAg loss [without hepatitis B e antibody (HBeAb)] after NAs cessation.

MATERIALS AND METHODS

Patients and methods

Patients: In our previous observational cohort, including CHB patients who discontinued NAs therapy and underwent regular follow-up, some discontinued NAs after achieving HBeAg loss (without HBeAb) for long periods. We included them in the current study (Cohort A). Before the decision to discontinue NAs, we informed all patients of the current guidelines, and we carefully evaluated all patients for potential risks. The patients decided to discontinue NAs according to their values and the state of their illness. We closely monitored them during follow-up after NAs cessation. Cumulative relapse rates were compared between HBeAg-positive patients with CHB who achieved HBeAg seroconversion (Cohort B) and Cohort A. Figure 1 describes the flowchart of patient inclusion. We recruited all patients from The Second Hospital, Cheeloo College of Medicine, Shandong University, between December 2001 and January 2020. All patients provided written informed consent. The Ethical Committee of The Second Hospital, Cheeloo College of Medicine, Shandong University, approved the study protocol. The study followed the principles of the Helsinki Declaration.

We previously published treatment criteria for NAs[5]. Cessation criteria required that patients in Cohort A receive at least 6 mo consolidation therapy after achieving HBeAg loss, with undetectable HBV DNA and normal alanine aminotransferase (ALT), and total therapy duration should last at least 18 mo. Patients in Cohort B should receive at least 6 mo consolidation therapy after achieving HBeAg seroconversion, with undetectable HBV DNA and normal ALT, and total therapy duration should be at least 12 mo. As per updated guidelines, consolidation therapy in these groups extended to at least 12 mo.

Exclusion criteria were signs of cirrhosis or decompensated liver disease and NAs resistance.

Data collection

We retrospectively collected pretreatment baseline data. After NAs cessation, the patients were followed-up at months 1, 2, 3, 4, 6, 9, and 12, and every 6 mo thereafter. The follow-up evaluation included liver biochemistry, serology of HBV, and quantification of HBV DNA.

The current study's observational endpoint was virological relapse, which was defined as serum HBV DNA > 10^4 copies/mL after NAs cessation confirmed 2 wk apart.

Statistical analyses

Variables were expressed as mean ± SD, median [interquartile range (IQR)], or number (percent) and were compared using the *t*-test, Mann–Whitney *U* test, chi-squared test, or Fisher's exact test. Cox proportional hazards model was used to identify predictors for response status after NAs cessation. The cut-off value of the consolidation period was confirmed using receiver operating characteristic curves; we confirmed the cut-off value of HBsAg according to a previous study[15]. The log-rank test was performed to compare cumulative relapse rates across groups. Propensity score matching (PSM) analysis was used to balance baseline characteristics between patients with HBeAg loss (Cohort A) and HBeAg seroconversion (Cohort B). We used 1:1 nearest-neighbor matching and a match tolerance of 0.10. All statistical analyses were performed using IBM SPSS version 22.0 (IBM Corp., Armonk, NY, United States). The statistical

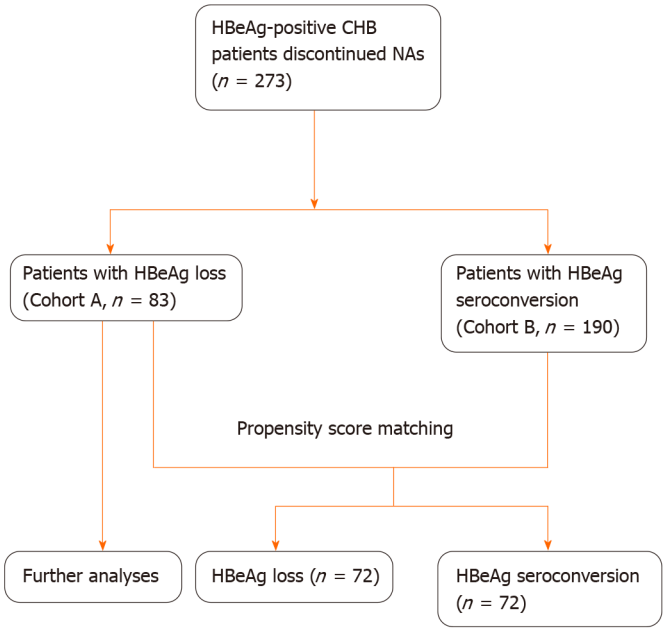


Figure 1 Flowchart of patient inclusion. CHB: Chronic hepatitis B; NAs: Nucleos(t)ide analogs.

procedure was reviewed by a biomedical statistician (Zhang Y).

RESULTS

Characteristics and virological response in patients with HBeAg loss (Cohort A)

There were 83 patients in Cohort A. The mean age of these patients was 32.1 ± 9.5 years, and the majority was male (56 patients, 67.5%). We treated patients for a median of 49 (IQR 36–61) mo. The median follow-up period for patients with SVR was 72 (IQR 36–108) mo. Patients received single NAs, including lamivudine in 45 patients, adefovir dipivoxil in 24 patients, telbivudine in nine patients, entecavir in four patients, and tenofovir in one patient. Table 1 displays the baseline characteristics of the patients. Figure 2 describes the distribution of ages and consolidation periods.

Thirty-eight patients relapsed and the cumulative relapse rates at month 3, 6, 12, 24, 36, 60, 120, and 180 were 22.9%, 36.1%, 41.0%, 43.5%, 45.0%, 45.0%, 45.0%, and 52.8%, respectively. Twenty-six (68.4%) patients relapsed in the first 3 mo after NAs cessation, and 35 patients (92.1%) relapsed in the first year after NAs cessation. The latest relapse occurred at month 129 after NAs cessation. Table 1 displays the baseline characteristics of relapsed patients and patients with SVR. All relapsed patients received close follow-up or salvage treatments according to their condition and treatment history. No liver failure or hepatic decompensation occurred.

Univariate and multivariate Cox regression determined predictors for virological relapse. Only consolidation period (≥ 24 mo *vs* < 24 mo) (HR 0.506, P = 0.043) and HBsAg at cessation (≥ 100 IU/mL *vs* < 100 IU/mL) (HR 14.869, P = 0.008) were significant predictors in multivariate regression (Table 2).

Among the 19 patients with HBsAg at cessation < 100 IU/mL, only one patient relapsed at month 3 after NAs cessation, and the cumulative relapse rates were significantly lower than those of patients with HBsAg at cessation ≥ 100 IU/mL (log-rank test, P < 0.001, Figure 3A). For the 51 patients with longer consolidation periods (≥ 24 mo), the cumulative relapse rates at month 3, 6, 12, 24, 36, 60, 120, and 180 were 17.6%, 27.5%, 31.4%, 31.4%, 33.8%, 33.8%, 33.8%, and 44.9%, respectively, which maintained significantly lower relapse rates than patients with shorter consolidation periods (< 24 mo) (log-rank test, P = 0.012, Figure 3B).

Patients with functional cure in Cohort A

Six of 83 patients achieved functional cure (HBsAg loss) at cessation. All these patients maintained SVR and HBsAg loss during follow-up. The other 11 patients, although they had detectable HBsAg at cessation, also achieved HBsAg loss during follow-up

Table 1 Clinical characteristics of the included patients

	Total (*n* = 83)	Relapser (*n* = 38)	Non-relapser (*n* = 45)	*P* values[2]
Age at cessation (yr)	32.1 ± 9.5	33.5 ± 9.8	31.0 ± 9.3	0.232
Male, *n* (%)	56 (67.5)	24 (63.2)	32 (71.1)	0.441
HBsAg < 100 IU/mL, *n* (%)	19 (22.9)	1 (2.6)	18 (40.0)	< 0.001
Pretreatment HBV DNA (log10 copies/mL)[1]	7.17 (6.71-7.68)	7.29 (6.93-7.69)	7.00 (6.10-7.68)	0.123
Total treatment period (mo)[1]	49 (36-61)	50 (36-63)	49 (37-61)	0.876
Pretreatment ALT[1]	151 (115-287)	149 (118-256)	163 (112 -305)	0.721
Pretreatment AST[1]	103 (68-180)	105 (69 -181)	102 (68 -169)	0.927
HBV DNA negativity last time (mo)[1]	44 (33-58)	44 (33-60)	44 (34-57)	0.964
Time to HBeAg loss (mo)[1]	14 (6-31)	19 (9-33)	12 (6-30)	0.241
Consolidation periods (mo)[1]	28 (19-40)	23 (18-38)	30 (21-40)	0.102

[1]median (IQR).
[2]*t* test, Mann-Whitney U test or χ^2 test. HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBsAg: Hepatitis B surface antigen.

Table 2 Determination of factors related to virological relapse in patients with hepatitis B e antigen loss

	Univariate			Multivariate		
	HR	95%CI	*P* values	HR	95%CI	*P* values
Age at cessation	1.021	0.987-1.056	0.235			
Gender	0.783	0.405-1.515	0.468			
Pretreatment ALT	0.999	0.998-1.001	0.451			
Pretreatment AST	1.000	0.997-1.002	0.716			
Pretreatment HBV DNA (log10 copies/mL)	1.391	0.942-2.054	0.097	1.256	0.816-1.933	0.300
Total treatment duration	1.001	0.989-1.013	0.826			
HBV DNA negativity last time	1.001	0.988-1.013	0.915			
Consolidation periods (≥ 24 mo *vs* < 24 mo)	0.469	0.247-0.891	0.021	0.506	0.262-0.978	0.043
HBsAg at cessation (≥ 100 IU/mL *vs* < 100 IU/mL)	15.515	2.119-113.575	0.007	14.869	2.027-109.084	0.008

HR: Hazard ratio; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

after cessation and maintained SVR. The median follow-up period of these 11 patients was 108 (IQR 78–180) mo.

Comparison of cumulative relapse rates between CHB patients with HBeAg loss (Cohort A) and HBeAg seroconversion (Cohort B)

We preliminarily screened 83 patients in Cohort A and 190 patients in Cohort B for PSM. We included 144 patients (72 pairs) in the PSM cohort (Figure 1). The baseline characteristics remained comparable between the two groups (Table 3). The log-rank test revealed lower cumulative relapse rates in patients with HBeAg seroconversion (P = 0.036, Figure 4).

DISCUSSION

The response status of patients with HBeAg-positive CHB with HBeAg loss after NAs cessation was evaluated. Despite the absence of HBeAb, these patients maintained

Table 3 Comparison of baseline clinical characteristics in the propensity score matching cohort

	HBeAg loss (*n* = 72)	HBeAg seroconversion (*n* = 72)	*P* values[2]
Age at cessation (yr)	31.8 ± 10.0	31.5 ± 11.8	0.873
Male, *n* (%)	49 (68.1)	48 (66.7)	0.859
Functional cure, *n* (%)	15 (20.8)	8 (11.1)	0.111
Pretreatment HBV DNA (log10 copies/mL)[1]	7.14 (6.70-7.65)	7.00 (6.67-7.67)	0.781
Pretreatment ALT[1]	151 (114-287)	200 (117-301)	0.394
Pretreatment AST[1]	102 (69 -188)	122 (72 -204)	0.327
Consolidation periods (mo)[1]	28 (21-40)	23 (15-37)	0.065
Time to HBeAg loss/conversion[1]	12 (6-25)	15 (7-27)	0.566

[1]median (IQR).
[2]*t* test, Mann-Whitney *U* test or χ^2 test. HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

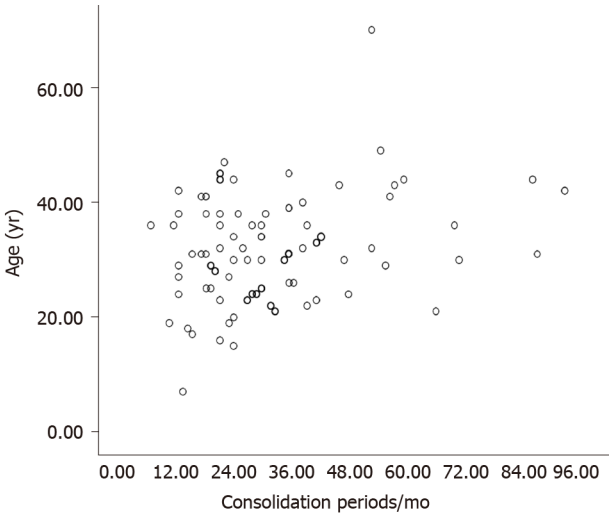


Figure 2 Distribution of age and consolidation periods of patients with hepatitis B e antigen loss.

acceptable virological response after relatively long-term consolidation therapy, especially in patients with HBsAg at cessation < 100 IU/mL. After PSM, the cumulative relapse rates were higher than those in patients with HBeAg seroconversion. More extended consolidation periods (≥ 24 mo) and low HBsAg at cessation (< 100 IU/mL) predicted better response after NAs cessation.

NAs cessation remains a matter of debate in clinical practice. Although a functional cure has been recognized as a feasible goal and an acknowledged treatment endpoint of NAs therapy[12,16,17], the low incidence rates are an essential obstacle to its clinical application. Furthermore, a considerable proportion of HBeAg-positive patients with CHB stopped NAs before achieving HBsAg loss; nevertheless, the response status remained acceptable[5,14]. For these reasons, NAs cessation following HBeAg seroconversion plus more than 1–3-year consolidation therapy is recommended by current guidelines for HBeAg-positive patients with CHB[1-3]. Because some patients in our cohort discontinued NAs after achieving HBeAg loss (without HBeAb) for long periods, the current study compared cumulative relapse rates between CHB patients with HBeAg loss and those with HBeAg seroconversion. Although the cumulative relapse rates remained acceptable in patients with HBeAg loss (45.0% at year 10), better outcomes were observed in patients with HBeAg seroconversion.

To achieve better outcomes for CHB patients with HBeAg loss, we recommend sufficient consolidation periods. In the present study, the consolidation period (≥ 24 mo) was considered an independent predictor for SVR. There were similar conclusions in previous studies exploring factors predicting drug withdrawal for patients with

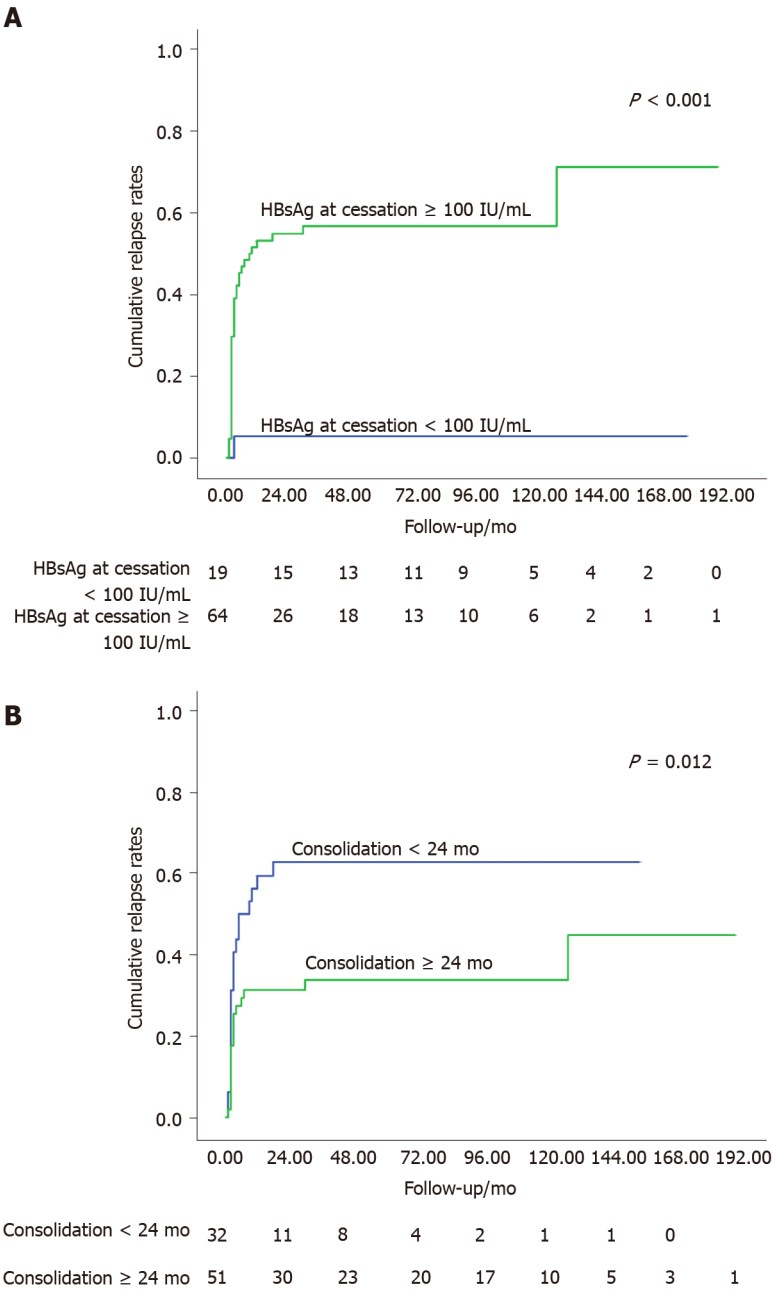


Figure 3 Cumulative virological relapse rates. Cumulative virological relapse rates in A: Hepatitis B e antigen (HBeAg) loss patients with hepatitis B surface antigen at cessation ≥ 100 IU/mL and < 100 IU/mL; B: HBeAg loss patients with consolidation periods ≥ 24 mo and < 24 mo. HBsAg: Hepatitis B surface antigen.

CHB achieving HBeAg seroconversion[5,10,18-21]. Nevertheless, the consolidation periods remain controversial. The three cut-off values (12 mo, 18 mo, and 36 mo) adopted by different cohorts might be attributed to various sample size distributions in different consolidation periods. In the current study, more than one-third of patients received consolidation for more than 36 mo (Figure 2), which ensured the cut-off value's credibility (≥ 24 mo). In addition, according to recommendations of early guidelines, three patients in Cohort A discontinued NAs with consolidation periods less than 12 mo. All these patients relapsed and were re-treated with good outcomes.

In addition to lengthier consolidation, several other factors in Cohort A patients may help obtain better outcomes. As mentioned above, HBsAg is an essential predictor of response status after NAs cessation[15]. Lower HBsAg at cessation predicted better outcomes[15], and our present results corroborate this finding. In Cohort A, seventeen patients achieved HBsAg loss (six patients at cessation and another 11 patients during follow-up) in the present study, a relatively high percentage (20.5%). All these patients maintained HBsAg loss and SVR during follow-up. Second, younger age predicted SVR in both HBeAg-positive and HBeAg-negative

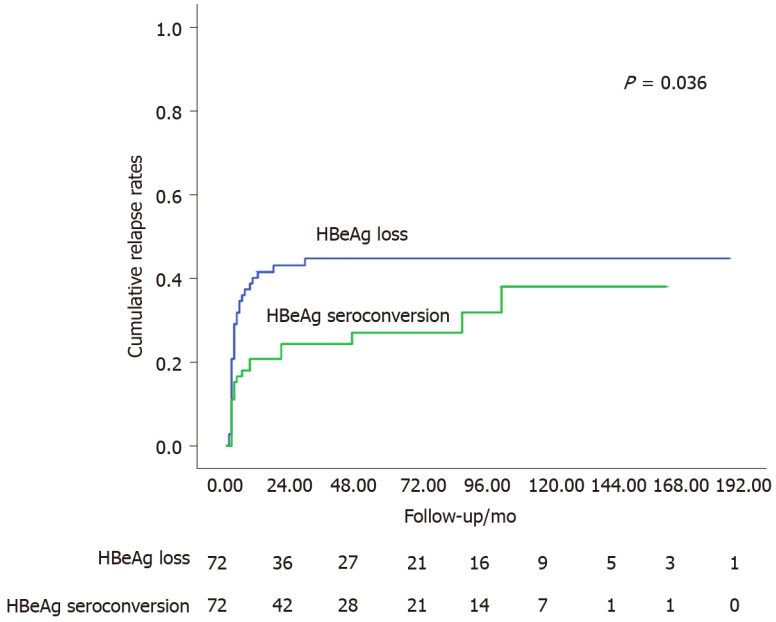


Figure 4 Cumulative virological relapse rates in patients with hepatitis B e antigen loss and seroconversion. HBeAg: Hepatitis B e antigen.

CHB patients[5,10,13,22,23]. However, age was not a predictor in the current study. The mean age of the patients in Cohort A was 32.1 ± 9.5 years, a relatively young sample. Furthermore, 82 patients (98.8%) were under 50 years old, while 65 patients (78.3%) were under 40 years old (Figure 2). The narrow age spectrum may also conceal the role of age in prognosis after cessation. Third, we excluded patients with cirrhosis and decompensated liver disease. The current guidelines recommend indefinite antiviral therapy for these patients[1-3]. We also excluded patients with NAs resistance to obtain better outcomes.

We followed patients in the current study regularly to identify relapse as early as possible. Most patients (92.1%) relapsed in the first year after NAs cessation, especially during the first 3 mo (68.4%), suggesting that more careful monitoring is critical in the early stages after cessation. These characteristics were similar to those in patients achieving HBeAg seroconversion[5,14].

There are some limitations to the current study. First, most patients did not receive first-line NAs because of their enrollment in a relatively early period (since 2001), and we followed them for relatively long periods. We excluded patients with NAs resistance to minimize the influence of low-genetic barrier NAs. Second, as an exploratory study, some features of the included patients made them more likely to achieve a sustained response, which might decrease the representativeness of the current study.

CONCLUSION

In conclusion, HBeAg-positive CHB patients with HBeAg loss may be able to discontinue NAs therapy after long-term consolidation. HBsAg at cessation < 100 IU/mL predicts SVR after NAs cessation. Careful monitoring, especially in the early stages after cessation, should be adopted to ensure favorable outcomes

ARTICLE HIGHLIGHTS

Research background

Nucleos(t)ide analogs (NAs) cessation in chronic hepatitis B (CHB) patients remains a matter of debate in clinical practice, especially in patients who fail to achieve hepatitis B e antigen (HBeAg) seroconversion after the long-term loss of HBeAg. Few studies were explicitly designed for this subpopulation.

Research motivation

To determine whether chronic hepatitis B patients with HBeAg loss could discontinue NAs after long-term consolidation.

Research objectives

We investigated the outcomes and factors associated with HBeAg-positive CHB patients with HBeAg loss [without hepatitis B e antibody (HBeAb)] after cessation of NAs.

Research methods

Patients who discontinued NAs after achieving HBeAg loss (without HBeAb) for long periods were included and predictive factors were explored. CHB patients who achieved HBeAg seroconversion were also included for controls.

Research results

HBeAg-positive CHB patients with HBeAg loss maintained acceptable virological response after NAs cessation, especially in patients with hepatitis B surface antigen (HBsAg) at cessation < 100 IU/mL. After PSM, the cumulative relapse rates were higher than those in patients with HBeAg seroconversion. More extended consolidation periods (≥ 24 mo) and low HBsAg at cessation (< 100 IU/mL) predicted better response after NAs cessation.

Research conclusions

HBeAg-positive CHB patients with HBeAg loss may be able to discontinue NAs therapy after long-term consolidation, especially in patients with HBsAg at cessation < 100 IU/mL.

Research perspectives

HBeAg-positive CHB patients with HBeAg loss may be able to discontinue NAs therapy after long-term consolidation and lower HBsAg at cessation is preferred. Careful monitoring, especially in the early stages after cessation, may ensure a favorable outcome.

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META-ANALYSIS

T-tube *vs* no T-tube for biliary tract reconstruction in adult orthotopic liver transplantation: An updated systematic review and meta-analysis

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Author contributions: All authors contributed to the study; Zhao JZ participated in the search strategy design and performed most statistical analyses and paper writing; Qiao LL, Du ZQ, and Zhang J did a literature search of the databases in parallel, built up selection criteria, and selected the potential studies; Wang MZ and Wang T defined the interventions and outcomes; Liu WM, Zhang L, and Dong J assessed the studies' quality and figured out the characteristics of the selected studies; Wu Z assisted with the design of the study; Wu RQ designed and supervised the study and revised the manuscript.

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Abstract

BACKGROUND

Whether to use a T-tube for biliary anastomosis during orthotopic liver transplantation (OLT) remains a debatable question. Some surgeons chose to use a T-tube because they believed that it reduces the incidence of biliary strictures. Advances in surgical techniques during the last decades have significantly decreased the overall incidence of postoperative biliary complications. Whether using a T-tube during OLT is still associated with the reduced incidence of biliary strictures needs to be re-evaluated.

AIM

To provide an updated systematic review and meta-analysis on using a T-tube during adult OLT.

METHODS

In the electronic databases MEDLINE, PubMed, Scopus, ClinicalTrials.gov, the Cochrane Library, the Cochrane Hepato-Biliary Group Controlled Trails Register, and the Cochrane Central Register of Controlled Trials, we identified 17 studies (eight randomized controlled trials and nine comparative studies) from January 1995 to October 2020. The data of the studies before and after 2010 were separately extracted. We chose the overall biliary complications, bile leaks or fistulas, biliary strictures (anastomotic or non-anastomotic), and cholangitis as outcomes. Odds

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ratios (ORs) with 95% confidence intervals (CIs) were calculated to describe the results of the outcomes. Furthermore, the test for overall effect (Z) was used to test the difference between OR and 1, where $P \leq 0.05$ indicated a significant difference between OR value and 1.

RESULTS

A total of 1053 subjects before 2010 and 1346 subjects after 2010 were included in this meta-analysis. The pooled results showed that using a T-tube reduced the incidence of postoperative biliary strictures in studies before 2010 ($P = 0.012$, OR = 0.62, 95%CI: 0.42-0.90), while the same benefit was not seen in studies after 2010 ($P = 0.60$, OR = 0.76, 95%CI: 0.27-2.12). No significant difference in the incidence of overall biliary complications ($P = 0.37$, OR = 1.41, 95%CI: 0.66-2.98), bile leaks ($P = 0.89$, OR = 1.04, 95%CI: 0.63-1.70), and cholangitis ($P = 0.27$, OR = 2.00, 95%CI: 0.59-6.84) was observed between using and not using a T-tube before 2010. However, using a T-tube appeared to increase the incidence of overall biliary complications ($P = 0.049$, OR = 1.49, 95%CI: 1.00-2.22), bile leaks ($P = 0.048$, OR = 1.91, 95%CI: 1.01-3.64), and cholangitis ($P = 0.02$, OR = 7.21, 95%CI: 1.37-38.00) after 2010. A random-effects model was used in biliary strictures (after 2010), overall biliary complications (before 2010), and cholangitis (before 2010) due to their heterogeneity ($I^2 = 62.3\%$, 85.4%, and 53.6%, respectively). In the sensitivity analysis (only RCTs included), bile leak ($P = 0.66$) lost the significance after 2010 and a random-effects model was used in overall biliary complications (before 2010), cholangitis (before 2010), bile leaks (after 2010), and biliary strictures (after 2010) because of their heterogeneity ($I^2 = 92.2\%$, 65.6%, 50.9%, and 80.3%, respectively).

CONCLUSION

In conclusion, the evidence gathered in our updated meta-analysis showed that the studies published in the last decade did not provide enough evidence to support the routine use of T-tube in adults during OLT.

Key Words: Orthotopic liver transplantation; T-tube; Biliary tract reconstruction; Biliary complications; Biliary strictures; Meta-analysis

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Core Tip: This is the first meta-analysis that compared the postoperative outcomes with or without a T-tube in biliary reconstruction during orthotopic liver transplantation (OLT) before and after 2010. Before 2010, T-tube was favorable for biliary stricture and had no influence on overall biliary complications, bile leaks, and cholangitis. After 2010, not using a T-tube was good for overall biliary complications and cholangitis; however, using a T-tube was not associated with biliary strictures and showed an unbeneficial trend for bile leaks. Therefore, the studies published in the last decade did not provide enough evidence to support the use of T-tube during adult OLT.

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INTRODUCTION

Orthotopic liver transplantation (OLT) is an effective treatment for end-stage liver diseases[1,2]. Although the overall outcome of OLT has improved significantly during the last decades, biliary complications, such as biliary strictures and bile leaks, remain the major causes of morbidity and mortality[3]. Biliary anastomosis was thought to be

the Achilles' heel of liver transplantation[4].

T-tube has been routinely used for biliary anastomosis during conventional hepatobiliary surgery[5,6]. However, its use in biliary reconstruction during OLT remains controversial. Several randomized controlled trials (RCTs) and comparative studies have been conducted to evaluate the value of using a T-tube in biliary reconstruction during OLT. The results, however, remained inconclusive. Even meta-analyses on this issue have provided different suggestions; Sun *et al*[7] indicated that T-tube was an excellent tool for biliary tract reconstruction while Sotiropoulos *et al*[8] suggested that abandonment of T-tube was better.

Some surgeons chose to use a T-tube during OLT because they believed that it reduces the incidence of biliary strictures. Recent advances in surgical techniques have significantly decreased the overall incidence of postoperative biliary complications. Whether using a T-tube during OLT is still associated with the reduced incidence of biliary strictures needs to be re-evaluated. Therefore, we performed this updated systematic review and meta-analysis to compare biliary complications with and without a T-tube during OLT in studies conducted before and after 2010[7-27]. We tried to find the current value of using a T-tube for biliary reconstruction during adult OLT.

MATERIALS AND METHODS

Search strategy and selection criteria

To find relevant studies, the electronic databases MEDLINE, PubMed, Scopus, ClinicalTrials.gov, the Cochrane Library, the Cochrane Hepato-Biliary Group Controlled Trails Register, and the Cochrane Central Register of Controlled Trials were used to retrieve papers in English literature from January 1995 to October 2020. The search terms included "OLT" (or "Liver Grafting" or "Liver Transplantations" or "Liver Transplant" or "Hepatic Transplantations") and "T-tube" (or "Stents") and "Biliary Tract Reconstruction" (or "Bile Ducts Reconstruction" or "Bile Duct"). Relevant comparative studies and RCTs on biliary tract reconstruction during OLT were identified. Manual search was still needed to seek further data from the article list.

Inclusion criteria

The inclusion criteria were: (1) Trails including OLT recipients (≥ 18 years); (2) Trials with primary reports of overall biliary complications and at least one biliary complication (bile leaks or fistula, anastomotic or non-anastomotic strictures, and cholangitis); and (3) Trials using choledochocholedochostomy (CCS) to reconstruct the bile duct. RCTs and comparative studies were considered, while news, commentaries, and case reports were excluded. The other exclusion criteria were as follows: (1) Liver recipient age less than 18 years; (2) Retransplantations; (3) Significant donor and recipient duct size mismatch; and (4) Diagnosis of some autoimmune diseases (*e.g.*, primary sclerosing cholangitis).

Data extraction

The data of the studies before and after 2010 were separately extracted. Three authors did a literature search of the above databases in parallel and reached consensus on most items. The differences in opinion were discussed by all authors and came to an agreement finally. The following variables were considered: Authors, year of publication, number of patients, sex, mean age of subjects, and cold ischemia time (CIT) (min). The following outcomes were evaluated: Overall biliary complications, bile leaks or fistulas, biliary strictures (anastomotic or non-anastomotic), and cholangitis.

Intervention and outcome definition

In case that a T-tube was used, a T-tube was inserted into the bile duct after the biliary tract reconstruction of end-to-end or side-to-side CCS. In case that T-tube was not used, there was not any T-tube used in the end-to-end or side-to-side CCS. The main outcomes were defined as bile leaks, biliary strictures, and cholangitis. Bile leaks were suspected in patients with abdominal pain and fever. Biliary strictures were indicated by the biochemical elevations of alkaline phosphatase and total bilirubin levels in patients without allograft rejection or hepatic artery thrombosis[28]. Bile leaks and biliary strictures were diagnosed by percutaneous transhepatic cholangiography, endoscopic retrograde cholangio-pancreatography, and T-tube cholangiogram[29]. In

addition, biliary strictures can also be diagnosed by computed tomography and magnetic resonance imaging. Cholangitis was suspected when the infectious symptoms with cholestatic parameters occurred, and the Charcot triad (right upper quadrant pain, jaundice, and fever) was also a characteristic of cholangitis[30].

Quality assessment

The quality of the studies in the systematic review and meta-analysis was determined using the Jadad composite scale[31]. The following parameters were evaluated: Randomized study, description of randomization, description of withdrawals and dropouts, double-blinded study, and description of double blinding. Each of these was scored as one point. The maximum of points was five. A greater score indicated better quality of selected studies.

Data analysis

This systematic review and meta-analysis were performed with STATA software (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC.). Outcomes evaluated were: Overall biliary complications, bile leaks or fistulas, biliary strictures, and cholangitis. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to describe the results of dichotomous outcomes. Furthermore, the test for overall effect (Z) was used to test the difference between OR and 1, where $P \leq 0.05$ indicated a significant difference between OR value and 1. Statistical heterogeneity was assessed with the statistics I^2, where $P \leq 0.05$ or $I^2 > 50\%$ were considered as having significant heterogeneity[32]. A fixed-effects model was suitable to estimate the cases with homogeneity ($I^2 \leq 50\%$) while a random-effects model should be used when significant heterogeneity ($I^2 > 50\%$) was detected. The Egger test (bias, P) was used to detect the publication bias, where $P \leq 0.05$ indicated a significant publication bias[33]. Sensitivity analysis based on ten RCTs was used to test the stability of the results of meta-analysis including all studies.

RESULTS

Characteristics of selected studies

There were 267 studies published between 1995 and 2020, of which 109 were excluded because they did not mention the use of a T-tube during OLT. Fourteen reviews, five case reports, and one letter were also excluded. After the full texts of the potential studies had been retrieved, only 20 studies met the inclusion criteria. A study was excluded because it was the preliminary result of a RCT while the final result of the RCT was already included[34]. Two studies were excluded because of the lack of no reference to patient selection or confounding variables[35] or the use of a T-tube in patients without a T-tube[36]. Finally, eight RCTs and nine comparative studies with 2199 subjects were included in our meta-analysis (Figure 1). Among them, 1053 subjects were from studies conducted before 2010 and 1146 were from studies conducted after 2010. Five RCTs got 3 points on the Jadad scale while three RCTs got only 2 points. The mean Jadad score of RCTs was 2.63 points. Seven comparative studies are retrospective, whereas two comparative studies are prospective. The observation period was at least 3 mo except for one with only 2 mo. In most studies, the T-tube was removed 3 mo after the biliary reconstruction operation, but in one comparative study, the T-tube was removed only 9 wk after operation. One study had selection bias for the use of a T-tube and another study was biased on the intention-to-treat[13]. There were no significant differences in age, CIT, and gender (data not shown). Detailed data and results are summarized in Tables 1 and 2, respectively.

Results of meta-analysis including all studies

The pooled resulted showed that in studies conducted between 1995 and 2010, the use of a T-tube appeared to reduce the incidence of biliary strictures ($P = 0.012$, OR = 0.62, 95%CI: 0.42-0.90, Figure 2A and Table 3), while for studies conducted after 2010, there was no significant difference between the "with T-tube" group and the "without T-tube" group in the occurrence of biliary strictures ($P = 0.60$, OR = 0.76, 95%CI: 0.27-2.12, Figure 2B). No significant differences in the incidences of overall biliary complications ($P = 0.37$, OR = 1.41, 95%CI: 0.66-2.98, Figure 3A), bile leak ($P = 0.89$, OR = 1.04, 95%CI: 0.63-1.70, Figure 4A), and cholangitis ($P = 0.27$, OR = 2.00, 95%CI: 0.59-6.84, Figure 5A) were observed between using and not using a T-tube in studies conducted before 2010. However, using a T-tube appeared to increase the incidences of

Table 1 Characteristics of all studies

Ref.	Study design	Year	T-tube	Number of patients	Gender[1]	Mean age (year)[3]	Mean CIT[2,3] (min)	Jadad score
López-Andújar *et al*[25]	CS	2019	Y	240	139/101	58 ± 18	268 ± 109	1
			N	165	89/76	63 ± 14	265 ± 105	
Ong *et al*[24]	CS	2018	Y	88	64/24	55	327	1
			N	37	27/10	51	316	
Santosh Kumar *et al*[23]	RCT	2017	Y	31	NR	43.4 ± 11.1	NR	2
			N	33	NR	48.5 ± 11.6	NR	
García Bernardo *et al*[22]	CS	2016	Y	23	21/2	55.3 ± 9.7	321 ± 108	1
			N	23	20/3	57.7 ± 6.6	343 ± 100	
T. Anila *et al*[21]	RCT	2015	Y	13	NR	NR	73.3 ± 25.1	2
			N	13	NR	NR	74.9 ± 31.5	
López-Andújar *et al*[20]	RCT	2013	Y	95	75/20	53.7 ± 7.68	316 ± 157	3
			N	92	66/26	53.2 ± 8.68	297 ± 143	
Weiss *et al*[19]	RCT	2009	Y	99	69/30	53.3 ± 9.8	597 ± 159	3
			N	95	60/35	55.3 ± 6.7	559 ± 178	
Lin *et al*[17]	CS	2007	Y	51	45/6	50.1 ± 10.0	NR	1
			N	53	47/6	52.0 ± 10.0	NR	
Li *et al*[18]	CS	2007	Y	33	30/3	47.2 ± 9.4	479 + 98	1
			N	51	46/5	42.5 ± 13.5	457 + 89	
Amador *et al*[16]	RCT	2005	Y	53	21/32	51.8 ± 9.4	362	3
			N	54	24/30	50.6 ± 10.6	390	
Elola-Olaso *et al*[15]	CS	2005	Y	50	29/71	51.93 ± 9.8	NR	1
			N	50			NR	
Scatton *et al*[14]	RCT	2001	Y	90	62/28	48.3 ± 9.9	604 ± 194	3
			N	90	58/32	49.2 ± 9.5	570 ± 153	
Shimoda *et al*[13]	CS	2001	Y	76	33/43	51.8 ± 11.7	NR	1
			N	71	32/39	52.9 ± 12.3	NR	
Rabkin *et al*[12]	CS	1998	Y	118	43/75	47.7	610	1
			N	44	7/37	48.0	522	
Nuño *et al*[11]	RCT	1997	Y	50	NR	NR	NR	2
			N	48	NR	NR	NR	
Vougas *et al*[10]	RCT	1996	Y	30	12/18	42.0	678	3
			N	30	13/17	45.0	696	
Randall *et al*[9]	CS	1996	Y	59	NR	NR	NR	1
			N	51	NR	NR	NR	

[1]Female/Male.
[2]Mean ± standard deviation or mean.
[3]Cold ischemia time.
NR: No reference; Y: Yes; N: No; CS: Comparative study; RCT: Randomized control trial; CIT: Cold ischemia time.

overall biliary complications (P = 0.049, OR = 1.49, 95%CI: 1.00-2.22, Figure 3B), bile leak (P = 0.048, OR = 1.91, 95%CI: 1.01-3.64, Figure 4B), and cholangitis (P = 0.02, OR = 7.21, 95%CI: 1.37-38.00, Figure 5B) in studies conducted after 2010. A random-effects model was used in biliary strictures (in studies after 2010), overall biliary

Table 2 Measurements of outcomes provided in all studies

Outcome	Ref.	Year	With T-tube	Without T-tube
Total biliary complications	López-Andújar *et al*[25]	2019	31/240	20/165
	Ong *et al*[24]	2018	26/88	3/37
	García Bernardo *et al*[22]	2016	7/23	5/23
	López-Andújar *et al*[20]	2013	24/95	18/92
	Weiss *et al*[19]	2009	27/99	50/95
	Li *et al*[18]	2007	10/33	6/51
	Lin *et al*[17]	2007	5/51	9/53
	Amador *et al*[16]	2005	32/53	6/54
	Scatton *et al*[14]	2001	30/90	14/90
	Shimoda *et al*[13]	2001	25/76	11/71
	Rabkin *et al*[12]	1998	43/118	11/44
	Nuño *et al*[11]	1997	5/50	16/48
	Vougas *et al*[10]	1996	5/30	6/30
	Randall *et al*[9]	1996	13/59	7/51
Bile leak	López-Andújar *et al*[25]	2019	10/240	2/165
	Ong *et al*[24]	2018	2/88	1/37
	Santosh Kumar *et al*[23]	2017	11/31	4/33
	García Bernardo *et al*[22]	2016	4/23	2/23
	T. Anila *et al*[21]	2015	0/13	1/13
	López-Andújar *et al*[20]	2013	4/95	5/92
	Weiss *et al*[19]	2009	5/99	9/95
	Li *et al*[18]	2007	4/33	1/51
	Lin *et al*[17]	2007	1/51	1/53
	Amador *et al*[16]	2005	2/53	3/54
	Elola-Olaso *et al*[15]	2005	3/47	0/50
	Scatton *et al*[14]	2001	2/90	2/90
	Shimoda *et al*[13]	2001	4/76	5/71
	Rabkin *et al*[12]	1998	3/118	1/44
	Nuño *et al*[11]	1997	3/50	8/48
	Vougas *et al*[10]	1996	1/30	0/30
	Randall *et al*[9]	1996	5/59	0/51
Biliary stricture	López-Andújar *et al*[25]	2019	11/240	14/165
	Ong *et al*[24]	2018	11/88	2/37
	Santosh Kumar *et al*[23]	2017	7/31	2/33
	García Bernardo *et al*[22]	2016	2/23	3/23
	T. Anila *et al*[21]	2015	1/13	2/13
	López-Andújar *et al*[20]	2013	2/95	14/92
	Weiss *et al*[19]	2009	7/99	8/95
	Li *et al*[18]	2007	5/33	4/51
	Lin *et al*[17]	2007	5/51	8/53
	Amador *et al*[16]	2005	6/53	3/54

	Elola-Olaso *et al*[15]	2005	6/44	4/46
	Scatton *et al*[14]	2001	3/90	6/90
	Shimoda *et al*[13]	2001	5/76	6/71
	Rabkin *et al*[12]	1998	7/118	10/44
	Nuño *et al*[11]	1997	1/50	8/48
	Vougas *et al*[10]	1996	2/30	6/30
	Randall *et al*[9]	1996	8/59	7/51
Cholangitis	López-Andújar *et al*[25]	2019	6/240	0/165
	Ong *et al*[24]	2018	1/88	0/37
	López-Andújar *et al*[20]	2013	6/95	0/92
	Weiss *et al*[19]	2009	5/99	11/95
	Li *et al*[18]	2007	1/33	1/51
	Amador *et al*[16]	2005	8/53	0/54
	Elola-Olaso *et al*[15]	2005	5/45	1/49
	Scatton *et al*[14]	2001	4/90	3/90
	Vougas *et al*[10]	1996	2/30	0/30

Bold typed studies indicate randomized control trials.

Table 3 Meta-analysis of outcomes (all studies included)

Period	Outcome	Studies	Participants	Effect size OR, 95%CI (fixed/random)	Heterogeneity	Test for overall effect (fixed/random)	Publication bias	Study quality[1]	Favors
1995-2010	Overall biliary complications	10	1246	1.41 [0.66, 2.98]	I^2 = 85.4% ([b]P = 0.001)	Z = 0.89/(P = 0.37)	Egger: Bias = 1.92; P = 0.60	3 × 4, 2 × 1, 1 × 5	None
	Bile leak	11	1346	1.04 [0.63, 1.70]	I^2 = 16.7% (P = 0.29)	Z = 0.14/(P = 0.89)	Egger: Bias = 2.43; [b]P = 0.003	3 × 4, 2 × 1, 1 × 6	None
	Biliary stricture	11	1346	0.62 [0.42, 0.90]	I^2 = 26.6% (P = 0.19)	Z = 2.51/([a]P = 0.012)	Egger: Bias = -1.09; P = 0.56	3 × 4, 2 × 1, 1 × 6	With T-tube
	Cholangitis	6	725	2.00 [0.59, 6.84]	I^2 = 53.6% (P = 0.06)	Z = 1.11/(P = 0.27)	Egger: Bias = 2.97; [a]P = 0.02	3 × 4, 1 × 2	None
2010-2020	Overall biliarycomplications	4	763	1.49 [1.00, 2.22]	I^2 = 31.9% (P = 0.22)	Z = 1.97/([a]P = 0.049)	Egger: Bias = 2.50; P = 0.22	3 × 1, 1 × 3	Without T-tube
	Bile leak	6	853	1.91 [1.01, 3.64]	I^2 = 6.0% (P = 0.38)	Z = 1.98/([a]P = 0.048)	Egger: Bias = -1.69; P = 0.30	3 × 1, 2 × 2, 1×3	Without T-tube
	Biliary stricture	6	853	0.76 [0.27, 2.12]	I^2 = 62.3% ([a]P = 0.02)	Z = 0.53/(P = 0.60)	Egger: Bias = 0.84; P = 0.70	3 × 1, 2 × 2, 1 × 3	None
	Cholangitis	3	717	7.21 [1.37, 38.00]	I^2 = 0.0% (P = 0.52)	Z = 2.33/([a]P = 0.02)	Egger: Bias = -12.51; P = 0.12	3 × 1, 1 × 2	Without T-tube

[1]Jadad scores × study number.
[a]P < 0.05.
[b]P < 0.01.
Odds ratio < 1 indicates the use of a T-tube. OR: Odds ratio; CI: Confidence interval.

complications (in studies before 2010), and cholangitis (in studies before 2010) due to their heterogeneity (I^2 = 62.3%, 85.4%, and 53.6%, respectively). Unfortunately, we failed to figure out the source of heterogeneity. Sensitivity analysis failed to reveal the main cause of the heterogeneity. Funnel plots detected a publication bias for bile leaks (Egger: Bias = 2.43, P = 0.003) and for cholangitis (Egger: Bias = 2.97, P = 0.02).

Results of sensitivity analysis including only RCTs

The meta-analysis with only RCTs showed that except for bile leaks which lost the significance in studies after 2010 (P = 0.66), the rest outcomes remained unchanged. Overall biliary complications and cholangitis were not evaluated because only one RCT was available to provide the information and the publication bias tests were not applicable (Table 4). A random-effects model was used in overall biliary complications (in studies before 2010), cholangitis (in studies before 2010), and biliary strictures (in studies after 2010) because of their heterogeneity (I^2 = 92.2%, 65.6%, 50.9%, and 80.3%, respectively). Funnel plots did not detect any publication bias among the outcomes.

DISCUSSION

Whether to use a T-tube or not during OLT remains a challenging question for today's transplant surgeons. The advantages of using a T-tube are the real-time monitoring of the color and quantity of bile. Therefore, using a T-tube can get the condition of transplanted liver and an access of the radiographic imaging of the biliary tree[10,12,14]. And the use of a T-tube has been thought to reduce intraductal pressure to protect biliary anastomosis[12,37]. However, the use of a T-tube may also contribute to the development of biliary leaks and cholangitis[15,16,25]. Although, in the period from 1995 to 2010, the data showed that the use of a T-tube could reduce the incidence of biliary strictures, the use of a T-tube had no influence on the occurrence of overall biliary complications, cholangitis, and bile leak. However, in the current updated (from 2010 to 2020) meta-analysis, we documented that the use of a T-tube did not reduce the incidence of biliary strictures and it appeared to increase the development of overall biliary complications and cholangitis and showed an unfavorable trend for bile leaks.

Reduction of the postoperative development of anastomotic or non-anastomotic strictures is considered an inherent trait of using a T-tube during OLT. Indeed, inserting a T-tube is still a useful way to decrease the use of invasive diagnostic techniques like endoscopic retrograde cholangiography[26,38], when a patient is suspected with bile leaks, just as the past decade data showed. However, unlike most studies, there was no significant difference between the "with T-tube" group and the "without T-tube" group in the occurrence of biliary strictures in the period from 2010 to 2020 in our analysis. Biliary stricture could occur in the non-anastomotic part due to the lack of the blood supply[39,40]. And bile leak, infection, and wound contraction may contribute to anastomotic biliary stricture[41-43]. The inflammatory reaction and fibrosis processes may occur in the biliary tract wall contacting with the T-tube due to the foreign body reaction to a T-tube[44,45]. If a T-tube was placed on the biliary tract for a long time, T-tube will alter the local host defense mechanism to increase the susceptibility to infection which may induce biliary fibrosis[46,47]. Fortunately, the recent advancement in surgical techniques has significantly improved the short-term outcomes after OLT[48,49].

Bile leaks commonly complicate liver transplantation. They usually occur in the early postoperative period or after the T-tube removal. And bile leaks prolonged the hospital stay, increased the medical costs, and disturbed the postoperative recovery[50]. Some physicians thought that the use of a T-tube plays an important role in development of bile leaks in recipients[51]. In the results of our study, there was no significant difference in the incidence of bile leaks between the "with T-tube" group and the "without T-tube" group in the period from 1995 to 2010. Nevertheless, we found that the use of a T-tube increased the risk of developing bile leaks in biliary reconstruction during OLT in the period from 2010 to 2020. Unfortunately, in the sensitivity analysis, bile leaks lost the significance, which lowered the statistic power of the results. Therefore, at least in part, our data demonstrated that T-tube showed an unbeneficial trend for bile leaks. Previous studies showed that 5% to 15% of patients suffered from bile leaks after T-tube removal[52,53]. And the biliary anastomosis or T-tube exit site was a major source of the bile leakage[54,55]. The delayed formation of a fibrous trajectory to allow safe removal of T-tube may play an important role in the incidence of bile leak[56]. In addition, multiple biliary reconstructions also contributed to the occurrence of the bile leaks and were associated with a poor prognosis of

Table 4 Sensitivity analysis of outcomes (only randomized control trials included)

Period	Outcome	Studies	participants	Effect size OR, 95%CI (fixed/random)	Heterogeneity	Test for overall effect (fixed/random)	Publication bias	Study quality[1]	Favors
1995-2010	Overall biliarycomplications	5	639	1.15 [0.28, 4.72]	I^2 = 92.2% ([b]P = 0.001)	Z = 0.19/(P = 0.85)	Egger: Bias = 2.63; P = 0.71	3 × 4, 2 × 1	None
	Bile leak	5	639	0.56 [0.28, 1.13]	I^2 = 0.0% (P = 0.72)	Z = 1.62/(P = 0.11)	Egger: Bias = 1.72; P = 0.08	3 × 4, 2 × 1	None
	Biliary stricture	5	639	0.45 [0.24, 0.85]	I^2 = 0.0% (P = 0.46)	Z = 2.47/([a]P = 0.014)	Egger: Bias= -2.78; P = 0.10	3 × 4, 2 × 1	With T-tube
	Cholangitis	4	541	1.83 [0.36, 9.41]	I^2 = 65.6% ([a]P = 0.03)	Z = 0.72/(P = 0.47)	Egger: Bias = 3.39; P = 0.054	3 × 4	None
2010-2020	Bile leak	3	277	1.38 [0.34, 5.65]	I^2 = 50.9% (P = 0.13)	Z = 0.45/(P = 0.66)	Egger: Bias = -1.94; P = 0.62	3 × 2, 2 × 1	None
	Biliary stricture	3	277	0.63 [0.06, 6.99]	I^2 = 80.3% ([a]P = 0.01)	Z = 0.38/(P = 0.71)	Egger: Bias = 1.08; P = 0.92	3 × 2, 2 × 1	None

[1]Jadad scores × study number.
[a]P < 0.05.
[b]P < 0.01.
Odds ratio < 1 indicates the use of a T-tube. OR: Odds ratio; CI: Confidence interval.

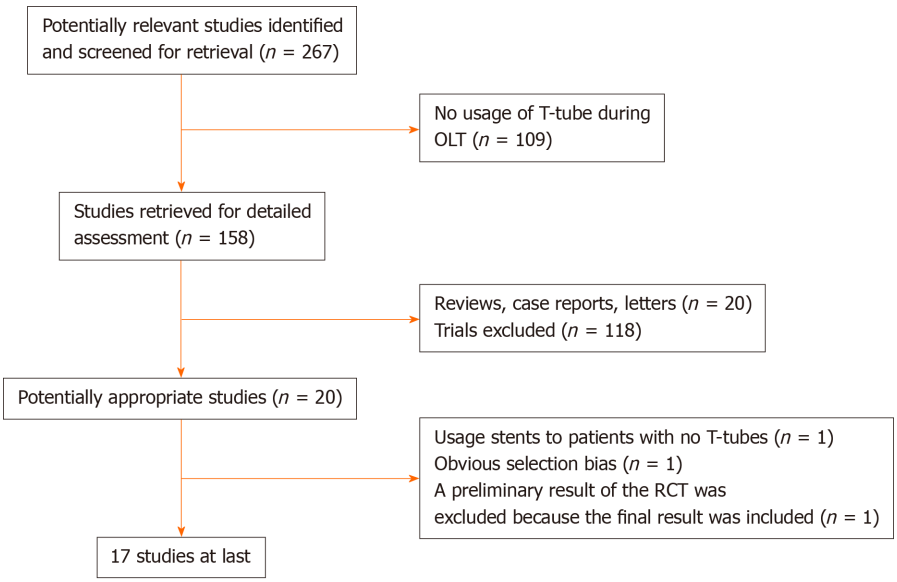


Figure 1 Flowchart of selection process of randomized control trials and comparative studies. RCT: Randomized control trials; OLT: Orthotopic liver transplantation.

patients[57].

Cholangitis always seems to be a direct outcome of usage of T-tube in biliary reconstruction during OLT. Our study documented that the development of cholangitis had a significant difference between the "with T-tube" group and the "without T-tube" group in the period from 2010 to 2020. However, in the period from 1995 to 2010, we found that cholangitis did not reach the significance between the two groups. There was a trend that T-tube increased the risk of cholangitis. A T-tube was a bridge between the abdominal cavity and external environment to facilitate entry of bacteria into human body. And the surface of a T-tube is a good platform for bacteria to implant so bacteria could form biofilm which help bacteria proliferate better[58,59]. The biofilm in T-tube plays an important role in occurrence of cholangitis[60,61]. If the T-tube was broken by the patient carelessly, the bile may flow out of the biliary tree to induce bacterial infection. Most patients were immunocompromised because of immunosuppressors after OLT, which made patients vulnerable to bacterial or viral infection. In another words, it is fatal for some patients suffering from infections such

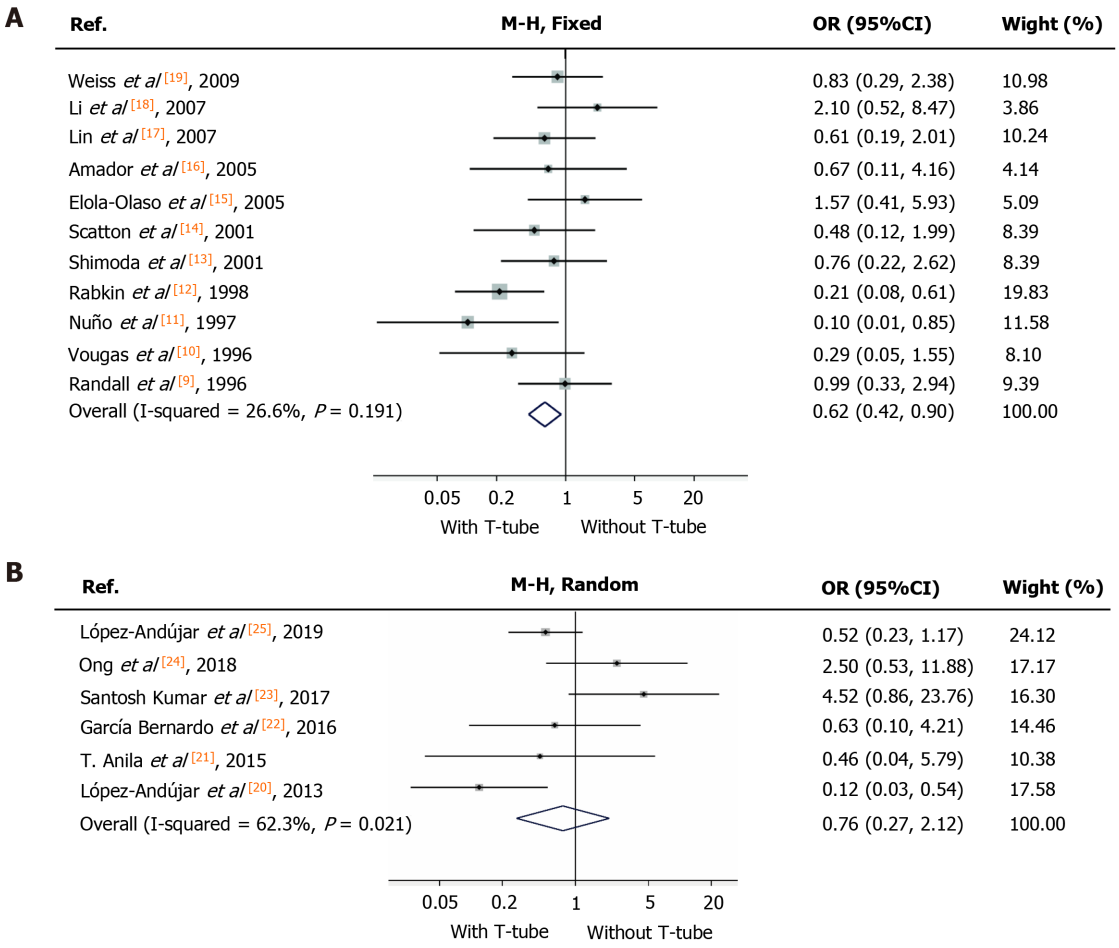


Figure 2 Biliary strictures. A: In the period from 1995 to 2010, odds ratio (OR) for biliary strictures between "T-tube" group and "without T-tube" group was calculated with a fixed-effects model. The shape of diamond represents the overall effect of OR with 95% confidence interval (CI). The use of T-tube decreased the incidence of biliary strictures; B: In the period from 2010 to 2020, OR for biliary strictures between "T-tube" group and "without T-tube" group was calculated with a random-effects model. The shape of diamond represents the overall effect of OR with 95%CI. There was no significant difference between the two groups. OR: Odds ratio; CI: Confidence interval.

as cholangitis. T-tube inserting also increased the risk of long-term biliary inflammation and biliary tract fibrosis[47].

As for overall biliary complications, there was no significant difference between the two groups in the period from 1995 to 2010. Interestingly, our document showed that the use of a T-tube did play a role in development of all kinds of biliary complications in the period from 2010 to 2020. The modern diagnostic devices and the advanced endoscopic technique revealed many latent complications (small thrombosis, local infections, mild internal hemorrhage, and so on), which were difficult to be detected in the past[10,13,14].

There may be a potential bias because several outcomes had significant publication bias. Since our meta-analysis contained nonrandomized studies and some comparative studies that had selection bias, both contributed to the article's limitation. Although we reduced the heterogeneity of the selected studies *via* using random-effects models, the inherent bias still partially existed. And almost all the comparative studies got just 1 point on the Jadad scale; however, the five RCTs got 3 points on the Jadad scale while three RCTs got 2 points, which indicated that the quality of the selected studies was not very high. In addition, the retrospective design of the comparative studies may introduce recall bias and reporting bias.

CONCLUSION

In conclusion, the data in our study showed that the use of a T-tube is a beneficial factor for biliary strictures before 2010, whereas no benefit was observed for overall biliary complications, biliary leak, and cholangitis in the same period. According to the

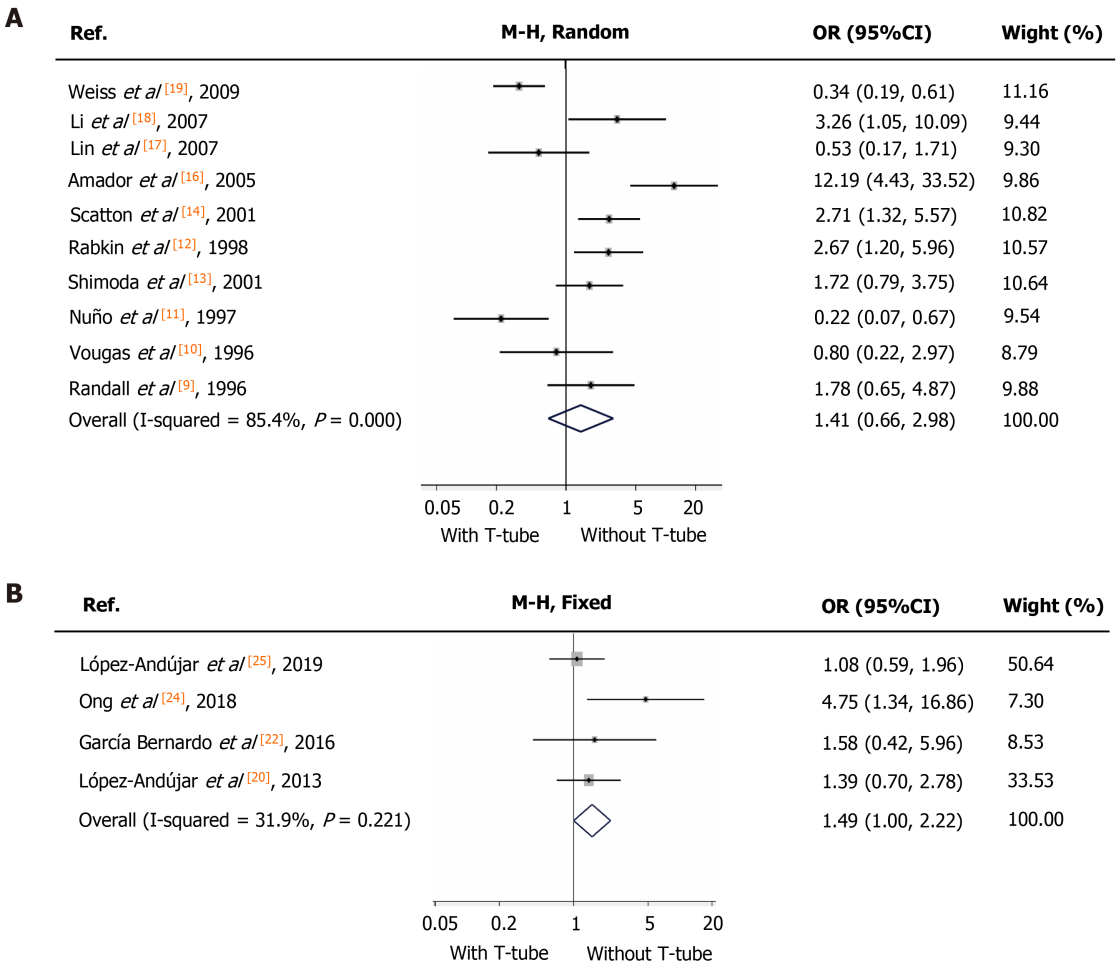


Figure 3 Overall biliary complications. A: In the period from 1995 to 2010, odds ratio (OR) for overall biliary complications between "T-tube" group and "without T-tube" group was calculated with a random-effects model. The shape of diamond represents the overall effect of OR with 95% confidence interval (CI). The use of T-tube had no influence on occurrence of overall biliary complication; B: In the period from 2010 to 2020, OR for overall biliary complications between "T-tube" group and "without T-tube" group was calculated with a fixed-effects model. The shape of diamond represents the overall effect of OR with 95%CI. The occurrence of overall biliary complications was significantly higher in "T-tube" group. OR: Odds ratio; CI: Confidence interval.

studies after 2010, not using a T-tube is beneficial in terms of overall biliary complications and cholangitis; however (after sensitivity analysis), the use of a T-tube is not associated with biliary strictures and shows an unbeneficial trend for bile leaks. Therefore, the evidence gathered in our updated meta-analysis showed that the studies published in the last decade did not provide enough evidence to support the routine use of T-tube in adults during OLT.

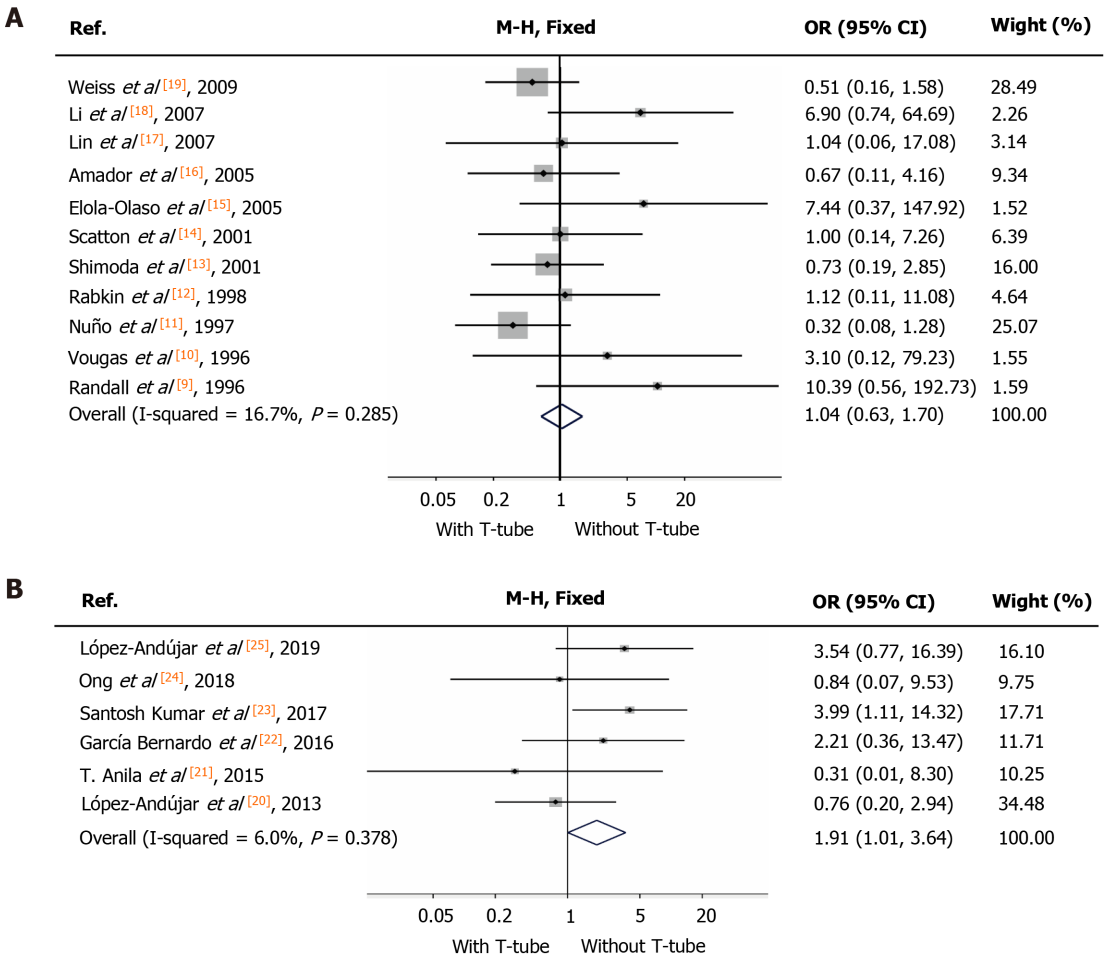


Figure 4 Bile leaks. A: In the period from 1995 to 2010, odds ratio (OR) for peritonitis between "T-tube" group and "without T-tube" group was calculated with the fixed model. The shape of diamond represents the overall effect of OR with 95% confidence interval (CI). There was no significant difference between the two groups; B: In the period from 2010 to 2020, OR for peritonitis between "T-tube" group and "without T-tube" group was calculated with the fixed model. The shape of diamond represents the overall effect of OR with 95%CI. The incidence rate of bile leaks was significantly higher in "T-tube" group. OR: Odds ratio; CI: Confidence interval.

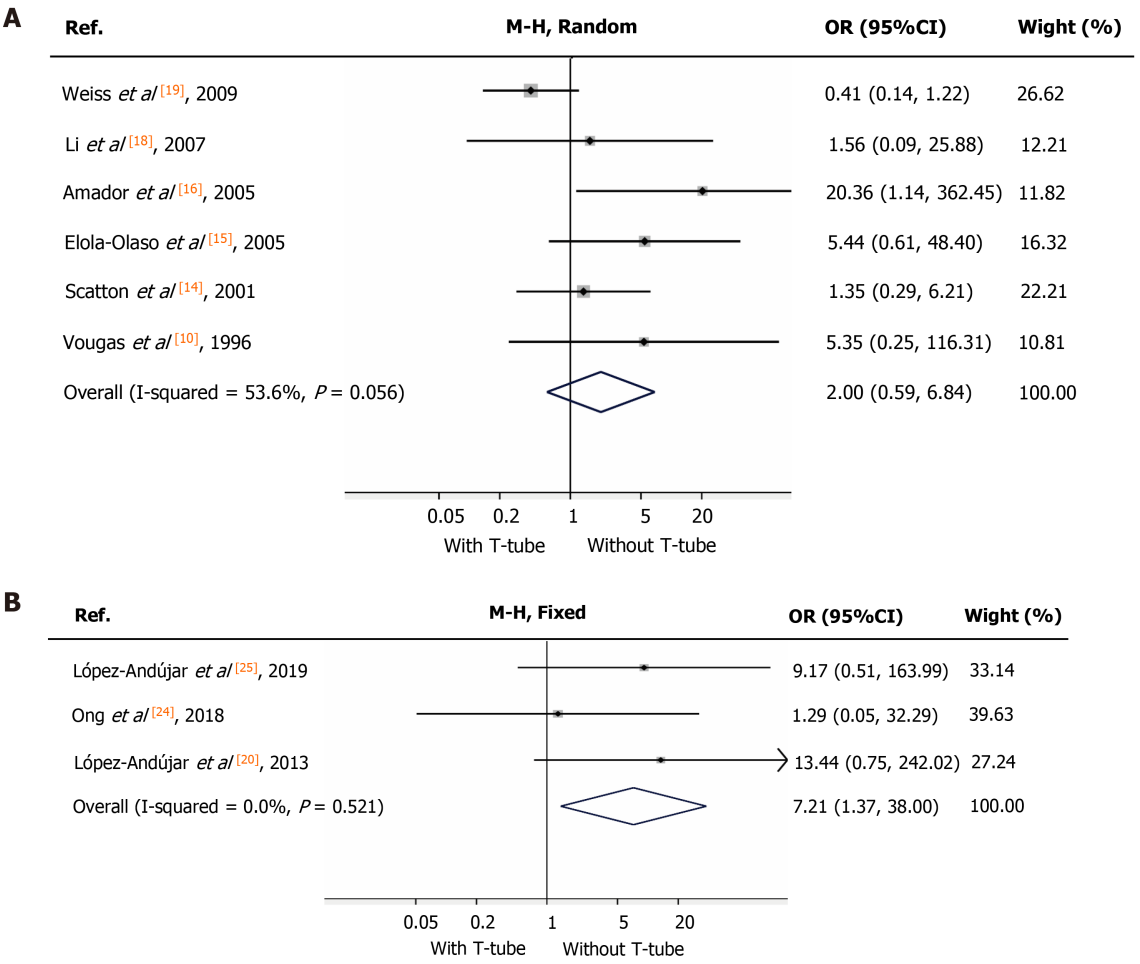


Figure 5 Cholangitis. A: In the period from 1995 to 2010, odds ratio (OR) for cholangitis between "T-tube" group and "without T-tube" group was calculated with a random-effects model. The shape of diamond represents the overall effect of OR with 95% confidence interval (CI). There was no significant difference between the two groups; B: In the period from 2010 to 2020, OR for peritonitis between "T-tube" group and "without T-tube" group was calculated with a fixed-effects model. The shape of diamond represents the overall effect of OR with 95%CI. The incidence rate of bile leaks was significantly higher in "T-tube" group. OR: Odds ratio; CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

The use of the T-tube in the reconstruction of the biliary tree during orthotopic liver transplantation (OLT) is still debatable.

Research motivation

Biliary complications after OLT, including bile leaks, cholangitis, and biliary strictures, prolonged the hospital stay, impaired the postoperative recovery, and increased the medical costs. T-tube was involved in the occurrence of these biliary complications. Therefore, it is helpful to identify the role of T-tube in the incidence of the biliary complications.

Research objectives

We performed a meta-analysis to evaluate whether patients benefit from the use of a T-tube during OLT.

Research methods

We calculated odds ratios with 95% confidence intervals to identify the role of a T-tube in the incidence of the overall biliary complications, bile leaks, cholangitis, and biliary strictures.

Research results

We discovered that T-tube had no influence on the risk of the overall biliary complications, bile leaks, and cholangitis and reduced the incidence of biliary strictures in the period from 1995 to 2010. However, in the recent decade (from 2010 to 2020), we found that T-tube did not affect the occurrence of biliary strictures and increased the incidence of overall biliary complications and cholangitis. And the use of a T-tube showed an unbeneficial trend for bile leak after 2010.

Research conclusions

In conclusion, the evidence gathered in our updated meta-analysis showed that the studies published in the last decade did not provide enough evidence to support the routine use of T-tube in adults during OLT.

Research perspectives

More T-tube-related outcomes should be included in the future meta-analysis so the advantages and disadvantages would be evaluated better.

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EDITORIAL

Textbook outcomes in hepatobiliary and pancreatic surgery

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Abstract

The concept of textbook outcome (TO) has recently gained popularity in surgical research and has been used to evaluate the quality or success of different surgical procedures, including hepatopancreatobiliary (HPB) operations. TO consists of individual outcome parameters that each reflect different domains of care including structure, process, and individual outcomes; in turn, the composite TO metric represents the optimal course after a surgical episode. TO can be used to assess patient-level outcomes, hospital performance, center designation and quality metrics. In addition to being an outcome measurement, TO may also be linked to healthcare costs. Future efforts should be directed towards establishing a universal definition of TO in HPB surgery so that surgeons and hospitals can assess and compare outcomes, identify shortcomings and improve real world patient outcomes.

Key Words: Textbook; Outcome; Hepatopancreatobiliary; Surgery

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Core Tip: The concept of textbook outcome (TO) has recently gained popularity in surgical research and has been used to evaluate the quality or success of different surgical procedures, including hepatopancreatobiliary (HPB) operations. TO can be used to assess patient-level outcomes, hospital performance, center designation and quality metrics. Future efforts should be directed towards establishing a universal definition of TO in HPB surgery so that surgeons and hospitals can assess and compare outcomes, identify shortcomings and improve real world patient outcomes.

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INTRODUCTION

Traditionally, quality assessment of complex surgery has focused on analyzing and evaluating individual outcome parameters, mainly complications, length of stay (LOS), in-hospital mortality, as well as 30-, 90-d mortality and readmission[1]. More recently, there has been a shift in quality assessment from individual outcome parameters to composite outcomes, which have been considered superior to assess patient outcomes and hospital performance[2-5]. A recently introduced concept, "textbook outcome" (TO) represents the optimal course following surgery that is better aligned with patient expectations around "optimal" care[2-5]. TO uses an "all-or-none" approach in which the optimal or "textbook" outcome is not achieved unless patients achieve all of the individual parameters comprising a TO[2-5].

The concept of TO has gained popularity in surgical clinical research and has been advocated as a measure to assess quality or success of complex surgical procedures, including abdominal aortic aneurysm repair, as well as hepatopancreatic, esophageal, gastric, sarcoma, colon cancer resection and transplantation[2-10]. Specifically, TO is a composite measure that aggregates various clinically important perioperative outcomes included in the definition of an "optimal" surgical episode. Such a composite measure encompasses determinants of quality of care from different domains including structure, process, and individual outcomes[2]. Indeed, each individual outcome may capture different domains of quality (*e.g.*, perioperative mortality might be related to operative volume, failure-to-rescue may be related to structure or processes of perioperative care, while LOS or readmissions may be related to discharge planning processes in place for each institution)[5]. In turn, TO is particularly relevant in the care of complex cancers, including hepatopancreatobiliary (HPB) malignancies, which require coordination across a number of specialties and through various phases of multidisciplinary treatment to achieve the best possible outcome[3,5,11]. In addition, TO can be used as a "benchmark" to track the quality of care delivered to patients and compare hospital performance across different institutions or health systems[12-14]. In this article, we highlight the existing and emerging literature on TOs in HPB surgery.

ASSESSING PATIENT-LEVEL OUTCOMES USING TO

Although the concept of TO was initially described in 2013 by a group of colorectal surgeons in Netherlands[2], it was used for the first time to assess outcomes of patients undergoing hepatopancreatic surgery in 2018[4]. Merath *et al*[4] first assessed TO rates after hepatopancreatic surgery among Medicare beneficiaries from 2013 to 2015[4]. TO was defined no postoperative complications, no prolonged LOS (*i.e.*, ≤ 75th percentile), no 90-d readmission and no 90-d postoperative mortality[4]. The study analyzed patients with all surgical indications (*i.e.*, both benign and malignant diseases) and demonstrated that TO was achieved in 44% of individuals undergoing hepatopancreatic surgery[4]. Of note, among patients who underwent pancreatic resection, TO was achieved in 47.8% following minor pancreatic resection (*i.e.*, distal pancreatectomy or other partial pancreatectomy) and 24.7% following major pancreatic resection (*i.e.*, proximal pancreatectomy, pancrea-ticoduodenectomy, total pancreatectomy)[4]. Similarly, while TO was achieved in 46.8% of patients undergoing minor hepatectomy, the incidence of TO decreased to 33.3% among patients who had undergone a major liver resection[4]. These data highlighted the fact that less than one-half of patients overall experienced an optimal or "textbook" outcome after hepatopancreatic surgery; in particular, achievement of TO was markedly lower among patients undergoing major pancreatic or liver resections.

Due to the inherent limitations of administrative billing databases, several important perioperative outcomes were not captured and assessed in the initial study by Merath *et al*[4]. Rather, the use single- or multi-center institutional databases that contain more granular data to provide more clinically important information and allow for a more thorough assessment of postoperative outcomes was needed. To this

end, Merath *et al*[3] published a subsequent study that assessed the incidence of TO following resection of intrahepatic cholangiocarcinoma (ICC) using a multi-institutional database that incorporated data from 15 major HPB centers[3]. In this study, the authors defined TO as R0 resection, no perioperative transfusion, no postoperative complications, no prolonged LOS, no 30-d readmission and no 30-d mortality[3]. Among 687 patients analyzed, TO was achieved only in 25.5% of patients undergoing curative-intent resection for ICC. Younger patient age (age < 60 years), absence of preoperative jaundice, earlier T-category disease (*i.e.*, T1a, T1b), node-negative disease, and no bile duct resection were independent predictors of TO[3]. Although 30-d mortality was less than 5%, a TO was achieved in only one-fourth of patients largely due to high rates of complications and LOS, highlighting the need for further improvements in managing patients undergoing complex HPB procedures[3].

Another study examined TO after curative-intent resection of hepatocellular carcinoma (HCC)[5]. TO was defined as R0 resection, no reoperation, no severe postoperative complications (Clavien-Dindo ≥ III), no prolonged LOS, no 90-d readmission and no 90-d mortality[5]. In analyzing 605 patients, the incidence of TO was 62.3% after curative-intent resection of HCC[5]. Patients with BCLC-0 HCC and albumin-bilirubin grade grade 1 (*i.e.*, good liver function) had higher odds of achieving a TO[5]. In addition to examining perioperative outcomes, the impact of TO on long-term outcomes was also assessed. Of note, patients who experienced a TO had a better 5-year overall survival (OS) of 69.6% compared with a 5-year OS of 56.9% among individuals who did not achieve a TO[5], which was in line with previous studies on esophageal and gastric cancer[6]. Similarly, Heidsma *et al*[15] reported an incidence of TO that was 49.3% among patients undergoing resection for pancreatic neuroendocrine tumors; the odds of TO varied based on the type of procedure performed[15]. In fact, the highest TO rates were noted among individuals undergoing distal pancreatectomy (56.7%) followed by those undergoing enucleation (52.0%) and then pancreatoduodenectomy (32.5%)[15]. TO was also an independent predictor of long-term outcomes, with 3-year disease-free survival ranging from 91.7% among patients who achieved a TO to 85.2% among patients who did not[15]. These data validated the clinical relevance of TO among cancer patients, aligning the importance of surgical quality (and outcomes) with the primary goal of achieving a potentially curative operation. As more investigators integrate TO into the field of surgical oncology, TO may evolve to incorporate other variables representing domains of multidisciplinary care delivery (*i.e.*, negative margin resection, adequate lymph node sampling, receipt of neoadjuvant/adjuvant chemotherapy) to create an oncologic composite outcome measure [*i.e.*, textbook oncologic outcome (TOO)][16,17]. Although consensus as to which individual components should define a TOO has not been reached (Table 1), the use of a TOO might be a better way to characterize patient and hospital-level outcomes among cancer patients.

ASSESSING HOSPITAL PERFORMANCE, DESIGNATIONS AND QUALITY METRICS USING TOs

Hospital performance remains an important topic among patients and stakeholders, such as insurance and pharmaceutical companies as well as the government. To date, different designations and ranking systems are available to evaluate hospital performance. Of note, teaching hospitals have been assigned with the mission to teach the next generation of physicians, support research and provide excellent patient care[18,19]. Previous studies had demonstrated contradictory results when examining individual outcome parameters (*i.e.*, mortality, morbidity, readmissions) relative to teaching hospital status[18,19]. To this point, Mehta *et al*[12] investigated the impact of teaching status on TO among Medicare beneficiaries undergoing hepatopancreatic surgery[12]. Of note, patients undergoing surgery for pancreatic cancer at a major teaching hospital were more likely to achieve a TO *vs* patients treated at a minor teaching hospital[12]. When assessing only high volume hospitals, the odds of a TO were comparable among patients treated at major *vs* minor teaching centers, highlighting that the beneficial effect of teaching hospital status was largely mediated by procedural volume and not necessarily by teaching status itself[12].

Another study investigated a commonly used ranking system, the United States News & World Report (USNWR) Best Hospital rankings, relative to TO[13]. Although the USNWR rankings are commonly used by patients and are thought to influence patient decision-making and choice of hospital to undergo treatment, a recent study demonstrated similar odds of achieving a TO among patients undergoing complex

Table 1 Existing literature around textbook outcome in hepatopancreatobiliary surgery

Ref.	Study period	Database	Indication	Components of TO	TO rates
Merath *et al*[3], 2019	2013-2015	Medicare	Benign and malignant hepatopancreatic lesions	No complications. No prolonged LOS. No 90-d mortality. No 90-d readmission	44.0%
Merath *et al*[4], 2020	1993-2015	Multi-institutional ICC database	Intrahepatic cholangiocarcinoma	R0 resection No transfusion. No complications. No prolonged LOS. No 30-d mortality. No 30-d readmission	25.5%
Tsilimigras *et al*[5], 2020	2000-2015	Multi-institutional HCC database	Hepatocellular carcinoma	R0 resection No reoperation. No complications (Clavien-Dindo ≥ III) No prolonged LOS. No 90-d mortality. No 90-d readmission	62.3%
Heidsma *et al*[15], 2020	2000-2016	US Neuroendocrine Tumor Study Group database	Pancreatic neuroendocrine tumors	R0 resection No complications (Clavien-Dindo ≥ III). No prolonged LOS. No 90-d mortality. No 90-d readmission	49.3%

TO: Textbook outcome; LOS: Length of stay; ICC: Intrahepatic cholangiocarcinoma; US: Ultrasonography; HCC: Hepatocellular carcinoma.

cancer surgery (*i.e.*, lung, colorectal, esophageal, liver and pancreatic resections) at honor roll (top 20 institutions) *vs* non-honor roll hospitals[13]. These data suggested that the USNWR ranking had only a minor impact on the likelihood of achieving a TO and, thus, should not necessarily been used to choose institutions to undergo complex cancer surgery. In addition, these data suggested that composite outcomes such as TO should perhaps been taken into consideration among policymakers to establish the methodology of hospital ranking systems for complex cancer surgery.

For oncologic patients, quality of care is of particular importance as patients seek a holistic, multidisciplinary care in the battle against cancer. To date, there are only 10 dedicated cancer centers (DCCs) that provide care to cancer patients[20]. Another important designation for hospitals providing cancer care is the National Cancer Institute cancer center (NCI-CC) designation, which approximately 70 institutions in the United States hold for outstanding efforts related to prevention, diagnosis and treatment of cancer[21]. In another study by Mehta *et al*[20], the authors compared outcomes of patients undergoing hepatopancreatic surgery at DCC *vs* NCI-CC for cancer[20]. Of note, patients who underwent hepatopancreatic surgery had increased odds of achieving a TO when treated at a DCC *vs* NCI-CC (pancreatic resections: 22% higher chance of a TO; liver resection: 31% higher chance of a TO), despite the fact that DCCs more frequently cared for patients with multiple comorbidities[20]. Higher TO rates translated into reduced Medicare expenditures at DCCs, which suggested a higher value proposition of DCC *vs* NCI-CC in the treatment of patients with hepatopancreatic malignancies[20].

Several other national quality metrics have been proposed to assess quality of care provided to patients. For example, hospital magnet recognition, established by the American Nurses Credentialing Center has been used to identify institutions with a focus on improving nursing care and, in turn, quality of care delivered to patients[22]. In addition, the Leapfrog Group has set a minimum annual hospital surgical volume for certain operations–including pancreatic resection (*i.e.*, > 20 pancreatic resections)–associated with improved outcomes[23]. Another quality indicator made available by the Leapfrog group is the Leapfrog safety grade which is solely focused on patient safety[23,24]. In analyzing data from 4853 Medicare beneficiaries, Merath *et al*[14] examined all 3 quality indicators (*i.e.*, leapfrog minimum volume standards, safety grade and magnet status) relative to TO following hepatopancreatic resections[14]. Of note, patients undergoing pancreatectomy at hospitals meeting all 3 quality metrics (*i.e.*, quality trifactor) had 28% higher odds of experiencing a TO compared with individuals undergoing pancreatectomy at non-trifactor hospitals[14]. When examining each of the quality indicators separately, magnet status and safety grade A were alone not enough to confer high TO rates. Rather, the positive effect of the quality trifactor was largely mediated by the compliance with the Leapfrog minimum volume standards that were associated with lower odds of mortality and serious complications and, in turn, greater odds of TO[14].

ASSESSING TRENDS IN TOs OVER TIME

TO has also been assessed relative to changes in practice and outcomes in HPB surgery over time. By analyzing the American College of Surgeons National Surgical Quality Improvement Program database, Beane *et al*[25] reported an increase in optimal or "textbook" outcomes after pancreatic surgery in North America[25]. There was a decrease in postoperative morbidity, mortality and LOS between 2013-2017 that resulted in an increase in the incidence of TO by 3% to 5% after pancreatic surgery on a nationwide level[25]. A number of reasons might be responsible for this improvement. First, there was a decrease in superficial and deep surgical site infections, and a decrease in the rates of shock/sepsis after pancreatic resection[25]. In addition, an increase in minimally invasive pancreatic resection was noted over time (mainly robotic resection) that may have contributed to a decrease in LOS. The broad dissemination and implementation of enhanced recovery after surgery protocols over the past 5 to 10 years likely also contributed to the observed reduction in LOS[26,27]. Furthermore, the increase in TO incidence over time may also have been to centralization of pancreatic cancer care at specialized centers and increased access to multidisciplinary oncologic teams. In turn, the modest improvement in the incidence of TO noted over time may be a reflection of the varied distribution of pancreatic cancer cases, and not an actual improvement in the majority of centers throughout the nation[25].

Indeed, by combining two multi-institutional datasets, Tsilimigras *et al*[11] analyzed the trends in TO rates after curative-intent resection of primary liver malignancies (*i.e.*, ICC and HCC) at major HPB centers over a 12-year period[11]. Overall, 62.0% of patients achieved a TO after ICC or HCC resection at major HPB centers. In assessing the trends of TO over the years, no significant improvement was noted over the study period examined (2005-2017) (P_{trend} = 0.90)[11]. When analyzing the individual components comprising TO, no specific factor demonstrated an increasing trend over time (all P_{trend} > 0.05). Perhaps more surprisingly, no increasing trends in TO rates were noted among patients undergoing either major (2005 to 2009: 49%; 2014 to 2017: 48%) or minor liver resection (2005 to 2009: 71%, 2014 to 2017: 71%) over the study period (both P_{trend} > 0.05)[11]. Apart from TO itself, the year of surgery was also not associated with improved long-term outcomes among patients with either ICC or HCC[11]. These data highlight the fact that despite advances in surgical techniques and perioperative care, only modest improvements in the outcomes of HCC and ICC patients occurred over the last decade. In turn, there is still a long way to optimize real world outcomes among patients with HPB malignancies.

FINANCIAL IMPACT OF TOs

Besides representing a quality outcome measure, TO may also have financial implications. In fact, the financial impact of TO has been investigated and data have suggested that a disproportionate amount of money is spent on patients who do not achieve a TO after hepatopancreatic surgery. For example, Merath *et al*[4] noted that among patients who achieved a TO, Medicare payments were approximately $11000 less following minor hepatopancreatic resections and $14000 less for patients undergoing major resection when compared with individuals who did not achieve a TO[4]. Similarly, Mehta *et al*[12] suggested that TO resulted in an average of $5000 less in Medicare expenditures after hepatopancreatic surgery at teaching hospitals (TO: $19191, *vs* no TO: $24165, $P < 0.001$)[12]. The association of decreased overall costs with the achievement of a TO was consistent across major and minor teaching hospitals, as well as among high and low volume institutions, highlighting that TO has financial implications irrespective of the hospital setting[12]. Collectively, the data suggest that TO might be a composite metric that reflects value—*i.e.*, high quality combined with lower costs. In turn, improving TO rates after hepatopancreatic surgery may not only optimize the quality of care provided to patients, but also decrease health care costs and lead to cost-effective and high-value care.

ADVOCATING FOR WIDER IMPLEMENTATION OF TO IN HPB SURGERY

Advocates of TO note that this composite metric provides a more comprehensive estimate of quality of perioperative care[28]. However, a primary criticism of TO is that there is no consensus in the literature as to what should be considered an "optimal"

outcome after a specific operation. Moreover, patient risk factors strongly influence outcomes and require comprehensive risk adjustment in order to make valid comparisons. With the thoughtful implementation of composite quality metrics in HPB surgery, surgeons and centers will hopefully gain a better understanding of the perioperative processes of care and develop insights to improve patient outcomes. Moreover, the optimization of expectation management will be facilitated, especially in high-risk patients. Improved understanding of these gaps through the use of TO can allow systems to identify patients who are high risk of failure to achieve optimal short- and long-term outcomes after surgery and redirect resources accordingly. Thus, fundamentally the concept of TO aligns with the patient best interest, which is the ideal outcome after surgery.

CONCLUSION

TO provides a more realistic assessment of patient-centered perioperative care and represents the optimal experience around a surgical episode. TO should be the ideal outcome that surgeons should strive to achieve for their patients. The use of TO in cancer populations is of paramount importance as a measure of both short- and long-term outcomes. TO can be used to assess performance across different institutions as well as assess quality metrics or hospital designations. Future efforts should be directed towards establishing a universal definition of TO in HPB surgery so that surgeons and hospitals can assess and compare outcomes, as well as identify shortcomings and improve real-world patient outcomes.

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REVIEW

Impact of cytokine storm and systemic inflammation on liver impairment patients infected by SARS-CoV-2: Prospective therapeutic challenges

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Abstract

Coronavirus disease 2019 (COVID-19) is a devastating worldwide pandemic infection caused by a severe acute respiratory syndrome namely coronavirus 2 (SARS-CoV-2) that is associated with a high spreading and mortality rate. On the date this review was written, SARS-CoV-2 infected about 96 million people and killed about 2 million people. Several arguments disclosed the high mortality of COVID-19 due to acute respiratory distress syndrome or change in the amount of angiotensin-converting enzyme 2 (ACE2) receptor expression or cytokine storm strength production. In a similar pattern, hepatic impairment patients co-infected with SARS-CoV-2 exhibited overexpression of ACE2 receptors and cytokine storm overwhelming, which worsens the hepatic impairment and increases the mortality rate. In this review, the impact of SARS-CoV-2 on hepatic impairment conditions we overviewed. Besides, we focused on the recent studies that indicated cytokine storm as well as ACE2 as the main factors for high COVID-19 spreading and mortality while hinting at the potential therapeutic strategies.

Key Words: COVID-19; Hepatic impairment; SARS-CoV-2; Cytokine storm; Immuno-modulators receptors

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Core Tip: Implications of fast coronavirus disease 2019 (COVID-19) outbreak are huge annoying problems that affected countries' health and economies around the world. Patients associated with hepatic impairment are considered at a high-risk target for severe acute respiratory syndrome namely coronavirus 2 severity and mortality. A good understanding of virus machinery provides excellent ideas about how to combat this monster. We provide detailed information about the recent mechanisms of COVID-19 pathogenesis, implications on several hepatic disorders, and potential therapeutic strategies.

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INTRODUCTION

Coronaviruses are classified into four categories, namely α, β, γ, and δ type. The α- and β-coronaviruses could infect mammals, while γ- and δ- coronaviruses appear to infect birds[1]. Six coronaviruses reported as human-affected viruses include α-coronaviruses NL63 and 229E, low-pathogenic β-coronaviruses OC43 and HKU1, these viruses cause mild respiratory symptoms as common cold[2]. The remaining two types were identified as β-coronavirus, which unusually induced fatal respiratory tract infections[3]. Between 2002 and 2003, severe acute respiratory syndrome (SARS) resulted in the outbreak of SARS0[4]. In 2012 the Middle East respiratory syndrome (MERS) originated and circulated in camels[5].

In 2019, a newly discovered coronavirus is a SARS coronavirus 2 (SARS-CoV-2), which induces a contagious disease coronavirus disease 2019 (COVID-19) and is currently spreading nationwide[6]. Consequently, the third identified zoonotic coronavirus detected after SARS and MERS cause potentially fatal respiratory tract infections[7,8]. The early cases of COVID-19 were linked to the Huanan seafood market, Wuhan, China[9,10].

On January 12, 2020, the World Health Organization (WHO) temporarily named this new virus the 2019 novel coronavirus (2019-nCoV)[11]. On January 30, 2020, the WHO declared the 2019-nCoV epidemic a public health emergency of international burden[11]. After that February 11, 2020, the WHO formally named the disease caused by 2019-nCoV as COVID-19[12]. On the same day, the international committee of taxonomy named this virus as SARS-CoV-2 and classified it as coronavirus under the family Coronaviridae, subfamily Orthocoronavirinae, based on the genotypic and serological characterization[11,13,14].

SARS-CoV-2 is a new generation of β-coronavirus which shares 79% and 50% genome sequencing with SARS and MERS, respectively[15]. This information confirmed some of the available evidence that SARS-CoV-2 was generated from bats and pangolins[14]. Other studies suggest that SARS-CoV-2 was a chimeric virus obtained from the fusion of a bat's coronavirus and a coronavirus of unknown origin[16]. In the same context, the study conducted by Sun *et al*[17] showed that the structure of SARS-CoV-2 was similar to the coronavirus isolated from Chinese chrysanthemum-headed bats in 2015. So far, no confirmation has been made of the zoonotic source of SARS-CoV-2 yet[18].

SARS-CoV-2 has a 29891-nucleotide genome (encoding 9860 amino acids)[19] with a diameter of about 50-200 nm[20]. Two-thirds of viral RNA, primarily in the first open reading frames (ORFs) (ORF1a/b), converts into two non-structural polyproteins pp1a, pp1ab, and encodes 16 non-structural proteins (NSP). In contrast, the remaining ORFs encode structural and accessory proteins[21,22]. Six ORFs of functions are arranged between 5′ to 3′ (ORF1a/ORF1b), spike glycoprotein (S), a small envelope protein (E), matrix protein (M), and nucleocapsid protein (N)[21]. Moreover, seven putative ORFs encoding accessory proteins are disseminated between the structural genes[23,24]. The glycoprotein S is responsible for the receptor-binding site and membrane fusion[25]. The envelope protein E has essential roles in releasing and accumulating viruses[26], besides protein M[27]. Additionally, a NSP plays many functions during the viral cycle[28].

SARS-CoV-2 AND ANGIOTENSIN-CONVERTING ENZYME 2

Only specific and permissive cells inside the host may be infected. Specifically, cells that generate proteins and viral factors which are required for virus replication. As soon as the virus enters the cell, the virus recruits cells to produce essential viral encoded proteins involved in replicating the virus's genetic material[29]. The viral replication process generally known as cytopathic effects, could induce biochemical changes resulting in cellular damage. Interestingly, SARS-CoV-2 relies on specific cellular receptors to invade the cells that carry these factors like other types of coronaviruses[30].

The surface of SARS-CoV-2 is covered by several types of glycosylated S proteins that facilitate the virus's attachment to the host angiotensin-converting enzyme 2 (ACE2), hence mediating the entry of the virus into the host cells[31]. This action could activate S protein by a specific type of serine protease located on the host cell membrane called TM protease serine 2. After the virus recruited the host cell, it releases its RNA and uses the host cells to replicate and synthesize its functional proteins[32].

SARS-CoV-2 is predicted to have organ tropism beyond the respiratory tract, also including the kidney, heart, skeletal muscle, central nervous system, liver, and gastrointestinal tract. The spread of infection through the body is reasonable for developing COVID-19 systemic symptoms and exacerbating pre-existing disorders[33,34]. Therefore, patients with severe COVID-19 illness show multiple organ damage, such as acute kidney injury, acute pulmonary injury, heart injury, and liver injury[35].

SARS-CoV-2 may induce liver injury *via* a similar mechanism. Furthermore, we cannot neglect angiotensin II's action; once SARS-CoV-2 binds to ACE2 receptors it stimulates angiotensin II to mediate the tissue injury[36]. These findings confirm that angiotensin II levels are increased in healthy patients with COVID-19 and are directly correlated with viral load[37].

Hepatic sharing of ACE2 receptors is unreasonable; it is widely distributed in the endothelial layer but not in the sinusoidal endothelium[38]. On the surface of cholangiocytes, ACE2 receptors have more significant expressions than hepatocytes[39]. Cholangiocytes ACE2 receptors expression was close to that of type II alveoli cells in the lungs, indicating that the liver may be a strong candidate for SARS-CoV-2[40]. In Contrast, Immunohistochemical stains for Kupffer cells and lymphocytes T and B for ACE2 are negative[41]. In fact, in patients with COVID-19, SARS-CoV-2 has been detected in their liver and can induce liver injury through different pathways[42]. The general mechanism of ACE2 mediated SARS-CoV-2 infection is outlined in Figure 1.

SARS-CoV-2 and devastating cytokine storm

Cytokine storm is an augmented cytokine output attributable to an irregular immune response to various stimuli like viral infections, rheumatic disorders, cancer, sepsis, multiple organ failure, and drugs that contribute to unnecessary immune cell activation[43]. Consequently, the rising aggressive systemic hyperinflammatory reaction is associated with the release of many pro-inflammatory cytokines responsible for critical illness[43,44]. Cytokine storm has been found in various viral infections, including influenza H5N1, H1N1 viruses, and two SARS-CoV and MERS-CoV coronaviruses[45-48]. Recent data reported that patients infected by SARS-CoV-2 show massive levels of cytokines and chemokines and are implicated with COVID-19 severity and high mortality rate[49].

The growing cytokine storm results in the continuous activation and expansion of innate and adaptive immune cells to produce a harmonized cascade of inflammatory responses, resulting in a devastating cytokine storm[50]. Indeed, the result of a mixture of several immune active molecules is the cytokine storm's product. The main components involved in forming the cytokine storm are interferons (IFNs) and interleukins (ILs)[51]. However, other inflammatory biomarkers such as c-reactive protein (CRP), fibroblast growth factor, macrophage-colony stimulating factor (M-CSF), interferon gamma-induced protein 10, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory proteins-1α and 1β, platelet-derived growth factor, tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF) are markedly detected in the COVID-19 sever case-patients[52-54]. Hence, the uncontrolled response in severe COVID-19 patients is correlated with unfavorable clinicopathological consequences.

During COVID-19 illness, several pathological factors have been reported to participate in the initiation and propagation of SARS-CoV-2-induced cytokine storm (Figure 2).

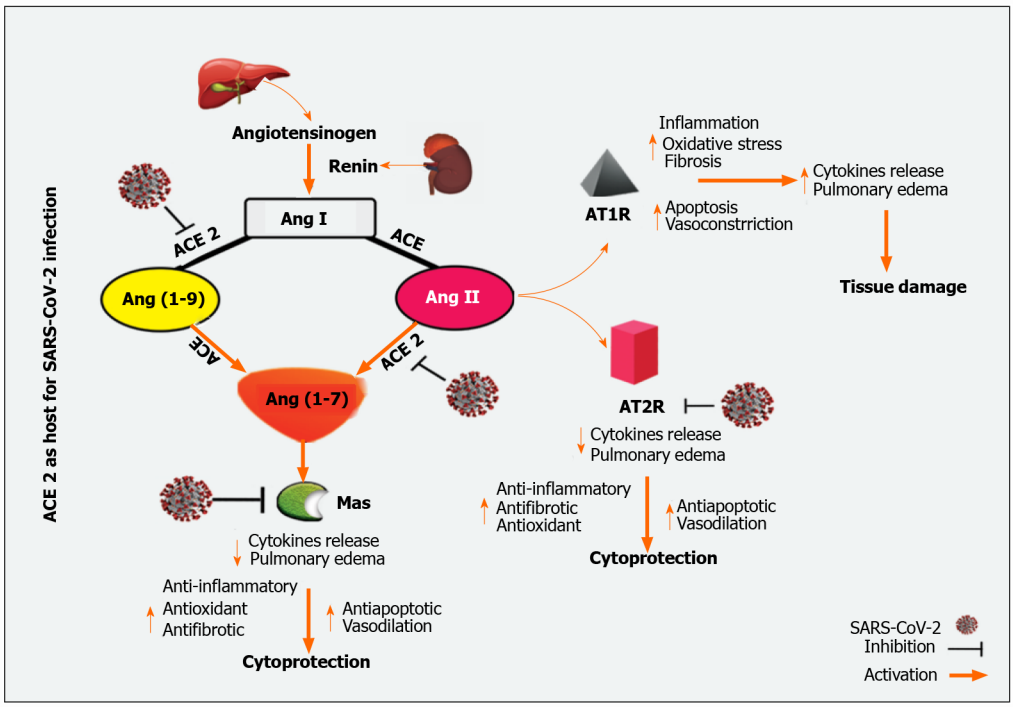


Figure 1 Angiotensin-converting enzyme 2 as a host for severe acute respiratory syndrome coronavirus 2 infection. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE: Angiotensin-converting enzyme; Ang: angiotensin; ATR: Angiotensin receptors type.

Renin angiotensin-aldosterone system

The Renin-angiotensin-aldosterone system (RAAS) is a significant regulator of several physiology and pathophysiology conditions such as maintenance of fluid and electrolyte homeostasis with the maintenance of vascular tone[55,56]. Recent evidence implies that RAAS has autocrine and paracrine effects in addition to the classical circulating endocrine effects. Hence, RAAS plays pivotal roles in cellular growth, migration, differentiation, apoptosis, inflammation, thrombosis, and fibrosis[11,57-59].

When renin is secreted directly into the bloodstream by juxtaglomerular cells, this secreted renin cleaves the liver-released substrate, angiotensinogen, to generate the inactive peptide, angiotensin (Ang) I, which is then converted by endothelial ACE to Ang II[56,60]. On the other hand, ACE2 cleaves Ang I to produce Ang (1-9) peptide. Besides, Ang II and Ang (1-9) can metabolize by ACE or other peptidases to form Ang 1-7[61,62].

The RAAS is a double-edged sword that acts through two distinct opposing arms: the ACE/Ang II/AT1 receptor axis, which is responsible for main actions, and the ACE-2/Ang-(1-7) /Mas receptor axis, the counter-regulatory arm[63,64]. The ACE catalytic activity contributes to an increase of Ang II levels and an increase in Ang-(1-7) catabolism, while ACE2 catalytic activity is primarily based on Ang I and Ang II and leads to the formation of Ang (1-7)[64,65]. Ang II plays a central role in the RAAS *via* angiotensin II type 1 and 2 receptors (AT1R and AT2R); thus, stimulating the ACE/Ang II/AT1 receptor axis which results in regulating vasoconstriction, fibrotic remodeling, inflammatory response, and production of reactive oxygen species (ROS)[66]. Ang II itself will directly activate the nuclear factor-kappaB (NF-κB) pathway through the phosphorylation of the p65 subunit of NF-κB; therefore, increases the output of IL-6, IL-1β, TNF-α, and IL-10[67,68]. Ang II also affects mitogen-activated protein kinases (MAPKs; extracellular signal-regulated kinases 1/2, c-Jun N-terminal kinases, p38-MAPK), which have a high impact on pro-inflammatory cytokines release[68,69]. Besides, a spontaneous association of Ang II with host immune cells, *e.g.*, neutrophils, T and B lymphocytes, and tissue-resident cells results in the release of pro-inflammatory cytokines, including prostaglandins, IL-6, IL-1β, TNF-α, VEGF, and IFN-γ, as well as the activation of kinase plethora [*e.g.*, Janus kinase (JAK) and p38 MAPK][70,71].

As soon as the SARS-CoV-2 spike (S)-protein binds to ACE2[72], the serum level of Ang II was significantly increased, which in turn mediated trans-signaling of the IL-6/soluble IL-6 receptor-a complex in to finally activate of signal transducer and activator of transcription 3 (STAT3)[73]. STAT-3 has been reported to up-regulated

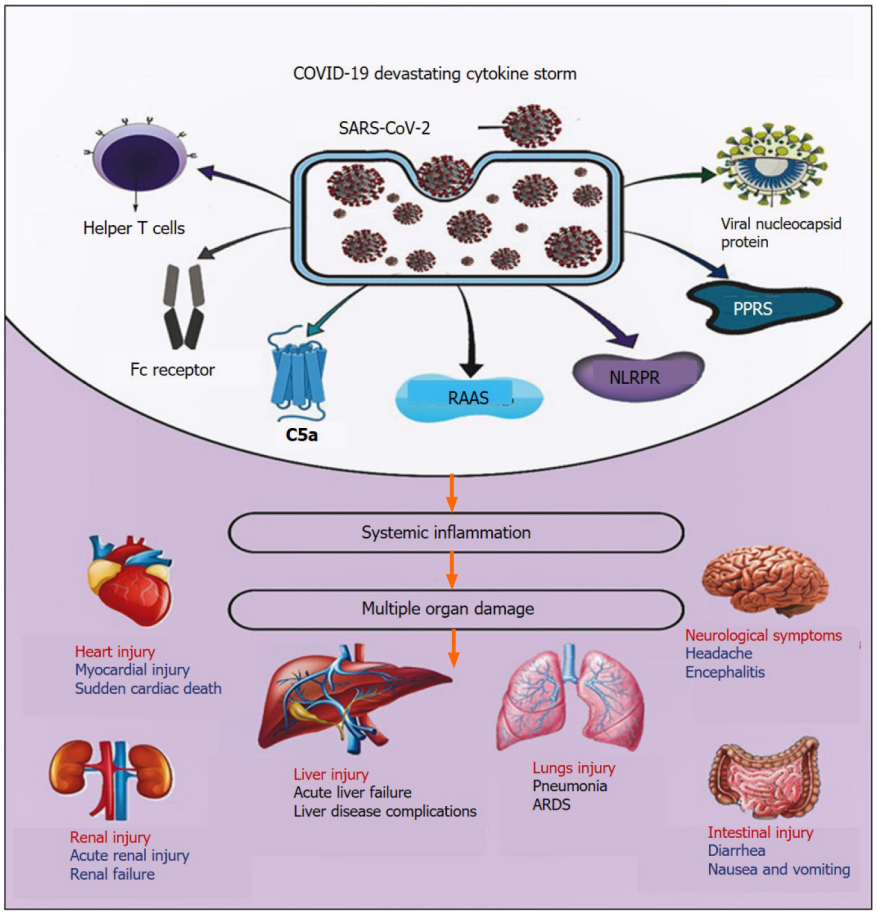


Figure 2 Coronavirus disease 2019 and devastating cytokine storm. COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; C5a: Complement component 5a; RAAS: Renin-angiotensin-aldosterone system; NLRPR: Nucleotide-binding oligomerization domain, Leucine-rich repeat and pyrin domain-containing receptor; PPRs: Pattern recognition receptors; ARDS: Acute respiratory distress syndrome.

several inflammatory genes like NF-κB. Besides activation of NF-κB by SARS-CoV-2 mediated STAT3 activation, SARS-CoV-2 itself can activate NF-κB by binding with pattern recognition receptors (PRRs). The net results trigger a cytokine storm accompanied by acute respiratory distress syndrome (ARDS) and multiple organ damage[68,74]. During COVID-19, ACE2 depletion and ACE/Ang II/AT1R axis activity are highly augmented[75]. Therefore, a therapeutic modality by administering exogenous ACE2 to patients with COVID-19 can potentially impact COVID-19 severity[76]. On the other hand, targeting the ACE/Ang-II/AT1R axis downstream, such as the IL-6/STAT3 axis, should be considered to avoid ARDS inflammation and end-organ damage induced by cytokine storm[68,76].

In contrast to the ACE/Ang II/AT1R pathway, the Ang (1-7) interacts with the Mas receptor to provide vasodilation and antiproliferative effects[77]. Indeed, activating Mas receptors induce activation of phospholipase A, phosphoinositide 3 kinases/protein kinase B axis, endothelial nitric oxide synthase, and intracellular calcium accumulation[78]. Additionally, Ang (1-7) down-regulates p38 MAPK and NF-κB expression harmony with the suppression of inflammatory markers such as IL-6, IL-8, and TNF-α[68]. Furthermore, Ang (1-7) *via* the Mas receptor activation attenuated Ang II-induced intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and MCP-1[68,79]. Together, activation of these pathways suggests that Ang (1-7) has antiproliferative, anti-thrombotic, and anti-inflammatory functions and ameliorates tissue damages[70,78].

Interestingly, down-regulation of the ACE-2/Ang (1-7)/Mas receptors axis was proposed to be increased during COVID-19; since the virus uses the enzyme's peptidase domain to enter into the cells, there is a down-regulation of ACE-2 with ACE up-regulation[63]. Thus, it contributes to a massive release of cytokines and inflammatory responses. Since Ang (1-7) plays a critical role in counteracting the pro-inflammatory activity of RAAS, it has been proposed that Ang (1-7) or one of its associated agents could be administered to patients with COVID-19 since it protects against activation of inflammatory mediators in a cytokine outbreak[68]. Additionally,

Ang (1-7) was previously reported to inhibit liver fibrogenesis[80]. Thus, it may be helpful to attenuate liver injury in patients with liver impairments infected with SARS-CoV-2.

Fc receptor

SARS-CoV-2 is a specific antibody with the Fc receptor. Indeed, SARS-CoV-2 recruits several immune cells, *e.g.*, granulocytes, monocytes, and macrophages, and activates the complement cascade, resulting in high virus reproduction and violent infection[81,82]. Since the liver includes several cells related to the immune response, so, antibody-dependent, ACE2-independent pathway was activated[83]. As a result, SARS-CoV-2 infection overactivated and secreted excessive amounts of cytokines and chemokines such as TNF-α, IFN-γ, IL-6, IL-8[82].

Viral nucleocapsid proteins

Viral nucleocapsid proteins fused with the infected cells' host cell membrane, which permits the viral nucleocapsid protein to stay on the surface of the cell membrane[84]. This fusion makes the proteins easily recognizable by antigen-presenting cells that activate the immune response[83]. The normal antigen-presenting cells are dendritic cells, macrophage cells, monocytes, plasma cells, *etc.*, introduced to viral particles CD8+ cytotoxic and CD4+ regulatory T lymphocytes, the major histocompatibility complexes[85].

PRRs

Several PRRs are the first line receptors that detect pathogenic infection like SARS-CoV-2. After the virus enters the body, it produces pathogen-associated molecular patterns (PAMPs), which recognizes by PRRs and fires the standard innate immune system[86]. The membrane-bound toll-like receptor (TLR) family among PRRs, predominantly recognizes PAMPs in the extracellular system and to a lesser degree in the intracellular milieu[87]. Activation of this signal contributes to increasing the expression of transcription factors that induce pro-inflammatory cytokine production, such as NF-κB, and activate immune defense against viral infection *via* the IFN type I pathway[85,88].

Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain-containing (nod-like receptor protein) receptor

Another family of pathogenic detecting receptors is nod-like receptor protein (NLRP) receptors (NLRP1, NLRP3, NLRP7, and NLRC4), which are the cytosol of endogenous danger-associated molecular patterns expressed inside the cell[89,90]. The activation of these receptors by SARS-CoV-2 has been reported to associate with the adhesion of molecules' activation, inflammatory response, and triggering innate immune cells[91]. This reaction contributes to the activation of pro-inflammatory mediators cytokines production such as TNF-α, IL-1, IL-6, IL-10, or type 1 interferons[92,93].

T-helper type 1 cell

The T-helper type 1 (Th1) cell response can be triggered by the elevation of inflammatory cytokines, which plays a significant role in providing a memory response against the virus and adaptive immunity. It is essential to coordinate humoral and cytotoxic T cell responses during viral infections[94]. Besides, elevated levels of Th2 cells secrete cytokines in patients with COVID-19, *e.g.*, IL-4 and IL-10, which inhibit inflammatory responses[95]. Furthermore, there was a significant increase in cytokines secreted by Th1 and Th2 cells, such as TNF-α, IL-6, IL-18, IL-4, and IL-10. Additionally, IL-2 and IL-6 are significantly elevated in patients with COVID-19 and correlate with the disease's seriousness[96]. Interestingly, hepatocytes, Kupffer cell, and hepatic stellate cells after being infected with SARS-CoV-2 immune cells after overactivation and secretion of excessive cytokines, *e.g.*, IL-6, IL-8, TNF-α, IFN-γ, which are involved in severe cytokine storm and causes tissue damage[85,97]. Compared to a reduced level of naive B cells, plasma B cells significantly increased in COVID-19 patients, several naive B-cell receptor isotypes [immunoglobulin heavy chain (IGHV) 3-15, IGHV3-30, and IGHV3-11] were previously used in the manufacture of other virus vaccines which were also reported and identified in patients[98]. Furthermore, the novel target genes in cytokine storm are IL-1β, IL-6, and the granulocyte-macrophage colony-stimulating factor (GM-CSF), while TNFSF13, IL-18, IL-2, and IL-4 seemed to help patients' recovery from COVID-19[99].

Complement activation

Viral infection has been reported to stimulate the complement cascade and consequently, initiate specific inflammatory responses[100]. Among these complements, complement factor 5a (C5a) is the most potent inflammatory peptide in the complement cascade, which increases the production of pro-inflammatory cytokines such as IL-6, IL-1, and TNF-α from macrophages under the effect of TLR-2, TLR-4, and TLR-9[101,102]. Terminal complement variable C5b-9 stimulates the release of IL-6 from vascular smooth muscle cells by stimulating the redox-sensitive transcription factor NF-κB, activator protein 1, and MCP-1[68,100]. Besides, C3a overexpression results in increased production of IL-1, IL-6, and TNF-α[103].

The complement cascade plays aberrant pivotal roles in the pathogenesis of SARS-CoV-2. It promotes viral nucleocapsid protein-mediated auto-activation of mannan-binding lectin serine peptidase-2 (MASP-2)[104]. Indeed, MASP-2 is considered the main serine protease in the lectin pathway. It binds to the mannose-binding lectin pathway to induce the downstream complement cascade, which in turn accelerates inflammatory responses. In two patients with COVID-19 who received an anti-C5a antibody, a positive clinical response was improved as evidence by enhancing lung oxygenation and alleviating systemic inflammation[105,106]. Hence anti-C5a antibodies could be a new therapeutic option for combating COVID-19 severity.

COVID-19 and hepatic dysfunction

Patients with digestive issues and co-infected with SARS-CoV-2 show a higher risk of mortality than patients without digestive problems. In severe cases of COVID-19, liver dysfunction was observed and associated with extensive activation of coagulative and fibrinolytic pathways as well as alteration of platelets, neutrophils, and lymphocytes profiles[8]. In parallel, patients with chronic liver disease (CLD), hepatitis viruses [hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus, and hepatitis E virus], hepatotropic viruses infection, non-alcoholic fatty liver disease, and non-alcoholic steatohepatitis are more susceptible to COVID-19 and may present worse outcomes from ARDS compared with the other critically ill patients[82,107]. Alqahtani *et al*[108] performed a meta-analysis that showed that patients with a previous history of liver disease have a 57.33% chance of severing COVID-19 infection and a 17.65 % higher mortality rate than other patients[108]. A recent multicenter cohort study showed that hepatic decompensation was positively associated with COVID-19 disease, which increasing the risk of death from 26.2% to 63.2%[109]. This rate could be related to low platelets and lymphocytes in those patients or due to cirrhosis-related immune dysfunction[97]. Besides, COVID-19 is markedly distinguished by an increase in cytokine secretion, which induces hepatocytes injury, contributing to the loss of hepatic regeneration and worse clinical outcome, especially in patients with CLD[97,110]. In contrast, Lippi *et al*[111] indicated that CLD plays a minor role in affecting patients' progression, severity, or mortality[111].

The pattern of liver damage in COVID-19 is essentially considered during COVID-19. The liver injury can be associated with the virus's immediate cytopathic effect, unregulated immune response, sepsis, hypoxia, and drug-inducing liver injury[112,113]. Also, COVID-19 causes underlying chronic hepatic disorders to exacerbate and contributes to liver decompensation with higher mortality[114]. Recent general studies show that about 2%-11% of COVID-19 patients have underlying CLD, and 14%-53% developed hepatic dysfunction with COVID-19[8,14,115,116]. In patients with severe COVID-19, the ratio of hepatic injury is highly set in contrast to mild patients' rate. Hence, hepatic injury frequency can represent 58.06%, 51%, and 78% in patients' death from COVID-19[14]. Cai *et al*[117] conducted a study on 417 patients with COVID-19 which showed that about 76.3% of patients had abnormal liver test results and 21.5% exhibited liver injury during hospitalization[117]. Li *et al*[118] indicated that patients with abnormal liver activity (58.8% and 66.7%) could be more likely to have moderate to severe COVID-19[118].

It is understood that cholangiocytes play an essential role in liver regeneration and immune response, and cholangiocytes ACE2 expression is greater than that of hepatocytes[38,116,119]. Thus, it was proposed that the liver injury that resulted in SARS-CoV-2 infection could be due to the damage caused by virus infection to bile duct cells and not liver cells[113]. The expression of ACE2 in cholangiocytes was also observed, it is about 20 times higher than in hepatocytes[13]. Additionally, gamma-glutamyl transferase, a diagnostic biomarker for cholangiocyte injury, has also been identified to be highly up-regulated in severe cases of COVID-19[120,121]. However, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are elevated predominantly in COVID-19. Generally, it is 1-2 times more than the normal range,

which averages to modestly elevated total bilirubin (TBIL) levels early in the disease process[112,115,116]. On the contrary, the study conducted by Chen *et al*[122] showed an elevation of ALT and AST serum levels to 7590 U/L and 1445 U/L, respectively, in patients with severe COVID-19[122].

Several studies have found that AST, ALT, and TBIL were substantially higher in the intensive care unit (ICU) patients than in non-ICU patients[123,124]. A retrospective cohort study performed by Hundt *et al*[125] reported abnormal liver tests [AST, ALT, Alkaline phosphatase (ALP), and TBIL] in the patients with COVID-19. Most patients with abnormal liver tests had an elevation limit of 1-2 times more than the normal limits. It has also been reported that abnormal liver tests were directly proportionated with poorer clinical outcomes[125].

Hypothesizes of hepatic injury during COVID-19 illness

Hepatic injury in COVID-19 patients could result from a cytokine storm rather than direct cytopathic effects of the virus itself[112,113,126]. The direct cytopathic effects of SARS-CoV-2 can induce stress on endoplasmic reticulum and mitochondrial dysfunction[127]. Moreover, the human body can initiate immune-mediated inflammation, such as cytokine storm resulting in liver damage or hepatic failure in critical COVID-19 patients[128-130]. Hypoxia is also a common characteristic of extreme COVID-19 illness that promotes ROS production and initiates the release of multiple pro-inflammatory factors to induce more liver damage[82]. Electron microscope examination of liver obtained from patients infected with SARS-CoV-2 revealed that SARS-CoV-2 causes endoplasmic reticulum tension that induces de novo lipogenesis. Lipogenesis could also contribute to the production of non-specific inflammatory changes, including hepatocyte swelling and steatosis, mild hepatic sinus cell proliferation, and Kupffer cell hyperplasia[82].

Effect of SARS-CoV-2 on several types of liver disorders

As we mentioned before, SARS-CoV-2 is remarkably impactful on patients with chronic diseases, including chronic hepatic impairments. The impact of SARS-CoV-2 on several types of liver disorders is outlined in Table 1.

Non-alcoholic fatty liver disease and COVID-19

Several studies have documented that obesity is a significant mortality factor in COVID-19 patients. The need for overall survival and mechanical ventilation correlated with obesity[131]. Typically the ACE2 expression level in adipose tissue is greater than that of lung tissue[97]. These clarify adipose tissue's susceptibility to invasion by SARS-CoV-2, and then the virus can spread to other organs[132]. Obese patients are at high risk for non-alcoholic fatty liver disease (NAFLD), which in turn has a higher chance of developing severe COVID-19 with a higher probability of abnormal liver function and a more extended viral shedding period[133]. NAFLD is associated with the development and severity of COVID-19[97,133]. NAFLD patients also elevate cytokine levels, rendering them more vulnerable to COVID-19 related to excessive cytokine production[134]. Experimentally, it has been shown that ACE2 expression increases in chronic liver damage and NAFLD[135]. Patients with COVID-19 exhibit increased serum levels of MCP-1, which exacerbate steatohepatitis; thus, the virus can increase NAFLD progression to Non-alcoholic steatohepatitis (NASH)[136]. Multicenter retrospective by Hashemi *et al*[137] study the clinical outcome of COVID-19 and CLD. Patients with high NAFLD require ICU admission and mechanical ventilation. There is no overall mortality among CLD patients mainly due to NAFLD[137]. According to the evidence available, NAFLD is an independent risk factor for extreme COVID-19, but most studies did not separate NAFLD from its more extreme NASH[138].

Liver cirrhosis and COVID-19

Patients with liver cirrhosis have a potentially higher risk of SARS-CoV-2 infection, a higher risk of a severe condition, and a higher risk of liver decompensation[139]. Liver cirrhosis in SARS-CoV-2 infected patients is described as a predictor of mortality[140,141]. A decrease in ACE2 *via* SARS-CoV-2 induced internalization is predicted to exacerbate liver fibrosis and augment the disease's severity, especially in the long term. Therefore, the impact of COVID-19 on long-term liver-related outcomes is also worth considering in patients with cirrhosis[142]. A multicenter matched cohort study by Kushner and Cafardi[139] patients with cirrhosis plus COVID-19 were observed to have mortality equal to cirrhosis patients alone higher than those with COVID-19 alone[139]. Sarin *et al*[143] The APCOLIS study of pre-existing liver disease indicated that in CLD patients,

Table 1 Implication of severe acute respiratory syndrome coronavirus 2 on patients with hepatic disorders

Hepatic disorders	Main findings of the study	Ref.
NAFLD	NAFLD is associated with a higher risk of symptomatic, severe, and progressive COVID-19	Hashemi *et al*[137], 2020
	SARS-CoV-2 infection in patients with NAFLD required ICU admission and mechanical ventilation concomitant with increased NAFLD progression to NASH	Sachdeva *et al*[212], 2020
Liver cirrhosis	Patients with liver cirrhosis and COVID-19 are related to worse clinical outcomes and a high mortality rate than patients with COVID-19 alone	Kushner and Cafardi[139], 2020
	SARS-CoV-2 co-infection augmented liver injury as evidenced by worsens decompensated clinical status	Sarin *et al*[143], 2020
	Cirrhotic patients with COVID-19 had a higher risk of mortality than COVID-19 patients alone, but they are equally mortality rate with cirrhosis patients without COVID-19	Bajaj *et al*[144], 2021
HCC	Patients with HCC consider a risk group, and HCC is positively related to deterioration symptoms and bad outcomes in COVID-19	Zhang *et al*[213], 2020
	Patients with cancer are more susceptible to infection and poorer prognosis of COVID-19	Liang *et al*[147], 2020
Hepatitis B	Patients infected with HBV tend to have a more severe form of COVID-19	Chen *et al*[150], 2020
	SARS-CoV-2 and HBV co-infection showed monocytopenia, lymphopenia, and thrombocytopenia, as well as metabolic disorders	Liu *et al*[151], 2021
	COVID-19 may induce HBV reactivation, but it rarely occurs	Aldhaleei *et al*[152], 2020
Hepatitis C	Patients infected with HBV or HCV showed a high risk of mortality and morbidity if co-morbid with COVID-19	Mirzaie *et al*[155], 2020
	Patient with hepatic c and COVID-19 has an undesirable clinical outcome	Mostardeiro *et al*[156], 2020
	HCV pre-existing was associated with high mortality	Mangia *et al*[157], 2020

NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; HBV: Hepatitis B virus; HCV: Hepatitis C virus; ICU: Intensive care units.

SARS-CoV-2 infection causes severe liver damage, decompensating one-fifth of the cirrhosis and worsening the already decompensated clinical status[143]. Bajaj *et al*[144] multicenter North American contemporaneously enrolled study, compared to patients with cirrhosis alone or cirrhosis and COVID-19 patients had similar mortality rate, but higher than patients with COVID-19 alone[144].

Hepatocellular carcinoma and COVID-19

In general, cancer patients, especially those who have recently received cancer treatment, have a greater chance of infection and a worse outcome. Besides, epidemiological data suggest that patients with cancer are more vulnerable to SARS-CoV-2 infection[145]. Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer globally, accounting for about 6% of all cancer incidences[146]. HCC patients are more susceptible than other cancers to the consequences of the COVID-19 pandemic since the liver damage caused by SARS-CoV-2 could complicate current hepatitis in most HCC patients[146]. A retrospective cohort study by Yang *et al*[96] confirmed that cancer patients had deteriorating symptoms and bad results from SARS-CoV-2 infection[96]. Besides, these patients have poor nutritional status-related anemia and hypoproteinemia that impair their immunity, rendering them more vulnerable to severe disease[96].

A prospective nationwide cohort study in China on cancer patients, including HCC patents and COVID-19, was more vulnerable to severe disease. They had an increased chance of mortality and ICU admission. Recent chemotherapy within a month also increased the risk of severe disease[147]. Most HCC and CLD patients fall into a high-risk group and are expected to have worse outcomes[114]. Vigorous screening for COVID-19 disease should be prescribed in cancer patients undergoing antitumor therapy, and medications that induce suppression of immunity should be stopped, or their dosages decreased in the event of COVID-19[148].

Hepatitis B and COVID-19

For patients with chronic hepatitis B and are in the immune tolerance phase, further tests are required to confirm whether these patients have active viral replication and repeated liver damage after co-infection with SARS-CoV-2[82]. Several Chinese clinical

trials have found patients with hepatitis B infection are also present among patients with severe COVID-19 cases. That suggests that patients infected with HBV tend to have a more severe form of SARS-CoV-2[85,149]. Chen *et al*[150] found that 47% of patients with HBV are observed with severe COVID-19 cases[150]. However, other studies have shown that chronic viral hepatitis does not seem to be proportional to the severity of COVID-19[115]. Liu *et al*[151] retrospective study reported that COVID-19 was not substantially affected by co-infection with HBV. However, at the onset of COVID-19, patients co-infected with SARS-CoV-2 and HBV showed more severe monocytopenia, thrombocytopenia, hypoalbuminemia, and hepatic deficiency in lipid metabolism[151]. SARS-CoV-2 and HBV co-infection produces lymphopenia that can cause HBV reactivation. However, it has been reported just in one case of a patient with potential HBV reactivation, which seems to be an unusual event[152]. Chinese medical association and Chinese society of hepatology indicated that patients with hepatitis B administrated antiviral therapy, discontinuation of anti-HBV drugs, or failure to receive anti-HBV treatment can lead to the reactivation of HBV, especially during SRSA-CoV-2 infection following high-dose hormone therapy[82].

Hepatitis C and COVID-19

HCV infection is a dominant factor for liver diseases, including liver cirrhosis, hepatocellular carcinoma, and a common liver transplantation cause[153]. All over the world, HCV infection is the primary cause of liver-related mortality and morbidity[154]. Similarly, the symptoms of hepatic impairments are widespread among patients with COVID-19 and HCV[155]. Therefore, the American associations of liver disease evaluated the underline liver diseases' status, *e.g.*, hepatitis A, hepatitis B, and hepatitis C, in COVID-19 patients and found that co-infection between HCV and SARS Cov-2 increased liver enzyme levels, especially in pediatric[112]. Besides, a systematic preprint review by Mirzaie *et al*[155] suggested that patients with hepatitis B and/or hepatitis C present a high risk of morbidity to COVID-19 and require further investigation to overcome a significant marker of mortality due to co-infection[155]. Mostardeiro *et al*[156] reported that in a case study patient with hepatitis C and COVID-19 co-morbidity has an undesirable clinical outcome[156]. Correspondingly, a cohort study by Mangia *et al*[157] has been elucidated that HCV pre-existing associated with high mortality; meanwhile, HCV antibodies suggestive as a protective agent against COVID-19[157].

On the other hand, countries co-operated and adapted to develop a screening model to detect HCV as a hepatitis elimination program[158]. Nowadays, due to the spreading of the COVID-19 pandemic, it extends beyond the direct morbidity and mortality associated with coinfection COVID-19 may act as a barrier to reduce hepatitis C care, result in a decrease in HCV serological testing and identification[159]. Hence, Blach *et al*[160] based on their mathematical models, predicted scenario of the 1-year delay which resulted in an additional 44800 liver cancers and 72300 deaths from HCV globally by 2030[160]. Thus, rapid HCV testing in the context of SARS-CoV-2 screening programs may be the only solution for achieving the WHO's 2030 HCV elimination target[161].

Therapeutic approaches for aborting COVID-19 cytokine storm

As mentioned before, SARS-CoV-2 infections may cause cytokine storm to result in hyperactivation of T lymphocytes and release massive amounts of cytokines such as IL-6, IL-1β, TNF-α, and others[162]. Neutralization of these cytokines offer an excellent therapeutic avenue in combating the COVID-19 outbreak. Here, several therapeutic options can be used for terminating SARS-CoV-2-induced cytokine storm.

IL-6 inhibitors

IL-6 acts as a critical catalyst during cytokine storm, specifically during COVID-19[163]. Hence, IL-6 may be the main accused acute phase response, including fever, the elevation of CRP and ferritinemia, ARDS, multiorgan damage in cases of severe COVID-19[90,164]. Anti-IL-6 biologics targeted the IL-6 receptor or IL-6 itself with anti-inflammatory properties[44]. So, anti-IL-6 agents like tocilizumab, siltuximab, and sarilumab are all humanized monoclonal antibodies that are generated to the IL-6 receptor[165,166]. Currently, tocilizumab (TCZ) is used in active rheumatoid arthritis, juvenile idiopathic arthritis, autoimmune rheumatic diseases[165,167], temporal arteritis, and giant-cell arteritis[168]. Recently, TCZ is used to attenuate the adverse effects of the cytokine storm-induced by chimeric antigen receptor T cell treatment[169,170]. Giving TCZ a potential therapeutic option for critically ill patients with COVID-19 who have a significant elevation in the level of IL-6[171]. However, the treatment of COVID-19 with TCZ is an off-label use[172]. However, based on existing evidence, it could be a right and

safe choice to compete with cytokine storm during COVID-19. A retrospective study by Nasonov and Samsonov[172] indicated that tocilizumab directly enhances clinical outcomes in severe and critical COVID-19 patients, no noticeable adverse reactions were observed, and it is effectively reducing mortality[172]. Another retrospective study by Luo *et al*[173] reported that in COVID-19 patients with a risk of cytokine storm, TCZ is an excellent treatment choice. They are also recommended a repeat dose of TCZ for severe COVID-19 patients with elevated IL-6[173]. Because of its common uses in rheumatoid arthritis and other auto-inflammatory disorders, TCZ hepatic side effect is well known. Typically mild elevations of serum aminotransferase are observed[174]. In contrast, TCZ may be correlated with HBV reactivation. Hence, HBV serological test could be a part of routine pretreatment work-up[175].

IL-1 inhibitors

IL-1 is an active pro-inflammatory cytokine secreted during cytokine storm[49]. It can lower pain thresholds, on the other hand it is one of the cytokines playing a dominant role in tissue damage[176,177]. The IL-1 receptor antagonist, anakinra, canakinumab, and Rilonacept are already indicated in conditions characterized by sustained fevers and systemic inflammatory response such as rheumatoid arthritis, familial Mediterranean fever, and cryopyrin-associated periodic syndrome[169]. Anakinra is the first biologic recombinant IL-1R antagonist. Anakinra inhibits both IL-1α and IL-1β *via* competitively IL-1R binding[178]. As previously reported, anakinra is effective in attenuating cytokine storm[169,178]. These promising results suggest the role of anakinra in combating CS in patients with COVID-19[179]. A retrospective cohort study by Huet *et al*[180] showed that in patients with COVID-19, anakinra decreased both the need for invasive mechanical ventilation in the ICU and mortality rate without significant side effects[180]. Anakinra is not a hepatotoxic agent but is more likely to indirectly trigger acute liver damage through activity against IL-1 or the immune system. Moreover, it has not been related to hepatitis B reactivation[181].

TNF-α inhibitors

The viral spike protein of SARS-Cov2 induced a TNF-α converting enzyme-dependent shedding of the ACE2 ectodomain, facilitating viral penetration into cells and promoting tissue injury *via* increased TNF-α production[90,182]. TNF-α is a crucial cytokine produced in all inflammatory conditions, and autoimmune diseases stimulate inflammation and oxidative stress production. TNF-α is mainly produced by monocytes, macrophages, B cells, and other tissues[93,183]. Activation of TNF-α increases IL-1 and IL-6 release[49]. TNF-α inhibitors such as adalimumab, etanercept, and infliximab are used to manage several conditions like rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, and inflammatory bowel disease[88,169]. Consequently, attenuate IL-6, IL-1, and VEGF levels as well as adhesion molecules and angiogenic factors. In patients with COVID-19, both plasma and tissues show an excessive amount of TNF-α[184]. Therefore, the anti-TNF-α antibody markedly reduces TNF-α in the blood, indicating that anti-TNF-α antibody could have potential anti-inflammatory benefits during COVID-19[185]. Additionally, it may cause downregulation of ACE2 expression and shedding[186,187]. So far, in treating patients with COVID-19, TNF-α blockers have not been proposed, but TNF-α blockers' effectiveness in treating patients with COVID-19 needs more priority[188,189].

JAK/STAT inhibitors

The JAK family and the adaptor-associated protein kinase 1 (AAK1) is a member of the numb-associated kinase family that plays a role in viral particles endocytosis, which act as a regulator of clathrin-mediated endocytosis[190,191]. Inhibitors of AAK1 can prevent viral particles entry into the cell[40,50]. Phosphorylated cytokine receptors recruit STAT transcription factors that modulate gene transcription. Furthermore, inhibiting activated cytokine receptors' phosphorylation through Janus kinase inhibitors, which could suppress cytokine signaling pathways[165]. Thus, AAK1 inhibitors have been suggested as possible candidates for the treatment of COVID-19. By inhibiting the JAK-STAT pathway, and cellular viral entry in COVID-19[192,193]. Janus kinase inhibitors such as baricitinib, filgotinib, fostamatinib, peficitinib, tofacitinib, and upadacitinib are previously indicated in managing the treatment of rheumatoid arthritis and autoimmune diseases[194,195]. Baricitinib is a JAK inhibitor with an extreme affinity to AAK1-binding[190]. Therefore, it inhibits the JAK-STAT pathway, that is used to suppress pro-inflammatory cytokine and attenuate the risk of cytokine storm[191,196]. Baricitinib is supposed to have dual effects in COVID 19 patients *via* reducing viral cell entry and inflammation[190]. One consideration related to the use of Baricitinib could be

superior to other JAK-STAT signaling inhibitors, as it is relatively safe[190,197]. However, baricitinib is in harmony with increased thromboembolic events, which affect the risk of developing these events in patients with COVID-19[198]. However, it is also probable that the JAK inhibitors might affect the activity of numerous cytokines, *e.g.*, INF-α, a potent mediator of antiviral response, may be impaired by JAK inhibitors[199]. Data by Bronte *et al*[200] suggests that baricitinib prevented the progression of COVID-19 by regulating patients' immune response, accordingly improving clinical outcomes[200].

Corticosteroids

Corticosteroids have a broad spectrum of cellular immune responses, different pharmacological activity, and wide therapeutic applications[201,202]. Glucocorticoids can inhibit innate and adaptive immune responses which are significantly used in inflammatory conditions and autoimmune disorders[203]. These anti-inflammatory and immunosuppressive effects occur in different mechanisms through direct actions on gene expression, transcription factors, and glucocorticoids' receptors' second messenger cascades[203]. Corticosteroids receptor complex induces gene transcription of many anti-inflammatory genes, involving IκB, which have immunosuppressive effects by inhibiting the activation of NF-κB signaling resulting in downregulation of IL-1β, IL-4, IL-10, IL-13, GM-CSF, TNF-α, and transforming growth factor-β[165,204,205].

Additionally, Corticosteroids reduce T cells and macrophages proliferation, activation, differentiation, and survival. Corticosteroids diminishes Th1 and macrophage pro-inflammatory cytokines IL-1β, IL-2, IL-6, TNF-α, and IL-17[206,207]. The uses of corticosteroids suppress the immune response, reduce viral clearance, and provoke viral replication[202]. Corticosteroids are widely used in critically ill patients with SARS and MERS infections[44]. Evidence has shown that corticosteroids' delayed their viral clearance[201]. The controversy on the use of corticosteroids remains far from definitive in COVID-19 patients. Corticosteroids decrease the pro-inflammatory cytokine's transcription, thus avoiding prolonged cytokine reaction and cytokine storm[208]. It allows corticosteroids in low doses and short duration and specific conditions with COVID-19 and requiring respiratory support the wat to survive[209]. In patients with COVID-19, trials indicated that dexamethasone in low dose, *i.e.*, 6 mg once daily orally for ten days reduced the death rate by one-third in patients who undergo ventilation and one-fifth in patients with oxygen supplementation[210]. Today, dexamethasone is widely used in severe COVID-19, including in patients with pre-existing CLD[211].

CONCLUSION

The outbreak of COVID-19 disease is the most annoying problem all over the world. This situation was developed due to a mutant version of the SARS virus called the SARS-CoV-2 virus. Studying the virus structure, mode of infection, and pathogenesis mechanism provided excellent hints about how we can fight it. In this review, we concluded that the most destructive power of the virus is the generation of violent cytokine storm, which is probably the cause of high mortality rates in hepatic and non-hepatic patients. Moreover, this review concluded that the possible ways to combat aggressive cytokine chaos might be a potential therapy for the COVID-19 patient co-morbid with liver disorders. Finally, this review encourages the scientists to study the SARS-CoV-2 virus cytokine storm in deep to find more effective and promising therapy for COVID-19 illness.

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MINIREVIEWS

Diagnostic approach to faecal incontinence: What test and when to perform?

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Abstract

Faecal incontinence (FI) is a debilitating common end result of several diseases affecting the quality of life and leading to patient disability, morbidity, and increased societal burden. Given the various causes of FI, it is important to assess and identify the underlying pathomechanisms. Several investigatory tools are available including high-resolution anorectal manometry, transrectal ultrasound, magnetic resonance imaging, and electromyography. This review article provides an overview on the causes and pathophysiology of FI and the author's perspective of the stepwise investigation of patients with FI based on the available literature. Overall, high-resolution anorectal manometry should be the first investigatory tool for FI, followed by either transrectal ultrasound or magnetic resonance imaging for anal internal sphincter and external anal sphincter injury, respectively.

Key Words: Incontinence; Manometry; Ultrasound; Rectal; Magnetic resonance imaging; Faecal

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Core Tip: Faecal incontinence (FI) is a debilitating symptom, which causes severe disability that deeply affect patients' quality of life. Given the various causes of FI, it is important for clinicians to recognize the available diagnostic investigatory tools and be familiar with the clinical approach for FI. Herein, we provide a concise overview of FI and recommend a stepwise algorithm for FI investigation.

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INTRODUCTION

Faecal incontinence (FI) is defined as the recurrent involuntary passage of rectal contents (solid or liquid stool) through the anal canal, and the inability to delay evacuation until socially convenient. Crucial factors to consider when defining FI include the duration of symptoms for at least 1 mo and age of onset of at least 4 years with previously attained control[1-3]. FI is considered to be a prevalent disorder with a considerable economic burden, but due to embarrassment, it is generally underreported; hence, its true prevalence is challenging to assess[4]. The prevalence of FI in the adult population of the United States is estimated to be 0.8%-6.2%[5]. The prevalence of FI increases with age from approximately 3% in the age group of 20 to 29 years to 16% in people aged ≥ 70 years[6]. A systematic review by Sharma *et al*[7] of over 30 studies estimated a prevalence of 1.4% to 19.5%. This discrepancy was clarified by variance in data collection methods applied and FI definition. Unsurprisingly, lower rates of FI were reported in personal interviews compared with anonymous questionnaires. The prevalence of FI is similar in both males and females, while the pathogenesis is often dissimilar between sexes.

FI has important consequences on social activities and quality of life as well as significant economic burden attributable to diagnostics, medications, instruments, procedures, and reduced work ability[8]. FI is regularly classified from a clinical point of view as: urge incontinence (discharge despite active efforts to constrict anal sphincters), passive incontinence (involuntary discharge with no awareness), and faecal seepage (leakage of stool with normal continence and evacuation)[2].

Numerous causes and associated conditions are linked to FI such as anal sphincter dysfunction, rectal disorders, neurological diseases, malignant diseases, psychiatric conditions, and other disease[8]. One major challenge in the management of FI is the hitch in correlating objective with subjective parameters.

In this review article, we shed light on the clinical evaluation and workup of patients with FI, from history taking to diagnostics including high-resolution anorectal manometry (HRAM), transrectal ultrasound (TRUS), perineal ultrasound, magnetic resonance imaging (MRI) of the pelvic floor including MR proctography, and nerve studies such as anal sphincter electromyography (EMG). The purpose of the review is to provide a practical tool box for clinicians to use when evaluating patients with FI.

CAUSES AND RISK FACTORS OF FAECAL INCONTINENCE

FI represents a final pathway of several traumatic and non-traumatic disorders. The most non-traumatic causes include anal sphincters and rectal dysfunctions as well as various inflammatory, congenital, structural, metabolic, neurological, muscular, psychological and functional diseases (Table 1). Alterations in bowel habits, such as diarrhoea for any reason (including inflammatory bowel disease, irritable bowel disease) and constipation with paradoxical diarrhoea and overflow incontinence are considered to be among the most established risk factors for FI[9]. The most common anal sphincters structural traumatic causes leading to FI are related to obstetrical trauma during vaginal delivery, anorectal surgical procedures including anorectal dilation, haemorrhoidectomy, fistulotomy, fistulectomy, sphincterotomy, rectal prolapse repair, and after ileo-anal reconstructive surgeries[10-12]. Many patients with neurological disorders affecting the brain, spinal cord or peripheral nervous system have FI because of impaired anal sphincter control, reduced or absent anorectal sensibility or abnormal anorectal reflexes. Patients with diabetes may have neuropathy of the anal canal and some have chronic diarrhoea. Connective tissue diseases, remarkable scleroderma, myopathy and atrophy of the internal anal sphincter (IAS) may lead to FI. Rectal surgery may disturb the reservoir function of the rectum as seen in patients after anterior resection surgery. Additionally, pelvic radiotherapy may lead to FI.

Table 1 Organic causes of faecal incontinence

Congenital neurological	**Spina bifida**
Acquired neurological	Pudendal neuropathy; Spinal injury/surgery; Demyelinating diseases; Degenerative diseases; Neurological malignancy
Congenital structural disease	Imperforate anus; Cloacal anomalies
Acquired structural disease	Vaginal delivery; Obstetric trauma; Ano-rectal surgery; Ano-rectal trauma
Gastrointestinal diseases	Inflammatory bowel disease; Irritable bowel syndrome; Radiation proctitis; Microscopic colitis; Malabsorption
Neoplastic	Hypersecretory tumours; Colorectal cancer; Ana cancer
Psychiatric	Psychiatric diseases
Medications	Diarrheal agents; Chemotherapy

INVESTIGATION FOR FAECAL INCONTINENCE

History taking and physical examination

It is important in the early stages of FI evaluation to search for possible causes and risk factors during careful detailed history taking in order to route diagnostics accordingly. The main symptoms of faecal incontinence are the progressive loss of control of rectal contents including solids stool and liquids. Urge incontinence is specified by the patient's awareness to defecation call but inability to constrict the external anal sphincter (EAS) sufficiently until reaching the toilet, and many patients report "accidents" within a few seconds or minutes. Involuntary discharge without awareness is categorized as passive incontinence and generally caused by disturbed sensation capability of the rectum and anal canal. It is imperative to identify discrete disparities in relation to various triggers such as daytime *vs* night time, physical activity, cough, stress, stool consistency, or food intake. Other secondary symptoms of FI may arise due to stool leakage including pruritus, perianal skin irritation, and infection as well as urinary tract infections. Notably, these secondary symptoms may be the chief complaints prompting patients to seek medical care without mentioning FI. Moreover, FI may have other important related conditions, which mandates direct exploration with the patient such as urinary incontinence, vaginal bulging (rectocele, cystocele), rectal prolapse, altered bowel habits, defecation disorders, and rectal bleeding.

Physical examination of the anorectal region is of paramount importance and starts with a visual inspection to search for leaked stool, skin irritation and infections, haemorrhoids, fistulas opening, and anal fissures[13]. During digital rectal examination, the experienced index finger is capable of affording a general estimation of the anal resting and squeeze pressures. Moreover, coordinated relaxation of the pelvic floor muscles during straining can be addressed as well during Valsalva manoeuver to search for possible defecation disorders (*i.e.* functional anismus)[14-16].

HRAM

According to American College of Gastroenterology guidelines, in cases of FI, it is worth testing with AM[17]. HRAM is an important diagnostic tool for the assessment of anorectal motor and sensory function[18]. The utilization of HRAM is a necessary diagnostic key tool in the evaluation of FI, as it enhances the understanding of the underlying pathophysiological bases of FI, permitting the delivering of optimal therapy for each specific patient. HRAM, which provides a dynamic recording of the anal sphincters and intraluminal rectal pressures, is considered to be the best-established diagnostic tool that permits an objective evaluation of several factors of anal and rectal function including basal tone and contractility, recto-anal coordination, and reflex function (such as recto-anal inhibitory reflux) as well as rectal sensation thresholds[18,19], which is an important predictor of response to biofeedback training[19]. The recently established international anorectal physiology working group published a consensus guideline paper that proposes a practical standardized protocol for the performance and analysis of anorectal manometry, named the London Classification[20]. Several manometric findings may be linked to FI such as impaired anal sphincter resting pressure tone (hypotension) and impaired squeeze pressure (hypo-contractility) (Figures 1 and 2). Moreover, HRAM enables the assessment of abnormal rectal sensitivity both hypersensitivity and hyposensitivity, two conditions that may cause FI in different manners. Rectal sensation is assessed by inflating a balloon in the rectum and recording balloon volumes needed to produce the first

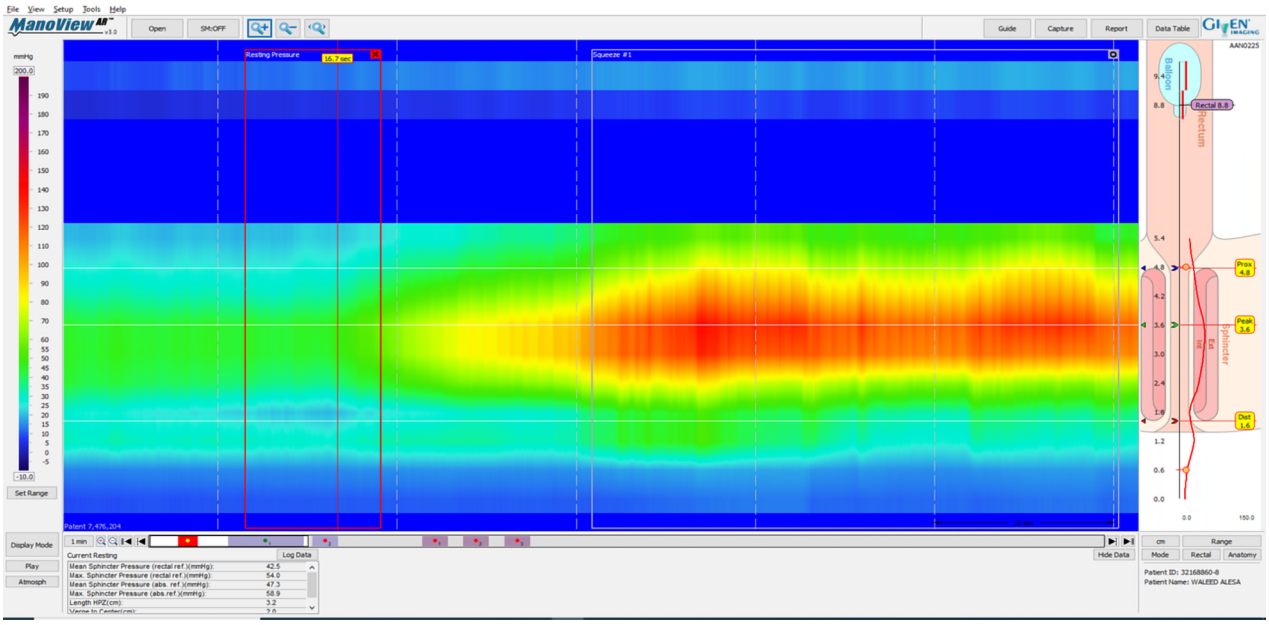


Figure 1 High-resolution anorectal manometry demonstrating normal resting and squeeze anal pressures. Supplied from the Gastroenterology and Endoscopy United, The Nazareth Hospital, EMMS, Nazareth, Israel.

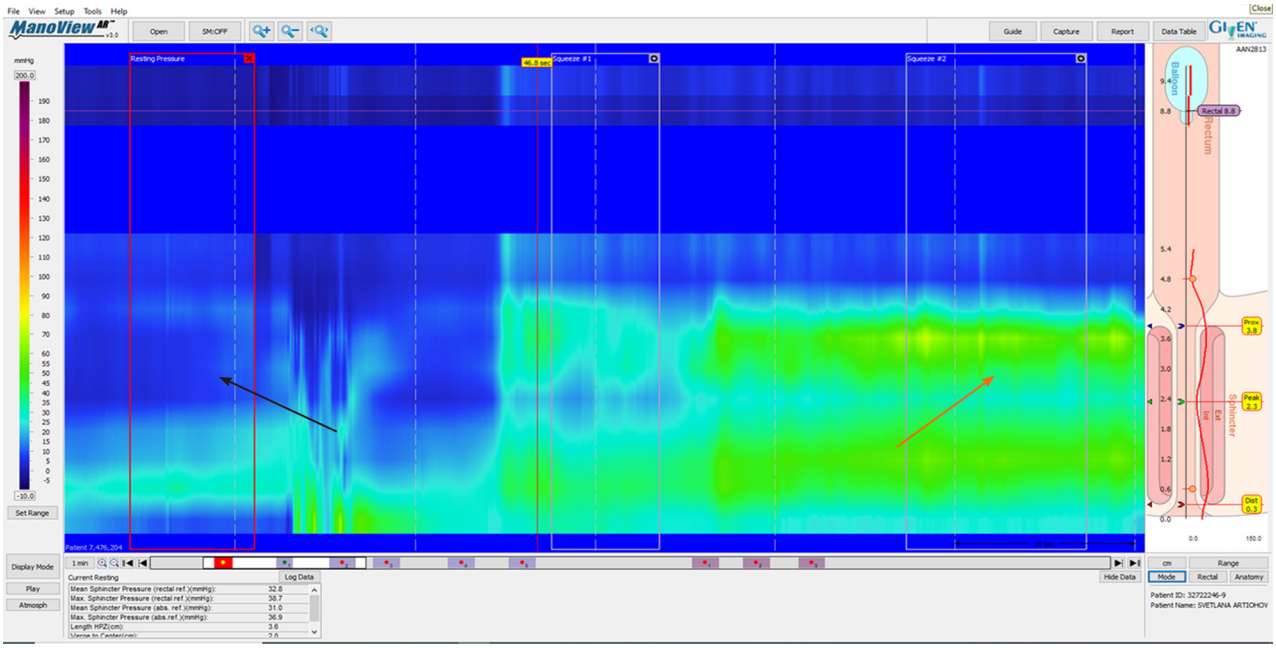


Figure 2 High-resolution anorectal manometry demonstrating low resting anal pressure (black arrow) and low squeeze pressure (orange arrow). Supplied from the Gastroenterology and Endoscopy United, The Nazareth Hospital, EMMS, Nazareth, Israel.

sensation, urge, and discomfort[20]. Rectal hyposensitivity can be caused by neuropathy, mucosal inflammation, or fibrosis and may lead to patient unawareness to the presence of stool in the rectum leading to accumulation of stool and incontinence. Rectal hypersensitivity may lead to urge incontinence and is common among patients with irritable bowel syndrome[13].

Pelvic floor and anal canal imaging

In incontinent patients with reduced anal pressures, especially when surgery is being considered, the anatomic integrity of the anal sphincters, rectal wall, and puborectalis muscle region should be evaluated by either endoscopic ultrasound or MRI[17]. Although, both tests are considered overlapping in identifying abnormalities including scars, defects, marked focal thinning or atrophy however, every test has its

uniqueness and diagnostic capabilities. While ultrasound is superior in identifying IAS tears, EAS atrophy is more identified by MRI[21,22]. Additionally, discriminating between an external anal sphincter tear and scar is better with MRI[17].

Endoscopic ultrasound: TRUS or endoanal ultrasound (EAUS) is a simple, well tolerated, widely available examination considered the gold standard in investigating the anal sphincter integrity[23,24] (Figure 3). However, its sensitivity and accuracy in identifying sphincter injuries is debated and operator-dependent[25]. Gold *et al*[26] reported that the interobserver agreement in diagnosing sphincter defects and IAS measurements by EAUS is very good, and better than EAS measurements. Common structural pathologies in patients suffering from FI that are easily identified by EAUS include IAS discontinuity (defect), IAS atrophy (identified by diffuse thinning of the sphincter to less than 1 mm thickness (Figure 4)[27], and EAS discontinuity (defect) or scar manifested as focal thinning (Figure 5)[19,28]. On the other hand, precise determination of EAS atrophy is difficult with EAUS due to its inferiority in determining the EAS boundaries and its inability to distinguish between normal muscle tissue and fatty infiltration[29]. Indeed, EAS atrophy is defined by diffuse reduction in muscle bulk on serial images below the level of the puborectalis, to the point where the muscle is barely identifiable[21]. The leading cause of FI is anal sphincter injury originating from vaginal delivery in females, and it may result from direct anal sphincter laceration or indirectly from sphincter innervation damage[29].

Two-dimensional EAUS, which allows cross sectional images only in the axial plane, remains the mainstay of sphincter inspection[24]; however it does not achieve accurate longitudinal measurements necessary for planning complete surgical intervention. On the other hand, three-dimensional EAUS enables the determination of length, thickness, area, and volume measurements by producing a digital volume that can be seen from any plane[29], and is displayed as either multiplanar images or tomographic slicing, thus allowing more accurate defect visualization[24].

MRI: MRI is expensive, not readily available, and not tolerated by claustrophobic patients and is unsuitable in patients with pacemakers, defibrillators, and other metal implants[29]. Two modalities of MRI are available in clinical practice: the invasive endoanal MRI using endoluminal coils and the non-invasive external phased-array. Both modalities are comparable in identifying EAS defects[30] and atrophy[31]. Compared to EAUS, the accuracy of endoanal MRI in diagnosing IAS defects is lower[32]. Several studies have reported that EAUS and endoanal MRI are equivalent in diagnosing EAS injuries[22,32,33]; however, endo-anal MRI allows better distinction between fat and muscle and in identifying scars[27]. This property underscores the diagnostic ability of EAUS in detecting focal EAS thinning and structural lesions[34-37]. Interestingly, only moderate agreement between EAUS and external phased-array MRI in diagnosing obstetric injuries, which represent the main aetiology for FI, has been reported[38]. Recently, magnetic resonance defecography has gradually emerged as a modern modality substituting the traditional X-ray based defecography. It is non-invasive test that uses MRI to obtain images at various stages of defecation to evaluate how well the pelvic muscles are working and provide insights into rectal function. Magnetic resonance defecography can provide important functional disorders related to the defecation process as well as various important structural abnormalities associated with FI and other pelvic floor disorders. These structural abnormalities include rectocele, enterocele, anorectal descent, and descending perineum[39]. The identification may impact the kind of surgical treatments[40].

Electromyography

A neurophysiological assessment of the anorectum includes an assessment of the conduction of the pudendal and spinal nerves using EMG examination of the sphincter. This test is of particular significance when surgery is being considered; however its availability is mainly reserved for research purposes and its clinical application has been declining recently in many countries[41,42]. However, EMG use has been advocated in patients with refractory symptoms, suspected to originate from neurogenic sphincter weakness especially when it is assumed to result from sacral root injury, it is advocated to assess rectal sensation and compliance by needle EMG of the anal sphincter[43].

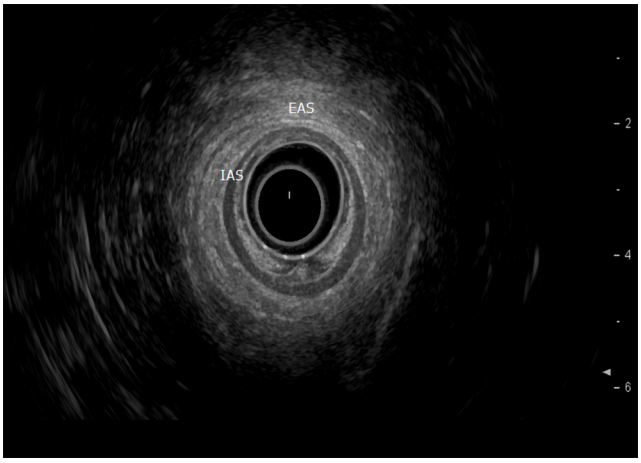


Figure 3 Normal internal and external anal sphincters (internal anal sphincter and external anal sphincter). Supplied from the Department of Gastroenterology, Galilee Medical Center, Nahariya, Israel. EAS: External anal sphincter; IAS: Internal anal sphincter.



Figure 4 Internal anal sphincter atrophy manifested as sphincter thinning. Supplied from the Department of Gastroenterology, Galilee Medical Center, Nahariya, Israel. EAS: External anal sphincter; IAS: Internal anal sphincter.

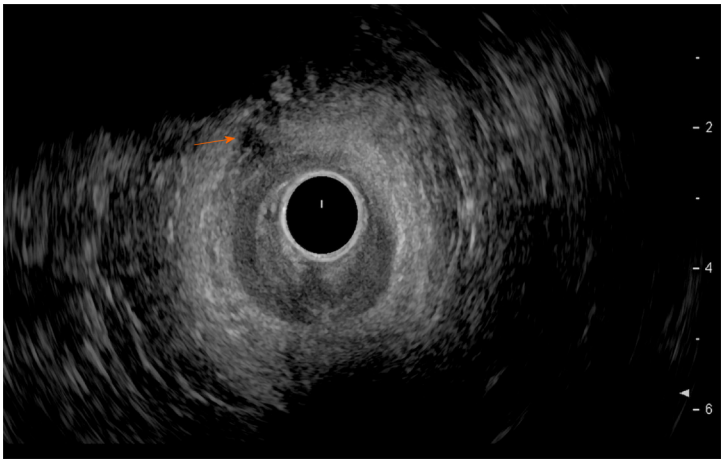


Figure 5 External anal sphincter tear. Supplied from the Department of Gastroenterology, Galilee Medical Center, Nahariya, Israel.

CONCLUSION

FI is considered to be a prevalent disorder with a considerable economic burden, but due to embarrassment it is generally underreported. FI impacts severely patients' quality of life, leading to various organic and psychological comorbidities. Detailed history taking and anamnesis by physician is mandatory to define the patient's main complaints with a focus on risk factors for FI and whether passive or urge FI is dominant. Moreover, digital rectal examination is of paramount importance to assess anorectal sensation, anal sphincter resting and importantly the squeeze pressures as well. HRAM is an important initial diagnostic modality to start with, as it can provide crucial information about the anal sphincter functions and can evaluate rectal sensation capability using balloon dilation in the rectum. The anatomic integrity of the anal sphincters, rectal wall, and puborectalis muscle region should be evaluated by either TRUS or MRI. Although, both tests are considered overlapping in identifying abnormalities including scars, defects, marked focal thinning or atrophy, every test has its own uniqueness and diagnostic capabilities. While TRUS is superior in identifying internal anal sphincter tears, external anal sphincter atrophy is more identified by MRI. Additionally, discriminating between an external anal sphincter tear and scar is better with MRI. Figure 6 demonstrates the authors' perspective of the structured, step-by-step approach for the evaluation and diagnosis of FI. Still, the place of EMG in the evaluation of FI needs to be explored. Finally, this recommended approach should aid clinicians in the stepwise management of patients with FI.

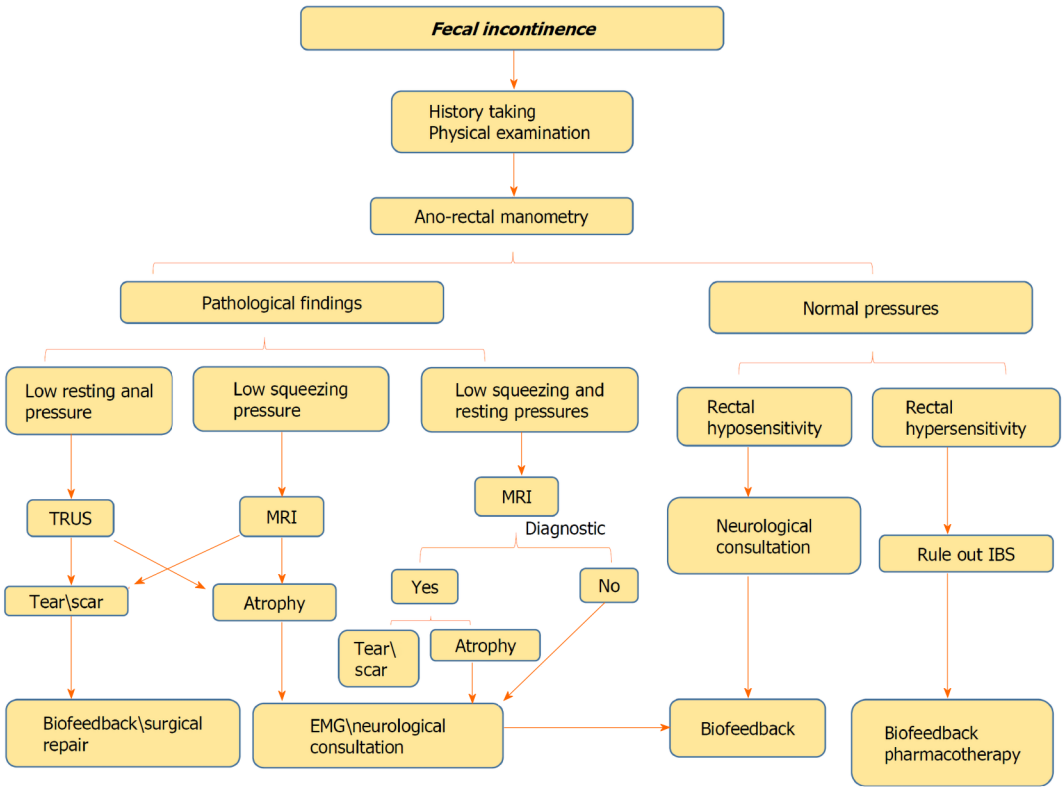


Figure 6 Stepwise approach of faecal incontinence investigation. EMG: Electromyography; MRI: Magnetic resonance imaging; TRUS: Trans-rectal ultrasound.

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MINIREVIEWS

Prevention of late complications with coverage agents in endoscopic resection of colorectal lesions: Current landscape in gastrointestinal endoscopy

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Abstract

Endoscopic removal of large (≥ 20 mm) non-pedunculated colorectal lesions (LNPCLs) may result in major adverse events, such as delayed bleeding (DB) and delayed perforation (DP), despite closure of the mucosal defects with clips. Topical application of a coverage agent refers to the creation of a shield with a biocompatible medical device (tissue or hydrogel) with proven bioactive properties. Coverage of the eschar after endoscopic resection provides shielding protection to prevent delayed complications. The aim of the present review was to systematically collect and review the currently available literature regarding the prevention of DB and DP with coverage agents after endoscopic mucosal resection or endoscopic submucosal dissection of LNPCLs.

Key Words: Large colorectal lesions; Delayed bleeding; Topical application; Endoscopic mucosal resection; Endoscopic submucosal dissection

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Core Tip: The use of coverage agents is the simplest and quickest technique to protect large mucosal defects. Published data have confirmed their efficacy in the prevention of delayed adverse events in patients with non-pedunculated colorectal lesions, especially in proximal lesions with an increased risk of bleeding of at least 2-fold. There are no comparative studies that address the best treatment. We herein review the

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INTRODUCTION

Endoscopic resection of precancerous colorectal lesions is one the most frequently performed medical interventions, which significantly decreases the risk of colorectal cancer incidence and death. Large (20 mm) non-pedunculated colorectal lesions (LNPCLs) show the highest cancer risk and their careful, complete, and timely removal is especially critical. Endoscopic removal of these lesions may result in major adverse events, such as delayed bleeding (DB) and delayed perforation (DP), especially in high-risk patients with a Spanish Endoscopy Society Endoscopic Resection Group score 6 or deep mural injury signs II-V, despite closure of the mucosal defects with clips[1-3]. Complete clip closure is not possible in 40% cases due to large size or poor accessibility[4]. The risk of DB ranges from 1% to 12% (1.5% with complete closure, 9% with partial closure and 12% with failed closure), whereas the risk of DP is around 1%[5,6]. The routine use of prophylactic clipping does not reduce the risk of post-procedural bleeding overall[7]. On the other, prophylactic endoscopic coagulation of visible vessels is not effective in the prevention of clinically significant DB[8].

Topical application of a coverage agent refers to the creation of a shield with a biocompatible medical device (tissue or hydrogel) with proven bioactive properties. Coverage of the eschar after endoscopic resection provides shielding protection to prevent delayed complications[9]. A comprehensive understanding of the pathogenic mechanisms of action involved is mandatory to address these challenges. The aim of the present review was to systematically collect and review the currently available literature regarding the prevention of DB and DP with coverage agents after endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) of LNPCLs.

SEARCH STRATEGIES

A comprehensive search of PubMed was performed to identify articles in English. Search strategies and key words were as follows: (1) ("Endoscopy" [All Fields] AND ("topical application" [All Fields]); (2) ("Large colorectal lesions" [All Fields] AND "EMR" [All Fields] OR "ESD" [All Fields]); and (3) ("Delayed bleeding" [All Fields] OR "delayed perforation" [All Fields]). In addition, manually inspected relevant articles that were missed by the above search strategy were also included.

CLINICAL DATA

Following our search, 8 studies were identified with 191 patients included in case series, which are summarized in Table 1. Tested agents were: Polyglycocolic acid sheets with fibrin glue (PGA-FG), Surgicel, platelet-rich plasma (PRP), Purastat and cyanoacrylate. All these measures present biological safety after experience in clinical practice. The first report was published in 2014 to evaluate the shielding technique after ESD in 10 patients with LNPCLs, placing PGA sheets on the mucosal defect with biopsy forceps and then spraying FG through a special double-lumen tube[10]. PGA is an absorbent and hydrophilic suture reinforcement material, hydrolysed *in vivo*, with a degradation and absorption period within approximately 15 wk[11]. To perform this technique, soft and elastic PGA sheets were cut into small pieces, held with biopsy forceps, and transported to the mucosal defect through the channel of the scope.

Table 1 Outcome of coverage agents to prevent delayed bleeding and perforation after endoscopic resection of colorectal lesions

Ref.	Year	Design	*n*	Size (mm)	Agent	Primary endpoint	Follow-up	Outcomes
Tsuji *et al*[10]	2014	SA	10	39.7	PGA-FG	Prevent late complications	2 wk	0% DB/0% DP
Myung *et al*[13]	2016	SA	49	38.8	Surgicel	Prevent late complications	1 wk	0% DB/0% DP/6% PPS
Lorenzo-Zúñiga *et al*[17]	2021	SA	4	53.7	PRP	Prevent late complications	4 wk	25% DB/0% DP/79% MHR
Pioche *et al*[18]	2016	SA	22	38.5	Purastat	Prevent delayed bleeding	4 wk	6.7% DB
Subramanian *et al*[19]	2019	SA	31	44.2	Purastat	Prevent delayed bleeding	4 wk	0% DB
Soons *et al*[20]	2020	SA	17	38.4	Purastat	Prevent delayed bleeding	4 wk	11.7% DB
Subramanian *et al*[21]	2021	RCT	43	33.7	Purastat	Prevent late complications	4 wk	5.5% DB
Martines *et al*[22]	2020	TA	15	25	NBCA-MS	Prevent delayed bleeding	-	0% DB

SA: Single-arm interventional case series study; TA: Two-arm interventional case series study; RCT: Randomized clinical trial; PGA-FG: Polyglycocolic acid sheet with fibrin glue; PRP: Platelet-rich plasma; NBCA-MS: N-butyl-2-cyanoacrylate with methacrylosulfolane (Glubran 2); DB: Delayed bleeding; DP: Delayed perforation; MHR: Mucosal healing rate.

Fibrinogen first and then thrombin were sprayed with different spray tubes to fix the sheets to the ulcer and to enhance the coating effect[12]. The use of PGA-FG in LNPCLs achieved a success rate of 100%, but required a long-procedure time (a mean of 19 min). During follow-up colonoscopy, 80% of patients showed persistence of PGA sheets at 2 wk.

Surgicel Fibrillar, an oxidized regenerated cellulose that swells into a gelatinous mass, was the second substance investigated to reduce late complications in a large case-series of 49 patients with colorectal ESD[13], using one layer of this agent diluted in 10 mL of normal saline through a special spraying catheter. Surgicel aids in clot formation after blood saturation, serving as a haemostatic adjunct, and has a localized bactericidal effect due to a low pH of 3.4-3.7[14]. To assess the effectiveness of Surgicel application, a retrospective comparison with another 52 patients with LNPCLs who underwent conventional ESD was performed. All lesions were successfully covered, and the covering procedure was less time-consuming (5 min). During the follow-up period, rebleeding occurred in 0 (0%) patients and 4 (7.7% in the control group) patients. Postpolypectomy syndrome (PPS) was observed in three patients (6.1%) who were treated with Surgicel, compared with 17 (32.7%) in the non-Surgicel group. In 20 patients treated with this product, a follow-up colonoscopy was performed the next day, and Surgicel remained on the defect in all cases. Based on this, the authors speculated that the reduction in the inflammatory reaction was associated with the shielding effect and reduced endotoxemia due to the bactericidal property of this agent, which acidifies the environment.

PRP, also known as autologous platelet gel, has confirmed robust healing properties over the eschar after EMR in preclinical models[15]. Platelets play a fundamental role in haemostasis and are a natural source of growth factors. PRP fluid contains at least a 2-fold peripheral blood platelet count and a large amount of pivotal growth factors for reepithelization, which are released from the alpha granules of activated platelets[16]. The use of PRP is justified in the exponential release of multiple pleiotropic factors, which enhances the physiological and haemostatic healing processes, with a very low risk of fibrotic healing or strictures. In clinical practice, PRP was used as a coverage agent to prevent late complications in a limited number of patients with very large lesions located in the rectum (mean size 54 mm)[17]. PRP was obtained from a sample of patient's blood (18-36 mL) drawn at the time of endoscopy. DB occurred in 1 of 4 lesions with blood transfusion or endoscopic treatment not required. PRP also showed a very high mucosal healing rate after 4 wk (79%), the time to apply PRP was very quick (2 min), and the force required to pass the composition was appropriate, comparable to saline. Nevertheless, patient number is too small to draw any conclusions on efficacy, and controlled data are lacking.

PuraStat has also been tested to prevent DB after endoscopic resection of LNPCLs. This agent is a fully synthetic matrix scaffold built from a chain of three types of amino acids than bond together to form a peptide. It forms a transparent gel when it comes into contact with blood or tissue fluids, comprising a network of nanofibers that form an extracellular matrix, providing a physical barrier to stop bleeding by blocking blood vessels. Three case series (single-arm interventional studies) and one randomized clinical trial have been reported in 113 patients with LNPCLs[18-21]. Total lesion surface was completely covered with a dose of 3 mL in a median time of 2 min. Clinically significant DB occurred in 4.4% of patients (range 0%-12%). The concerns with this gel are that it has to be applied through a special catheter, it is affected by gravity, and slowly slides from the ulcer bed after covering. Exsufflation after application seems to be effective in applying the gel to the whole area with less migration.

Recently, cyanoacrylate has been evaluated in a two-arm study[22]. Two groups of fifteen patients with LNPCLs were compared to evaluate early and DB after EMR in association with a modified cyanoacrylate glue (N-butyl-2-cyanoacrylate + methacryloxysulfolane-Glubran 2®) *vs* EMR alone. Cyanoacrylate is a strong and fast-acting synthetic glue with sealing, adhesive and haemostatic properties, which rapidly polymerizes in the presence of water to form long and strong chains. Based on these properties it has been widely used in surgery, and for primary and secondary prophylaxis of bleeding from gastric varices[23]. This substance has been applied using a 7 Fr spray Teflon catheter. No case of early bleeding was reported in both groups. Two cases (13.3%) of DB with readmission to hospital and redo endoscopy with apposition of haemostatic clips were performed in patients with EMR alone, as compared with no cases of DB in the shielded group (P = 0.48).

DISCUSSION

Endoscopic shielding with coverage agents is a very promising method to prevent late complications in patients with LNPCLs, especially in proximal lesions with an increased risk of bleeding of at least 2-fold. The protective effect of clips is limited to those cases where complete closure was achieved (57% of cases), and the median number of clips to completely close the resection defect was four[4-6]. The absence of efficacy in many cases of the clipping technique, with its cost and technical difficulties, induced the appearance of new endoscopic approaches to solve this unmet need.

The use of coverage agents is the quickest and simplest technique to cover large mucosal defects, and published data seem to confirm their efficacy in the prevention of late complications. However, most of the reports are case series, without a control arm and with a relatively short follow-up. There is a lack of randomized controlled trials and of head-to-head comparative studies of shielding products. Moreover, none of the published series can incorporate blinding, with considerable bias therefore inevitable.

Regarding the type of active treatment used, there is no ideal treatment, and all have pros and cons (Table 2). As options developed to prevent DB and DP it is important to consider the cost-effectiveness of each treatment. The overall rate of delayed adverse events is assessed as 10%, and the cost for management of these complications, including admission and additional therapies, is estimated at 5000 $ per patient. The cost for an economical prophylactic measure for each patient without adding to the overall financial cost is around 500 $ per cushion. Commercially available data show the price range per 1 mL to be 10-150 $; thus, it is necessary to consider the economics, the upfront cost for the added prophylactic intervention, and the downstream cost savings for an avoided hospitalization. If the mean used volume is 3 mL, we can estimate the cost-effectiveness of each tested agent, from PRP, the cheapest, to cyanoacrylate, the most expensive.

Apart from efficacy, mucosal healing activity is another important issue to consider. All these prophylactic measures help and accelerate mucosal reepithelialisation, but the healing process has only been measured with PRP. The ideal coverage agent should have a chemical structure and physical properties showing an appropriate adhesion capacity to avoid migration against gravity and adherence failure, some refractoriness to bacterial degradation in order to increase the bioactive period and reduce the incidence of PPS, and healing activity to increase mucosal healing rate. Application should be straight with minimal force to pass the agent, ideally using standard devices accessible to all endoscopy units, inducing a small increase in the time of the resection procedure and with a short learning curve. Ideally, it should also be able to release bioactive drugs to treat specific conditions such as colorectal cancer

Table 2 Pros and cons of coverage agents based on ideal properties

Property	PGA-FG	Surgicel	PRP	Purastat	Cyanoacrylate
Appropriate adhesion capacity	+	+	+	+	+
Absence of special device	-	+	+	-	+
Not time-consuming	-	+	+	+	+
Refractory to bacterial degradation	+	+	-	-	+
Healing activity	+	+	+	+	+
Price range of 1 mL ($)	20-25	15-20	10-12	100-150	150
Drug-release	-	-	-	-	-

PGA-FG: Polyglycocolic acid sheet with fibrin glue; PRP: Platelet-rich plasma.

or inflammatory bowel disease. All these properties are still to be confirmed in proof of concept studies with robust data.

To obtain the ideal agent, larger prospective studies with control groups and a comparison of the different substances are needed.

CONCLUSION

The use of coverage agents is the quickest and simplest technique to cover large mucosal defects, and published data seem to confirm their efficacy in the prevention of late complications. However, most of the reports are case series, without a control arm and with a relatively short follow-up. There is a lack of randomized controlled trials and of head-to-head comparative studies of shielding products. Moreover, none of the published series can incorporate blinding, with considerable bias therefore inevitable.

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MINIREVIEWS

Current status of diagnosis and therapy for intraductal papillary neoplasm of the bile duct

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Abstract

Bile duct epithelial tumours showing papillary neoplasm in the bile duct lumen are present in the intrahepatic and extrahepatic bile ducts. Clinicopathological images of these tumours are distinctive and diverse, including histological images with a low to high grade dysplasia, infiltrating and noninfiltrating characteristics, excessive mucus production, and similarity to intraductal papillary mucinous neoplasm (IPMN) of the pancreas. The World Health Organization Classification of Tumours of the Digestive System in 2010 named these features, intraductal papillary neoplasm of the bile duct (IPNB), as precancerous lesion of biliary carcinoma. IPNB is currently classified into type 1 that is similar to IPMN, and type 2 that is not similar to IPMN. Many of IPNB spreads superficially, and diagnosis with cholangioscopy is considered mandatory to identify accurate localization and progression. Prognosis of IPNB is said to be better than normal bile duct cancer.

Key Words: Intraductal papillary neoplasm of the bile duct; Intraductal papillary mucinous neoplasm of the pancreas; Peroral cholangioscopy; Cholangioscopy

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Core Tip: Intraductal papillary neoplasm of the bile duct (IPNB) is classified into type 1 that is similar to intraductal papillary mucinous neoplasm (IPMN) and type 2 that is not similar to IPMN. Many of IPNB spreads superficially, and diagnosis with

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INTRODUCTION

Presence of intraductal tumours showing a pattern of spurring papillary neoplasm has been known since before[1-4]. Although the names of these tumours varied depending on the country and region, it is known that the tumours have a better postoperative prognosis than normal infiltrating bile duct cancer. Also known are the facts that quite a few pre-infiltrating phases can be found in these tumours; cases with a low grade dysplasia are present; and some cases have excessive mucus production. Many similarities with intraductal papillary mucinous neoplasm (IPMN) of the pancreas are also pointed out. A recommendation has been made in recent years to call these tumours intraductal papillary neoplasm of the bile duct (IPNB)[1]. Diagnosis of IPNB and the current status of its treatment are hereby reported.

HISTORY OF IPNB

Epithelial tumours demonstrating a variety of images develop in the bile duct. Of these tumours, bile duct epithelial tumours indicate papillary neoplasm in the bile duct lumen[1-4]. These tumours have better prognosis than normal bile duct cancer, and often present with distinguishing and diverse clinical pathophysiology. Yet, no specific name was given to these tumours. In the United States and Europe, these tumours are called biliary papilloma if they are solitary, and biliary papillomatosis if they are multiple[5,6]. These are slow growing, low grade tumours with potential malignancy, and some cases of infiltration and metastasis have been also reported. Separately, hepatobiliary cystadenoma and adenoma also exist, and differences among these diseases were not necessarily clear-cut.

Intrahepatic stone-related intrahepatic bile duct cancer was examined in 2001. There are quite a few bile duct epithelial tumours showing papillary growth in the bile duct, with excessive mucus production. These tumours are morphologically and characteristically similar to IPMN of the pancreas; thus, we consider these cases as neoplastic lesion and reported it as intraductal papillary neoplasm of the liver[7]. As similar tumours were also noted thereafter in the extrahepatic bile duct, the tumours were renamed IPNB[8]. This lesion was officially named IPNB in the revised World Health Organization Classification of Tumours of the Digestive System in 2010[9].

PATHOLOGICAL DIAGNOSIS OF IPNB

IPNB is epithelial tumour with intraductal papillary proliferation, and defined as having a histologically thin fibrovascular stem[1-4]. Excessive mucus production is often noted but this is not a decisive element for diagnosis of IPNB. IPNB is reported to be a lesion similar to IPMN. Specific lesions are: (1) Papillary proliferation of tumours in the bile duct lumen or in the bile duct; (2) Intestinal, gastric, pancreatobiliary, and oncocytic subtypes noted in IPMN are also common in IPNB; (3) As in IPMN, cancerous cells show intraepithelial and intramucosal progression along the intraductal wall adjacent to the primary site, and some are multiple cases with asynchronous and simultaneous tumours; (4) IRNB has better prognosis compared with normal bile duct cancer or normal pancreatic carcinoma; (5) Some IPNB cases show excessive mucus production; however, the incidence is lower than that of IPMN. In some cases, no clear excessive mucus production is noted. Patients with excessive

mucus production often show cystic expansion of the bile duct at the main site of papillary growth; and (6) In the case of infiltrating growth of IPNB, either normal tubular adenocarcinoma or bile duct mucinous adenocarcinoma may result. Similar infiltrating images are also known in IPMN.

The World Health Organization Classification of Tumours of the Digestive System in 2010 added the definition of IPNB as one of precancerous, pre-infiltrating lesions of the bile duct cancer[9]. However, studies since then have revealed that some cases with intraductal noninfiltrating papillary tumours are slightly different from those cases similar to IPMN. These different cases include biliary papillary adenocarcinoma that has been previously reported by Albores-Saavedra[10,11]. Based on these results, IPNB was classified into two types. Type 1 is IPNB similar to IPMN, and mostly classified into gastric and intestinal subtypes. Type 1 shows homogeneous tissue image, and often occurs in the intrahepatic bile duct, with excessive mucus production often experienced by patients. Many of type 1 IPNB is considered a high grade dysplasia; however, some regions often show a low grade dysplasia, and some cases have only a low grade dysplasia. Type 1 includes many of patients undergoing resection in noninfiltrating phase, and those who show cystic expansion in the bile duct with lesion. Type 2 IPNB is slightly different from IPMN; however, it is experienced as IPMN in rare cases. Type 2 IPNB includes lesions previously reported by Albores-Saavedra as bile duct papillary adenocarcinoma and papillary bile duct cancer[7,8]. Tissue images of type 2 IPNB are slightly more complicated, showing some solid sites. Almost all cases are considered a high grade dysplasia, often occurring in the extrahepatic bile duct. Many cases already show infiltrating images at the time of resection, but some cases show noninfiltration. Most of the cases are classified into intestinal and pancreatobiliary subtypes, with few cases of excessive mucus production. Examination of IRNB with macroscopic mucus production revealed that many of tumours with mucus production are noninfiltrating, similar to IPMN, and those with no mucus production tended to show infiltration[12]. IPNB classification is still far from perfect. After classifying into two types, we find it difficult to classify into type 1 IPNB and type 2 IPNB in some cases, or to differentiate progressed papillary bile duct cancer from type 2 IPNB. Further consideration is necessary.

IPNB DIAGNOSIS

Image diagnosis plays an important role in giving a diagnosis of IPNB. If clinical symptoms show abdominal pain, pyrexia, and elevation of hepatobiliary enzymes in a blood test, an image diagnosis of the abdominal region will be made. In this case, abdominal ultrasonography, contrast computed tomography (Figure 1), Magnetic resonance imaging (Figure 2), and endosonography will enable us to identify the main focus[13]. However, these modalities may not ensure an accurate diagnosis because the presence of mucus, which is characteristics of IPNB, must be verified, and superficial expansion and progression of IPNB are often to the extent beyond our expectation. This is where direct cholangiography, intraductal ultrasonography (IDUS), and cholangioscopy come in[14-16]. The presence of mucus can be identified by checking papillary dilation and emission of mucus with endoscopy, or by outlining the mucous plug with cholangiography (Figure 3). Even when cholangiography contrasts the bile duct dilated with mucus, it is difficult in some cases to identify the main focus itself owing to mucus, let alone superficial expansion and progression. Meanwhile, IDUS enables us to identify the main focus and depict mucus; it can be easily conducted at the time of ERCP by following the guide wire; and it allows us to capture a wide scope of the region for observation. The presence of mucus, however, makes it difficult to evaluate the bile duct wall in IDUS. Thus, cholangioscopy plays a central role in the diagnosis of IPNB[14-16]. Cholangioscopy enables us to observe with narrow band imaging in addition to usual white light imaging, and is considered useful for the diagnosis of benignancy and malignancy as well as progression staging[14-16]. As mentioned earlier, the main focus of papillary bulge may be captured by computed tomography and magnetic resonance imaging, but the evaluation of superficial spread and progression from the main focus is difficult. Cholangioscopy allows us to directly observe the superficial spread and progression and to perform an open biopsy, thereby enabling accurate diagnosis of the progression (Figure 4). Depending on a route of approach, percutaneous transhepatic cholangioscopy (PTCS) and peroral cholangiosccopy (POCS) are available in cholangioscopy. Generally, PTCS where percutaneous biliary drainage is performed, has a bigger channel diameter and a shorter effective length. These features make it easy to wash and superior in removing



Figure 1 Contrast computed tomography revealed a 70-mm cystic lesion with a papillary bump in the lumen of left hepatic lobe.

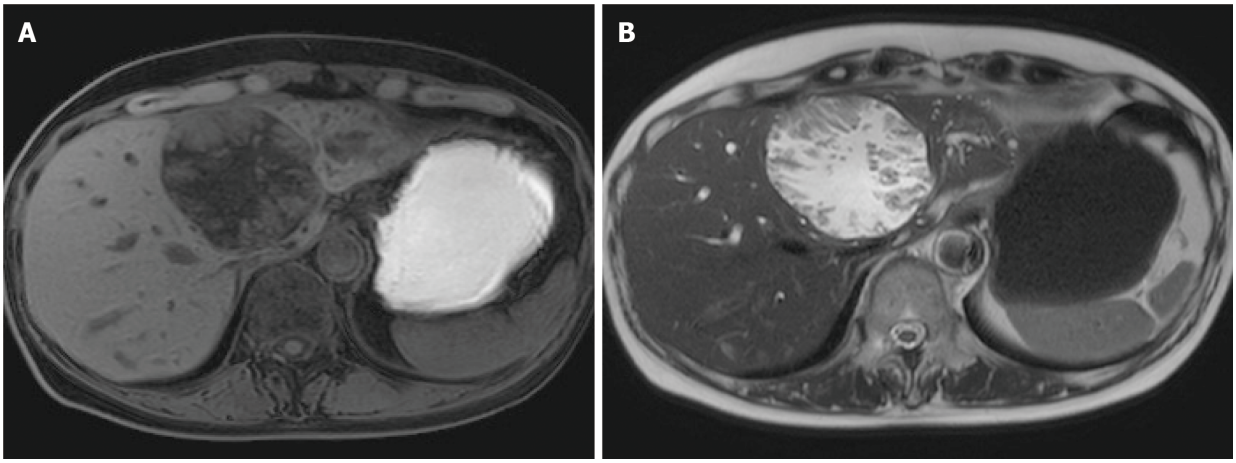


Figure 2 Magnetic resonance imaging. A: T1-weighted image; and B: T2-weighted image papillary solid lesion protuberating from cystic wall is noted and the cystic component showed the same signals as water. Transportation between the cyst and the root of left hepatic duct is noted.



Figure 3 Translucency caused by mucus is noted from hepatic portal region to lower bile duct.

mucus. With great operability, PTCS ensures a sufficient visual field and enables a wider scope of observation and more detailed monitoring. Weak points of PTCS are that it takes time until we can observe as time to fistula formation is required, and that dissemination *via* fistula has been also reported[15]. Therefore, the current indications for PTCS are limited, and POCS is mostly performed except for the following cases:

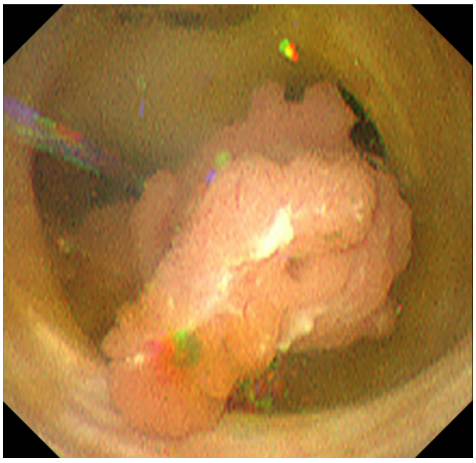


Figure 4 The lesion originates from the root of left hepatic duct, and pedunculated and elevated lesion protruded toward the extrahepatic bile duct, with no clear superficial spread.

Difficulty in biliary cannulation with ERCP; difficulty in reaching duodenal papilla after Billroth-2 method or Rou-X-en Y anastomosis; and difficulty in removing mucus with POCS. Benefits of POCS are that it does not require fistula formation, allows direct observation from the initial ERCP, and entails no risk for fistula dissemination. On the other hand, POCS may limit the scope of observation since its approach route is longer and operation is cumbersome and difficult. Nonetheless, sufficient evaluation with POCS is possible because the resection line of distal site of the tumour is important in determining the scope of resection at the time of operation on IPNB (Figures 5 and 6).

RESULTS OF SURGICAL TREATMENT OF IPNB

Treatment results in dysplasia

Kubota *et al*[17] examined 119 patients with intrahepatic IPNB, and reported the 5-year survival: 84.6% in low or intermediate-grade neoplasia group, 90.9% in high-grade neoplasia group, and 79.2% in invasive carcinoma group. When complete resection was performed, no statistical significance was noted in overall survival between the group of infiltrating cancer and the other group of noninfiltrating cancers. As further examinations of infiltrating cancer, Onoe *et al*[18] reported the ratio of infiltrating site relative to the lesion and its prognosis on 184 patients with intrahepatic and extrahepatic bile duct cancer exhibiting intraductal papillary proliferation. The 5-year survival in four groups: Noninfiltrating cancer, infiltrating site < 10%, infiltrating site = 10%-50%, and infiltrating site > 50%, were 92%, 74%, 64%, and 33%, respectively. The former three groups showed no statistical between-group differences; however, the infiltrating site > 50% group showed significantly poor prognosis, almost equivalent to the 5-year survival of 35% in 460 patients with non-papillary bile duct cancer. Onoe *et al*[18], pointed out that there still was a moot point in the disease concept of IPNB but indicated that if bile duct cancer exhibiting intraductal papillary proliferation was an infiltrating cancer, with an infiltrating site accounting for not more than 50% of the entire cancer, its good prognosis was more significant than non-papillary bile duct cancer. Rocha *et al*[19] reported that 29 of 39 IPNB patients (74%) had infiltrating cancer. Significantly poor prognostic factors were infiltration exceeding 5 mm, and infiltration beyond the bile duct wall, exceeding 10% of the entire tumour. The ratio of infiltrating cancer in IPMN is 21%-48%[19,20] , while that in IPNB is 70%-80%, slightly higher[21,22]. It is reported that prognoses of IPMN-derived infiltrating cancer and infiltrating pancreatic ductal carcinoma are almost the same[23,24], or prognosis of IPMN-derived infiltrating cancer is slightly better[25,26]. Meanwhile, better prognosis is reported in IPNB as compared with normal bile duct cancer[27]. This is due to characteristics of IPNB tumour itself, and because of relatively early diagnosis that is possible with obstructive jaundice caused by its proliferation into the intraductal lumen.



Figure 5 Resected specimen. Left lobectomy, caudal lobectomy, resection of extrahepatic bile duct, and choledochojejunostomy were performed.

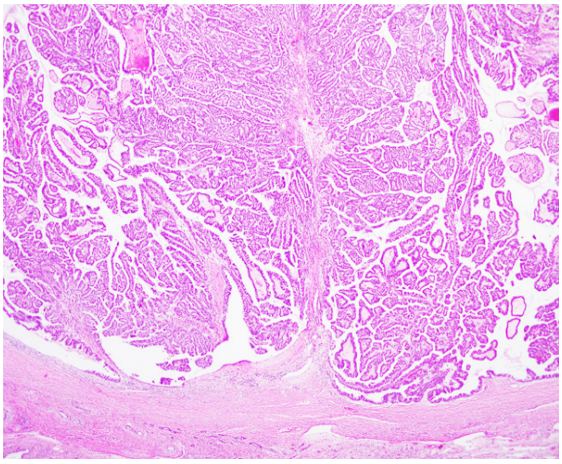


Figure 6 Pathological diagnosis is intraductal papillary neoplasm of the bile duct with an associated invasive carcinoma (Hematoxylin-Eosin Stain) with no superficial spread.

PROGNOSTIC FACTORS

Presence or absence of complete resection is an important prognostic factor, and reported in great numbers[18,19,28,29]. Luvira *et al*[28] reported that the bile duct resection stump tested positive for invasive carcinoma, and overall survival significantly decreased, while testing positive for dysplasia and carcinoma in situ did not impact prognosis. In contrast, Jung *et al*[29] reported that overall survival and recurrence-free survival of the group in which the bile duct resection stump tested positive for a low to intermediate grade dysplasia, significantly decreased to the extent similar to the overall survival of carcinoma in situ and invasive carcinoma, as compared with the group that tested negative. International consensus guidelines for the management of IPMN specifies that additional resection is indispensable if a clear high grade dysplasia or infiltrating cancer is present in the pancreatic resection stump while additional resection is not necessary even when a low grade dysplasia is present on the stump[30]. In IPNB, no guideline is available for a low grade dysplasia in the bile duct resection stump. Unlike IPMN where total pancreatectomy is therapeutic option depending on a patient, there is a resection limit for the bile duct, and thus, additional resection is performed as much as possible at each institution.

Presence or absence of metastases to lymph nodes is a major prognostic factor along with complete resection[18,28,29]. Luvira *et al*[28] reported that the 5-year survival of patients tested negative for metastases to lymph nodes was 51.2% while that of those tested positive was 11.1%, suggesting significantly poor prognosis. Of all 124 patients with IPNB, 98 patients underwent lymph node dissection. No difference in prognosis

was noted between these 98 patients and 26 patients without lymph node dissection, and thus, the results failed to support the usefulness of lymph node dissection. Patients with infiltrating cancer include those patients with lymph node dissection, and lymph node dissection may seem necessary. However, metastases to lymph node rarely occur in an intraductal growth type of intrahepatic bile duct cancer. As there are type 1 and type 2 for IPNB, more meticulous consideration may be needed to discuss the necessity of lymph node dissection in the future.

RECURRENCE OF IPNB AFTER SURGICAL TREATMENT

IPNB is known to recur in the bile duct after surgical resection. Despite that the initial resection stump of the bile duct tested negative, intraductal papillary tumour presenting with tissue image similar to the initial resection recurred. This is the characteristics similar to IPMN. IPNB recurs after more than 10 years in some cases, appearing as if it were new carcinoma. This suggests a multicentric growth of IPMN[31]. Therefore, observation of clinical course is necessary, with postoperative intraductal recurrence in mind.

CONCLUSION

IPNB is classified into type 1 that is similar to IPMN and type 2 that is not similar to IPMN. Prognosis of IPNB is said to be better than normal bile duct cancer. Cholangioscopy is useful for diagnosis of localization and progression. It is important to determine an operative method based on information obtained through cholangioscopy and to observe clinical course.

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ORIGINAL ARTICLE

Basic Study

Leucine-rich repeat-containing G protein-coupled receptor 5 marks different cancer stem cell compartments in human Caco-2 and LoVo colon cancer lines

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Abstract

BACKGROUND

Colon cancer cell lines are widely used for research and for the screening of drugs that specifically target the stem cell compartment of colon cancers. It was reported that colon cancer carcinoma specimens contain a subset of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)-expressing stem cells, these so-called "tumour-initiating" cells, reminiscent in their properties of the normal intestinal stem cells (ISCs), may explain the apparent heterogeneity of colon cancer cell lines. Also, colon cancer is initiated by aberrant Wnt signaling in ISCs known to express high levels of LGR5. Furthermore, *in vivo* reports demonstrate the clonal expansion of intestinal adenomas from a single LGR5-expressing cell.

AIM

To investigate whether colon cancer cell lines contain cancer stem cells and to characterize these putative cancer stem cells.

METHODS

A portable fluorescent reporter construct based on a conserved fragment of the LGR5 promoter was used to isolate the cell compartments expressing different levels of LGR5 in two widely used colon cancer cell lines (Caco-2 and LoVo). These cells were then characterized according to their proliferation capacity, gene expression signatures of ISC markers, and their tumorigenic properties *in vivo* and

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in vitro.

RESULTS

The data revealed that the LGR5 reporter can be used to identify and isolate a classical intestinal crypt stem cell-like population from the Caco-2, but not from the LoVo, cell lines, in which the cancer stem cell population is more akin to B lymphoma Moloney murine leukemia virus insertion region 1 homolog (+4 crypt) stem cells. This sub-population within Caco-2 cells exhibits an intestinal cancer stem cell gene expression signature and can both self-renew and generate differentiated LGR5 negative progeny. Our data also show that cells expressing high levels of LGR5/enhanced yellow fluorescent protein (EYFP) from this cell line exhibit tumorigenic-like properties *in vivo* and *in vitro*. In contrast, cell compartments of LoVo that are expressing high levels of LGR5/EYFP did not show these stem cell-like properties. Thus, cells that exhibit high levels of LGR5/EYFP expression represent the cancer stem cell compartment of Caco-2 colon cancer cells, but not LoVo cells.

CONCLUSION

Our findings highlight the presence of a spectrum of different ISC-like compartments in different colon cancer cell lines. Their existence is an important consideration for their screening applications and should be taken into account when interpreting drug screening data. We have generated a portable LGR5-reporter that serves as a valuable tool for the identification and isolation of different colon cancer stem cell populations in colon cancer lines.

Key Words: Colorectal cancer; Colon cancer cell lines; Intestinal stem cell; Cancer stem cell; Leucine-rich repeat-containing G protein-coupled receptor 5; Heterogenicity

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Core Tip: The intestinal epithelium harbors two distinct pools of putative stem cells, the leucine-rich repeat-containing G protein-coupled receptor 5$^+$(LGR5) stem cell population and the B lymphoma Moloney murine leukemia virus insertion region 1 homolog$^+$ stem cell population. Colon cancer cell lines such as Caco-2 and LoVo are extensively used in colon cancer research, and express high levels of LGR5. Here, we aimed to investigate whether colon cancer cell lines contain cancer stem cells and characterized these cells. Using an LGR5 reporter, we characterized LGR5$^+$ cells and revealed that Caco-2 cell line contains a classical intestinal stem cell-like population (LGR5$^+$). However, in LoVo cell lines, stem cell-like population is more akin to the B lymphoma Moloney murine leukemia virus insertion region 1 homolog$^+$ stem cells.

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INTRODUCTION

Colorectal cancer (CRC) remains a leading cause of morbidity and mortality worldwide[1], highlighting the need for more effective therapeutics. It is now well established that both blood and solid cancers are initiated and propagated from a subset of rare cells, called cancer stem cells (CSCs), which are resistant to chemotherapy or radiation[2-4]. Current evidence indicates that CRC is indeed a disease of colon stem cells[2,5,6] that are resistant to current therapeutics and can rapidly proliferate to re-establish the tumor[7]. The intestinal crypt-villous structure harbors two distinct pools of putative stem cells[8]. One pool is located at the crypt base and is characterized by the expression of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), and the other pool resides at +4 position and consists of B lymphoma Moloney murine

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leukemia virus (Mo-MLV) insertion region 1 homolog (Bmi-1) and telomerase reverse transcriptase (TERT) expressing cells[9-11]. The hierarchy of these stem cell pools in the normal crypt and their respective contributions to colon cancer and relapse following therapy is under debate. Some suggest both pools contribute equally to maintenance of the crypt, following a pattern of neutral drift[12], while others propose that Lgr5$^+$ stem cells (SCs) comprise the active population of the crypt and that Bmi-1$^+$ or TERT$^+$ cells are quiescent SCs that represent a reserve pool of SCs with the ability to replace Lgr-5$^+$ cells in case of loss or injury[8,13]. The existence of CSCs within intestinal tumors was most elegantly demonstrated by Schepers *et al*[14] using lineage re-tracing with a multicolor Cre-reporter R26R-confetti targeted to a single adenomatous polyposis coli allele in mouse intestinal stem (LGR5$^+$) cells, demonstrating that these intestinal adenomas were maintained by CSCs that exist in a cellular hierarchy. LGR5 is a receptor for R-spondins and activates potent Wnt signal enhancers such as Rnf43 and Znrf3[15]. LGR5 is present in various stem cell compartments throughout the body[16], including the intestine, and is an exquisite marker of intestinal stem cells (ISCs) capable of forming the entire intestinal mucosa[9]. LGR5$^+$ ISCs persist in adenoma and can initiate CRC through the activation of the Wnt/β-catenin signaling pathway[17].

Human colon cancer cell lines such as Caco-2 and LoVo are extensively used in drug screening and colon cancer research and express high levels of LGR5[18-20]. To establish criteria for the selection of suitable cell lines for drug discovery, several studies have investigated variability between the colon cell lines, identifying characteristic differences in gene expression signature, epigenetic and genetic make-up, migratory abilities, and proliferative capacities between different intestinal cancer and cell lines, including Caco-2 and LoVo[21,22]. However, the CSC population(s) within these cell lines remains to be fully characterized. This is important since anticancer drugs often attempt to target these CSCs[23,24].

To isolate the CSC compartment from colon cancer cell lines we constructed a reporter based on a conserved promoter fragment of the *LGR5* gene. After validating the fidelity of this LGR5 reporter, we showed that it can be used as a genetic tool to isolate LGR5-expressing CSCs from Caco-2 and LoVo human colon cancer cell lines. Surprisingly, our data revealed that these two widely used colon cancer lines possess different stem cell compartments. We conclude that this portable LGR5 reporter constitutes a valuable tool for the development of colon cancer therapeutics specifically designed to ablate these CSCs.

MATERIALS AND METHODS

Cell culture

Human colon cancer cell lines (LoVo, Caco-2, and SW480), a human fibroblast cell line (CRL-2429), a mouse fibroblast cell line (3T3), and a mouse motor neuron cell line (NSC-34) were obtained from ATCC and maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, United States) supplemented with fetal bovine serum (FBS, Bovogen Biologicals, Keilor East, Australia) to a final concentration of 10% and passaged every 3-4 d at 80% confluency. The human neuroblastoma cell line (SH-SY5Y from ATCC) was maintained in DMEM supplemented with 20% FBS and passaged every 2-3 d. All cell lines used for experiments were at early (a maximum of 20-25) passage number.

Construction of LGR5 promoter-based reporter constructs

Conservation of the *LGR5* gene and its promoter was assessed using evolutionary conserved region browser and multiple alignments of several vertebrate genomes. The human *LGR5* gene is located on chromosome 12 and has a conserved region upstream of the main promoter. This gene segment [LGR5 promoter element (983 bp)] was amplified from RP11-59F15 bacterial artificial chromosome clones (obtained from the Australian Genome Research Facility, Ltd., Melbourne, Australia) by polymerase chain reaction (PCR) (Supplementary Figure 1A) using a set of primers (Table 1). Sequences for specific restriction sites (*Nde* I, *Nhe* I, *Sac* I, and *Xho* I) were added to allow the insertion of the amplicon into the pEYFP-N1 vector (Clontech, Mountain View, CA, United States). Subsequently, this purified deoxyribonucleic acid (DNA) fragment was A-tailed and ligated into pGEM®-T Easy Vector System I (Madison, WI, Promega). White colonies were selected and plasmid DNA isolated, purified, and sequenced. Verified plasmids containing the DNA of interest, which is also free of PCR-introduced mutations, were then digested and ligated into the pEYFP-N1 vector. A negative control clone was constructed by the deletion of the promoter insert from the

Table 1 Primer sequences

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	TGAAGCTGGAGAAGGAGAAG	ATCGGCCTGTGTATATCCC
LGR5	CCTCTGCTGGCTTTTAGGTG	AGCAGTTTTCAGGCCTTTCA
OLFM4	ACTGTCCGAATTGACATCATGG	TTCTGAGCTTCCACCAAAACTC
ASCL2	ACCTGCGTACCTTGCTTTGG	GAAATCTGCGAGTTCCCGGT
Amplification/cloning of the *LGR5* promoter element	CATATGCTAGCTCGAGCTCACTTCGACTTCCTCACCCCGC	AAGCTTGGTGCCCGAAGTAGGGGGCCA

promoter-pEYFP-N1 clone. The original pEYFP-N1 vector (Clontech) with the CMV promoter was used as a positive control.

Transient transfection

Cell lines at 50%-60% confluence was transfected with LGR5 promoter-pEYFP-N1 DNA using FuGENE® HD Transfection Reagent (Roche, Basel, Switzerland) following the manufacturer instructions. LoVo, Caco-2, SW480, human fibroblast CRL2923, 3T3, 4T1, and EMT6 cell lines were transfected in 3:1 transfection reagent to DNA ratio in serum-free medium. For the SY5Y cell line, the ratio was 3:2. Eight to ten hours post-transfection, serum was added to the culture medium. All cultures were kept at 37 °C in a 5% CO_2 atmosphere until microscopic analyses were performed using an inverted fluorescence microscope (IX51, Nikon, Tokyo, Japan) 24-48 h post-transfection to examine enhanced yellow fluorescent protein (EYFP) expression levels.

Flow cytometry

For flow cytometry and fluorescence-activated cell sorting (FACS), cultured cells were harvested by incubation in a non-enzymatic cell dissociation buffer (Gibco® Life Technologies, Waltham, MA, United States) for 7-10 min. Next, cells were washed in phosphate buffer saline (PBS) and resuspended in 0.5 mL PBS for analysis. Flow cytometric analysis of EYFP expression of live cells was conducted using an Accuri flow cytometer (BD Biosciences, San Jose, CA, United States). FACS sorting was performed using an Influxcell sorter (BD Biosciences). Negative controls were used in every analysis to set the background fluorescence. Propidium Iodide Staining Solution (Invitrogen) was added to discriminate dead cells. Data analysis was performed on at least 10000 cells per sample as assessed by CFlow software (BD Biosciences). Fluorescence gates for positive cells were set to attain the false-positive rates of < 1%.

Colony PCR

A single colony of bacterial cells was suspended in a reaction volume of 50 µL, containing 1 µL of PCR primers (10 µmol/L each), 5 µL of 10 × PCR Buffer Minus Mg^{2+}, 1 µL of 10 mmol/L dNTP mixture, 1.5 µL of 50 mmol/L $MgCl_2$, and Milli-Q water. The cycle parameters of the reaction were as follows: Initial denaturation 95 °C for 3 min; 35 cycles of 30 s for denaturation at 95 °C, 30 s for annealing at 58 °C, and 1 min for the extension at 72 °C then final elongation at 72 °C for 2 min.

Quantitative real-time PCR

RNA extraction and complementary DNA (cDNA) synthesis: RNA was extracted from approximately 5 × 10^6 cells using RNeasy mini kit (Qiagen, Valencia, CA, United States) following the manufacturer's instructions. DNAse I treatment (on column) was performed during the extraction procedure as recommended by the kit manual. RNA quantification was performed using Nanodrop 1000. One microgram of RNA was used for cDNA synthesis using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, United States) following the manufacturer's instructions. As a control for genomic DNA contamination, a 'no reverse transcriptase reaction' was carried out for every batch of cDNA synthesized.

Quantitative real-time PCR analysis: SsoFast™ EvaGreen® Supermix was used as the Master-mix and quantitative real-time PCR (qPCR) was carried out with a C1000™ Thermal Cycler (Bio-Rad Laboratories, Inc.). Each gene was amplified in triplicate in a reaction volume of 10 µL, containing 10 µL qPCR primers (Table 1), 5 µL of 2 × SsoFast EvaGreen Supermix, 0.2 µL cDNA, and Milli-Q water. The cycling parameters of the reaction were as follows: 95 °C for 3 min for enzyme (Sso7-fusion polymerase)

activation; 40 cycles of 10 s for denaturation at 95 °C, and 30 s for annealing and extension at 60 °C followed by a Melt Curve analysis to ensure reaction specificity. Glyceraldehyde-3-phosphate dehydrogenase was amplified in parallel and used for normalization. The amplification product was then analyzed using the 2-ΔCt method. 'No reverse transcriptase' and water controls were included in each qPCR run to exclude genomic DNA or sample cross-contamination.

Soft agar assay

To test anchorage-independent growth in soft agar, a layer of 0.6% agar (Agar Noble; Difco Laboratories, Detroit, MI, United States) in 2 × DMEM containing 20% FBS was plated in 35-mm dishes and placed in the fridge overnight. The next day, Geneticin resistant (400-500 μg/mL of Geneticin) cells were sorted for EYFP expression into high, low, and no-EYFP expressing cells using the Influxcell sorter (BD Biosciences). Cells were next washed with PBS and pelleted by centrifugation at 300 *g* for 3 min. Cells (2×10^4) were suspended in 2 mL of equal volumes of 0.6% agar (Agar Noble; Difco Laboratories) and 2 × DMEM containing 20% FBS and overlaid as a second layer. This was overlaid over a layer of 3 mL of 0.3% agar in the same medium, in 35 mm dishes. Once a week, 300 μL of medium containing 0.3% agar was added. After 21 d, plates were stained with 0.5 mL of 0.005% Crystal Violet for an hour. Colonies larger than 0.25 mm in diameter were then counted using ImageJ software.

In vivo tumorigenicity assay

Six- to eight-week-old non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice were obtained from Animal Resources Centre (Perth, Australia) and were housed in the animal house facility of the Australian Institute for Bioengineering and Nanotechnology, The University of Queensland. All procedures were approved by the University of Queensland Animal Ethics Committee (UQCCR/115/13/NHMRC) and conformed to the Animal Care and Protection Act Qld (2002) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). Sorted cells were suspended in a 1:1 mixture of medium and Matrigel (BD Biosciences) and injected subcutaneously into NOD/SCID mice (2×10^5 cells per animal in a total volume of 50 μL) into the ventral side of the flank (n = 3 animals per group). Tumor growth was monitored and measured with calipers daily. Three weeks after the injection, mice were anesthetized by CO_2 inhalation, and tumors were excised, measured, weighed, and fixed in 4% paraformaldehyde for paraffin embedding. Tissue sections were stained with hematoxylin-eosin.

RESULTS

A conserved LGR5 enhancer-based promoter fragment recapitulates endogenous LGR5 expression

Due to the lack of a reliable anti-LGR5 antibody, isolation of LGR5 expressing cells *via* FACS or magnetic-ACS was not possible in our hands. We, therefore, constructed an EYFP reporter driven by a conserved LGR5 promoter fragment (Supplementary Figure 1A). To test whether the *LGR5* promoter-based construct correctly reported on LGR5 expression, we transiently transfected the LGR5-EYFP reporter as well as a negative control vector (promoter-less vector or empty-EYFP vector) and a positive control vector (CMV-EYFP) into colon cancer cell lines Caco-2, LoVo, and SW480 that display a high endogenous expression of *LGR5* and into CRL2429, 3T3, NSC-34 and SH-SY5Y cells that do not express LGR5 messenger RNA (mRNA). Transient transfection of the LGR5 reporter construct in these LGR5-expressing and LGR5-negative cell lines revealed that EYFP expression correlated well with endogenous LGR5 mRNA expression level (Supplementary Figure 1B). As expected, transfection of the promoter-less construct (empty-EYFP vector) did not result in YFP expression, whereas the CMV-driven EYFP vector showed widespread (30%-60% of cells) expression (Supplementary Figure 1B), indicating that the absence of fluorescence in LGR5 negative lines was not due to ineffective delivery of plasmid DNA.

To generate stably expressing reporter lines, Caco-2 and LoVo cell lines were transfected with a linearized LGR5 reporter vector, single-cell seeded, and maintained under Geneticin selection (400 μg/mL for LoVo cell line and 500 μg/mL for Caco-2 cell line) for 1 mo. Two single cell-derived clones from each cell line were picked, expanded, and cryopreserved. Fluorescence microscopy analysis revealed that these clonal LGR5/EYFP Caco-2 and LGR5/EYFP LoVo cultures contained a subset of cells

with a range of EYFP fluorescence, including small EYFP fluorescence bright cells (Figure 1A and B; orange arrows), which was validated using FACS (Figure 1C and D). Cells transfected with promoter-less plasmid showed no EYFP expression in any of the surviving clones, whereas the majority of CMV-promoter transfected clones homogeneously expressed EYFP, as expected. We hypothesized that cells expressing high levels of LGR5/EYFP in the colon cancer cell lines would constitute the stem cell compartment of Caco-2 and LoVo cell lines. To test this hypothesis, we sorted the cells into three populations according to their EYFP expression (EYFP^{negative}, EYFP^{low}, and EYFP^{high} cells). As anticipated, the qPCR analysis showed that LGR5 mRNA expression was 8-fold higher in EYFP high cells than in the EYFP negative fraction in both LGR5/EYFP Caco-2 and LGR5/EYFP LoVo clonal lines (Figure 1E and F, respectively).

In agreement with the idea that LGR5 marks a stem cell compartment, flow cytometric analysis of sorted EYFP^{negative}, EYFP^{low}, and EYFP^{high} cells from each of the lines revealed that over the course of several days, EYFP^{high} cells gave rise to EYFP negative "daughter" cells (Figure 1G and H). Time-lapse immunofluorescence and phase-contrast images of cultured EYFP^{high} sorted Caco-2 cells confirmed that EYFP negative cells emerged from the small EYFP^{high} cells over time (Figure 1G). No cells acquired EYFP expression amongst derivatives of FACS-sorted EYFP-negative cells (Figure 1I). LGR5-EYFP LoVo clones exhibited identical behaviors (Figure 1H and J). We interpreted these data to mean that LGR5^{high} (EYFP^{high}) cells possess stem cell-like properties, given they are capable of self-renewal and generate a distinct EYFP-negative cell population, incapable of re-acquiring expression of the LGR5 reporter.

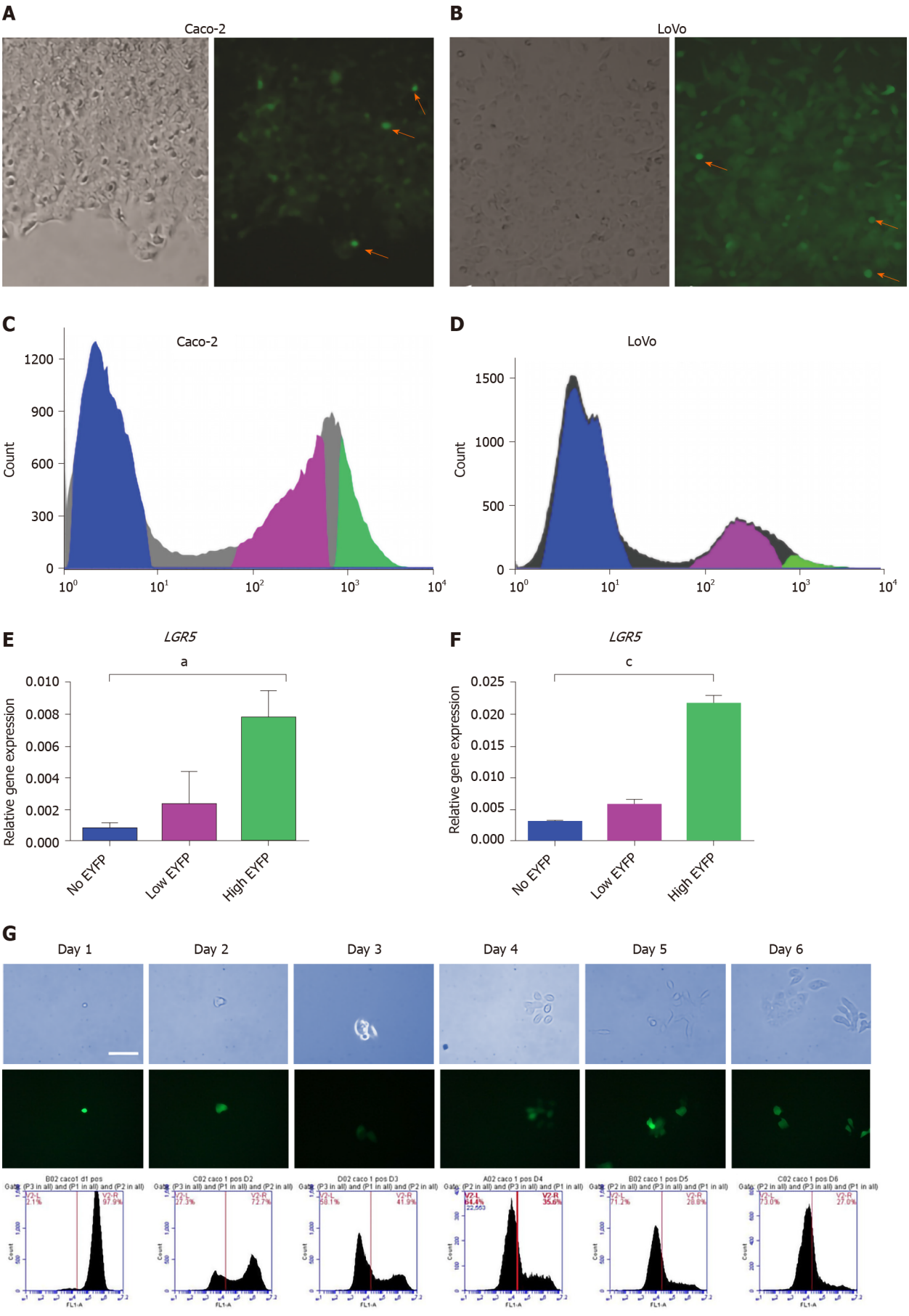
EYFP^{high}-expressing cells of Caco-2 and LoVo CRC cell lines show an ISC gene expression signature

If LGR5/EYFP^{high} cell populations in Caco-2 and LoVo clones represent an ISC compartment, then these cells should show higher mRNA expression of typical ISC marker genes compared to LGR5/EYFP^{low} and LGR5/EYFP^{negative} fractions. Our data (Figure 2A) show that EYFP^{high} cells in the Caco-2 cell line indeed display significantly increased expression of the stem cell markers LGR5, olfactomedin 4 (OLFM4), TERT, leucine-rich repeated and immunoglobulin-like domains 1 (LRIG1), AFAP1I1, TNFRF19, organic cation transporter 1 (OCT1), and achaete-scute complex homolog 2 as compared to cells with low or no EYFP expression, but conspicuously do not express B lymphoma Moloney murine leukemia virus insertion region 1 homolog (Bmi1). Surprisingly, in the LoVo cell line (Figure 2B) EYFP^{high} cells expressing high LGR5 reporter activity possessed low levels of OLFM4, AFAP1I1, and LRIG1 compared to EYFP^{low} and EYFP^{negative} fractions. EYFP^{high} cells in the LoVo cell line instead robustly express Bmi1 as compared to EYFP^{negative} cells but exhibit lower hTERT as compared to EYFP^{negative} and EYFP^{low} fractions. These data suggest that in the LoVo cell lines LGR5 marks a different cancer cell compartment than in Caco-2 cells. Collectively, our data indicate that the LGR5/EYFP reporter faithfully marks a population of LGR5 expressing CSCs in the Caco-2 cell line but that this stem cell population is distinct from the EYFP^{high} fraction in the LoVo cell line that appears to mark a Bmi1^{high}/LGR5 TERT^{low} expressing a subset of cells.

LGR5/EYFP^{high} cells in Caco-2 colon cancer cell line exhibit increased colony-forming ability

To assess whether LGR5 expressing subsets in the Caco-2 and LoVo lines exhibit functional differences we employed the anchorage-independent tumor stem cell colony-forming assay[25]. Using this assay, we assessed the *in vitro* anchorage-independent growth of LGR5/EYFP^{high}, LGR5/EYFP^{low}, and LGR5/EYFP^{negative} cell populations of the LGR5/EYFP Caco-2 and LGR5-EYFP LoVo lines (Figure 3A and B). Our data showed that after 3-wk culture in soft agar, the number of colonies formed by the three cell populations of Caco-2 and LoVo cell lines was comparable (Figure 3C and E) but that the colony sizes of the LGR5/EYFP^{high} cell populations in Caco-2 line were considerably larger (> 0.25 mm in diameter) than those in LGR5/EYFP^{low} and LGR5/EYFP^{negative} cell populations (Figure 3D and F), suggesting LGR5^{high} cells in Caco-2 cell line exhibit increased proliferation and/or reduced cell death rates.

To assess further the tumorigenicity of the LGR5/EYFP^{high} cell compartments of Caco-2 and LoVo lines, LGR5/EYFP^{high} and EYFP^{negative} sorted cells were injected subcutaneously into the right and left flanks, respectively, of NOD-SCID mice (n = 3), and tumor growth was monitored daily before excision of tumors after 3 wk. As shown in Figure 4A, LGR5/EYFP^{high} Caco-2 cells were able to form tumors when injected into NOD-SCID mice. LGR5/EYFP^{high} formed larger tumors than the LGR5/


A
Caco-2
B
LoVo
C
Caco-2
Count
1200
900
600
300
0
10^0
10^1
10^2
10^3
10^4
D
LoVo
Count
1500
1000
500
0
10^0
10^1
10^2
10^3
10^4
E
LGR5
a
Relative gene expression
0.010
0.008
0.006
0.004
0.002
0.000
No EYFP
Low EYFP
High EYFP
F
LGR5
c
Relative gene expression
0.025
0.020
0.015
0.010
0.005
0.000
No EYFP
Low EYFP
High EYFP
G
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6

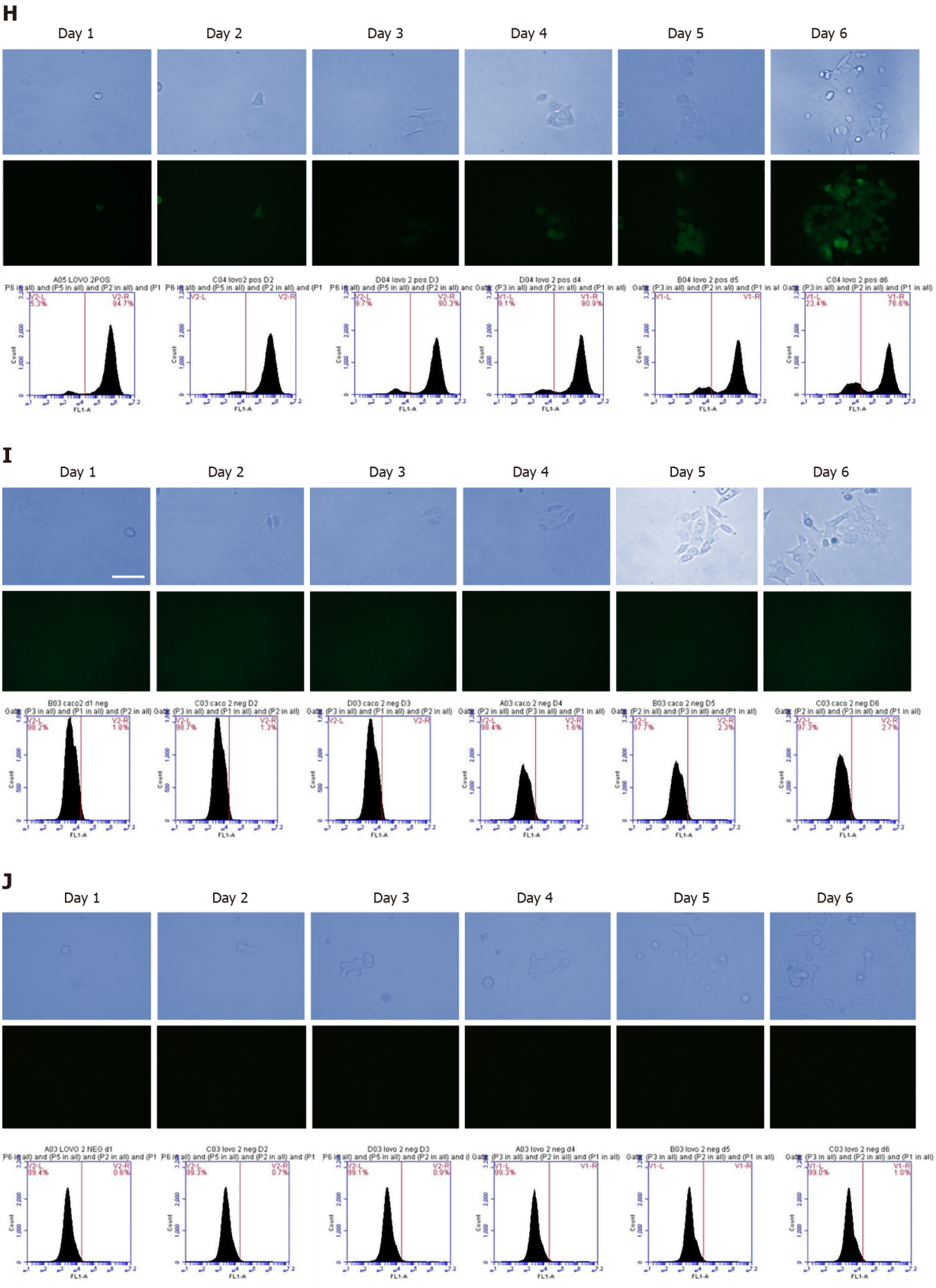


Figure 1 Proliferation and differentiation analysis of the stem cell compartment in stably transfected clones. A and B: Phase contrast and fluorescence images show colonies of stably transfected Caco-2 (A) and LoVo (B) cells expressing the enhanced yellow fluorescent protein (EYFP) reporter (red arrows show small cells expressing high level of enhanced yellow fluorescent protein, EYFP); C and E: Fluorescence-activated cell sorting (FACS) sorting of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)/EYFP expression in Caco-2 clone (C) and LoVo clones (E) into EYFP^{high}, EYFP^{low}, and EYFP^{negative} populations; D and F: Quantitative real-time polymerase chain reaction analysis of *LGR5* messenger ribonucleic acid expression in EYFP^{high}, EYFP^{low}, and

EYFP^{negative} cells relative to glyceraldehyde-3-phosphate dehydrogenase gene in Caco-2 clones (D) and LoVo clones (F); G: Phase contrast images show proliferation of EYFP^{high} cells from LGR5/EYFP Caco-2 clone; fluorescence images show self-renewal of LGR5/EYFP stem cells and down-regulation of LGR5/EYFP in differentiated progeny in LGR5/EYFP Caco-2. Sequential FACS analysis shows EYFP expression in LGR5/EYFP Caco-2 clone over 6 d in culture; H: Phase contrast images show proliferation of EYFP^{negative} and cells from LGR5/EYFP Caco-2 clone; fluorescence images show self-renewal of LGR5 stem cells and down-regulation of LGR5 in differentiated progeny in LGR5/EYFP Caco-2. Sequential FACS analysis shows EYFP expression in LGR5/EYFP Caco-2 clone over 6 d in culture; I and J: Phase contrast images show proliferation of EYFP^{high} (I) and EYFP^{negative} (J) cells from LGR5/EYFP LoVo clone; fluorescent pictures showing self-renewal of LGR5 stem cells and down-regulation of LGR5/EYFP in differentiated progeny in LGR5/EYFP LoVo; sequential FACS analysis of EYFP expression in LGR5/EYFP LoVo clone over 6 d in culture. LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; EYFP: Enhanced yellow fluorescent protein.

EYFP^{negative} cells (Figure 4B and C).

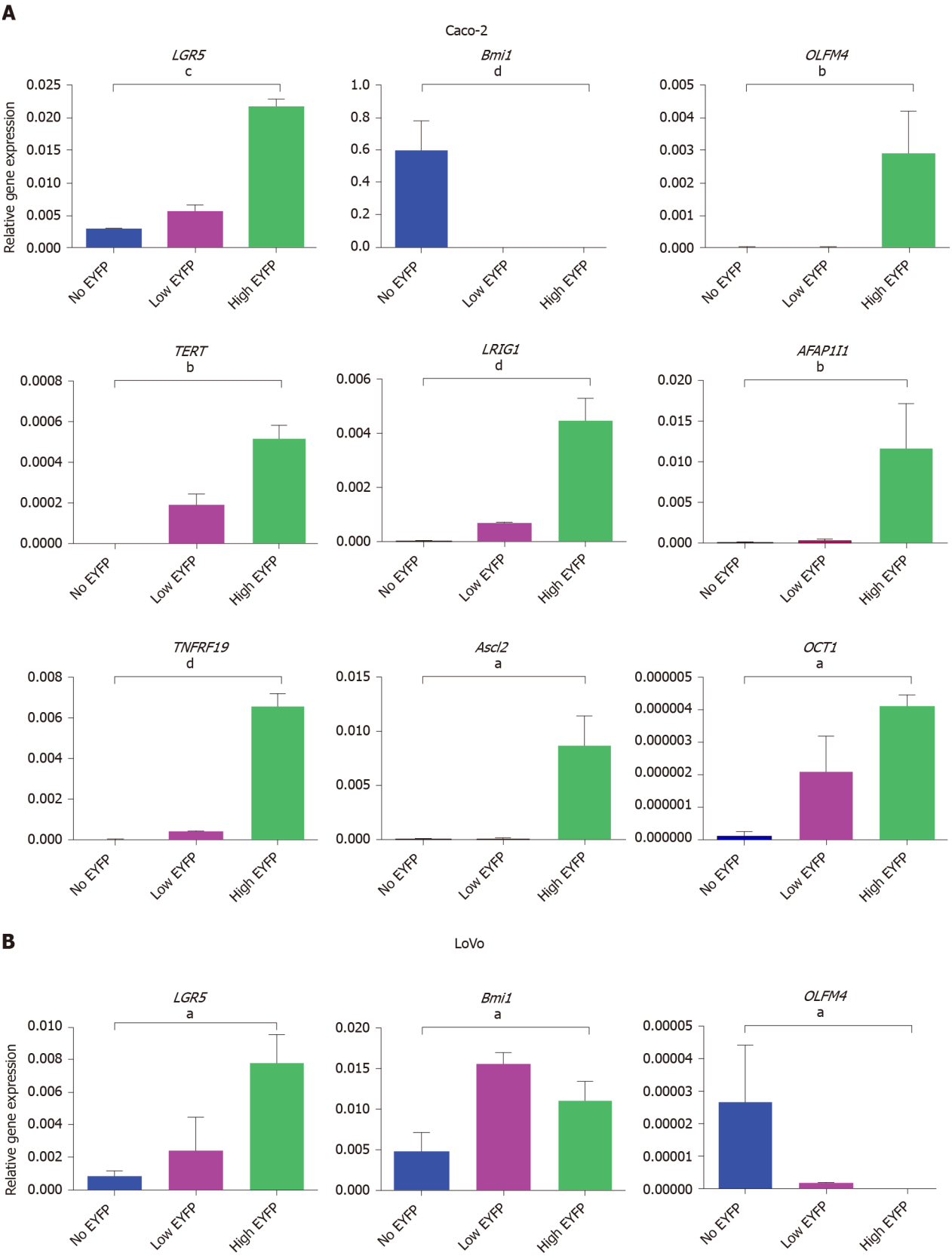
In the LoVo cell line, on the other hand, both LGR5/EYFP^{high} cells and LGR5/EYFP^{negative} sorted cells had almost comparable tumorigenic properties (Supplementary Figure 2).

DISCUSSION

The heterogeneity of cancer cell lines has been recognized, but it was not until the 1980s that it was shown that the human DLD-1 colon cancer cell line, originally established from a single human colon carcinoma[26], contains subpopulations of cells with different morphology, karyotype, and cloning efficiency in soft agarose that produce histologically distinct tumors when injected into athymic mice. Recent studies have explained this apparent heterogeneity of colon cancer cell lines when it was discovered that colon cancer carcinoma contains a subset of stem cells expressing LGR5, termed "tumor-initiating" cells specimens[27]. This was strongly supported by lineage tracing studies[28] showing that colon cancer is initiated by aberrant Wnt signaling in ISCs, known to express high levels of LGR5. Furthermore, *in vivo* lineage tracing experiments on intestinal adenomas demonstrated the clonal expansion of these adenomas from a single LGR5-expression cell[14].

In light of this evidence, we decided to investigate whether colon cancer cell lines widely used for drug-screening[21] contain CSCs and developed a reporter that would allow their isolation and characterization. Here, we showed that a 983 bp fragment of the LGR5 promoter faithfully reports on endogenous LGR5 expression in the colon cancer cell lines LoVo and Caco-2. Interestingly, we revealed that the ISC populations in these two cell lines exhibit different gene expression and functional properties. Our data show that the Caco-2 cell line contains a population of highly proliferative cells that exhibit a classic intestinal CSC gene expression signature that is marked by a high expression of LGR5, in agreement with previous findings[28,29]. These cells can both self-renew and generate more differentiated LGR5 negative progeny and also exhibit an *in vitro* tumorigenic potential. In support of the notion that LGR5/EYFP^{high} cells within Caco-2 colon cancer cell line represent the CSC compartment, our limited gene expression analysis revealed that LGR5/EYFP^{high} cells display a transcriptional signature very similar to that of the ISC, including *LGR5*, *Olfm4*, and *Ascl2*[29-31]. Our data are also in agreement with Sato *et al*[9] who showed that only very high LGR5-expressing cells are the ISCs in normal intestinal tissue. In agreement with a study by Maddox *et al*[32], our data further show that the LGR5/EYFP^{high} fraction of the Caco-2 cell line exhibits a high expression of OCT1, a known ISC marker. The LGR5/EYFP^{high} fraction of the Caco-2 colon cancer cells also showed the highest telomerase expression (TERT), which is a characteristic of most CSCs, associated with the ability to proliferate indefinitely[33,34]. Conspicuously, the LGR5/EYFP^{high} fraction of the Caco-2 acquires Bmi1 expression upon loss of LGR5 expression, in agreement with the proposed cellular differentiation hierarchy of the intestinal crypt[10]. Collectively, these data suggest that the LGR5/EYFP^{high} fraction of the Caco-2 colon cancer cell line indeed represents a CSC-like population. These data are in close agreement with a similar study in SW480 and HT-29 human colon cancer cell lines in which LGR5 was also found to mark a CSC population[35].

In contrast to the Caco-2 cell line, in the LoVo colon cancer cell line, LGR5/EYFP^{high} cells showed the lowest expression of the ISC markers hTERT, LRIG1, AFAP1I1, and , and low expression of the CSC marker OCT1, suggesting that in LoVo cells LGR5 marks a different cell population, *i.e.* not a stem cell-like population. Indeed, our data show that in the LoVo cell line, cells with high LGR5/EYFP expression possess a gene expression signature more akin to Bmi-expressing stem cells of the crypt. In LoVo cells it is rather the LGR5^{negative} fraction that highly expresses ISC marker OCT1[32],


A
Caco-2
LGR5
c
Bmi1
d
OLFM4
b
TERT
b
LRIG1
d
AFAP1I1
b
TNFRF19
d
Ascl2
a
OCT1
a
Relative gene expression
No EYFP
Low EYFP
High EYFP
B
LoVo
LGR5
a
Bmi1
a
OLFM4
a

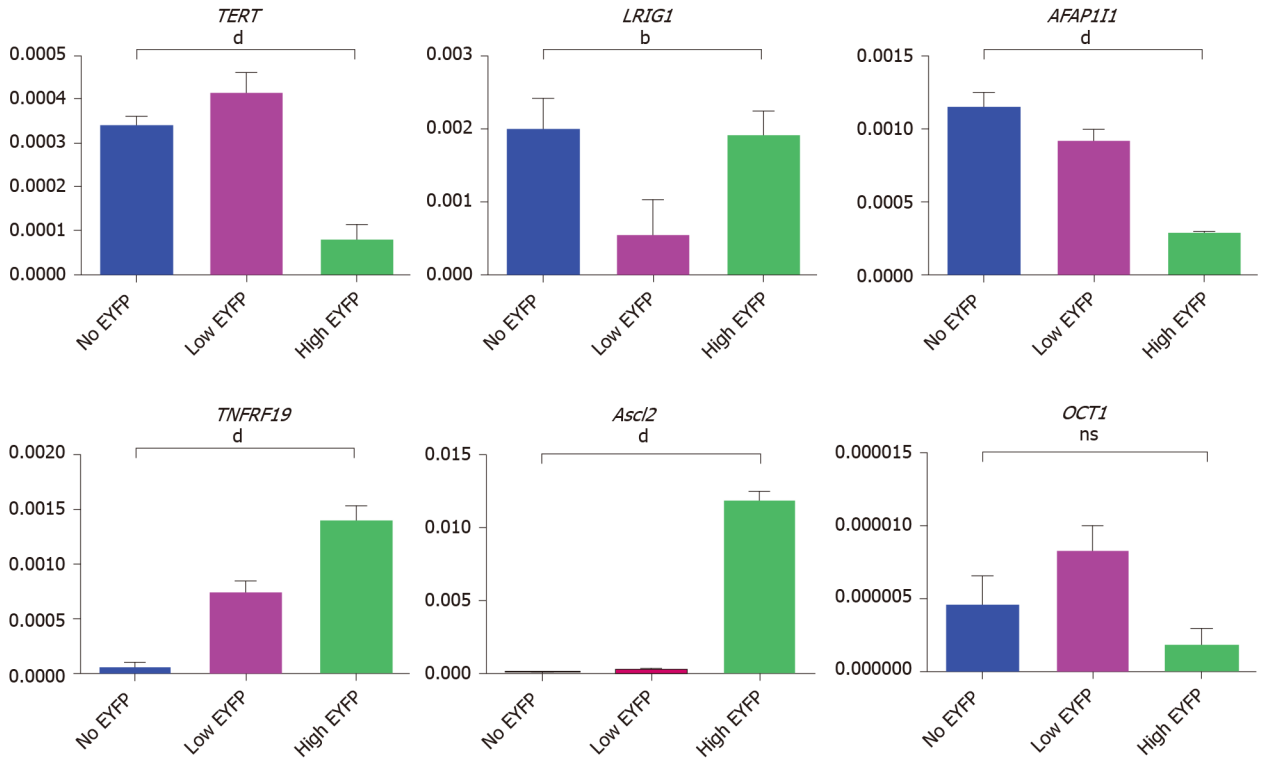


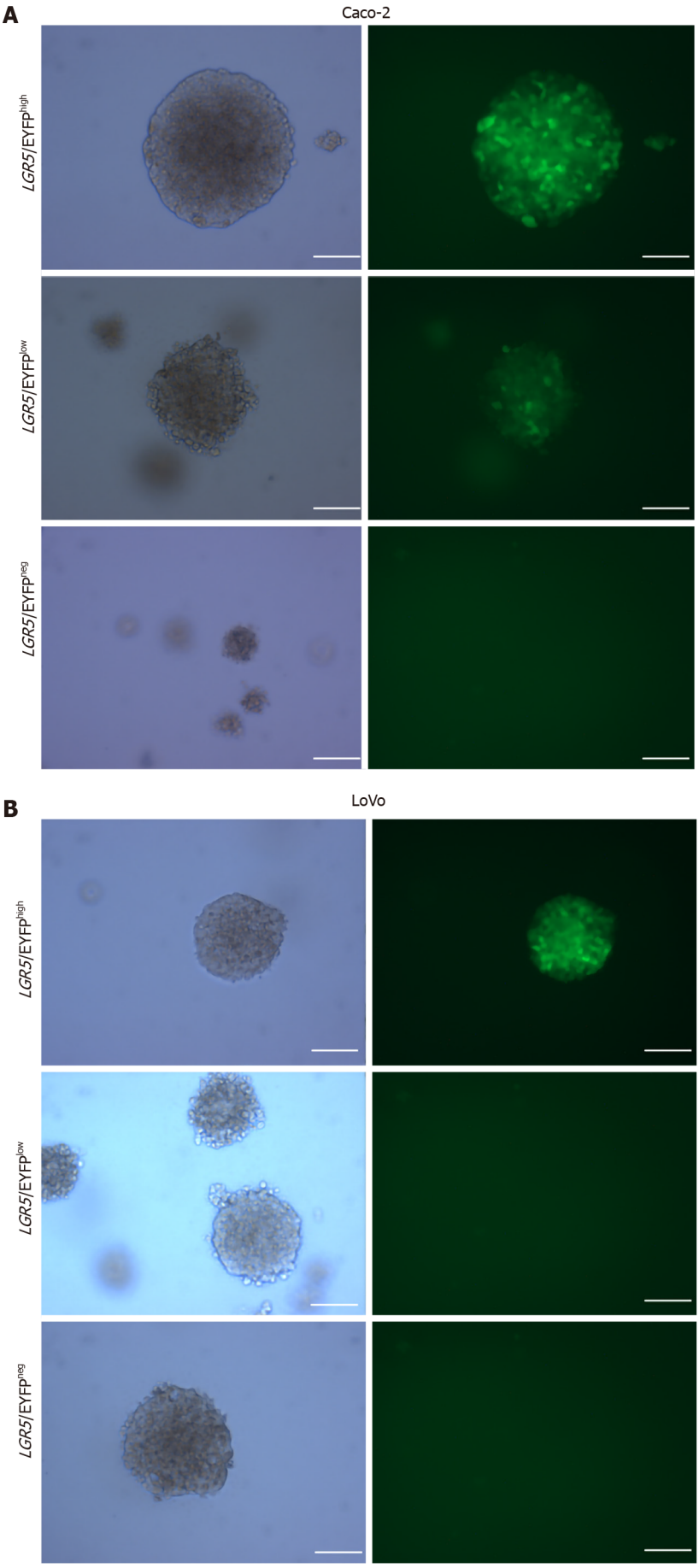
Figure 2 Quantitative polymerase chain reaction analysis. A and B: Quantitative polymerase chain reaction analysis of intestinal stem cell markers and the cancer stem cell marker organic cation transporter 1 in leucine-rich repeat-containing G protein-coupled receptor 5/enhanced yellow fluorescent protein (LGR5/EYFP)^{high}, LGR5/EYFP^{low}, and LGR5/EYFP^{negative} fractions of the Caco-2 cell line (A) and the LoVo cell line (B). The expression value of each gene was normalized to glyceraldehyde-3-phosphate dehydrogenase gene. Data were then analyzed by one-way analysis of variance. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; EYFP: Enhanced yellow fluorescent protein; Bmi1: B lymphoma Moloney murine leukemia virus insertion region 1 homolog; OCT1: organic cation transporter 1; TERT: Telomerase reverse transcriptase; ASCL2: Achaete-scute complex homolog 2.

OLFM4[36,37], and Bmi1[10], suggesting that this cell line originated from a CSC population other than LGR5^{+}cells. Supporting this suggestion, in a recent review on the intestinal hemostasis and plasticity in humans, the writer argued that there is a wide range of cell types in the intestinal epithelium that can revert to stem cell fate and regenerate the epithelium post-injury[38]. Moreover, Bmi1 marks a distinct pool of ISCs that mainly reside above the crypt base[39]. Lineage tracing has shown that this Bmi1^{+} stem cell pool, the so-called reserve stem cell pool, is able to regenerate intestinal epithelial tissue in the absence of LGR5^{+} ISCs[10,39] and also give rise to LGR5^{+} cells in normal and injured intestinal epithelium[40]. In support of this idea, a number of other human colon cancer cell lines (such as LIM1899, LIM1215, LIM2537, and LIM1863)[41] have previously been shown to exhibit increased anchorage-independent growth and enhanced tumorigenicity in xenograft experiments upon ablation of LGR5.

CONCLUSION

We conclude that we have generated a portable LGR5-reporter that should prove a valuable tool for the identification and isolation of different colon CSC populations in colon cancer lines. The characterization of such populations is important for drug screening and design. Since LGR5 marks adult stem cell populations in a number of tissues throughout the body[42], it will be interesting to determine whether our LGR5 promoter reporter can also be used to isolate such LGR5 expressing CSCs from other human cancer cell lines such as glioblastoma[43], esophageal adenocarcinomas[44], ovarian primary tumors[45], gastric tumors[46], and hepatomas[47-50] that are also marked by high LGR5 expression.

Our data further indicate that LGR5/EYFP^{high} expression marks two different intestinal crypt stem cell population in the Caco-2 and LoVo cell lines. Since Caco-2 and LoVo cell lines are widely used for screening of colon cancer drugs[51], our findings of different stem cell populations in these lines likely has important implications for


A
Caco-2
LGR5/EYFP^high
LGR5/EYFP^low
LGR5/EYFP^neg
B
LoVo
LGR5/EYFP^high
LGR5/EYFP^low
LGR5/EYFP^neg

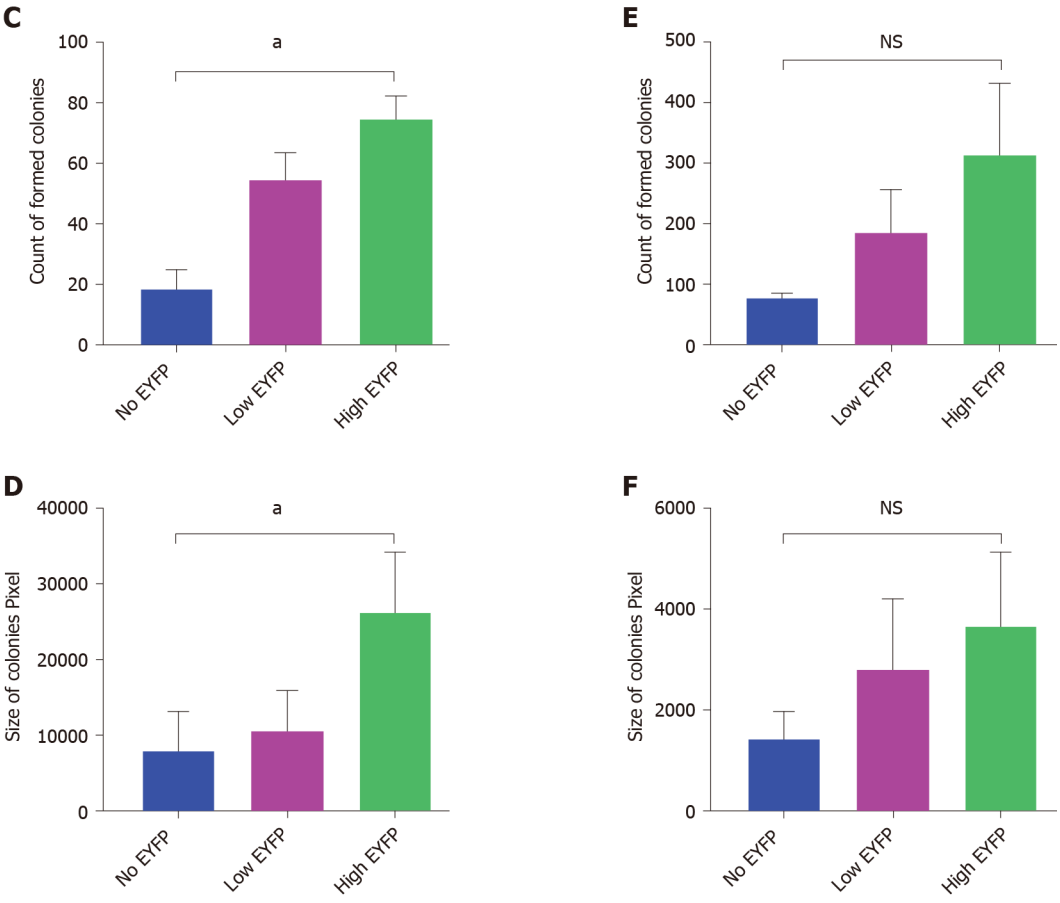


Figure 3 *In vitro* anchor independent assay of Caco-2 and LoVo colon cancer cell line. Sorted cells were cultured for 2 wk in soft agar before analysis. A-F: Representative images of colonies formed by cells with different leucine-rich repeat-containing G protein-coupled receptor 5/enhanced yellow fluorescent protein levels in Caco-2 cell line (A) and in LoVo cell line (B). Scale bar, 50 μm; the (C) and (E) number, and (D) and (F) size of colonies formed by Caco-2 and LoVo clones respectively were assessed using crystal violet staining. Colonies larger than 0.25 mm in diameter were then counted. Columns and error bars represent means ± SD of two independent experiments using duplicate measurements in each experiment. $^{a}P < 0.05$. LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; YFP: Yellow fluorescent protein; EYFP: Enhanced yellow fluorescent protein.

such drug screening programs.

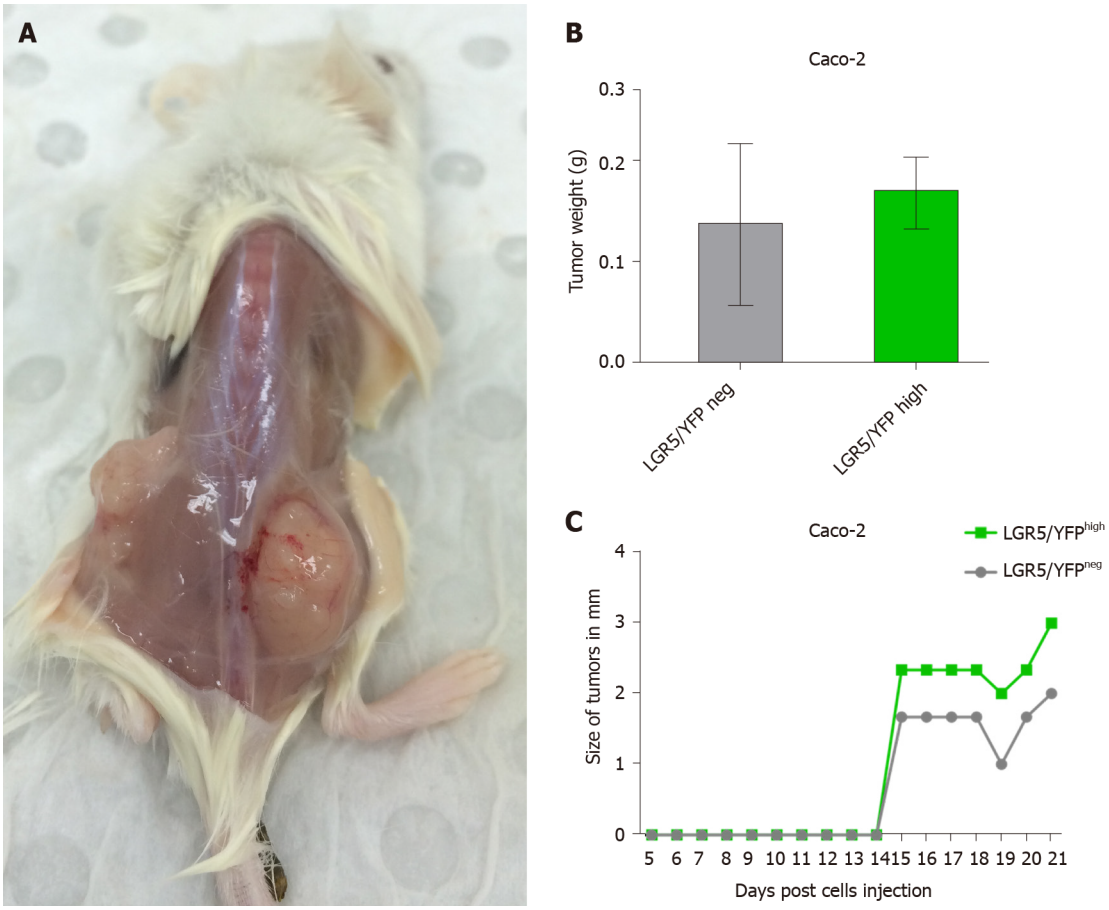


Figure 4 Stable leucine-rich repeat-containing G protein-coupled receptor 5/enhanced yellow fluorescent protein^{high} expressing Caco-2 cells line possesses stem cell-like properties *in vivo*. Sorted leucine-rich repeat-containing G protein-coupled receptor 5/enhanced yellow fluorescent protein (LGR5/EYFP)^{negative} and LGR5/EYFP^{high} cells were injected subcutaneously in left and right flanks (respectively) of non-obese diabetic- severe combined immunodeficient mice and monitored for 3 wk. A: Representative images of tumors formed by LGR5/EYFP^{high} (right flank) and LGR5/EYFP^{negative} (left flank) cells; B and C: Tumor weights (B) and size (C) resulting from LGR5/EYFP^{negative} and LGR5/EYFP^{high} sorted cells. Columns and error bars represent means ± SD of two independent experiments using duplicate measurements for each experiment. neg: Negative; LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; YFP: Yellow fluorescent protein.

ARTICLE HIGHLIGHTS

Research background

The intestinal crypt-villus structure harbors two distinct pools of putative stem cells, the active [leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)$^+$ cells] and the quiescent stem cell populations (B lymphoma Moloney murine leukemia virus insertion region 1 homolog$^+$ or telomerase reverse transcriptase$^+$ cells). Current evidence indicates that CRC is indeed a disease of colon stem cells that are resistant to current therapeutics and can rapidly proliferate to re-establish the tumor. Furthermore, it was reported that colon cancer carcinoma specimens contain a subset of LGR5-expressing stem cells. Human colon cancer cell lines, such as Caco-2 and LoVo, are extensively used in drug screening and colon cancer research and express high levels of LGR5.

Research motivation

To establish criteria for the selection of suitable cell lines for drug discovery and reveal potential variability between colon cell lines, we here identify the cancer stem cell (CSC) population(s) within colon cancer cell lines, quantify differences in gene expression signatures in these cells, and assess their proliferative and tumorgenicity capacities.

Research objectives

The present study aimed to isolate cells expressing LGR5 in Caco-2 and LoVo colon cancer cell lines and investigate whether these cells constitute the CSC compartments

in these cell lines. This was achieved through the creation of a transgenic proliferating stem cell-specific reporter construct based on the proximal promoter of *LGR5* gene.

Research methods

Using a portable fluorescent reporter construct based on a conserved fragment of the LGR5 promoter, subpopulations of cells from the colon cancer cell lines (Caco-2 and LoVo) were sorted into three cell compartments expressing different levels of LGR5 (high-, low-, and no-expression of LGR5). Next these cell compartments were characterized based on their gene expression signatures, proliferation, and tumorgenicity properties.

Research results

Cells expressing high levels of LGR5 with Caco-2 colon cancer cell line appear to represent a CSC-like population. In contrast, in the LoVo cell line, the LGR5 negative fraction possessed some features of the intestinal stem cells, *e.g.*, a specific gene expression signature, suggesting that this cell line was likely derived from a CSC population other than LGR5$^+$ cells.

Research conclusions

LGR5 marks the stem cell compartments of the Caco-2, but not LoVo, cell lines. Thus, it would appear that different colon cancer lines possess properties of different stem cell compartments. The portable LGR5 reporter outlined in this study constitutes a valuable tool for the identification and purification of LGR5-expressing cells from mixed cell populations.

Research perspectives

The portable LGR5 reporter described in this study constitutes a valuable tool for the identification and purification of LGR5-expressing cells from mixed cell populations.

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ORIGINAL ARTICLE

Basic Study

Abelson interactor 1 splice isoform-L plays an anti-oncogenic role in colorectal carcinoma through interactions with WAVE2 and full-length Abelson interactor 1

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Author contributions: Li K, Peng YF, and Guo JZ contributed equally to this work and performed the majority of experiments; Li M, Zhang Y, Chen JY, Lin TR, and Yu X performed minor experiments, provided vital reagents, and were also involved in revising the manuscript; Peng YF and Yu WD designed the study, wrote the manuscript, and provided financial support; all authors read and approved the final manuscript.

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Abstract

BACKGROUND

Expression of the full-length isoform of Abelson interactor 1 (ABI1), ABI1-p65, is increased in colorectal carcinoma (CRC) and is thought to be involved in one or more steps leading to tumor progression or metastasis. The ABI1 splice isoform-L (ABI1-SiL) has conserved WAVE2-binding and SH3 domains, lacks the homeodomain homologous region, and is missing the majority of PxxP- and Pro-rich domains found in full-length ABI1-p65. Thus, ABI1-SiL domain structure suggests that the protein may regulate CRC cell morphology, adhesion, migration, and metastasis *via* interactions with the WAVE2 complex pathway.

AIM

To investigate the potential role and underlying mechanisms associated with

Hospital Institutional Review Board (No. 2020-11).

Conflict-of-interest statement: The authors declare that they have no conflicts of interest to disclose.

Data sharing statement: No additional data are available.

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ABI1-SiL-mediated regulation of CRC.

METHODS

ABI1-SiL mRNA expression in CC tissue and cell lines was measured using both qualitative reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR. A stably ABI1-SiL overexpressing SW480 cell model was constructed using Lipofectamine 2000, and cells selected with G418. Image J software, CCK8, and transwell assays were used to investigate SW480 cell surface area, proliferation, migration, and invasion. Immunoprecipitation, Western blot, and co-localization assays were performed to explore intermolecular interactions between ABI1-SiL, WAVE2, and ABI1-p65 proteins.

RESULTS

ABI1-SiL was expressed in normal colon tissue and was significantly decreased in CRC cell lines and tissues. Overexpression of ABI1-SiL in SW480 cells significantly increased the cell surface area and inhibited the adhesive and migration properties of the cells, but did not alter their invasive capacity. Similar to ABI1-p65, ABI1-SiL still binds WAVE2, and the ABI1-p65 isoform in SW480 cells. Furthermore, co-localization assays confirmed these intermolecular interactions.

CONCLUSION

These results support a model in which ABI1-SiL plays an anti-oncogenic role by competitively binding to WAVE2 and directly interacting with phosphorylated and non-phosphorylated ABI1-p65, functioning as a dominant-negative form of ABI1-p65.

Key Words: Colon cancer; Abelson interactor 1 isoform-L; Cell adhesion; Cell migration; WAVE2

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Core Tip: The purpose of this study was to investigate the role and mechanism of Abelson interactor 1 splice isoform-L (ABI1-SiL) in the metastatic behavior of colorectal carcinoma cells. Our results showed that ABI1-SiL played a key role in cell surface area, adhesion, and migration, but not in proliferation and apoptosis in SW480 cells. ABI1-SiL may have anti-oncogenic roles by competitively binding to WAVE2, and directly interacting with phosphorylated and non-phosphorylated ABI1-p65, functioning as a dominant-negative molecule towards ABI1-p65.

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INTRODUCTION

Globally, colorectal carcinoma (CRC) is the third most commonly diagnosed cancer and the fourth most common cause of cancer death[1]. Despite treatment advances for CRC over the past two decades, novel molecular therapeutic strategies are required to generate informative biomarkers and identify new targets[2,3].

Abelson interactor protein-1 (ABI1) is increased and/or phosphorylated in many malignant tumors and has been proposed as a potential tumor promoter[4-11]. By forming complexes with Eps8-Sos1[6], NWasp[12], and WAVE2[13,14], ABI1 has been shown to play critical roles in the regulation of cell proliferation, apoptosis, adhesion, and migration in breast cancer, Bcr-Abl-induced leukemia, and melanoma, both *in vitro* and *in vivo*[4-15]. Recent findings have indicated that ABI1-p65 is increased and involved in colonic tumorigenesis and that it mediates the invasive properties of

CRC[7,8]. Recently, using a CRC tissue chip, we showed that increased expression of ABI1-p65 is significantly correlated with infiltration and associated with shorter overall survival[16]. Taken together, this evidence strongly suggests that ABI1 is involved in one or more of the steps leading to tumor progression or metastasis.

Alternative splicing is one of the main engines that drive proteome diversity, and up to 94% of genes are estimated to be alternatively spliced in humans[17,18]. There are many abnormal mRNA splice isoforms specifically associated with cancer progression and metastasis, some of which exert antagonistic functions[2,19,20]. Numerous structurally distinct isoforms of ABI1 exist in mammalian cells[12,21]. The ABI1-p65 isoform is increased in CRC and is thought to be involved in one or more of the steps leading to tumor progression and metastasis. However, the specific role, if any, that other splice isoforms of ABI1, such as ABI1 splice isoform-L (ABI1-SiL), play in CRC development remains unknown. As Figure 1 shows, ABI1-SiL is a splice isoform of ABI1 resulting from the alternative splicing of exons III, IV, and VIII through X. ABI1-SIL has conserved WAVE2-binding and SH3 domains, as well as several phosphorylated sites, such as pY213, pY421/pY435, and pY506, but lacks the homeodomain homologous region (HHR) and the majority of the PxxP- and Pro-rich domain[16,21]. The domain structure of ABI1-SiL suggests that it might regulate cell morphology, adhesion, migration, and even metastasis of CRC cells, similar to the ABI1-p65 isoform, by interacting with WAVE2 or other proteins.

In this study, we generated polymerase chain reaction (PCR) primers capable of specific detection of the ABI1-SiL isoform. We demonstrated that, compared with ABI1-p65, the expression of ABI1-SiL was significantly decreased in CRC tissues and cell lines, and overexpression of ABI1-SiL repressed migration and adhesion in SW480 colon cancer cells. We showed that both ABI1-p65 and ABI1-SiL were able to interact and co-localize with WAVE2 and ABI1-p65 in SW480 cells. Taken together, this evidence is the first to demonstrate that ABI1-SiL is an antagonistic splice isoform of ABI1-p65 that functions as a potential anti-oncogene.

MATERIALS AND METHODS

Cell lines and reagents

The CRC cell lines HCT-116, HT-29, LoVo, LS174T, SW480, and SW620 (the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China.), as well as the normal colon cell line CRL-1541 (ATCC, Rockville, MD, United States), were cultured in DMEM (HyClone, Beijing, China) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, United States). HEK-293T cells (ClonTech, Mountain View, CA, United States) were grown in DMEM (HyClone, Beijing, China) supplemented with 10% FBS. Plasmid-mediated transfection of SW480 cells with pCMV6 and pCMV6-ABI1-GFP (green fluorescence protein) transcript variant 12 (ABI1-Tv12) was performed as previously described using Lipofectamine (Invitrogen, CA, United States)[4,22]. Both pCMV6 and the vector containing human ABI1 cDNA (NM_001178125.1, ABI1-Tv12, encoding ABI1-SiL) were purchased from OriGene Technologies, Inc. (Rockville, MD, United States). G418 (500 μg/mL) was used to select stable cell lines overexpressing ABI1-SiL.

Patient tissue collection

CRC and matched adjacent tissue samples were obtained from 24 patients. All CRC cases were diagnosed using enteroscopy, and primary carcinoma of the colon-rectum was histologically identified. All samples were anonymized prior to this study. The local ethics committee of the Peking University, People's Hospital approved the study.

Qualitative and quantitative PCR analysis

Tissue samples, as well as the cell lines described above, were analyzed for the expression of transcript variant-12 (ABl1-SiL)[21]. Total RNA was isolated from patient samples, CRC cell lines, and transfected SW480 cells using an RNeasy mini kit (QIAGEN, Valencia, CA, United States) as previously described[23]. cDNA was subsequently generated using oligo-(dT) primers and the SuperScript III First-strand Synthesis System (Invitrogen, Carlsbad, CA, United States). PCR was performed to confirm the expression of ABI1-SiL[21] using the following primers: Forward, 5'-CCTCTCAGCTTCGGAGAATGG-3' and reverse, 5'-AGGAGGATTTGT TCTCGACAGTGT-3'. A 101 bp-amplified fragment in the PCR products was shown to be ABI1-SiL (confirmed by DNA sequencing). Quantitative PCR (qPCR) was performed using either human ABI1 universal 1 (ABI1-U1) (transcript variant 1-12)

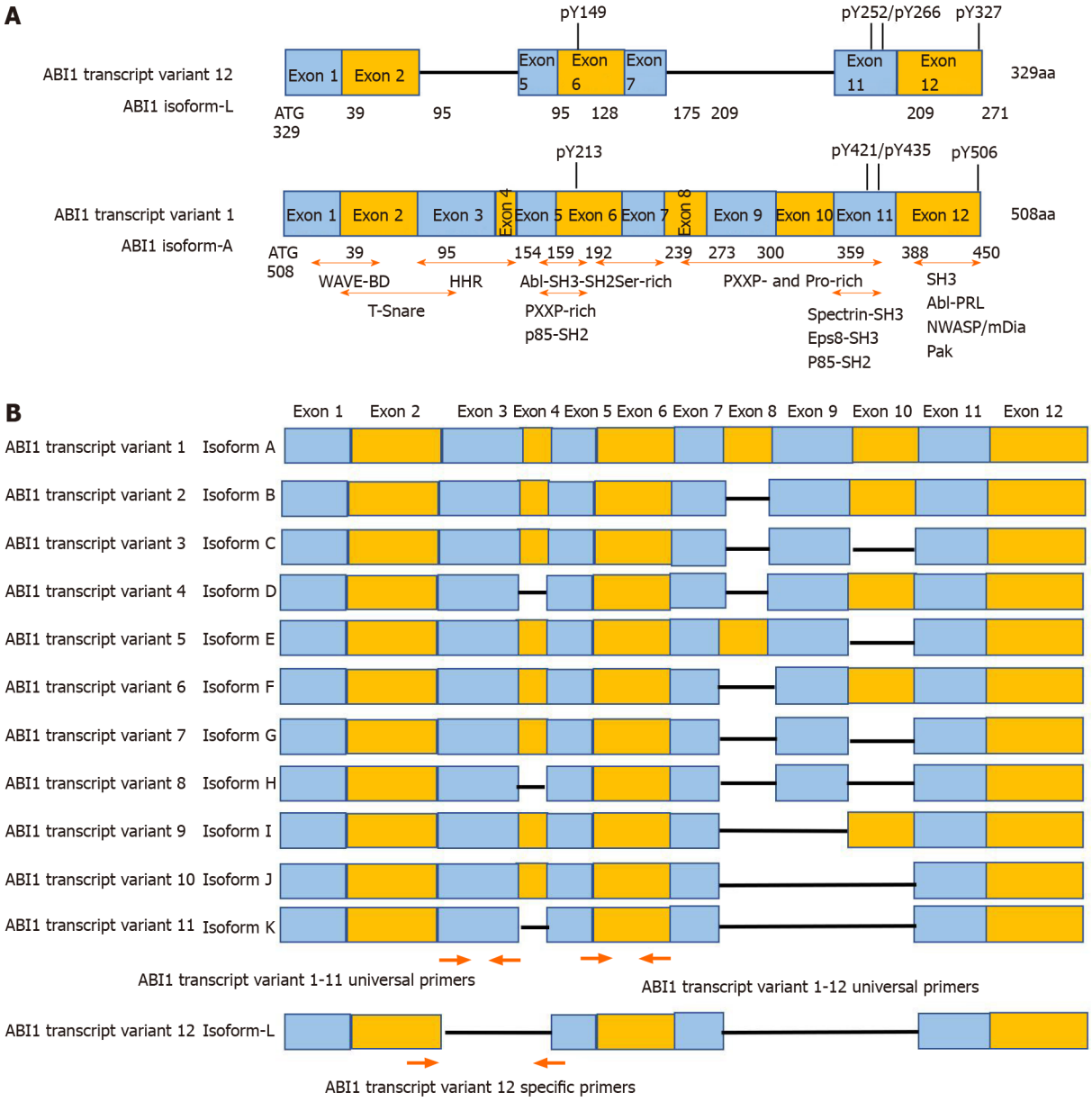


Figure 1 Diagram of Abelson interactor 1 isoforms and polymerase chain reaction primers used to measure expression levels of Abelson interactor 1. A: Schematic comparison of the exons and functional domains of two major isoforms of Abelson interactor 1 (ABL1): ABL1 splice isoform L (ABI1-SiL) and ABL1 splice isoform A (ABI1-p65). ABI1-SiL has conserved WAVE2-binding and SH3 domains, but lacks the homeo-domain homologous region and the majority of the PxxP- and Pro-rich domain; B: Schematic showing locations of ABI1 universal 1 (ABI1-U1)-, ABI1 universal 2 (ABI1-U2)-, and ABI1-SiL-specific primers. Primers were selected to target exon 3, as it represents a major structural difference between ABI1-SiL and other ABI1 variants. Three sets of primers were designed to analyze the expression of ABI1-SiL in colorectal carcinoma cells and tissues. Transcript variant 12-specific primers were used for qualitative analysis, while transcript variant 1-12 universal (ABI1-U1) and transcript variant 1-11 universal (ABI1-U2) primers were used for quantitative analysis of ABI1-SiL expression. ABI1: Abelson interactor 1; HHR: Homeo-domain homologous region.

primers (forward: 5′-ACACTGGGACGGAATACTCCTTAT-3′; reverse: 5′-CTACCACTGTTTTCTCGACTTCCA-3′) or human ABI1 universal 2 (ABI1-U2) (transcript variant 1-11) primers (forward: 5′-AGTGGCACGAAGAGAGATTGG-3′; reverse: 5′-CTGTGTAATCGATAGGTTTCCGAAT-3′) and SYBR Green Master Mix (Applied Biosystems, Foster City, CA, United States). PCR reactions began with a 10 min incubation at 95 °C for AmpliTaq Gold activation, followed by 40 cycles at 95 °C for 15 s for the denaturation step and 60 °C for 1 min for the primer annealing and extension step. qPCR was performed using the DNA Engine Option 2 System (Bio-Rad, CA, United States). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous housekeeping control for relative quantification of transcription (forward primer: 5′-AACGACCCCTTCATTGAC-3′, reverse primer: 5′-TCCACGACATACTCAGCAC-3′). The percentage of ABI1-SiL among all variants was calculated as $(1\text{-}2^{-[\text{Ct-ABI1-U2-Ct-ABI1-U1}]}) \times 100\%$.

To examine the potential roles of ABI1-SiL overexpression on differentiation and invasion of SW480 cells, a series of additional genetic markers (including POU, CD44, KRT20, MMP1-4, and WAVE2) were also analyzed by qPCR (primers sequences are not shown).

Cell culture and transfection

Lipofectamine-mediated transfection of SW480 cells was performed as previously described[4]. Cells were plated in six-well plates 24 h prior to transfection, and 2 μg of plasmid DNA was used for each transfection. To overexpress ABI1-SiL, a pCMV6 vector specifically coding for ABI1-SiL or a control pCMV6 empty vector was used for stable transfection of SW480 cells or transient transfection of 293T cells. Forty-eight hours after transfection, transfected cells were treated with G418 (500 μg/mL). Individual G418-resistant clones were picked after 3-4 wk. Clones were analyzed by qPCR and Western blot for ABI1-SiL expression, and clones with the highest ABI1-SiL expression were chosen for further studies. Clones 3 and 6 overexpressing ABI1-SiL were named SW480-ABI1-SiL3 and SW480-ABI1-SiL6, respectively. SW480 cells transfected with the empty pCMV6 vector were named SW480-V.

To analyze the subcellular localization of ABI1-SiL in SW480 cells and to test for co-localization with ABI1-p65 and/or WAVE2, a pCMV6 vector encoding a GFP-ABI1-SiL fusion protein was used for both transient and stable transfections. In transient experiments, 48 h after transfection, the 293T cells underwent subcellular localization studies by fixing the cells in 4% paraformaldehyde in phosphate buffer saline (PBS) for 10 min, followed by fluorescence microscopy analysis.

Western blot and co-immunoprecipitation

Western blot analyses were performed as previously described[4]. We used the following antibodies: GAPDH (1:2000 dilution; #CB100999, Proteintech, IL, United States), ABI1-p65 (1:1000 dilution; D147-3, BML, MA, United States), WAVE2 (1:500 dilution; 07-410, Upstate, NY, United States), GFP (1:200 dilution; 1G6; Pregene Co. Beijing, China), and NAP-1 (1:1000 dilution; #12140-1-AP, Proteintech, IL, United States) protein A/G (Sc-2003, Santa Cruz, CA, United States). The protease inhibitor cocktail was purchased from Sigma (St. Louis, MO, United States). Cells were lysed in lysis buffer (20 mmol/L HEPES, pH 7.2; 150 mmol/L NaCl; 1% Triton X-100; and 10% glycerol). Total protein lysates (150 μg each) from all tested cell lines were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to a nitrocellulose membrane, and immunoblotted with appropriate antibodies. Immunoprecipitation analysis was performed as described previously[4]. Briefly, SW480-V and/or SW480-ABI1-SiL6 cells were lysed as described above and incubated with appropriate antibodies bound to sepharose beads. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with appropriate antibodies. Membranes were then washed with TBST buffer and incubated with secondary antibodies conjugated to HRP (horseradish peroxidase) (Jackson ImmunoResearch Laboratories Beijing, China). Bound antibodies were detected using the SuperSignal West Pico Trial Kit (Thermo Scientific, Rockford, IL, United States), and images were acquired using an ImageQuant 350 system (GE Healthcare, Piscataway, NJ, United States) and analyzed using ImageJ software (NIH, Bethesda, MD, United States).

Cell surface area assay

To measure the surface area, all four tested cell lines were routinely cultured and images were captured using a Cannon EOS600D camera. ImageJ was used to measure the cell surface area of 100 cells per condition. The measured values were normalized to the mean value of the SW480 parental cells.

Cell adhesion, migration, and invasion assays

For adhesion assays, 2.5×10^5 cells from each cell line were plated in a six-well plate (2.5 mL/well) coated with or without either 0.2% BSA or 0.1% gelatin, or fibronectin (5 μg/mL) (BD Biosciences, Bedford, MA, United States) and incubated at 37 °C/5% CO_2 for 6 h. Non-adherent cells were removed, and adherent cells were washed three times with 1 mL pre-warmed DMEM medium. To determine the number of adherent cells, the number of adherent cells within five randomly selected microscopic fields was counted in three independent experiments.

Cell migration assays were performed using a 24-well transwell chamber (Corning, New York, NY, United States) with 8.0-μm pore polycarbonate filter inserts, and cell invasion assays were performed using inserts of Transwell plates (8.0-μm pores,

Corning Costar Corp., Cambridge, MA, United States) coated with Matrigel (Costar, Cambridge, MA, United States)[22]. Cells (2.5×10^4 cells/well) suspended in serum-free DMEM containing 0.2% BSA were placed in the upper chamber of each transwell. In each lower chamber, 600 μL of DMEM supplemented with 10% FBS was added. The inserts were then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h (migration) and 48 h (invasion), respectively. Cells that had not penetrated the filters were removed using cotton swabs. Cells that migrated to the bottom of the insert were fixed in 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet for 30 min, followed by rinsing with PBS and examination under a bright-field microscope equipped with a 100 × objective. Migratory or invasive activity was expressed as the average number of migrated cells per microscopic field over five fields in each assay from three independent experiments.

Cell proliferation and apoptosis assays

Cell proliferation assays were performed using Cell Counting Kit-8 (CCK-8; Dojindo, Rockville, MD, United States) according to the manufacturer's instructions. All tested cell lines were seeded at 2.5×10^4 cells per well of a 96-well plate and cultured in 100 μL of DMEM supplemented with 10% FBS. At the indicated time points, culture medium was exchanged for 110 μL of DMEM supplemented with CCK-8 reagent (10 μL CCK-8 and 100 μL DMEM), and cells were incubated for an additional 2 h. Absorbance was measured for each well at a wavelength of 450 nm, with the reference wavelength set at 600 nm. An increase or decrease in absorbance values at 450 nm in the experimental wells relative to the initial value was indicative of cell growth or death, respectively. Cell growth was monitored at 24 h, 48 h, 72 h, and 96 h, and was repeated in replicates of three wells per time point.

Apoptosis assays were performed immediately after selection with G418. Cells were washed twice and seeded at a density of 2×10^5 cells/mL in DMEM and 10% FBS in the presence of G418 (for SW480-V, SW480-ABI1-SiL3, and SW480-ABI1-SiL6). After 48 h of culture, apoptosis was monitored using the Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotech, #KGA107, Shanghai, China). For FACS analysis, 1×10^5 cells were co-stained with Annexin V-EGFP and with the fluorescent dye propidium iodide (PI). Cells that stained negative for both PI and annexin V were considered viable cells; PI-negative and annexin V-positive stained cells were considered early apoptotic cells; PI-positive and annexin V-positive stained cells were considered to be in the later stages of apoptosis. All assays were carried out independently, in triplicate.

Immunofluorescence microscopy

Samples of 293T cells were cultured on fibronectin-coated coverslips and transiently transfected with either the ABI1-SiL-GFP plasmid or a control plasmid, as well as SW480-ABI1-SiL6. The cells were fixed in 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.2% Triton X-100 in PBS for 8 min. Non-specific binding was blocked by incubation of the coverslips with 3% bovine serum albumin in PBS, followed by incubation with primary antibodies (anti-ABI1-p65 and or anti-WAVE2) in 1% bovine serum albumin/PBS. After extensive washing with PBS, cells were incubated with appropriate Alexa-conjugated secondary antibodies. Cytoskeleton was detected by Phalloidin staining. The slides were examined under a Nikon Eclipse TE2000-U fluorescence microscope, and images were captured and analyzed using Nikon NIS Elements imaging software.

Statistical analysis

SPSS version 16.0 software (SPSS Inc., Chicago, IL, United States) was used for statistical analyses. Data from all quantitative assays are expressed as the mean ± SD and were analyzed statistically using one-way analysis of variance (ANOVA) followed by Dunnett test and general linear model analysis (univariate). *P* values less than 0.05 were considered statistically significant.

RESULTS

Establishment of qualitative and quantitative real-time reverse transcriptional polymerase chain reaction assays for detection of ABI1-SiL

The *ABI1* gene encodes a member of the Abelson interactor family of adaptor proteins. At least 12 alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene[21]. There are significant differences in the exons and

functional domains contained in the different isoforms of *ABI1*, which may result in structural differences in the proteins that lead to differing functions between ABI1-SiL and other transcript variants (Figure 1A). To detect the expression of ABI1-SiL, we designed a set of qualitative primers and two sets of universal quantitative primers that detect this isoform specifically, as shown in Figure 1B. Using qualitative PCR, two visible bands were amplified, and we verified through sequencing that the 101-bp band represents the transcript variant-12 specific PCR product (Figure 2A). We demonstrated that ABI1-SiL was expressed in all tested CRC cells and control CRL-1541 cells.

Next, we tested the reliability of the primers designed for quantitative analysis of ABI1-SiL expression. ABI1-SiL and 12 plasmids (single and/or mixtures), as well as the cDNA from CRL-1541 cells and HCT116 cells, were used as templates to perform qPCR. PCR amplified products were examined by 1.5% agarose gel electrophoresis (Figure 2B). Taken with the analysis of the amplification curve, the melting curve, and the sequencing data for the PCR products (data not shown), these observations indicated that we have successfully established a quantitative assay specific for detection of ABI1-SiL.

The proportion of ABI1-SiL isoform is significantly decreased, relative to other ABI1 splice isoforms, in colon cancer tissues and cell lines

In previous studies, we and other groups have shown that the full-length isoform of ABI1 (ABI1-p65) is increased in colon cancer cells and tissues and involved in the progression and development of colon cancer[7,8,22]. To analyze the potential role of ABI1-SiL in colon cancer, we examined the expression level of ABI1-SiL in CRC cells and tissues using qPCR. As Figure 2D and E shows, the ratio of the ABI1-SiL isoform to other ABI1 splice isoforms was significantly decreased in all CRC cells compared with control CRL-1541 cells and was also decreased in 16 of 24 CRC tissues compared with adjacent tissues. These observations indicated that ABI1-SiL may play an anti-oncogenic role in colorectal tissues.

Successful creation of a human colon cancer SW480 cell model stably overexpressing ABI1-SiL

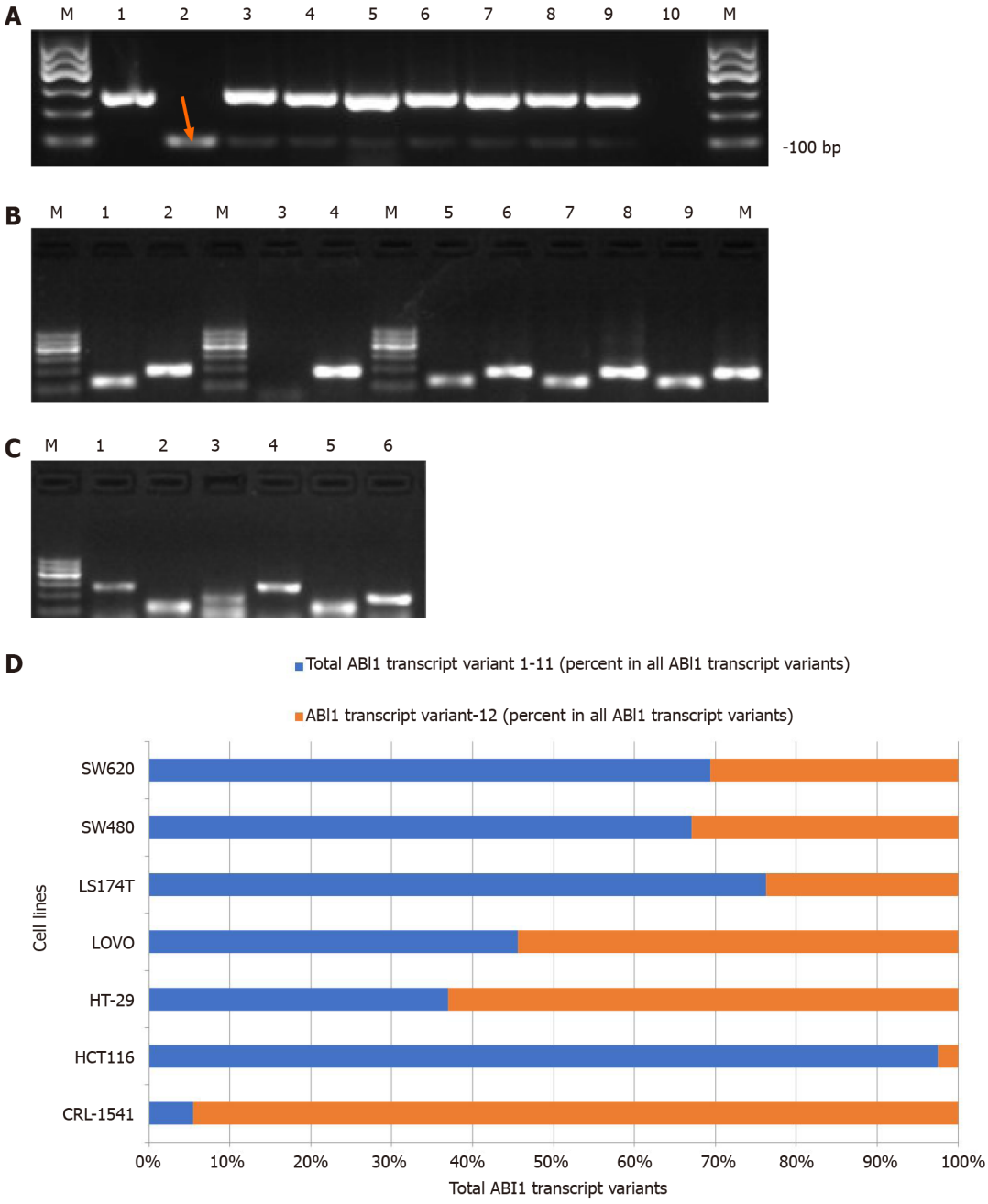
To test the function of ABI1-SiL in cellular phenotypes of colon cancer, we overexpressed ABI1-SiL in SW480 colon cancer cells. The full-length cDNA of ABI1-SiL, or an empty vector control plasmid, was introduced into SW480 by Lipofectamine-mediated transfection. We confirmed ABI1-SiL expression in two stable cell lines, SW480-ABI1-SiL3 and SW480-ABI1-SiL6 (Figure 3A and B). As shown in Figure 3C, the expression of the cDNA in SW480 cells resulted in a significant increase in the proportion of the ABI1-SiL isoform relative to other isoforms by about 30%, as compared to the parental SW480 and SW480-V cell lines. We also demonstrated through Western blot that the ABI1-SiL-GFP fusion protein was expressed in SW480-ABI1-SiL3 and SW480-ABI1-SiL6 cells, but not in parental SW480 and SW480-V cells (Figure 3D). These data confirm that we successfully constructed a SW480 colon cancer cell model overexpressing ABI1-SiL.

Overexpression of ABI1-SiL increases the surface area of human colon cancer SW480 cells

We observed that the SW480-ABI1-SiL3 and SW480-ABI1-SiL6 stable cell lines were substantially larger and were more similar in morphology to epithelial cells than the parental and empty vector transfected cells (Figure 4A-D, see arrows). To quantitatively assess this difference in cellular morphology, Image J and SPSS software were used to examine the cell surface area of the transfected and control cell lines. As Figure 4E shows, the cellular surface area was significantly increased by about 40% in SW480-ABI1-SiL3 and SW480-ABI1-SIL6 cells compared with the parental and control SW480 cells ($P < 0.01$). To determine whether ABI1-SiL-induced changes in cellular morphology are due to effects on differentiation of SW480 cells, we used qPCR to analyze the expression of POUS, CD44, and KRT20, but we observed no difference in the expression of these genes, except for KRT20 in SW480-SiL3 (Figure 4E).

Overexpression of ABI1-SiL inhibits adhesion and migration, but not invasion, of SW480 human colon cancer cells

We performed adhesion, migration, and invasion assays to test whether ABI1-SiL overexpression activated these cellular mechanisms. In the adhesion assay, we discovered that ABI1-SiL overexpression significantly impaired the adhesive


A
M 1 2 3 4 5 6 7 8 9 10 M
-100 bp
B
M 1 2 M 3 4 M 5 6 7 8 9 M
C
M 1 2 3 4 5 6
D
Total ABl1 transcript variant 1-11 (percent in all ABl1 transcript variants)
ABl1 transcript variant-12 (percent in all ABl1 transcript variants)
SW620
SW480
LS174T
LOVO
HT-29
HCT116
CRL-1541
Cell lines
0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
Total ABI1 transcript variants

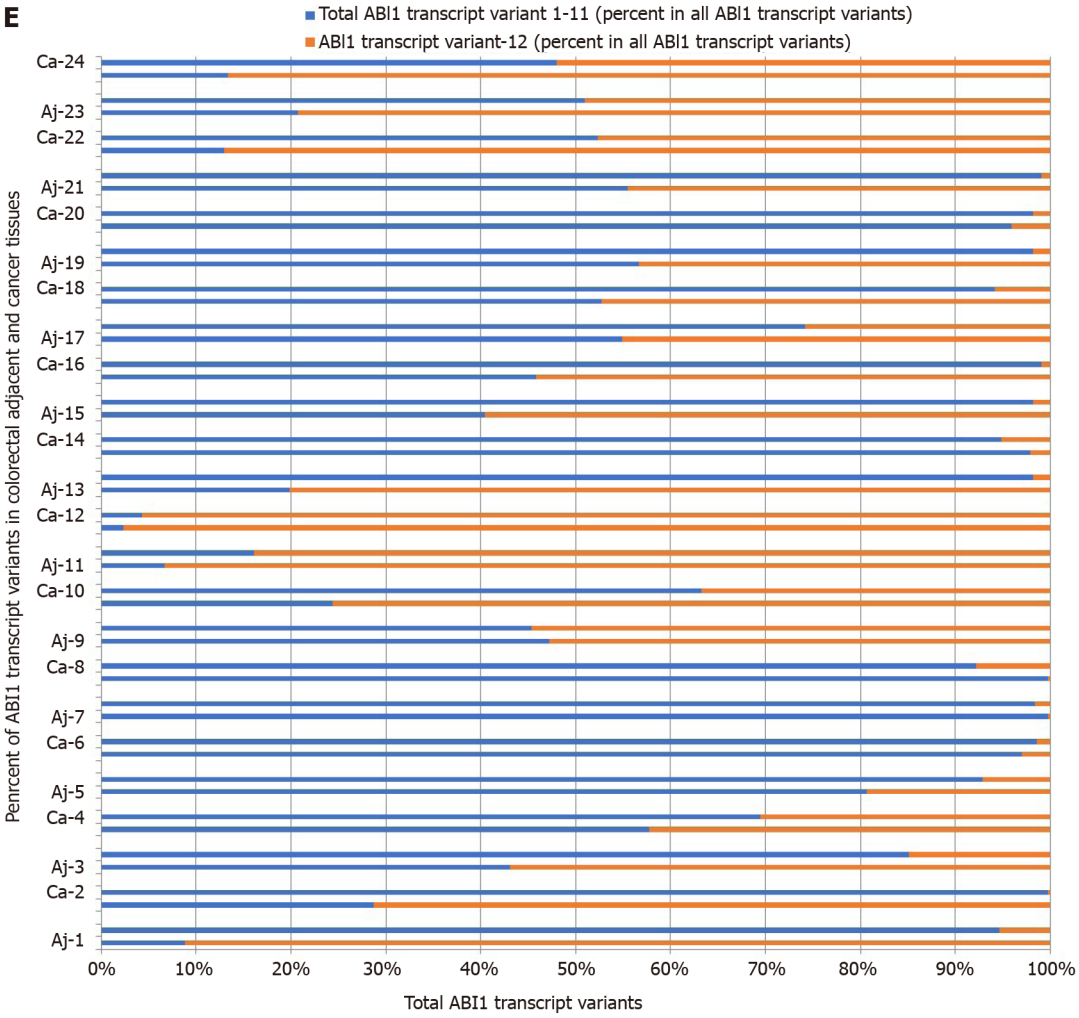


Figure 2 The proportion of Abelson interactor 1 splice isoform-L is significantly decreased in colon cancer tissues and cell lines compared to other Abelson interactor 1 splice isoforms. A: Expression of endogenous Abelson interactor 1 splice isoform-L (ABI1-SiL) in colorectal carcinoma cells. M, 100 bp DNA Marker; 1, ABI1-Tv1 positive Ctrl (278 bp); 2, ABI1-SiL positive control (101 bp, orange arrow); 3, CRL-1541; 4, HCT-116; 5, HT-29; 6, LoVo; 7, LS174T; 8, SW480; 9, SW620; 10, negative control; B: ABI1-Tv1, ABI1-SiL, or a mixture of ABI1-Tv1 and ABI1-SiL were used as templates to confirm the feasibility of the ABI1-SiL quantitative polymerase chain reaction assay using the ABI1-U2 (lanes 1, 3, 5, 7, and 9) and ABI1-U1 primers (lanes 2, 4, 6, 8, and 10); C: ABI1-U1- (lanes 2 and 5) and ABI1-U2-specific (lanes 3 and 6) polymerase chain reaction products amplified from cDNA from CRL-1541 (lanes 1-3) and HCT116 (lanes 4-6) cells. Lanes 1 and 4 contain GAPDH (glyceraldehyde-3-phosphate dehydrogenase); D: The ratio of ABI1-SiL relative to all other ABI1 isoforms was significantly decreased in colorectal carcinoma cells compared with control CRL-1541 cells; E: The ratio of ABI1-SiL relative to other ABI1 isoforms was significantly decreased in 66.7% (16/24) of all tested colorectal tissues compared with adjacent tissues. M: Maker; ABI1: Abelson interactor 1; Aj: Adjacent; Ca: Cancer.

capability in all tested conditions (0.2% BSA, gelatin, fibronectin, and no coating) (P < 0.01, Figure 5A). Among the tested coating conditions, the adhesion assay showed that ABI1-SiL had the strongest inhibitory effect on adhesion in the presence of fibronectin. In the migration assay, ABI1-SiL overexpression also greatly inhibited the migration of SW480 cells *in vitro* (P < 0.01) (Figure 5B-F). Interestingly, ABI1-SiL overexpression showed no inhibitory effect on invasion of SW480 cells (P > 0.05; Figures 5G-K). Furthermore, expression analysis using qPCR revealed that ABI1-SiL overexpression had no effect on expression of matrix metalloproteases 1, 2, 3, or 4 (Figure 5L).

Overexpression of ABI1-SiL does not alter either proliferation or apoptosis of the human colon cancer cell line SW480

It has been reported that the full-length isoform of ABI1 (ABI1-p65) promotes proliferation and inhibits apoptosis of colon cancer cells[24-26], but the action of ABI1-SiL in colon cancer cells is unknown. To further determine the effect of ABI1-SiL overexpression on the proliferation and apoptosis of SW480 cells, we performed CCK-8 cell viability and apoptosis assays. As Figure 6A and B shows, overexpression of ABI1-SiL had no effects on proliferation or apoptosis of SW480 cells. These observations confirmed that changes in migration and invasion did not result from alterations in cell proliferation and apoptosis.

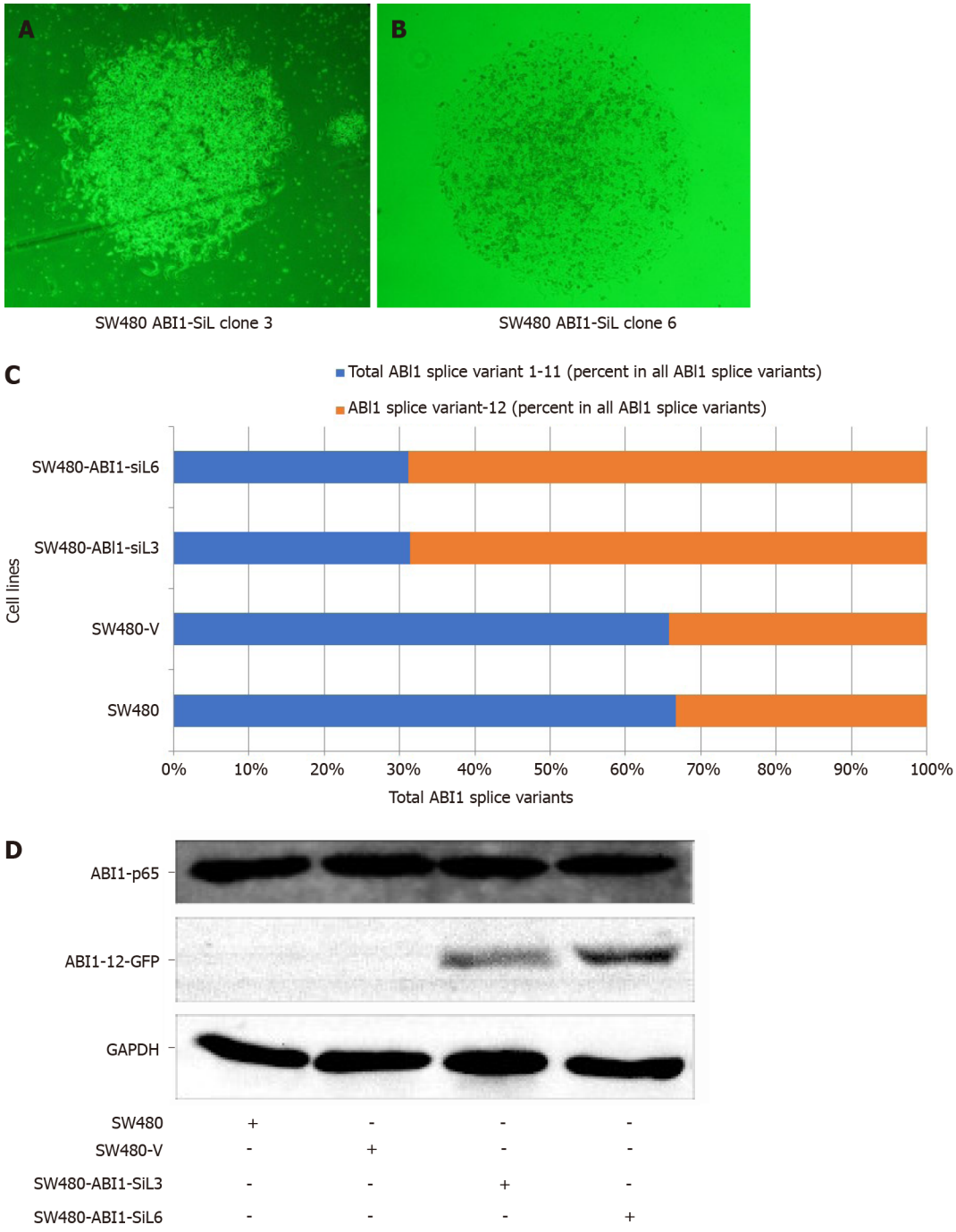
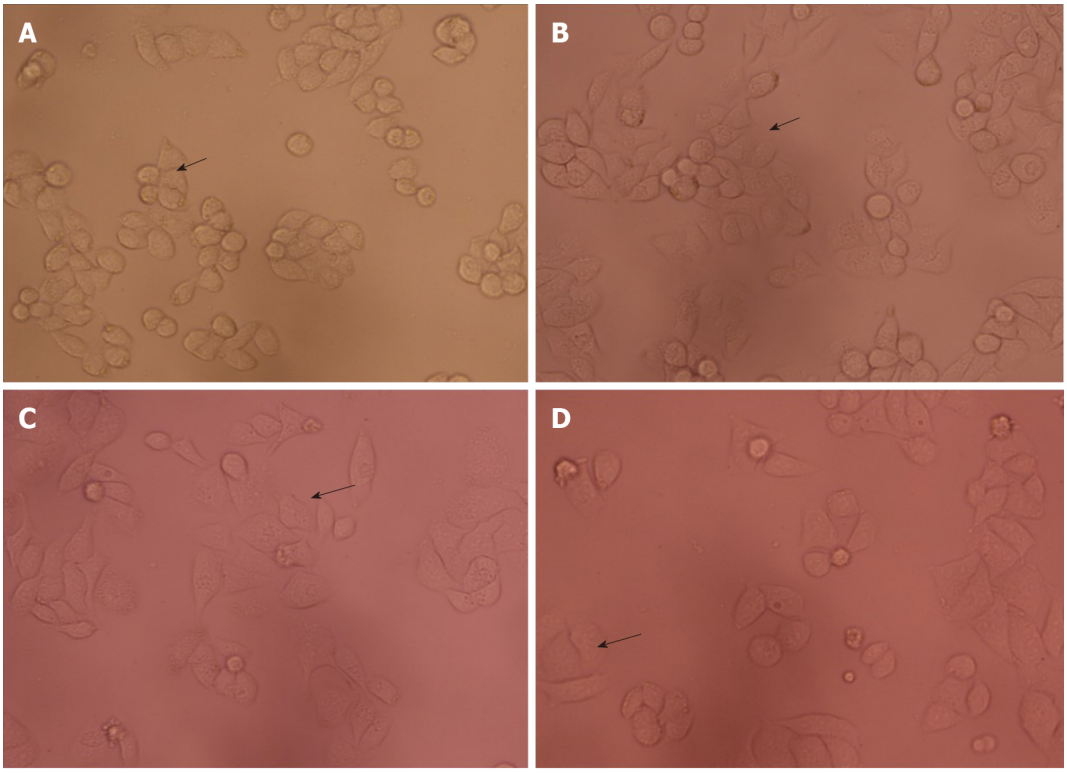
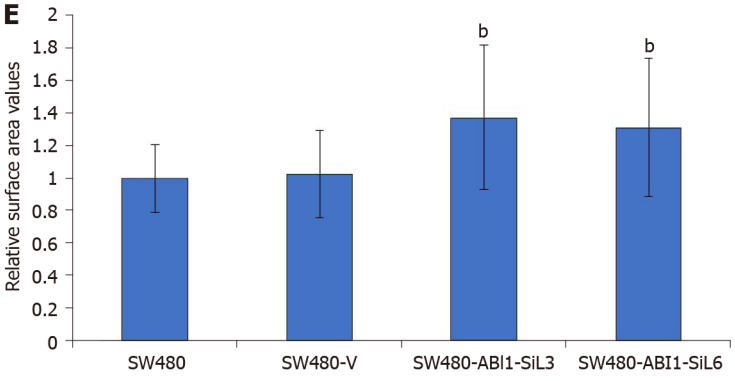


Figure 3 Construction of a human colon cancer SW480 cell model stably overexpressing Abelson interactor 1 splice isoform-L. A: SW480 clone #3 overexpressing Abelson interactor 1 splice isoform-L (ABI1-SiL); B: SW480 clone #6 overexpressing ABI1-SiL; C: The ratio of ABI1-SiL to all Abelson interactor 1 (ABI1) isoforms was determined by quantitative polymerase chain reaction using ABI1 universal 1 and ABI1 universal 2 primers; D: Western blot demonstrating efficacy of ABI1-SiL overexpression. Lysates from 1 × 10^6 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was probed with the indicated antibodies. ABI1-SiL: Abelson interactor 1 splice isoform-L; ABI1: Abelson interactor 1; GFP: Green fluorescence protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Potential mechanism underlying the effects of ABI1-SiL on cell morphology, adhesion, and migration of SW480 cells

It has been widely accepted that ABI1 regulates cancer cell morphology, adhesion, and migration by directly interacting with WAVE2 and forming the ABI1-WAVE2-NAP-1 complex[27-29]. We confirmed the expression of ABI1-p65, WAVE2 and NAP-1 in all tested cell lines. As Figures 3D, 7A, and 7B demonstrate, ABI1-SiL overexpression did not alter the expression of ABI-p65 and WAVE2 at either the mRNA or protein level, as well as NAP-1 at the protein level. The SW480-Vector and SW480-ABI1-SiL6 cell lines were used to analyze potential mechanisms underlying the effect of ABI1-SiL expression on cell morphology, adhesion, and migration of SW480 cells. Western blot





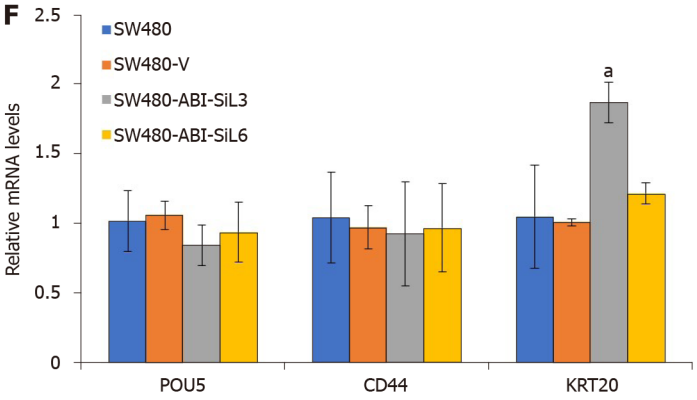
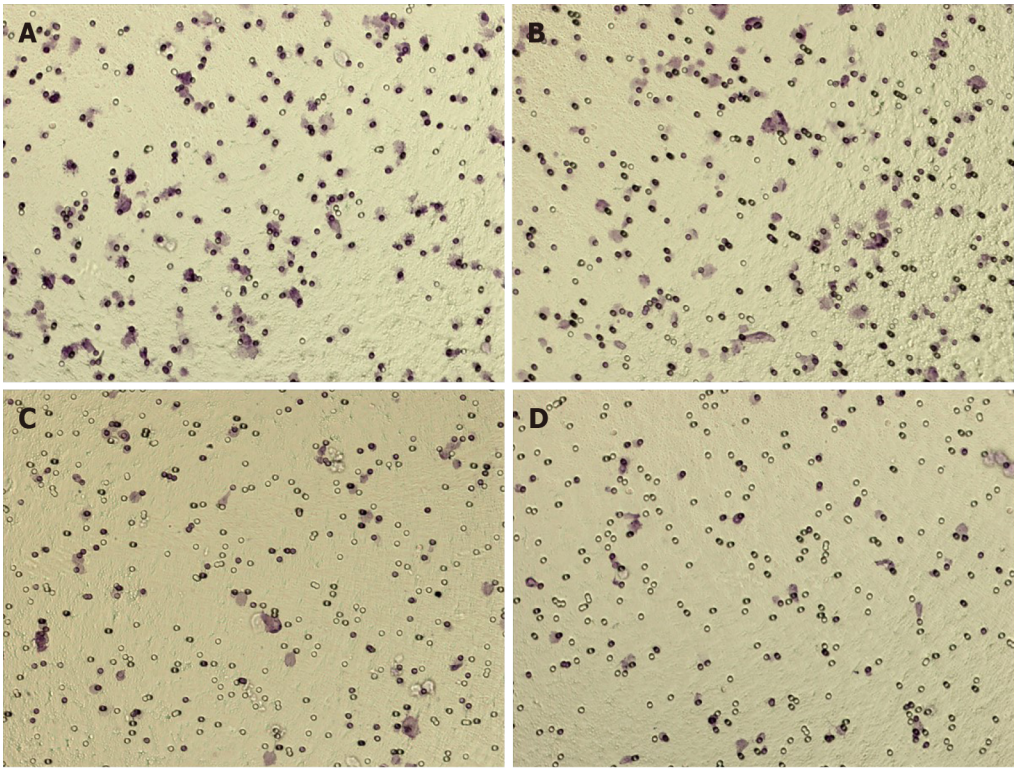
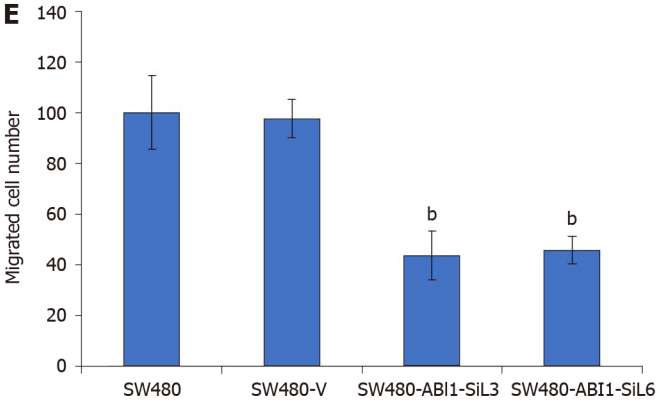
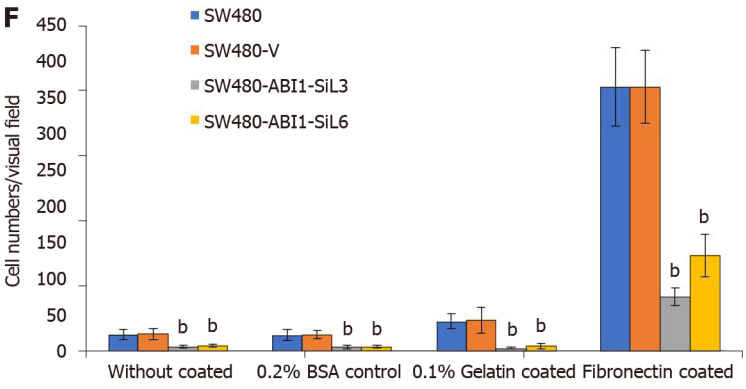


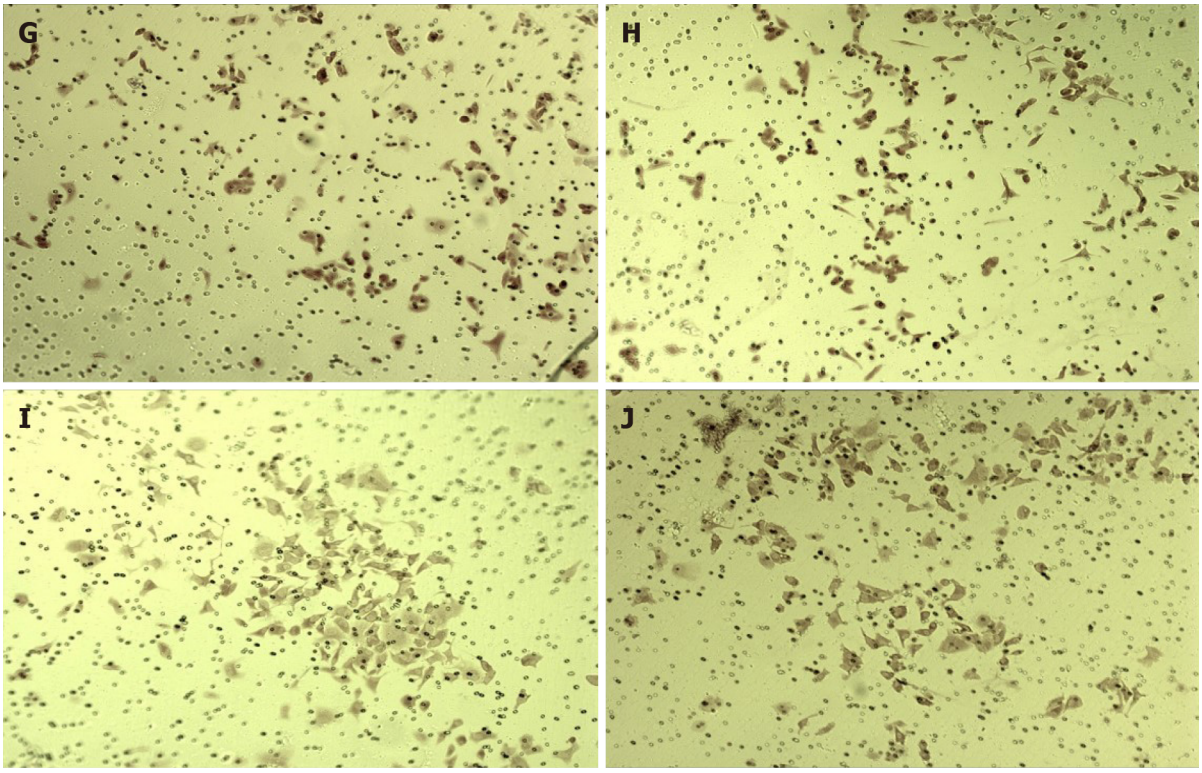
Figure 4 Alterations in cellular morphology occur with exogenous Abelson interactor 1 splice isoform-L expression in SW480 colon cancer cells. A: SW480; B: SW480-V; C: SW480-ABI1-SiL3; D: SW480-ABI1-SiL6 cells; E: Cell surface area measured using ImageJ software. The mean values are normalized to those of parental SW480 cells. Data represent the mean ± SD of triplicate experiments. SW480 cells overexpressing Abelson interactor 1 splice isoform-L (ABI1-SiL) showed a 1.4 folds increase in cell surface area, $^{b}P < 0.01$; F: Expression levels of stem-cell and cell differentiation markers determined by quantitative polymerase chain reaction using gene-specific primers. No significant differences were observed between SW480-ABI1-SiL (3 and 6) cells and parental SW480 cells or SW480-V cells ($P > 0.05$), except for KRT20 in the SW480-ABI1-SiL3 cells ($^{a}P < 0.05$).

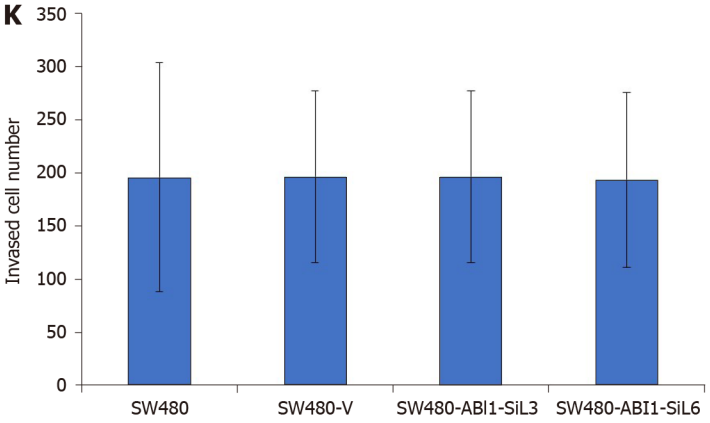
and co-immunoprecipitation (co-IP) revealed that both ABI1-p65 and ABI1-SiL-GFP were able to interact with WAVE2 in SW480-ABI1-SiL6 cells, as determined by successful bidirectional co-IP of ABI1-p65, ABI1-SiL-GFP, and WAVE2 (Figure 7C and


A
B
C
D


E
Migrated cell number
140
120
100
80
60
40
20
0
b
b
SW480
SW480-V
SW480-ABI1-SiL3
SW480-ABI1-SiL6


F
Cell numbers/visual field
450
400
350
300
250
200
150
100
50
0
SW480
SW480-V
SW480-ABI1-SiL3
SW480-ABI1-SiL6
b
Without coated
0.2% BSA control
0.1% Gelatin coated
Fibronectin coated





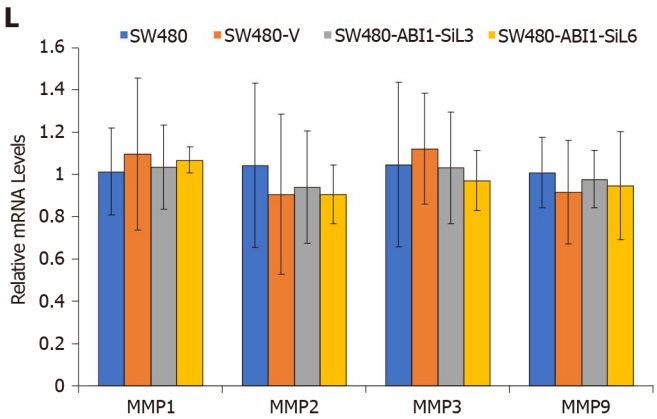


Figure 5 Overexpression of Abelson interactor 1 splice isoform-L inhibits migration and adhesion, but not invasion, of SW480 human colon cancer cells. A-F: Effect of Abelson interactor 1 splice isoform-L (ABI1-SiL) overexpression on migration of SW480 cells *in vitro*. A migration assay was conducted over 24 h with 2.5 × 10^4 cells using a transwell chamber. Cells on the lower surface of the filter were imaged. SW480 (A), SW480-V (B), SW480-ABI1-SiL3 (C), and SW480-ABI1-SiL6 (D) were used. The number of migrating cells that penetrated the transwell chamber is expressed as the mean number of cells from five random fields. Representative results from one experiment of three are shown (E). The effects of ABI1-SiL overexpression on SW480 cell adhesion on 0.2% BSA, 0.1% gelatin, or fibronectin-coated surfaces were assessed (F). All tested cell lines were cultured in 0.2% BSA, 0.1% gelatin, or 5 μg/mL fibronectin-coated six-well plates (2.5 × 10^5 cells per well) for 6 h. The total number of cells that were adherent in each condition were counted. The vertical axis shows the number of the

adherent cells per field (from five random fields) and is expressed as the mean ± SD of triplicate wells; G-L: Effect of ABI1-SiL overexpression on invasion of SW480 cells *in vitro*. An invasion assay was conducted over 48 h with 2.5 × 10 ^{4} cells using Matrigel filters. Results shown here are for one representative experiment. Cells on the lower surface of the filter were imaged. SW480 (G), SW480-V (H), SW480-ABI1-SiL3 (I), and SW480-ABI1-SiL6 (J) were used. The number of migrated cells that penetrated through Matrigel-coated filters is expressed as the mean number of cells in five random fields (K). ABI1-SiL overexpression in SW480 cells has no effect on the mRNA expression of MMPs (L). $^aP < 0.05$ and $^bP < 0.01$ indicate significant differences between SW480, SW480-V, SW480-ABI1-SiL3, and SW480-ABI1-SiL6 cells. ABI1-SiL: Abelson interactor 1 splice isoform-L.

D). Interestingly, we also observed evidence of a direct interaction between ABI1-p65, phosphorylated ABI1-p72, and ABI1-SiL-GFP (Figure 7D). Additionally, we showed that ABI1-SiL does not bind to NAP-1, but ABI1-p65 and phosphorylated ABI1-p72 do in SW480-ABI1-SiL6 cells (Figure 7E). Finally, 293T cells, transfected with ABI1-SiL-GFP and stable SW480-ABI1-SiL6, were used to examine the co-localization of ABI1-SiL-GFP and WAVE2 or ABI1-p65. As shown in Figures 8 and 9, ABI1-SiL co-localizes with both WAVE2 and ABI1-p65. These observations suggested that the ABI1-SiL isoform inhibits the adhesion and migration of SW480 cells *in vitro* by competitively interacting with WAVE2 and directly binding to ABI1-p65. These results provide a novel molecular mechanism explaining the functions of distinct ABI1 isoforms in CRC cells.

DISCUSSION

In this study, we have identified a novel function for ABl1-SiL in adhesion and migration of colon cancer SW480 cells and propose a mechanism underlying this function. Our model is based on a combination of gene expression and mechanistic studies of ABI1-SiL in colon cancer cells and tissues. We and other groups have previously shown that overexpressed non-phosphorylated ABI1-p65 functions as a pro-oncogene in development and progression of CRC[7,8,22]. Now, we wanted to address the question of whether there are other ABI1 isoforms that exist in colon tissues and cells that play additional roles in colon cancer. Identification of novel alternatively spliced isoforms with dysregulated expression in cancer could lead to the development of tumor-specific molecular targets for prognosis and therapy[30].

At present, there is no commercial antibody available that can detect the ABI1-SiL protein. Here, we report a set of PCR-based methods that can specifically measure ABI1-SiL mRNA expression. Unlike the full-length ABI1-p65 isoform, ABI1-SiL was significantly decreased in colorectal tissues and cells compared with the adjacent tissues and CRL-1541 normal colon cells (Figure 2). This observation, coupled with the structural differences between ABI1-SiL and other isoforms, especially ABI1-p65 (Figure 1A), led us to postulate that ABI1-SiL might function as an anti-oncogene.

ABI1-SiL mRNA expression was significantly decreased in SW480 cells, so we selected this cell line to investigate the effects of ABI1-SiL expression on cellular phenotypes in colon cancer cells. We successfully constructed SW480 cell lines that stably overexpress ABI1-SiL. We observed that ABI1-SiL overexpression in SW480 cells resulted in enlarged cell size and impaired adhesion and migration, but had no effect on invasion, proliferation, apoptosis, or transcriptional regulation (Figures 3-6). ABI1-SiL overexpression did not alter the mRNA expression levels of ABI1-p65, WAVE2, MMPs, POU, CD44, or KRT20. In combination with its decresion in CRC tissues and cells, these observations suggest that ABI1-SiL could function as an anti-oncogene and that the underlying mechanism needs to be explored.

It is widely accepted that an ABI1-WAVE2 complex is involved in epithelial morphogenesis[31], adhesion, migration, and invasion of cancer cells[13,28,32]. As shown in Figure 1A, the conserved WAVE2-binding and SH3 domains of ABI1-SiL provide the same sites as ABI1-p65 to competitively bind to WAVE2 and/or the proline-rich domain (PRD) of other molecules, including the PRD of ABI1-p65, but the lack of the HHR and the majority of the PxxP- and Pro-rich domain makes it likely to function as a native dominant negative that suppresses the pro-oncogenic functions of ABI1-p65. Bidirectional co-IP experiments confirmed interactions between ABI1-SiL, WAVE2, and ABI1-p65. In control SW480 cells, ABI1-p65 interacts with WAVE2, while in SW480 ABI1-SiL6 cells, both ABI1-p65 and ABI1-SiL are able to bind to WAVE2 (Figure 7C and D). These observations suggested a possible mechanism by which ABI1-SiL competitively binds to WAVE2. Increased ABI-SIL expression could inhibit ABI1-p65-WAVE2 complex formation, resulting in reduced adhesion and migration. Competitive binding of WAVE2 to ABI1 isoforms suggests the possibility of fine

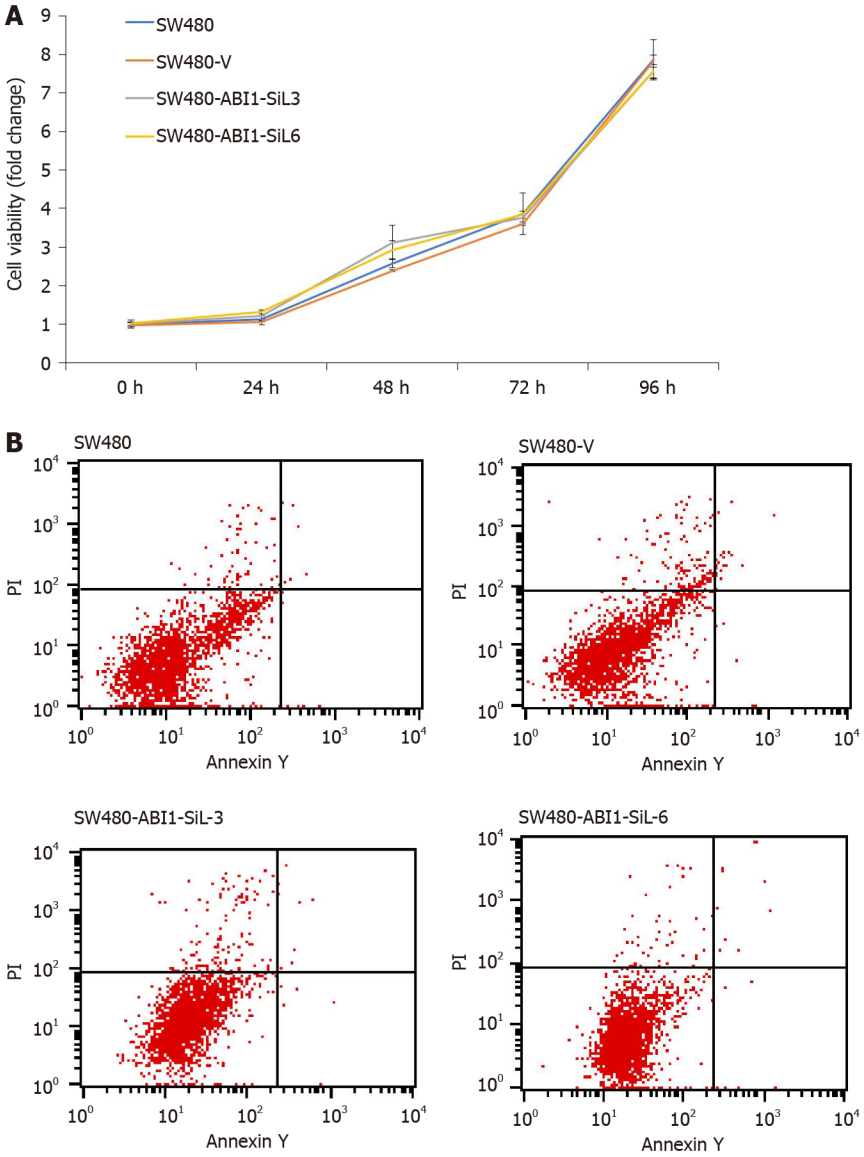


Figure 6 Overexpression of Abelson interactor 1 splice isoform-L does not alter proliferation or death of SW480 cells. Abelson interactor 1 splice isoform-L (ABI1-SiL) overexpression did not impair the proliferation of SW480 cells *in vitro*. Proliferative assay was performed with a Cell Counting Kit-8. A: Cell viability (fold change) is shown in the vertical axis as the mean ± SD of triplicate wells (n = 3). ABI1-SiL overexpression did not induce the apoptosis of SW480 cells *in vitro*; B: Cell apoptosis was assessed by Annexin-V assay. ABI1-SiL: Abelson interactor 1 splice isoform-L.

regulation of WAVE2 complex function in an ABI1 isoform-dependent manner. Interestingly, we also found that ABI1-SiL might interact directly with ABI1-p65, thus indirectly decreasing the formation of the ABI1-p65-WAVE2 complex. These intermolecular interactions were further confirmed by the co-localization of ABI1-SiL-GFP with WAVE2 and ABI1-p65 (Figures 8 and 9). WAVE2, ABI1, and NAP-1 are required for the WAVE regulatory complex (WRC) which activates the Arp2/3 complex to control branched actin polymerization in response to Rac activation[33-35]. As Figure 7E shows, the fact that ABI1-SiL (lacks a HHR domain required for binding to Nap1) does not bind to NAP-1, but ABI1-p65 and phosphorylated ABI1-p72 do in SW480-ABI1-SiL6 cells, suggests that ABI1-SiL overexpression disturbed WRC assembly and further impaired SW480 adhesion and migration. Additionally, all of the above observations were also observed in leukemia K562 and gastric cancer NCI-N87 cells (data not shown).

The present results indicate possible functions of ABI1-SiL in the regulation of SW480 morphogenesis, adhesion, and migration through interactions with WAVE2 and ABI1-p65, respectively. Although the overexpression of ABI1-SiL results in increased cell surface area and a more epithelial-like morphology, we did not find alterations in expression levels of any mRNA involved in differentiation of SW480 cells. Future studies will explore correlations between ABI1 splicing-isoform expression and *KRAS* or *BRAF* mutations or *MSI* in patients with colon cancer.

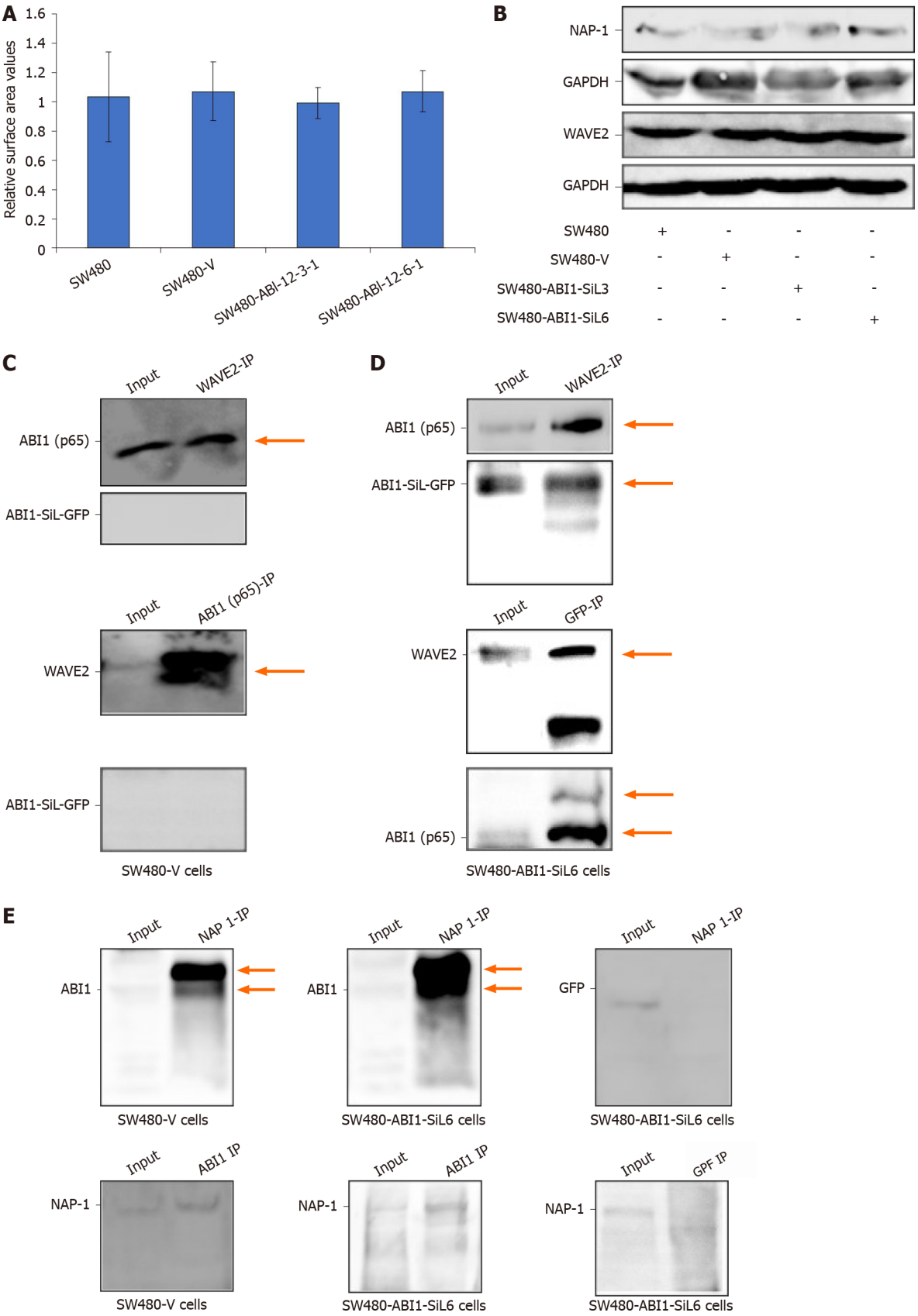


Figure 7 Abelson interactor 1 splice isoform-L interacts with WAVE2, phosphorylated or non-phosphorylated Abelson interactor 1-p65, and NAP-1 in SW480 cells. A and B: Abelson interactor 1 splice isoform-L (ABI1-SiL) overexpression does not change the mRNA (A) or protein (B) expression of WAVE2; C and D: Co-immunoprecipitates (IP) were subjected to Western blot using the indicated antibodies. Bidirectional co-IP and Western blot analysis show that both ABI1-p65 and ABI1-SiL bind to WAVE2 in SW480-V (C) and SW480-ABI1-SiL6 cells (D); E: ABI1-SiL interacts with phosphorylated and non-phosphorylated ABI1-p65 in SW480 cells (arrows). ABI1-SiL does not bind to NAP-1, but ABI1-p65 does. ABI1-SiL: Abelson interactor 1 splice isoform-L; ABI1: Abelson interactor 1; GFP: Green fluorescence protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

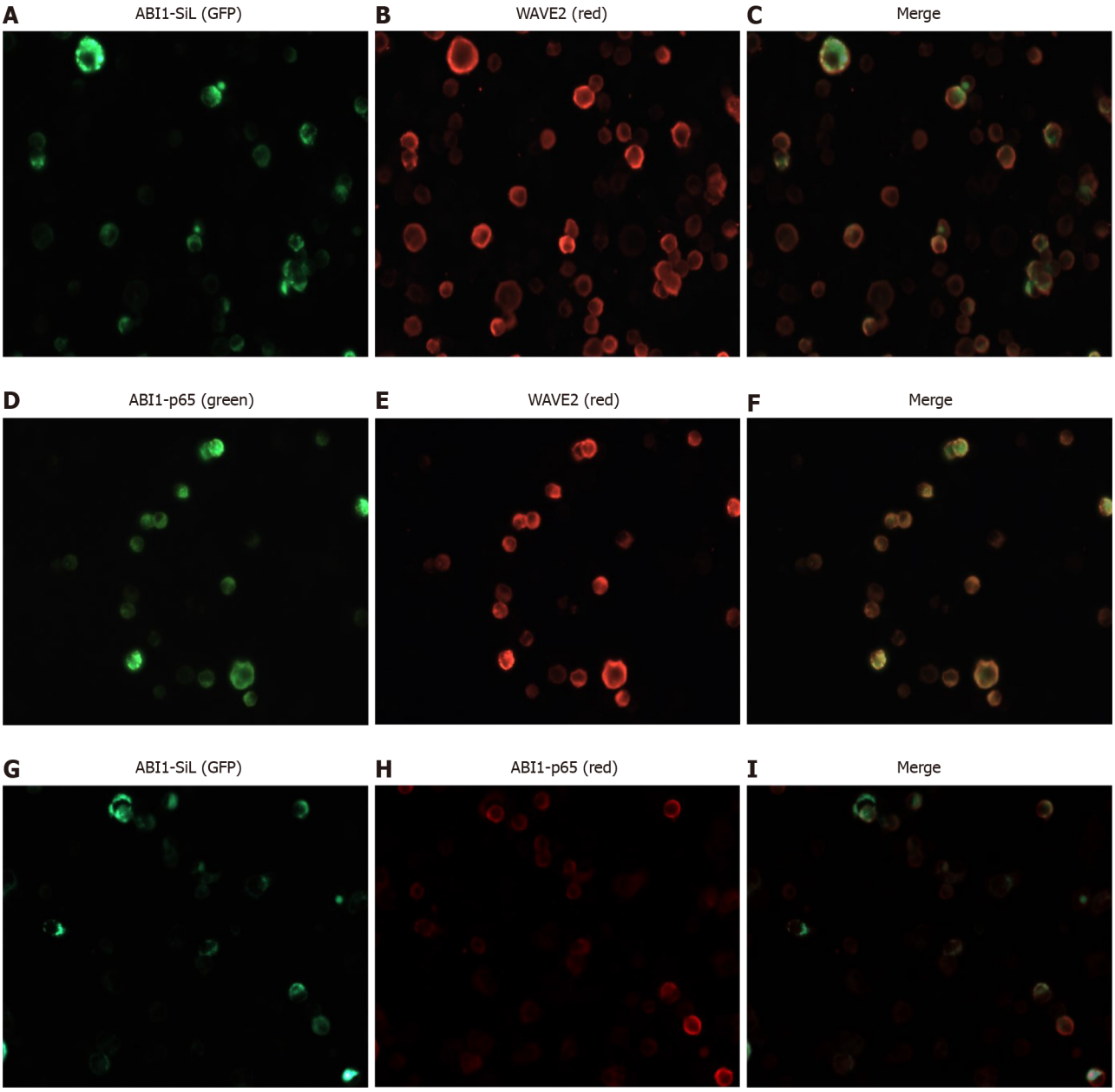


Figure 8 Abelson interactor 1 splice isoform-L co-localizes with WAVE2 and Abelson interactor 1-p65 in 293T cells. A-I: Abelson interactor 1 splice isoform-L-GFP (green fluorescence protein) co-localizes with WAVE2 and ABI1-p65. Samples of 293T cells transiently transfected with Abelson interactor 1 splice isoform-L were probed with WAVE2 (A, B, and C) and Abelson interactor 1-p65 (G, H, and I) antibodies, respectively, while 293T cells were probed for WAVE2 and Abelson interactor 1-p65 (D, E, and F). Magnification × 400. ABI1-SiL: Abelson interactor 1 splice isoform-L; ABI1: Abelson interactor 1; GFP: Green fluorescence protein.

Similarly, relationships and underlying mechanisms will be investigated to connect ABI1-SiL overexpression with mesenchymal-epithelial transition, and determine the roles of other ABI1 isoforms in the cellular and molecular phenotypes underlying colon cancer. Furthermore, ABI1 is present in several intrinsic protein complexes that regulate actin cytoskeletal remodeling, and the existence of several ABI1 isoforms raises the possibility of isoform-specific roles in other ABI1-specific functions[36]. In conclusion, we have shown for the first time herein that ABI1-SiL regulates adhesion and migration in SW480 cells *via* a mechanism involving fine regulation of WAVE2 complex activity, providing a putative biomarker and novel therapeutic target for colon cancer.

In addition, there are still some other limitations in the current study. First, we need more specific and accurate quantitative methods[37,38] to investigate the expression pattern of ABI1-SiL in a large-scale population in the future, and evaluate its potential application value as a clinical molecular diagnostic marker. Second, we need to systematically analyze the exon sequences of all ABI1 transcriptional variants, and use

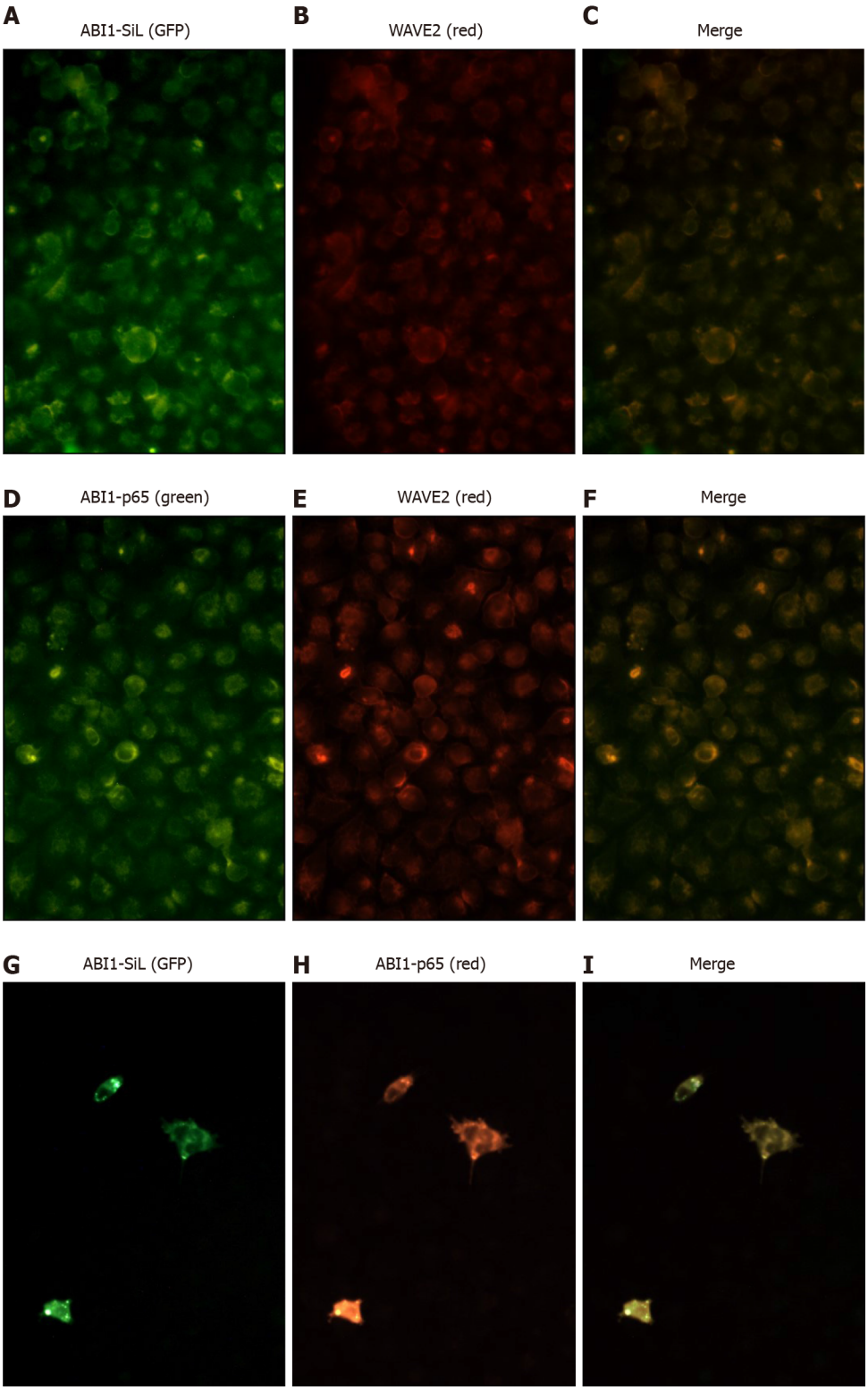


Figure 9 Abelson interactor 1 splice isoform-L co-localizes with WAVE2 and Abelson interactor 1-p65 in SW480-ABI1-SiL6 cells. A-I: Abelson interactor 1 splice isoform-L (ABI1-SiL)-GFP (green fluorescence protein) co-localizes with WAVE2 and ABI1-p65. SW480-ABI1-SiL6 cells were probed with WAVE2 (A, B, and C) and ABI1-p65 (G, H, and I) antibodies, while SW480 cells were similarly probed for WAVE2 and ABI1-p65 (D, E, and F). Magnification × 400. ABI1-SiL: Abelson interactor 1 splice isoform-L; ABI1: Abelson interactor 1; GFP: Green fluorescence protein.

splice switching oligonucleotides[39] and/or gene modification techniques[40] to explore clinical interventions targeting ABI1-SiL. Finally, we also need to rely on animal models to further verify the underlying mechanism of the anti-oncogenic role of ABI1-SiL.

CONCLUSION

These results support a model in which ABI1-SiL plays an anti-oncogenic role by competitively binding to WAVE2 and directly interacting with phosphorylated and non-phosphorylated ABI1-p65, functioning as a dominant-negative form of ABI1-p65.

ARTICLE HIGHLIGHTS

Research background

Abnormally expressed and/or phosphorylated Abelson interactor 1 (ABI1) participates in the metastasis and progression of colorectal carcinoma (CRC), but the roles of ABI1 splice isoforms (ABI1-SIs) are unknown. Exploring the roles and underlying mechanism of ABI1-SIs has important theoretical and practical significance for molecular diagnosis and targeted therapy of CRC.

Research motivation

ABI1-SIs-L (ABI1-SiL) plays an anti-oncogenic role in CRC, and it may be a potential molecular marker and therapeutic target in CRC.

Research objectives

To exploring the roles and underlying mechanism of ABI1-SiL in CRC.

Research methods

We report a set of polymerase chain reaction-based methods that can specifically measure ABI1-SiL mRNA expression and constructed an ABI-SiL overexpressing SW480 cell model. CCK8, transwell, immunoprecipitation, Western blot, and co-localization assays were used to identify the anti-oncogenic role of ABI-SiL and the underlying mechanism in CRC.

Research results

ABI-SiL had a potential anti-tumor effect in CRC. Overexpression of ABI-SiL did not affect the proliferation and invasion of SW480 cells, but can increase the surface area and migration of SW480 cells by competitively binding WAVE2 and ABI1-p65.

Research conclusions

ABI1-SiL plays an antioncogenic role in CRC cells by competitively binding WAVE2 and ABI1-p65.

Research perspectives

Targeting ABI1-SIL expression is a potential treatment for colorectal cancer metastasis.

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ORIGINAL ARTICLE

Basic Study

Effects of CXCL12 isoforms in a pancreatic pre-tumour cellular model: Microarray analysis

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Abstract

BACKGROUND

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of death among cancers, it is characterized by poor prognosis and strong chemoresistance. In the PDAC microenvironment, stromal cells release different extracellular components, including CXCL12. The CXCL12 is a chemokine promoting the communication between tumour and stromal cells. Six different splicing isoforms of CXCL12 are known (α, β, γ, δ, ε, θ) but their role in PDAC has not yet been characterized.

AIM

To investigate the specific role of α, β, and γ CXCL12 isoforms in PDAC onset.

METHODS

We used hTERT-HPNE E6/E7/KRasG12D (Human Pancreatic Nestin-Expressing) cell line as a pancreatic pre-tumour model and exposed it to the α, β, and γ CXCL12 isoforms. The altered expression profiles were assessed by microarray analyses and confirmed by Real-Time polymerase chain reaction. The functional enrichment analyses have been performed by Enrichr tool to highlight Gene Ontology enriched terms. In addition, wound healing assays have been carried out to assess the phenotypic changes, in terms of migration ability, induced by the α, β, and γ CXCL12 isoforms.

RESULTS

Microarray analysis of hTERT-HPNE cells treated with the three different CXCL12 isoforms highlighted that the expression of only a few genes was altered. Moreover, the α and β isoforms showed an alteration in expression of different genes, whereas γ isoform affected the expression of genes also common with α and β isoforms. The β isoform altered the expression of genes mainly involved in cell cycle regulation. In addition, all isoforms affected the expression of genes

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associated to cell migration, adhesion and cytoskeleton. In vitro cell migration assay confirmed that CXCL12 enhanced the migration ability of hTERT-HPNE cells. Among the CXCL12 splicing isoforms, the γ isoform showed higher induction of migration than α and β isoforms.

CONCLUSION
Our data suggests an involvement and different roles of CXCL12 isoforms in PDAC onset. However, more investigations are needed to confirm these preliminary observations.

Key Words: CXCL12; Splicing isoforms; Pancreatic cancer; Microarray; Migration; Wound healing assay

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Core Tip: In the microenvironment of pancreatic ductal adenocarcinoma (PDAC), stromal cells release different extracellular components, including CXCL12, in order to communicate with cancer cells. Here, we investigated the specific role of α, β, and γ CXCL12 splicing isoforms in PDAC onset, by using a pre-tumour model. Microarray analysis suggested a role of CXCL12 in cell migration, and wound healing assays confirmed this hypothesis. In particular, γ isoform showed the highest promotion of migration. Our results shed light on the molecular basis of PDAC onset and progression.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive gastrointestinal tumours with a 5-year survival rate of 7%[1]. Due to the lack of early symptoms and specific diagnostic markers of early-stage disease, PDAC is often diagnosed at an advanced stage. At the time of diagnosis, the 90% of patients already presents advanced tumour progression and distant metastasis, therefore the surgical treatment is no longer applicable. This tumour also shows a high resistance rate to anti-tumour drugs, so chemio- and radio-therapy are not effective[2,3]. Progressive accumulation of genetic mutations, causing activation of different signalling pathways, drives tumour growth and development[4]. The earliest genetic event in the progression of the normal ductal epithelial cells to premalignant pancreatic intraepithelial neoplasia (PanIN) is the mutation of the K-Ras oncogene[5]. The activation of K-Ras protein triggers various downstream effector proteins which promote proliferation, metabolic reprogramming, anti-apoptosis, evasion of the immune response and remodelling of the microenvironment[6]. Recently, increasing interest has been focused on the PDAC microenvironment. The PDAC microenvironment is composed of a large portion of stroma surrounding cancer cells and contains cells such as cancer-associated fibroblasts (CAFs), T cells, stellate cells, macrophages, regulatory T cells, endothelial cells and others[7-9]. To maintain a favourable microenvironment for cancer cell survival, stromal cells secrete extracellular components, such as extracellular matrix (ECM), matrix metallo-proteinases, growth factors, transformation growth factor-β and cytokines.

One of the above-mentioned molecules is the chemokine CXCL12 (C-X-C motif chemokine ligand 12), a low molecular weight protein (about 12 kDa), belonging to the CXC chemokine family. In PDAC, CXCL12 is a key messenger in the intercellular communication between tumour and stromal cells[10]. In fact, CXCL12 promotes tumour proliferation, epithelial to mesenchymal transition, metastases, angiogenesis and immunosurveillance[10,11]. In PDAC setting, it acts through highly expressed

receptors namely CXCR4 and CXCR7[11]. Although six different CXCL12 splicing variants have been described (α, β, γ, δ, ε, θ), only some properties of α, β and γ isoforms are known[12]. The sequence of the isoforms differ significantly one from another, thus also the function of the isoforms which are not yet characterized could be different or even contrary[12,13]. In breast cancer the role of the isoforms α, β, γ, and to a lesser extent δ, was studied[14,15], whereas in PDAC no information is available about the specific role played by each isoform of CXCL12. Moreover, often the papers focusing on CXCL12 role in PDAC actually report the results without stating which isoform in particular is under investigation[12].

The hTERT-HPNE E6/E7/KRasG12D (Human Pancreatic Nestin-Expressing) is a pancreatic pre-tumour cell line. These cells derive from normal pancreas duct epithelial cells and harbour the classical KRas (G12D) mutation, furthermore it bears inactivated p53 and Rb tumour suppressor genes. This status mimics the cells that have acquired the cancer predisposing mutations, but are not yet fully transformed. The phenotype of this cell line is similar to the third phase of the premalignant lesions, called PanIN-3[5,6,16].

Here, we investigated the transcriptomic alterations induced by treatments with the α, β and γ CXCL12 isoforms (the only ones commercially available) in a pancreatic pre-cancerous model in order to study PDAC onset. To this aim, we treated hTERT-HPNE cells with different CXCL12 isoforms, and assessed the gene expression profiles of these cells by microarray analyses. Since microarray analyses indicated several deregulated genes involved in cell migration, we also performed wound healing assays.

MATERIALS AND METHODS

Cell culture

The human pancreatic pre-tumour cell line hTERT-HPNE E6/E7/K-RasG12D (ATCC® CRL-4038™) was purchased from American Type Culture Collection. These cells are adherent and have an epithelial-like morphology. Furthermore, they lack fundamental features of malignant pancreatic cancer cells, such as the anchorage independent growth in soft agar and the ability to engraft as tumours in athymic mice. This cell line was developed from human pancreatic duct cells by transduction of human telomerase gene (*hTERT*) in order to escape from cellular senescence. Further immortalization steps included the introduction of the human papillomavirus 16 E6 and E7 proteins, which are able to impair the function of key tumour suppressors p53 and Rb. In these cells, the KRAS *G12D* mutation is sufficient to induce formation of PanIN lesions, without inducing a fully malignant phenotype. Cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (Lonza, Milan, Italy) supplemented with 10% fetal bovine serum, 1% L-Glutamine and 1% Penicillin/Streptomycin (EuroClone, Milan, Italy). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cell treatments

A total of 2 × 10^6 hTERT-HPNE cells were seeded in p100 dishes for cell treatments. Once cells reached the right confluence, they were treated for 24 h with 100 ng/mL of each of the following isoforms: Human recombinant CXCL12-α (PHC1346, Thermo Fisher Scientific, Milan, Italy), human recombinant CXCL12-β (2716-SD, R&D Systems) and human recombinant CXCL12-γ (6448-SD-025, R&D Systems, Minneapolis, MN, United States). Cells cultured similarly for 24 h have been used as control. All experiments were carried out in biological triplicates.

RNA isolation and cDNA synthesis

Total RNA was isolated from harvested cells (12 samples in total) using the RNeasy Protect Cell Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. The RNA concentration in isolated samples was determined by ultraviolet absorption at 260 nm. The quality of total RNA was first assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, United States). Biotin-labelled cDNA targets were synthesized starting from 150 ng of total RNA. Double stranded cDNA synthesis and related cRNA was performed with GeneChip® WT Plus Kit (Affymetrix, Thermo Fisher Scientific, Milan, Italy). Same kit was used to synthesize the sense strand cDNA before fragmenting and labelling. All steps of the labelling protocol were performed as suggested by Affymetrix, starting from 5.5 μg of ssDNA.

DNA microarray hybridization and image acquisition

Hybridization was performed using the GeneChip® Hybridization, Wash and Stain Kit (Affymetrix, Thermo Fisher Scientific, Milan, Italy). In particular, fragmented and labelled sscDNA were diluted in hybridization buffer at a concentration of 23 ng/μL for a 2.3 μg total and denatured at 99 °C for 5 min, incubated at 45 °C for 5 min and centrifuged at maximum speed for 1 min prior to introduction into the GeneChip® cartridge. A single GeneChip® Human Clariom S was then hybridized with each biotin-labelled sense target (12 samples in total). Hybridizations were performed for 16 h at 45 °C in a rotisserie oven (60 rpm). GeneChip® cartridges were washed and stained with GeneChip® Hybridization, Wash and Stain Kit in the Affymetrix Fluidics Station. GeneChip arrays were scanned using an Affymetrix GeneChip® Scanner 3000 7G using default parameters. Affymetrix GeneChip® Command Console software was used to acquire GeneChip® images and generate .DAT and .CEL files, which were used for subsequent analysis.

Bioinformatics analysis of microarray data

Analyses of the raw expression data (probe-level .CEL files) were carried out within R/Bioconductor environment (version 3.6 and 3.9, respectively). In particular, we applied the background correction and quantile normalization by employing the Robust Multi-array Average method available in the *oligo* R package[17]. Then, ComBat function from *sva* R package[18] was used to remove potential batch effects. Principal component analysis was used for the data quality control, before and after batch effect correction. For differential expression analysis, *genefilter* and *limma* R packages have been used[19]. In particular, for the identification of differentially expressed genes (DEGs), we considered the Benjamini-Hochberg corrected *P* value cut-off (FDR), set at 0.05, and a $\log_2$ Fold Change $|\log_2 FC| > 0.7$.

Functional and pathway enrichment analyses

Enrichr tool (http://amp.pharm.mssm.edu/Enrichr/)[20] was used to perform functional enrichment analysis of the DEGs, *i.e.*, the identification of the most over-represented (enriched) Gene Ontology (GO) terms. In this tool, we selected the panels GO Biological Process, GO Molecular Function and GO Cellular Component. Only statistically significant results are reported ($P < 0.05$).

Real-Time polymerase chain reaction

To confirm the microarray results, we assessed the expression of some DEGs by Real-Time polymerase chain reaction (PCR). We selected six genes (CXCL8, RRP12, ADH1B, UBA7, FEM1C, and CASP1) based on the commonly used criteria, that is their fold-changes in relative expression and *P* values[21]. For this purpose, we isolated RNA from hTERT-HPNE cells as described previously for microarray analysis. Then, RNA was reverse transcribed with HyperScript First Strand Synthesis Kit (GeneAll Biotechnology, Korea) using random primers to obtain complementary DNA (cDNA). The selected genes were run in duplicate by Real-Time PCR, using SYBR Green chemistry. The primer sequences are reported in Supplementary Table 1. All samples were tested in triplicate using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the reference gene for data normalization to correct for variations in RNA quality and quantity. Threshold cycle (C_T) values of genes of interest were normalized against C_T values of GAPDH, and a relative fold change in expression with respect to a reference sample was calculated by the $2^{-\Delta\Delta Ct}$ method.

Monolayer wound healing assay

To evaluate the cell migration capacity, hTERT-HPNE cells were seeded into a 24-well plate (2×10^4 cells/well) and allowed to attach and grow until reaching the 90%-100% confluence. Cell monolayers were scratched with a sterile 1000 μL pipette tip to make a wound. Then, wounded cell monolayers were washed with phosphate-buffered saline 1× to remove cell debris, and incubated for 24 h in DMEM with 100 ng/mL of each CXCL12 isoform. Cells were monitored under the Eclipse Ti2E microscope (Nikon, Tokyo, Japan) equipped with a camera (Hamamatsu Photonics, Japan) and photographed at 0 h, 12 h and 24 h. Each experiment was performed in triplicate and the data were presented as mean ± SD.

Statistical analysis

For Real-Time PCR and migration assays, differences in gene expression levels between the treated and untreated cells or difference in cell migration ability were determined using the *t*-test. *P* values less than 0.05 were considered statistically

significant. All statistical analyses were performed by using the Stat6 Software for Windows (Stat6 Software, San Diego, CA, United States).

RESULTS

Identification of DEGs

Gene expression alterations in pancreatic hTERT-HPNE cells treated with α, β or γ CXCL12 isoforms, compared to the untreated cells, have been assessed by microarray analyses on Affymetrix Human Clariom S Array. After data processing (*i.e.*, quality control, normalization, batch effect removal), only a few genes resulted to be differentially expressed between treated and untreated cells. Indeed, pairwise comparisons between each treatment and the controls highlighted 17, 24 and 16 DEGs for α, β and γ isoforms, respectively (Table 1). This observation suggests that treatments with CXCL12 did not dramatically alter the gene expression of hTERT-HPNE cells and that, among tested isoforms, the β isoform may induce the highest effect on the transcriptome. Among DEGs regulated by α isoform, 7 were overexpressed and 10 were underexpressed; regarding β isoform, 12 genes were upregulated and 12 were downregulated; finally, for γ isoform, 2 genes were overexpressed and 14 were underexpressed. By hierarchical clustering, the expression levels of these DEGs allow to easily highlight control samples, but homogeneous clusters of samples treated with each CXCL12 isoform are not so clearly distinguishable (Figure 1). This result suggests that, although CXCL12 treatments alter gene expression in hTERT-HPNE cells, the specific effect of each isoform is quite difficult to identify. Therefore, we compared DEGs among different treatments, revealing common and exclusive genes (Figure 2). In particular, 10, 11 and 1 (*i.e.*, LGR5) DEGs were exclusive for α, β and γ isoforms, respectively, suggesting that the effects on the transcriptome of γ isoform is intermediate between those induced by α and β isoforms. Indeed, α and β isoforms induced expression alteration in many different genes, whereas γ isoform affected the expression of genes also common with α and β isoforms (Figure 2).

Quantitative PCR validation

Real-Time PCR analysis was carried out on treated and control samples in order to validate microarray data. Among significant DEGs identified by microarray analysis, we chose 3 up-regulated (UBA7, FEM1C, CASP1) and 3 down-regulated (CXCL8, RRP12 and ADH1B) genes for Real-Time PCR validation. As reported in the Figure 3, the selected genes showed no discrepancies in their expression profiles, for each CXCL12 isoform, between microarray and Real-Time PCR. Indeed, for all isoforms, CXCL8, RRP12 and ADH1B genes resulted to be downregulated also in Real-Time PCR results. In particular, CXCL8, RRP12 and ADH1B genes showed an average fold change reduction of 2.01, 1.78 and 1.72, respectively. Regarding UBA7, FEM1C and CASP1 genes, Real-Time PCR confirmed that they were upregulated, on average, of 1.74, 1.55 and 1.60 times, respectively, after all CXCL12 isoform treatments.

Functional and pathway enrichment analyses

The three lists of DEGs (*i.e.*, for each CXCL12 isoform) have been submitted to Enrichr tool in order to provide an interpretation of the biological processes associated with these genes. This gene enrichment analysis has been performed considering GO terms, regarding GO Biological Process, GO Molecular Function and GO Cellular Component (Supplementary Table 2-4). Our analyses showed that β isoform seems to alter, more than α and γ isoforms the expression of genes involved in cell cycle regulation, DNA replication, G2/M checkpoints, p53 signalling pathway, regulation of apoptosis and senescence. In addition, enrichment analyses highlighted that, for all CXCL12 isoforms, most enriched GO terms involved cell migration and adhesion. Indeed, we often found terms such as "intermediate filament", "cytoskeleton", "microtubule", "kinesin complex", "motor activity", "tubulin binding", " positive chemotaxis", "hemidesmosome assembly", "cell-substrate junction assembly", "microtubule polymerization or depolymerisation", "regulation of cell motility", "regulation of actin filament depolymerisation" (Supplementary Tables 2-4). Since these terms seem to be uniformly distributed among the three CXCL12 isoforms, we decided to carry out *in vitro* assays to evaluate better the specific effect of each CXCL12 isoform on cell migration.

Table 1 List of differentially expressed genes upon treatment of pancreatic hTERT-HPNE cells with 3 different isoforms of CXCL12

Probe name	Log_2FC	*P* value	Adjusted *P* value	Gene name
CXCL12 α isoform *vs* control				
TC1100012755.hg.1	-1.203589	3.12×10^{-8}	0.000052	KIRREL3
TC1700010680.hg.1	-1.036293	1.56×10^{-6}	0.001479	KRT14
TC0200015894.hg.1	-1.009543	1.37×10^{-6}	0.001358	DOCK10
TC1700007330.hg.1	-0.937268	9.64×10^{-6}	0.007801	KSR1
TC0200007399.hg.1	-0.920275	1.26×10^{-5}	0.009220	PLEKHH2
TC1000011549.hg.1	-0.908378	2.40×10^{-5}	0.016397	RRP12
TC1100009990.hg.1	-0.841133	5.45×10^{-5}	0.032188	OR6A2
TC1000011923.hg.1	-0.827117	7.17×10^{-5}	0.038532	GFRA1
TC0300013528.hg.1	-0.824444	7.59×10^{-5}	0.038532	GMNC
TC1800009242.hg.1	-0.818366	1.03×10^{-4}	0.049741	SERPINB2
TC0300007393.hg.1	0.807450	1.12×10^{-4}	0.049980	UBA7
TSUnmapped00000150.hg.1	0.812168	1.05×10^{-4}	0.049741	OBP2B
TC0400008134.hg.1	0.841986	5.91×10^{-5}	0.033939	MMRN1
TC1300010008.hg.1	0.854761	4.73×10^{-5}	0.028684	GAS6-AS1
TC0100008815.hg.1	0.864724	1.12×10^{-4}	0.049980	IFI44L
TC1600010467.hg.1	0.873123	2.89×10^{-5}	0.019144	CNGB1
TC0400011618.hg.1	0.958292	5.08×10^{-6}	0.004269	NEUROG2
CXCL12 β isoform *vs* control				
TC0400007836.hg.1	-1.317647	3.40×10^{-9}	0.000008	CXCL8
TC0400011408.hg.1	-1.007967	2.72×10^{-6}	0.002057	ADH1B
TC2100006784.hg.1	-1.000559	9.12×10^{-7}	0.000839	JAM2
TSUnmapped00000478.hg.1	-0.944922	2.60×10^{-6}	0.002052	ZNF502
TC0400006661.hg.1	-0.799480	1.01×10^{-4}	0.033702	STK32B
TC0200014802.hg.1	-0.797931	7.39×10^{-5}	0.028291	SCN3A
TC1000011549.hg.1	-0.796631	8.77×10^{-5}	0.030275	RRP12
TC0200015894.hg.1	-0.792474	9.80×10^{-5}	0.033299	DOCK10
TC0X00010399.hg.1	-0.779084	1.41×10^{-4}	0.042386	BEX1
TC0600014250.hg.1	-0.776865	1.08×10^{-4}	0.034696	ZBED9
TC0300007220.hg.1	-0.767719	1.30×10^{-4}	0.040552	ZNF502
TC0400009879.hg.1	-0.764228	1.70×10^{-4}	0.049472	CYTL1
TC0600013231.hg.1	0.768484	1.61×10^{-4}	0.047319	SGK1
TC1300007248.hg.1	0.803510	8.35×10^{-5}	0.029556	CKAP2
TC1100013221.hg.1	0.818187	4.63×10^{-5}	0.019311	CASP1
TC0500012030.hg.1	0.819484	4.61×10^{-5}	0.019311	GDF9
TC0300007393.hg.1	0.838000	3.14×10^{-5}	0.014151	UBA7
TC0300012670.hg.1	0.853540	2.67×10^{-5}	0.012705	ATR
TC0100013700.hg.1	0.868308	2.28×10^{-5}	0.011426	CLSPN
TC0600007138.hg.1	0.884415	1.49×10^{-5}	0.008040	E2F3
TC0600010241.hg.1	0.895254	9.02×10^{-6}	0.005389	KIF25
TC0100008101.hg.1	0.951395	3.50×10^{-6}	0.002479	KIF2C

TC0500010150.hg.1	1.068352	1.53×10^{-7}	0.000178	DNAH5
TC0500011752.hg.1	1.070992	1.39×10^{-7}	0.000171	FEM1C
CXCL12 γ isoform *vs* control				
TC1700010680.hg.1	-1.080970	5.93×10^{-8}	0.000105	KRT14
TC0400011408.hg.1	-0.986416	1.69×10^{-5}	0.011437	ADH1B
TC0200015894.hg.1	-0.932303	1.47×10^{-6}	0.001696	DOCK10
TC0400007836.hg.1	-0.928065	6.86×10^{-6}	0.005275	CXCL8
TC0400006661.hg.1	-0.899192	5.58×10^{-6}	0.004432	STK32B
TC2100006784.hg.1	-0.897234	3.99×10^{-6}	0.003542	JAM2
TC1000011549.hg.1	-0.840455	1.83×10^{-5}	0.012077	GFRA1
TC1100012755.hg.1	-0.836071	2.44×10^{-5}	0.015179	KIRREL3
TC1200008176.hg.1	-0.818598	6.33×10^{-5}	0.032429	LGR5
TC1700007330.hg.1	-0.815796	2.93×10^{-5}	0.017802	KSR1
TSUnmapped00000478.hg.1	-0.787902	4.44×10^{-5}	0.025573	ZNF502
TC1100009990.hg.1	-0.785292	4.90×10^{-5}	0.027540	OR6A2
TC0600014250.hg.1	-0.774330	6.12×10^{-5}	0.032045	ZBED9
TC0X00010399.hg.1	-0.769031	1.00×10^{-4}	0.047072	BEX1
TC0500011752.hg.1	0.893861	4.79×10^{-6}	0.004094	FEM1C
TC1100013221.hg.1	0.922373	2.14×10^{-6}	0.002246	CASP1

Only significant differentially expressed genes are reported (adjusted *P* values < 0.05). FC: Fold change.

Monolayer wound healing assay

To assess the biological influence of each CXCL12 isoform on cell migration, hTERT-HPNE cells have been subjected to monolayer wound healing assay. Compared with untreated control, the migration ability of treated hTERT-HPNE cells significantly ($P < 0.05$) increased at 12 h and 24 h time-points after CXCL12 isoforms administration. In particular, the γ isoform increased (37% at 24 h) the hTERT-HPNE migration more than α (16% at 24 h) and β (22% at 24 h) CXCL12 isoforms (Figure 4). Our results confirmed the functional enrichment analyses on DEGs identified by microarray experiments.

DISCUSSION

The strong interaction between stroma and tumour cells is a typical characteristic of PDAC microenvironment. CAFs, representing the 50% of PDAC stroma, are involved in malignant progression, by releasing several chemokines such as CXCL12[12]. In cancer cells presenting the CXCL12 receptor (CXCR4), numerous signalling pathways are activated, which promote cell growth, proliferation, migration, invasion, metastasis and drug resistance[12]. In this study, we investigated the potential role of three different CXCL12 splicing isoforms in a pancreatic pre-tumour model. In particular, we used immortalized, epithelial-like pancreatic duct cells bearing a mutation in the KRAS gene (*G12D*), which is known to be present in 90% of low-grade PanIN-1 lesions. The *G12D* mutation triggers conformational changes that result in KRAS protein activation, which, in turn, can constitutively stimulate several effector pathways involved in tumour development. However, in pancreas, it leads only to a pre-malignant phenotype; indeed, further mutations, amplifications or inactivation of other genes are necessary for the tumour formation[5,6,16]. Therefore, this model has a great value as control in functional *in vitro* assays.

Our microarray data identified some DEGs in hTERT-HPNE cells upon treatment with different CXCL12 isoforms. The DEGs of each isoform were associated with cell migration, adhesion, and cytoskeleton. In vitro wound healing assays confirmed these gene expression results for all isoforms. Our data also showed that the treatment with

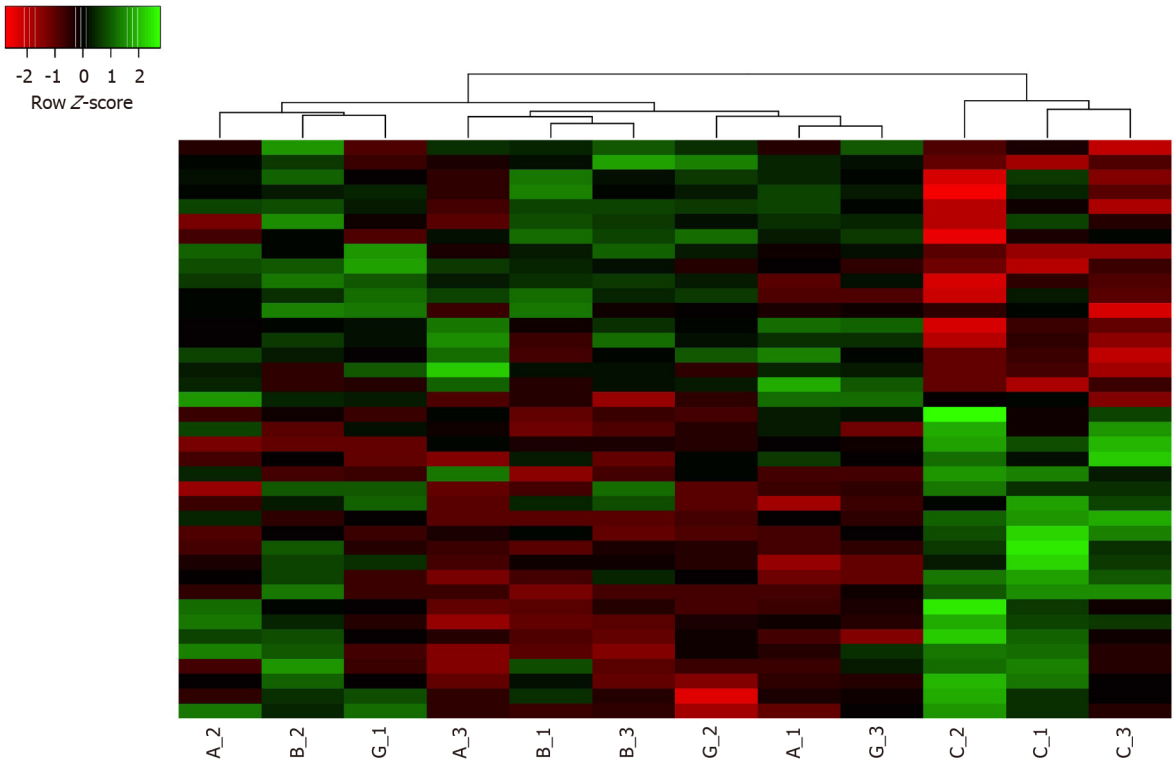


Figure 1 Hierarchical cluster heatmap of treated (α, β and γ isoforms) and control (C1, C2, C3) samples based on the obtained differentially expressed genes. Distance measurement method: Pearson; Clustering method: Average linkage.

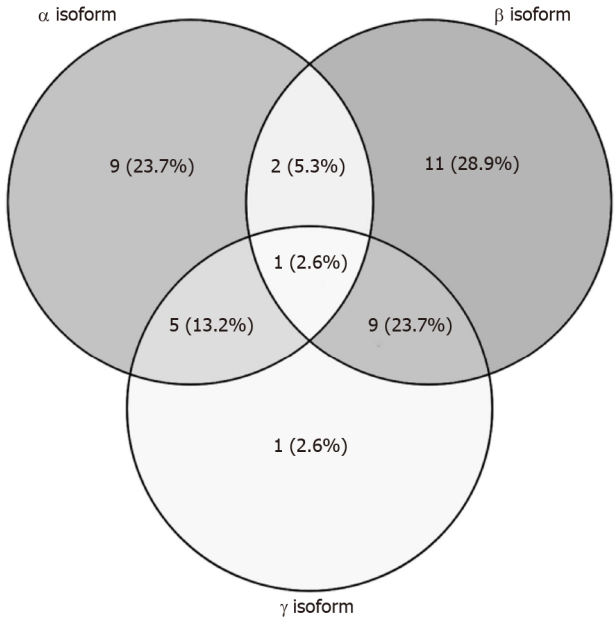


Figure 2 Comparison of the number of differentially expressed genes in all treatment conditions (α, β and γ CXCL12 isoforms) in hTERT-HPNE cells. Differentially expressed genes were detected using an FDR-corrected *P* value threshold of $P < 0.05$.

CXCL12 γ isoform on hTERT-HPNE cells caused enhanced wound healing repair than α and β isoforms.

Similar previous results are not available, since the different CXCL12 isoforms have been usually evaluated in terms of their chemoattractant abilities, that is in *in vitro* chemotaxis assays with a CXCL12 gradient. In a study of Yu *et al*[22], β isoform was more efficient than the others, while in some other studies the isoform with the highest attractant ability was α isoform[23,24]. Furthermore, there are in other studies which determined that γ isoform induced greater migration than other isoforms[25,26]. In

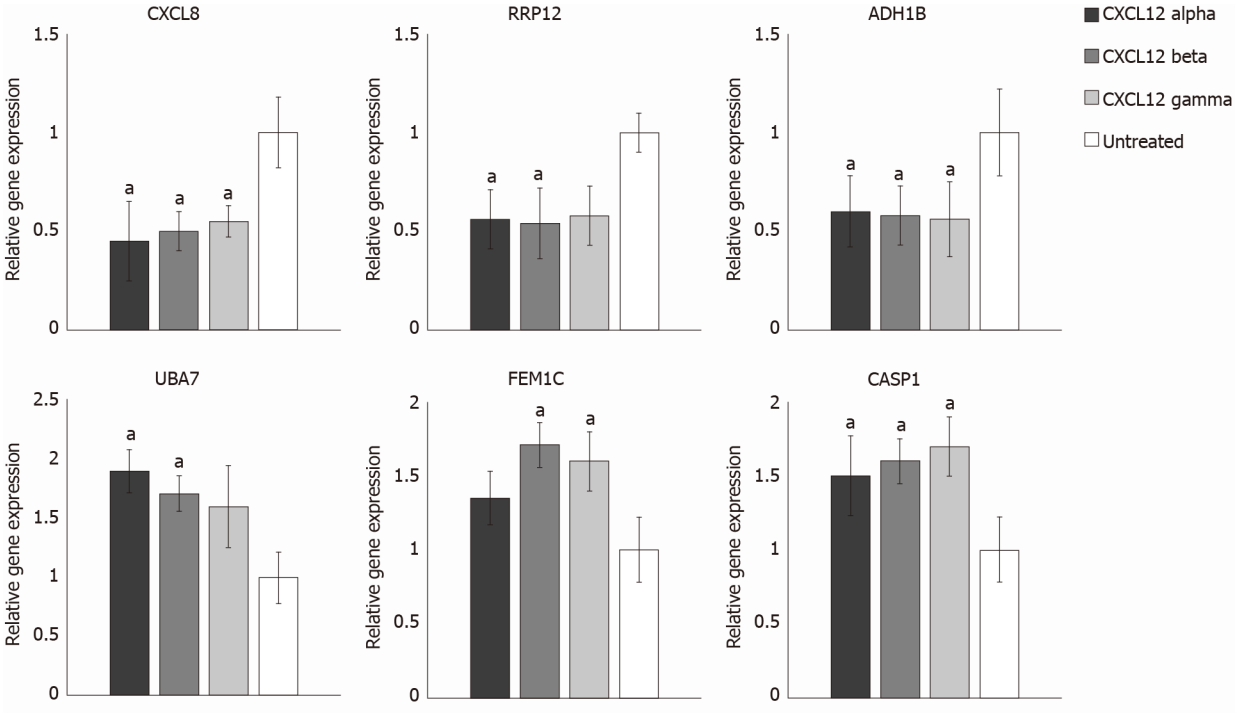


Figure 3 Real-Time polymerase chain reaction analysis of differentially expressed genes. Expression levels of CXCL8, RRP12, ADH1B, UBA7, FEM1C, and CASP1 in treated and untreated hTERT-HPNE cells. All values are expressed as the mean ± SD. Values were considered to be significantly different respect to the control at $^{a}P < 0.05$.

another *in vitro* chemotaxis assay, the use of different concentrations allowed to realize that α isoform induced greatest migration at low concentrations, whereas γ isoform at high concentrations[27]. It was also observed that the chemotactic responses of cells toward CXCL12 can be drawn as a bell-shaped curve with very low effects at both high and low concentrations of CXCL12 isoforms, maybe due the CXCR7 scavenging which alters the CXCL12 gradient to facilitate migration[25]. On the contrary, γ isoform drives chemotaxis to a much greater extent than α and β in *in vivo* studies. Indeed, γ isoform binds to the ECM components, with an extremely greater affinity than α isoform, allowing its local increase and so its activation by oligomerization[26,27].

Among genes with significantly altered expression upon treatments with the different CXCL12 isoforms, there are many genes already known to be involved in cell migration. Interestingly, this is the first study highlighting the possible genes targeted by different CXCL12 isoforms. For example, all CXCL12 isoforms were able to reduce the expression of DOCK10 gene, involved in several cellular processes, including the cellular migration. In particular, it regulates amoeboid motility[28] and it is able to induce filopodia and membrane ruffles[29]. In many tumour types, the overexpression of the receptor GFRA1 Leads to enhanced cancer cell proliferation and migration[30]. The GFRA1 downregulation caused by α and γ CXCL12 isoforms could highlight their potential anti-tumoral role. Both β and γ isoform affect the expression of the alcohol dehydrogenase gene (*ADH1B*), known to take part in several pathways promoting ovarian cancer cell infiltration[31]. Since CXCL12 β and γ isoform downregulate ADH1B gene, it suggests their possible anti-tumoral effect. Also JAM2 expression is downregulated by CXCL12 β and γ isoforms and, interestingly, JAM2 is known to affect cell invasion and migration abilities in pancreatic cancer cells[32]. GAS6-AS1 (GAS6 antisense RNA 1), overexpressed only upon treatments with the α isoform, promotes cancer cell growth, migration, and invasion ability in breast[33] and gastric cancer[34]. The CXCL12 β treatment induced overexpression of GDF9, CKAP2 and KIF2C. While GDF9 overexpression is correlated with a loss of the invasiveness, growth, and migration in human kidney cancer[35], CKAP2 overexpression increased cell proliferation, migration and invasion in HeLa cells[36]. Similarly to CKAP2, KIF2C mediates the cell migration in gastric cancer[37] and hepatocellular carcinoma[38]. Moreover, in KRas mutated cells, the knock down of KIF2C reduced the cell migration[39]. Our results showed that only CXCL12 γ isoform reduced the expression of LGR5. It is able to alter actin cytoskeleton, and in turn, reduce cell migration[40]. However, other studies demonstrated the central role of LGR5 in the tumour

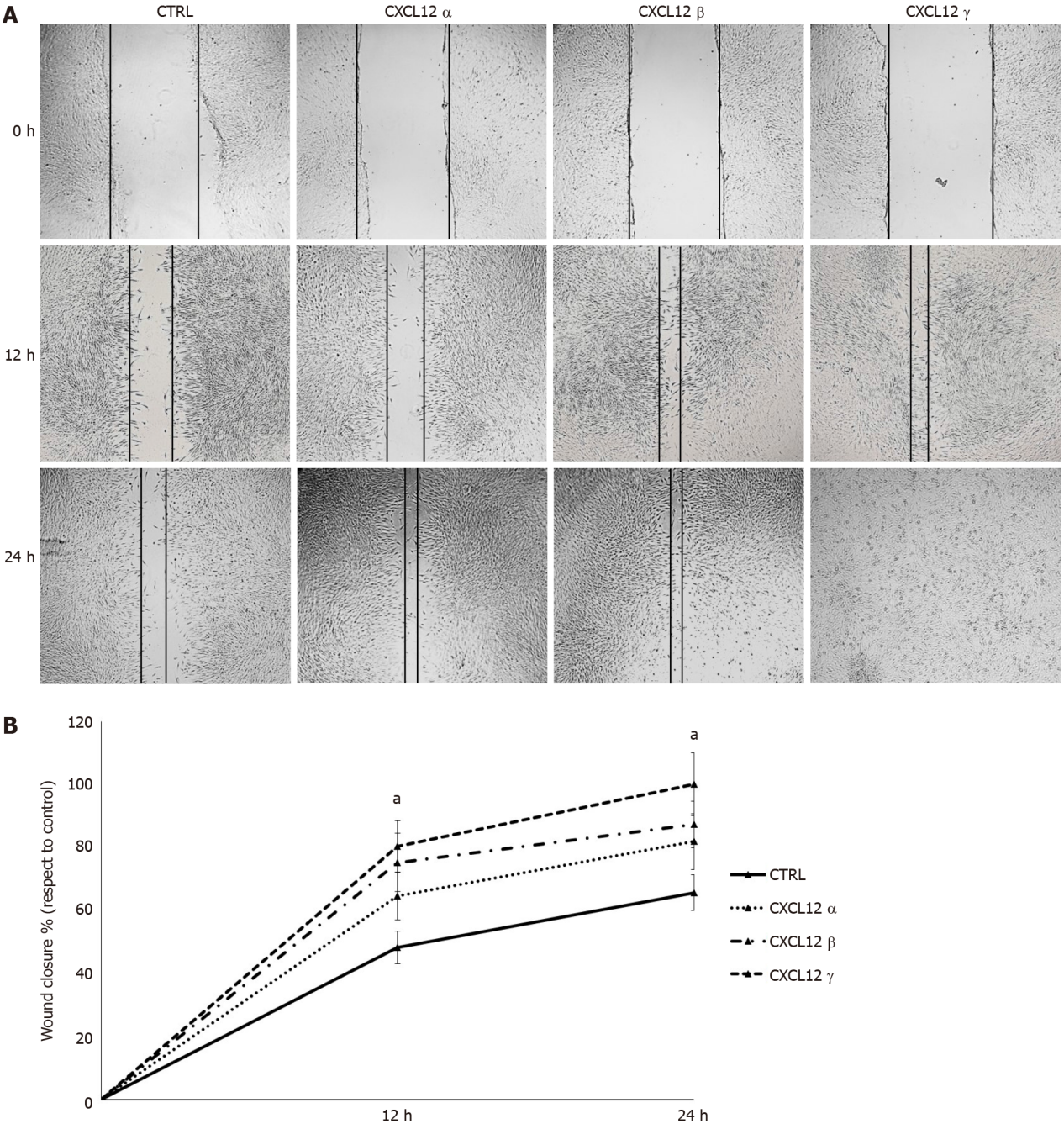


Figure 4 Migration ability of hTERT-HPNE cells. Wound healing assay was used to assess the effect of CXCL12 isoform treatments on cell migration. A: The cells were photographed immediately (0 h) after wounding by a pipette tip and at different time points (12 h and 24 h); B: The migration ability of the treated cells was evaluated by measuring their efficiency in wound repair compared with that of the control. All values are expressed as the mean ± SD. Values were considered to be significantly different respect to the control at $^{a}P < 0.05$.

physiopathology, although its utility as a marker of cancer stem cells is still controversial[41].

Besides cell migration, CXCL12 treatments affected the expression of genes involved in cell cycle regulation, genome integrity, stroma remodelling, and inflammation. For example, only the CXCL12 β treatment induced overexpression of *E2F3* and *ATR* genes. The upregulation of *E2F3* gene, involved in regulation of cell cycle, promotes proliferation and progression of pancreatic cancer cells[42]. The DNA damage sensing by the *ATR* kinases plays critical role in the resistance to chemotherapeutic drugs. Indeed, the inhibition of ATR increased pancreatic cancer cells' sensitivity to gemcitabine and radiation *in vitro*[43,44]. The *SERPINB2* gene expression is downregulated only upon treatments with α isoform. Interestingly, since *SERPINB2* mediates the remodelling of PDAC stroma, leading to the suppression of tumour growth and local invasion[45], α isoform may have a pro-tumoral activity in our hTERT-

HPNE cell model. The expression of the pro-tumour *KSR1* (Kinase suppressor of Ras-1) gene in our KRAS mutated pancreatic cells was downregulated upon treatments with CXCL12 α and γ isoforms. Interestingly, deletion of *KSR1* prevented cell signalling, leading to the block of the transformation induced by mutated KRAS[46]. The inhibition of *KSR1* gene determined a significant growth reduction of pancreatic tumours in vivo, as observed after injection of Panc-1 cells, a cellular model of KRAS-dependent PDAC, in nude mice[47]. Finally, CXCL12 β and γ isoforms downregulated the expression of the major metastasis-promoting inflammatory chemokine CXCL8 (also known as interleukin-8, IL-8). Indeed, KRAS mutation induces overexpression of CXCL8, which is necessary for cancer growth, vascularization and stromal remodelling[48]. Unlike CXCL12 β and γ isoforms, tumor necrosis factor-α, leukemia inhibitory factor, IL-1β, IL-6, and interferon-β induced the expression of CXCL8 in PDAC cells[49]. On the contrary, Matsuo *et al*[50] demonstrated that CXCL8 secretion by pancreatic cancer cell lines was significantly induced by CXCL12. However, unlike our experiments, these authors performed assays in a tumour model and without information about the involved CXCL12 isoform.

CONCLUSION

In conclusion, we showed that treatments with different CXCL12 isoforms prompt cell migration to different extents, probably due to different genes identified by our microarray analysis. Our results may facilitate the elucidation of the role of some CXCL12 isoforms in pancreatic cancer onset, and its underlying molecular mechanisms. In addition, the identified genes may represent novel candidates for diagnostic biomarkers and therapeutic targets for pancreatic cancer.

ARTICLE HIGHLIGHTS

Research background

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal cancer type, since it is usually diagnosed late, it has a very poor prognosis and strong chemoresistance. In the tumour microenvironment, cancer cells and other cell types co-exist and communicate by exchanging several molecules, including the chemokine CXCL12.

Research motivation

CXCL12 pre-mRNA can be alternatively spliced into different isoforms (α, β, γ, δ, ε, θ). However, their specific roles in PDAC have not yet been fully described.

Research objectives

Here, we aim to evaluate the specific roles of the main CXCL12 isoforms (α, β, and γ) in PDAC onset.

Research methods

We administered α, β, and γ CXCL12 isoforms to a pre-tumour model of PDAC, *i.e.*, the hTERT-HPNE E6/E7/KRasG12D cells. Then, we performed microarray analysis and Real-Time polymerase chain reaction validation in order to evaluate the global gene expression alterations. We also carried out wound healing assays in order to evaluate the effect of α, β, and γ CXCL12 isoforms on the cell migration ability.

Research results

The transcriptomic analyses showed that the expression of only few genes was affected by the treatment with the three isoforms. In particular, α and β isoforms affect different genes, whereas γ isoform altered the expression of genes already affected by the other isoforms. Since many genes affected by all isoforms are involved in cell migration and cytoskeleton remodelling, we performed cell migration assays, which confirmed the role of CXCL12 in migration, mainly caused by the γ isoform.

Research conclusions

Our results suggest that α, β and γ CXCL12 isoforms can trigger different responses in a pancreatic pre-tumour model. The γ isoform induced the highest level of cell migration.

Research perspectives

Although our data shed light on the molecular basis of PDAC onset and progression, further studies are necessary for a deeper characterization of CXCL12 isoforms.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Resection of pancreatic cystic neoplasms in recurrent acute pancreatitis prevents recurrent pancreatitis but does not identify more malignancies

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Abstract

BACKGROUND

Recurrent acute pancreatitis (RAP) may be a presenting feature of and an indication for resection of pancreatic cysts, including intra-ductal papillary mucinous neoplasm (IPMN). Few data are available regarding the prevalence of malignancy and post-operative RAP in this population.

AIM

To study the role of resection to help prevent RAP and analyze if presentation as RAP would be a predictor for malignancy.

METHODS

This retrospective study assessed 172 patients who underwent surgical resection of pancreatic cystic neoplasms at a university hospital between 2002 and 2016. The prevalence of preoperative high-risk cyst features, and of neoplasia was compared between patients with and without RAP. To identify the cause of pancreatitis, all the patients had a detailed history of alcohol, smoking, medications obtained, and had cross-sectional imaging (contrast-enhanced computed tomography/magnetic resonance imaging) and endoscopic ultrasound to look for gallstone etiology and other structural causes for pancreatitis. The incidence of RAP post-resection was the primary outcome.

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RESULTS

IPMN accounted for 101 cases (58.7%) {[branch duct (BD) 59 (34.3%), main duct (MD) 42] (24.4%)}. Twenty-nine (16.9%) presented with RAP (mean 2.2 episodes): 15 had BD-IPMN, 8 MD-IPMN, 5 mucinous cystic neoplasm and 1 serous cystic neoplasm. Malignancy was similar among those with *vs* without RAP for all patients [6/29 (20.7%) *vs* 24/143 (16.8%)] and IPMN patients [6/23 (26.1%) *vs* 23/78 (29.5%)], although tended to be higher with RAP in BD-IPMN, [5/15 (33.3%) *vs* 3/44 (6.8%), $P = 0.04$]. At mean follow-up of 7.2 years, 1 (3.4%) RAP patient had post-resection RAP. The mean episodes of acute pancreatitis before *vs* after surgery were 3.4 *vs* 0.02 ($P < 0.0001$).

CONCLUSION

Malignancy was not increased in patients with pancreatic cystic neoplasms who have RAP compared to those without RAP. In addition, specific cyst characteristics were not clearly associated with RAP. The incidence of RAP was markedly decreased in almost all patients following cyst resection.

Key Words: Pancreatic cyst; Pancreatic neoplasm; Pancreatitis; Malignancies

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Core Tip: Our findings from this original study support international consensus guidelines recommendations for surgical resection of pancreatic cystic neoplasms presenting with acute pancreatitis but more for the effective treatment of recurrent acute pancreatitis (RAP) rather than for the identification of malignancy. The objective of this research was to study the role of resection to help prevent RAP and analyze if presentation as RAP would be a predictor for malignancy.

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INTRODUCTION

Acute pancreatitis is among the most frequent gastrointestinal causes of hospital admission in the United States with nearly 280000 hospitalizations annually[1]. While pancreatic cysts in the setting of pancreatitis are frequently assumed to be pseudocysts, neoplastic pancreatic cysts are recognized as a cause of acute pancreatitis. Intra-ductal papillary mucinous neoplasms (IPMN) are the most common cystic pancreatic neoplasms. They arise within the main pancreatic duct or side-branches and are characterized by production of thick mucinous fluid. Additional pancreas cystic neoplasms include mucinous cystic neoplasm and serous cystic neoplasm. Pancreatic cysts may cause acute pancreatitis by compression of the pancreatic duct or by obstruction of the pancreatic duct with mucus. The reported rate of acute pancreatitis among patients with IPMN varies between 7% and 67%[2-5]. A careful clinical history could help to differentiate such neoplastic cysts from a mature, encapsulated fluid collection without necrosis (pseudocyst) or with necrosis (walled-off necrosis, WON) that may occur as a cystic lesion after 4 wk from the onset of acute pancreatitis[6]. The presence of a pancreatic cyst in imaging at diagnosis of index pancreatitis is probably a neoplastic cyst, while the detection of a cyst in the follow-up of pancreatitis with a normal first imaging exam likely a post inflammatory pseudocyst or WON.

The diagnosis of acute pancreatitis is based on the presence of two of the following three features: (1) Abdominal pain consistent with acute pancreatitis (acute onset of a persistent, severe, epigastric pain often radiating to the back); (2) Serum lipase activity (or amylase activity) at least three times greater than the upper limit of normal; And (3) Characteristic findings of acute pancreatitis on contrast-enhanced computed

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tomography (CECT) or magnetic resonance imaging (MRI) or transabdominal ultrasonography[6]. Recurrent acute pancreatitis (RAP), defined as two or more definite episodes of acute pancreatitis, occurs in approximately 17% to 29% of patients who present with a first episode of acute pancreatitis[5,7-9]. Approximately 80% of RAP is related to alcohol and gallstones. Other etiologies include medications, pancreas divisum, sphincter of Oddi dysfunction, pancreatic cancer, hypertriglyceridemia, hypercalcemia, infections, and genetic predisposition[10,11].

Few data are available regarding the prevalence of malignancy and on the course of RAP associated with pancreatic cystic neoplasms, such as IPMN, and the potential benefit of surgical resection in patients with acute pancreatitis due to pancreatic cysts. We, therefore, performed a retrospective study to assess the characteristics of patients with pancreatic cystic neoplasms who present with unexplained RAP and the effect of surgical resection on the natural history.

MATERIALS AND METHODS

We conducted a retrospective cohort study at the Yale-New Haven Hospital to identify patients who underwent surgical resection of pancreatic cystic neoplasms between January 1, 2002 and December 1, 2016. Those who had RAP, defined as 2 or more episodes of acute pancreatitis without an identifiable cause prior to resection[12], and imaging [computed tomography, magnetic resonance imaging, and/or endoscopic ultrasound (EUS)] documenting the cyst pre-operatively were defined as the RAP cohort. To identify the cause of pancreatitis, all the patients had detailed history of alcohol, smoking, medications obtained and had cross-sectional imaging (CECT/MRI) and EUS to look for gallstone etiology and other structural causes for pancreatitis. The diagnosis was double-checked. Patients with resection who did not have prior RAP served as the control cohort. Patient follow-up extended until September 2018. The study was approved by the institutional review board at Yale University.

Preoperative classification was based on preoperative imaging and results of fine-needle aspiration, if performed. Cysts were classified as IPMNs and non-IPMNs. The IPMNs were sub-classified as main-duct (MD) and branch-duct (BD). Mixed-type IPMNs were included in the MD group. Preoperative diagnosis of IPMNs were based on clinical findings from the images obtained. Histopathology from post-surgical resection specimens was used as the gold standard to ascertain the final diagnosis. The pathological classification and pre-surgical classification were made on the basis of World Health Organization criteria and current literature[13-16]. Performing metastatic urothelial cancer (MUC) staining or gene sequencing

Malignancy was defined as the presence of invasive carcinoma or high-grade dysplasia on final surgical pathology. Patients with no follow-up data post-operatively, known pseudocysts and predominantly solid pancreatic masses were excluded. Patient demographic, clinical, radiographic, and pathologic data were extracted from the medical record. In patients with multiple cysts, the features of the largest cyst or the cyst with the most concerning high-risk features (*e.g.*, mural nodules) were the characteristics recorded for the patient. Patient data post-resection were also extracted, with the primary goal of identifying episodes of acute pancreatitis. On surgically resected specimens, MUC staining, histologic examination and gene sequencing were used for pathologic subclassification[17,18].

Statistical analysis

The primary analysis involved a comparison of the number of episodes of acute pancreatitis pre- and post-resection of pancreas cyst in patients who had RAP. We also compared baseline patient and cyst characteristics between patients with RAP and those without RAP. Comparisons between the RAP cohort and control (non-RAP) cohort and between the preoperative and post-operative time period were performed with the Mantel-Haenszel risk difference calculation for continuous variables and Mantel-Haenszel risk ratio for proportions. Statistical analysis was performed by using the statistical algorithms in Cochrane Review Manager Software 5.3.

RESULTS

A total of 172 patients with pancreatic cysts underwent surgery between 2002 and

2016. The indication for surgery was RAP along with cyst characteristics in 11 patients and among all other patients, the indication for surgery was based on the cyst morphology. IPMNs accounted for 101 (58.7%) of these 172 cases {BD-IPMN [59 (34.3%)] and MD-IPMN [42 (24.4%)]} (Table 1 and Figure 1). The next most common types of lesions were mucinous cystic neoplasms [32/172 (18.6%)] and serous cystic neoplasms [16/172 (9.3%)] (Table 1). Since preoperative diagnosis on cysts were made using radiologic findings, the final diagnosis might have varied based on surgical findings. Twenty-nine (16.9%) patients had RAP without other identifiable cause prior to resection, while the remaining 143 (83.1%) patients had no episodes of acute pancreatitis prior to resection. No patient had evidence of chronic pancreatitis. Among the 29 patients with RAP with cystic neoplasms, the surgical procedure performed was pancreaticoduodenectomy (n = 18) or distal pancreatectomy (n = 11) with surgical mortality 0%, morbidity 3/29 (10%) (one patient had mesenteric bleeding requiring immediate exploratory laparotomy, one had afferent limb syndrome 2 wk later required exploratory laparotomy for adhesion lysis, and other with a delayed abscess in left upper quadrant managed with external drainage.

Comparison of pre-resection and post-resection period in the RAP cohort

The mean number of pancreatitis episodes *per* patient prior to resection in the RAP cohort was 2.2 (range 2-32 episodes). The median elapsed time between the first episode of acupuncture (AP) and surgery was 17 mo (range 1.5 mo to 7 years). The mean follow-up after surgery was 7.2 years (range 1.9-13.8 years).

Only 1 (3%) of 29 RAP patients (with BD-IPMN) had acute pancreatitis documented post-resection. The mean number of recurrent episodes of acute pancreatitis *per* patient-year before *vs* after surgery was 3.40 *vs* 0.02 (absolute difference = 3.38, 95%CI 1.76 to 5.00; $P < 0.0001$)). All the episodes were mild acute pancreatitis and none of the patients in this cohort had severe acute pancreatitis[3]. A marked decrease was seen in all types of cysts (Table 2). The one patient with recurrent pancreatitis after resection had BD-IPMN; he had 8 episodes before resection and 32 after resection and progressed to chronic pancreatitis with ductal calcification at 6 years after resection. No patient without prior RAP developed acute pancreatitis after resection.

Comparison of RAP and control cohort

Selected characteristics of the RAP and control cohorts are shown in Table 3. Median age and proportion who were male were comparable. No clear differences were identified between the cohorts in characteristics of the cystic lesions, including in size, or proportion with malignancy except for location in the tail of pancreas where most cyst patients were without RAP. IPMNs tended to be more common among patients with RAP than in those without RAP (79.3% *vs* 54.5%; risk difference = 0.25; 95%CI: 0.08, 0.41, $P = 0.004$) with most of the difference seen in the BD-IPMN patients.

Table 4 shows the results for the RAP and control cohorts in all patients with IPMN. Patients with RAP were more likely to have intestinal and less likely to the gastric IPMN cysts (intestinal: 34.8% *vs* 12.8%; risk difference: 0.22 (95%CI: 0.01, 0.43); $P = 0.04$; gastric: 13% *vs* 53.9%; risk difference: -0.41 (95%CI: -0.58, -0.23); $P < 0.0001$). The pathological subtypes of 21.8% of RAP patients and 10.3% of non-RAP patients was unknown.

Table 5 is restricted to patients with BD-IPMN. Again, no clear differences were seen between cohorts in patient or cyst characteristics with the possible exception of a tendency to a higher prevalence of malignancy in the RAP group *vs* non-RAP group with BD-IPMNs {33.3% *vs* 6.8%, [risk difference = 0.27 (95%CI: 0.02, 0.52); $P = 0.04$]} although the numbers in each cohort were small. A similar difference in pathological subtypes were noted. Patients with RAP were more likely to have BD-IPMN of an intestinal subtype and less likely to have a gastric subtype *vs* the control cohort [intestinal: 46.7% *vs* 9.1%; risk difference = 0.38 (95%CI: 0.11, 0.64); $P = 0.006$; gastric: 0% *vs* 68.2%; risk difference = -0.68 (95%CI: -0.84, -0.52); $P < 0.001$]. However, it should be noted that the pathological subtype of 26.7% of BD-IPMNs was not recorded. Based on the significant results from Univariate analysis, multivariable analysis logistic regression performed, however, the results did not vary in any significance (Tables 6-8).

DISCUSSION

Surgical resection has been recommended for patients with pancreatic cystic neoplasms who present with acute pancreatitis[16], regardless of cyst type. Reasons for

Table 1 Types of pancreatic cysts

IPMN cysts (total)	101
BD-IPMN	59
MD-IPMN	42
Non-IPMN cysts (total)	71
Mucinous cystic neoplasm	32
Serous cystic neoplasm	16
Pancreatic neuroendocrine tumor	11
Solid pseudopapillary epithelial neoplasm	6
Squamoid	3
Benign	2
Cystic degeneration	1

IPMN: Intra-ductal papillary mucinous neoplasm; MD-IPMN: Main duct-IPMN; BD-IPMN: Branch duct-IPMN.

Table 2 Incidence of recurrent acute pancreatitis episodes in recurrent acute pancreatitis patients

	Episodes *per* patient-year (mean + SD)		Mean difference [95%CI]	*P* value
	Pre-resection	Post-resection		
All cysts (n = 29)	3.40 (4.46)	0.02 (0.08)	3.38 [1.76, 5.00]	< 0.0001
IPMN (n = 23)	2.32 (1.69)	0.03 (0.09)	2.29 [1.60, 2.98]	< 0.0001
BD-IPMN (n = 15)	2.06 (1.85)	0.04 (0.11)	2.02 [1.08, 2.96]	< 0.0001
MD-IPMN (n = 8)	2.81 (1.32)	0 (0)[1]	2.81 [1.83, 3.79]	< 0.0001
Non-IPMN (n = 6)	7.52 (8.53)	0 (0)[1]	7.52 [0.69, 14.35]	0.03

[1]In order to calculate mean differences and risk ratios, a standard deviation of 0.0001 was substituted for zero. IPMN: Intra-ductal papillary mucinous neoplasm; MD-IPMN: Main duct-IPMN; BD-IPMN: Branch duct-IPMN; SD: Standard deviation; CI: Confidence interval.

resection include identification and removal of underlying malignancy, prevention of the development of malignancy, and prevention of additional attacks of pancreatitis[16]. The characteristics of cystic neoplasms associated with acute pancreatitis and the impact of surgical resection on the prevention of malignancy and future episodes of RAP, however, have not been well defined. We found that cyst characteristics, such as location, size, the proportion with solid component, and prevalence of malignancy were not clearly different between patients with *vs* without RAP. IPMN was the most common cystic neoplasm among patients with a history of RAP undergoing surgical resection. Although a prior report found MD/combined type IPMN to be more frequently associated with RAP (%)[3] and another study found no difference[5], we found that among patients with IPMN, RAP was more often associated with BD-IPMN (65%) than MD-IPMN (35%). Our results are different from Jang *et al*[3] where MD/combined IPMN resulting more RAP was thought to be due to mechanical obstruction of the main pancreatic duct by thick mucin, resulting in ductal hypertension, thereby premature activation and release of pancreatic enzymes[3]. However, BD-IPMN leading to AP/RAP is assumed primarily due to the obstruction of the main pancreatic duct by the migration of mucin from the BD or may not be related to mucin but just to the viscosity of cyst fluid[3]. Patients with RAP also were more likely to have intestinal histological subtype *vs* the control cohort, although histologic subtype was unknown in a third of patients.

Malignancy was present in approximately one-fifth of patients undergoing surgical resection of pancreatic cysts, with similar proportions in those with and without RAP. Malignancy tended to be more common in BD-IPMN patients with RAP (33.3% *vs* 6.8% without RAP), although given relatively small numbers and multiple comparisons, this finding must be viewed as hypothesis-generating. Prior studies vary

Table 3 Characteristics of all patients (*n* = 172)

	With RAP (*n* = 29) *n* (%)	Without RAP (*n* = 143) *n* (%)	Risk difference [95%CI]	*P* value
Median age	61 (IQR 18)	65 (IQR 15)	NA	0.65
Sex (M)	13 (44.8)	58 (40.6)	NA	0.67
Location of cyst				
Head	16 (55.2)	65 (45.5)	0.10 [-0.10, 0.30]	0.34
Neck	2 (6.9)	7 (4.9)	0.02 [-0.08, 0.12]	0.69
Body	8 (27.6)	32 (22.4)	0.05 [-0.12, 0.23]	0.56
Tail	3 (10.3)	37 (25.9)	-0.16 [-0.29, -0.02]	0.02
Diffuse	0 (0)	2 (1.4)	-0.01 [-0.06, 0.04]	0.59
Main pancreas duct > 10	4 (13.8)	10 (7.0)	0.07 [-0.06, 0.20]	0.31
Cyst size > 3	6 (20.7)	36 (25.2)	-0.04 [-0.21, 0.12]	0.59
Solid component	7 (24.1)	46 (32.2)	-0.08 [-0.25, 0.09]	0.36
Malignancy	6 (20.7)	24 (16.8)	0.04 [-0.12, 0.20]	0.63
Pathological subtype				
Intestinal	10 (34.5)	11 (7.7)	0.27 [0.09, 0.45]	0.003
Gastric	3 (10.3)	43 (30.1)	-0.20 [-0.33, -0.06]	0.004
Pancreatobiliary	7 (24.1)	15 (10.5)	0.14 [-0.03, 0.30]	0.10
Oncocytic	0 (0)	3 (2.1)	-0.02 [-0.07, 0.03]	0.43
Unknown	9 (31.0)	71 (49.7)	-0.19 [-0.37, 0.001]	0.05
Cyst type				
IPMN total	23 (79.3)	78 (54.5)	0.25 [0.08, 0.41]	0.004
BD-IPMN	15 (51.7)	44 (30.8)	0.21 [0.01, 0.41]	0.04
MD-IPMN	8 (27.6)	34 (23.8)	0.04 [-0.14, 0.22]	0.67
Non-IPMN	6 (20.7)	65 (45.5)	-0.25 [-0.42, -0.08]	0.004

RAP: Recurrent acute pancreatitis; IPMN: Intra-ductal papillary mucinous neoplasm; MD-IPMN: Main duct-IPMN; BD-IPMN: Branch duct-IPMN; IQR: Interquartile range; CI: Confidence interval.

in the reported association of malignancy within pancreatic cysts in patients presenting with acute pancreatitis[19]. Some report no increased risk[20-22]. However, a recent study identified IPMN presenting with acute pancreatitis to be associated with an increased risk of malignancy as compared to IPMN without pancreatitis on multivariable analysis[23]. Morales-Oyarvide *et al*[23] also identified a significant association of AP with IPMN of intestinal subtype (OR = 4.7, 2.5-8.8)[23]. The intestinal phenotype of IPMN has a propensity for mucin hypersecretion, which may promote AP due to ductal obstruction[24-27]. In addition, the intestinal phenotype has been associated with an increased risk of malignancy and with colloid carcinoma[25].

Among the 15 BD-IPMN patients with RAP who had surgical resection in our study, pre-operatively only 1 (6.7%) demonstrated all 3 high-risk features for malignancy (dilated main pancreatic duct > 10 mm, size > 3 cm, solid component)[28] and 3 (20%) had 2 high-risk features. One-third, however, had malignancy identified on surgical pathology. Based on the presence of high-risk stigmata/worrisome features alone, *per* international revised consensus guidelines of 2017[16,29] or European guidelines[30], resection would not have been indicated in 14 of the 15 BD-IPMN patients if they had not presented with acute pancreatitis. And based on American Gastroenterological Association guidelines, if these patients did not have RAP, only 3 of 15 would have had EUS based on the requirement for at least 2 high-risk features[28]. American College of Gastroenterology guidelines recommends EUS or referral to a multidisciplinary team for pancreatic cystic lesions in the presence of pancreatitis regardless of high-risk

Table 4 Characteristics of patients with intra-ductal papillary mucinous neoplasm with and without recurrent acute pancreatitis

	With RAP (*n* = 23) *n* (%)	Without RAP (*n* = 78) *n* (%)	Risk difference [95%CI]	*P* value
Median age	64 (IQR 17)	67 (IQR 12)	NA	0.64
Sex (M)	13 (56.5)	38 (48.7)	NA	0.51
Location of cyst				
Head	16 (69.6)	46 (59)	0.11 [-0.11, 0.32]	0.34
Body/Tail	7 (30.4)	32 (41)	-0.11 [-0.32, 0.11]	0.34
Main pancreas duct > 10	4 (17.4)	10 (12.8)	0.05 [-0.13, 0.22]	0.60
Cyst size > 3 cm	6 (26)	36 (46.2)	-0.20 [-0.41, 0.01]	0.06
Solid component	5 (21.7)	20 (25.6)	-0.04 [-0.23, 0.16]	0.69
Malignancy	6 (26.1)	23 (29.5)	-0.03 [-0.24, 0.17]	0.75
Pathological subtype				
Intestinal	8 (34.8)	10 (12.8)	0.22 [0.01, 0.43]	0.04
Gastric	3 (13)	42 (53.9)	-0.41 [-0.58, -0.23]	< 0.0001
Pancreatobiliary	7 (30.4)	15 (19.2)	0.11 [-0.10, 0.32]	0.29
Oncocytic	0 (0%)	3 (3.8)	-0.04 [-0.11, 0.03]	0.30
Unknown	5 (21.8)	8 (10.3)	0.11 [-0.07, 0.30]	0.22

RAP: Recurrent acute pancreatitis; IQR: Interquartile range; CI: Confidence interval.

features[31]. While observational studies have suggested an increased risk of pancreatic cancer in all patients presenting with acute pancreatitis[19,32], further studies are needed to evaluate whether acute pancreatitis may be an independent predictor of malignancy within pancreatic cysts.

The primary goal of our study was to determine the post-resection course of patients with RAP associated with pancreatic cystic neoplasms. We found that episodes of RAP are rare following surgical resection of pancreatic cystic neoplasms, suggesting that the pancreatic cystic neoplasms were indeed the cause of RAP in almost all cases. Only 1 (3%) of 29 patients presenting with RAP had RAP after resection, and the rate of acute pancreatitis *per* patient-year decreased from 3.4 to 0.02 ($P < 0.0001$), with marked reductions in all types of pancreatic cystic lesions. It is also important to note that as the pancreatic exocrine function decreases after pancreas resection, the mere resection itself may reduce the frequency of RAP[33]. Pancreatic surgery for an indication of RAP alone is a very invasive procedure and therefore, risk/benefits should be considered while making the decision. In such cases, since the pancreatic ductal occlusion at the site of the papilla has been postulated to be the main reason for RAP in IPMN, performing an endoscopic pancreatic sphincterotomy with or without stenting may be effective in preventing pancreatitis recurrence[34].

We recognize several limitations to this study, including the retrospective review of data. In addition, this cohort is limited to patients who have undergone surgical resection of pancreas cystic neoplasms, which makes it a selected population not representative of the overall group of patients with pancreatic cystic neoplasms. For example, most patients with BD-IPMN or serous cystic neoplasms do not undergo resection. Patient cases were reviewed to exclude other identifiable causes of acute pancreatitis; however, given the study's retrospective nature, the evaluation was not standardized. All of the patients in our surgical cohort had RAP, with 2 or more episodes. This is reflective of clinical practice for BD-IPMN, as resection in the absence of multiple high-risk features is typically not pursued following a single episode of idiopathic pancreatitis. Although, this study clearly shows the risk reduction of RAP after the pancreatic resection, further investigation with large population prospective study is needed to prove causal relationship between the cause of RAP and pancreatic resection.

Table 5 Characteristics of patients with branch-duct intra-ductal papillary mucinous neoplasm with and without recurrent acute pancreatitis

	With RAP (*n* = 15) *n* (%)	Without RAP (*n* = 44) *n* (%)	Risk difference [95%CI]	*P* value
Median age	60 (IQR 23)	65 (IQR 12.5)	NA	0.50
Sex (M)	8 (53.3)	24 (54.6)	NA	0.94
Location of cyst				
Head	11 (73)	28 (64)	0.10 [-0.17, 0.36]	0.47
Body/Tail	4 (9.1)	16 (36)	-0.10 [-0.36, 0.17]	0.47
Main pancreas duct > 10	2 (13.3)	2 (4.6)	0.09 [-0.10, 0.27]	0.35
Cyst size > 3 cm	6 (40)	21 (47.7)	-0.08 [-0.37, 0.21]	0.60
Solid component	2 (13.3)	4 (9.1)	0.04 [-0.15, 0.23]	0.66
Malignancy	5 (33.3)	3 (6.8)	0.27 [0.02, 0.52]	0.04
Pathological subtype				
Intestinal	7 (46.7)	4 (9.1)	0.38 [0.11, 0.64]	0.006
Gastric	0	30 (68.2)	-0.68 [-0.84, -0.52]	< 0.001
Pancreatobiliary	4 (26.7)	7 (16)	0.11 [-0.14, 0.36]	0.4
Oncocytic	0	0	NA	NA
Unknown	4 (26.7)	3 (6.8)	0.20 [-0.04, 0.43]	0.10

RAP: Recurrent acute pancreatitis; IQR: Interquartile range; NA: Not available; CI: Confidence interval.

CONCLUSION

In conclusion, we found that malignancy is not increased in patients with pancreatic cystic neoplasms who have RAP compared to those without RAP. In addition, specific cyst characteristics were not clearly associated with RAP, although intestinal histological subtype appeared to be more common in patients with RAP. Most importantly, episodes of RAP were markedly reduced after cyst resection, with only one of 29 patients having recurrent episodes. Our findings support international consensus guidelines recommendations for surgical resection of pancreatic cystic neoplasms presenting with acute pancreatitis but more for the effective treatment of RAP rather than for the identification of malignancy[16].

Table 6 Multivariable analysis of all patients (*n* = 172)

	With RAP (*n* = 29)	Without RAP (*n* = 143)	*P* value
	Odds ratio	95%CI	
Location of cyst			
Head	-		
Neck	1.240013	[0.19933, 7.713993]	0.818
Body	1.736854	[0.570778, 5.285173]	0.331
Tail	0.686678	[0.155679, 3.028833]	0.62
Diffuse	-		
Pathological subtype			
Intestinal			
Gastric	0.059886	[0.013402, 0.267608]	< 0.001
Pancreatobiliary	0.454606	[0.122387, 1.688636]	0.239
Oncocytic	-		
Unknown	0.355592	[0.088518, 1.428472]	0.145
Cyst type			
IPMN total	3.462462	[0.782586, 15.31927]	0.102
BD-IPMN	1.660612	[0.558816, 4.934779]	0.361
MD-IPMN	-		

RAP: Recurrent acute pancreatitis; IPMN: Intra-ductal papillary mucinous neoplasm; MD-IPMN: Main duct-IPMN; BD-IPMN: Branch duct-IPMN; CI: Confidence interval.

Table 7 Multivariable analysis of patients with intra-ductal papillary mucinous neoplasm with and without recurrent acute pancreatitis

	With RAP (*n* = 29)	Without RAP (*n* = 143)	*P* value
	Odds ratio	95%CI	
Location of cyst			
Head	-	-	
Neck	1.265446	[0.169046, 9.472912]	0.819
Body	0.506621	[0.111835, 2.295024]	0.378
Tail	0.572817	[0.090659, 3.619267]	0.554
Diffuse	-	-	
Pathological subtype			
Intestinal	-	-	
Gastric	0.096091	[0.020977, 0.440165]	0.003
Pancreatobiliary	0.773074	[0.199147, 3.001027]	0.71
Oncocytic	-		
Unknown	1.034683	[0.214488, 4.991279]	0.966
Cyst type			
IPMN total			
BD-IPMN	1.571892	[0.530571, 4.65695]	0.414
MD-IPMN	-		

RAP: Recurrent acute pancreatitis; IPMN: Intra-ductal papillary mucinous neoplasm; MD-IPMN: Main duct-IPMN; BD-IPMN: Branch duct-IPMN; CI: Confidence interval.

Table 8 Multivariable analysis patients with branch-duct intra-ductal papillary mucinous neoplasm with and without recurrent acute pancreatitis

	With RAP (*n* = 29)	Without RAP (*n* = 143)	*P* value
	Odds ratio	95%CI	
Location of cyst			
Head	-	-	
Neck	-	-	
Body	0.61173	[0.059835, 6.254136]	0.679
Tail	0.537057	[0.050552, 5.705601]	0.606
Diffuse	-	-	
Pathological subtype			
Intestinal	-	-	
Gastric	-	-	
Pancreatobiliary	0.461648	[0.063565, 3.352753]	0.445
Oncocytic	-	-	
Unknown	1.458186	[0.15039, 14.13861]	0.745

RAP: Recurrent acute pancreatitis; CI: Confidence interval.

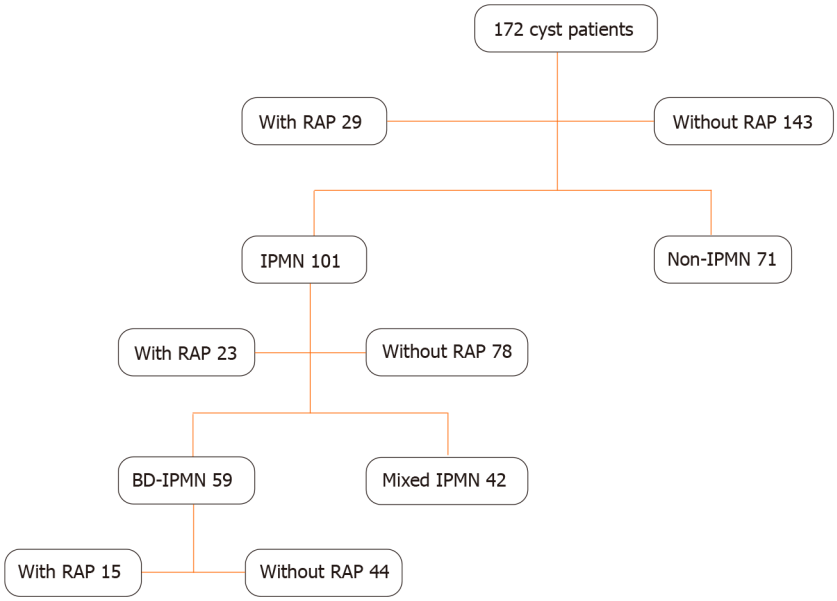


Figure 1 Flow chart of patient population with cyst resection. RAP: Recurrent acute pancreatitis; IPMN: Intra-ductal papillary mucinous neoplasm; BD-IPMN: Branch duct--IPMN.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cystic neoplasms may present recurrent acute pancreatitis (RAP). Little is known on the role of resection for preventing RAP and if any correlation of higher prevalence of malignancy is seen among these patients.

Research motivation

Predicting malignancy among the pancreatic cystic neoplasms and management of RAP is challenging.

Research objectives

The objective of this research was to study the role of resection to help prevent RAP and analyze if presentation as RAP would be a predictor for malignancy.

Research methods

We adopted a retrospective cohort study model, enrolling all the patients with pancreatic cystic neoplasms who underwent surgical resection and compare between those who presented with RAP and without RAP. Incidence of RAP after resection and prevalence of malignancy among those who presented with RAP were the primary outcomes.

Research results

Malignancy was similar among those with *vs* without RAP for all patients 20.7% *vs* 16.8%. The mean episodes of acute pancreatitis before *vs* after surgery were 3.4 *vs* 0.02 ($P < 0.0001$). These findings clearly contribute much to this area of science. Although, this study clearly shows the risk reduction of RAP after the pancreatic resection, further investigation with large population prospective study is needed to prove a causal relationship between the cause of RAP and pancreatic resection.

Research conclusions

When patients with RAP are noted to have pancreatic cysts, it should be emphasized to differentiate if those cysts are the cause or the effect of RAP, *i.e.*, if cystic neoplasms are causing the RAP or if cysts are pseudocysts as a result of RAP. Larger data is needed to see if there exists a causal relationship. Similar to the presentation of jaundice as a predictor for malignancy among pancreas cystic neoplasms, RAP may have a role as a predictor of high-risk cysts.

Research perspectives

Future research to be focused on a larger prospective cohort to answer the above two questions.

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ORIGINAL ARTICLE

Retrospective Study

Intestinal bacterial overgrowth in the early stage of severe acute pancreatitis is associated with acute respiratory distress syndrome

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Author contributions: Liang XY made substantial contributions to the conception and design of the study, acquisition, analysis, and interpretation of data, and drafting the article; Jia TX made substantial contributions to the acquisition and analysis of data; Zhang M made substantial contributions to the conception, design and guide of the study; all authors read and approved the final manuscript.

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Abstract

BACKGROUND

In the early stage of acute pancreatitis (AP), a large number of cytokines induced by local pancreatic inflammation seriously damage the intestinal barrier function, and intestinal bacteria and endotoxins enter the blood, causing inflammatory storm, resulting in multiple organ failure, infectious complications, and other disorders, eventually leading to death. Intestinal failure occurs early in the course of AP, accelerating its development. As an alternative method to detect small intestinal bacterial overgrowth, the hydrogen breath test is safe, noninvasive, and convenient, reflecting the number of intestinal bacteria in AP indirectly. This study aimed to investigate the changes in intestinal bacteria measured using the hydrogen breath test in the early stage of AP to clarify the relationship between intestinal bacteria and acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). Early clinical intervention and maintenance of intestinal barrier function would be highly beneficial in controlling the development of severe acute pancreatitis (SAP).

AIM

To analyze the relationship between intestinal bacteria change and ALI/ARDS in the early stage of SAP.

METHODS

A total of 149 patients with AP admitted to the intensive care unit of the Digestive Department, Xuanwu Hospital, Capital Medical University from 2016 to 2019 were finally enrolled, following compliance with the inclusion and exclusion criteria. The results of the hydrogen breath test within 1 wk of admission were collected, and the hydrogen production rates at admission, 72 h, and 96 h were calculated. The higher the hydrogen production rates the more bacteria in the small intestine. First, according to the improved Marshall scoring system in the 2012 Atlanta Consensus on New Standards for Classification of Acute

Data sharing statement: No additional data are available.

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Pancreatitis, 66 patients with a PaO_2/FiO_2 score ≤ 1 were included in the mild AP (MAP) group, 18 patients with a PaO_2/FiO_2 score ≥ 2 and duration < 48 h were included in the moderately SAP (MSAP) group, and 65 patients with a PaO_2/FiO_2 score ≥ 2 and duration > 48 h were included in the SAP group, to analyze the correlation between intestinal bacterial overgrowth and organ failure in AP. Second, ALI (PaO_2/FiO_2 = 2) and ARDS (PaO_2/FiO_2 > 2) were defined according to the simplified diagnostic criteria proposed by the 1994 European Union Conference. The MSAP group was divided into two groups according to the PaO_2/FiO_2 score: 15 patients with PaO_2/FiO_2 score = 2 were included in group A, and three patients with score > 2 were included in group B. Similarly, the SAP group was divided into two groups: 28 patients with score = 2 were included in group C, and 37 patients with score > 2 were included in group D, to analyze the correlation between intestinal bacterial overgrowth and ALI/ARDS in AP.

RESULTS

A total of 149 patients were included: 66 patients in the MAP group, of whom 53 patients were male (80.3%) and 13 patients were female (19.7%); 18 patients in the MSAP group, of whom 13 patients were male (72.2%) and 5 patients were female (27.8%); 65 patients in the SAP group, of whom 48 patients were male (73.8%) and 17 patients were female (26.2%). There was no significant difference in interleukin-6 and procalcitonin among the MAP, MSAP, and SAP groups (P = 0.445 and P = 0.399, respectively). There was no significant difference in the growth of intestinal bacteria among the MAP, MSAP, and SAP groups (P = 0.649). There was no significant difference in the growth of small intestinal bacteria between group A and group B (P = 0.353). There was a significant difference in the growth of small intestinal bacteria between group C and group D (P = 0.038).

CONCLUSION

Intestinal bacterial overgrowth in the early stage of SAP is correlated with ARDS.

Key Words: Acute respiratory distress syndrome; Hydrogen breath test; Intestinal bacterial overgrowth; Severe acute pancreatitis; Interleukin-6; Acute lung injury

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Core Tip: Our retrospective study included 149 patients with acute pancreatic disease. The changes in intestinal flora were detected by the hydrogen breath test. We found that the change in intestinal flora in patients with severe acute pancreatitis was related to acute respiratory distress syndrome, which can aggravate the disease. The results of the hydrogen breath test can be used as a warning signal for severe acute pancreatitis.

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INTRODUCTION

Acute pancreatitis (AP) is a common form of acute abdomen. The mortality rate of severe AP (SAP) with early organ failure can be as high as 36%-50%[1]. The earliest SAP-related organ dysfunction occurs in the respiratory system with the main symptoms of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)[2,3]. In the early stage of AP, a large number of cytokines is induced by local pancreatitis to enter the bloodstream, causing systemic inflammatory response syndrome (SIRS) through the cytokine cascade reaction; the large number of released cytokines can seriously damage the intestinal barrier function and increase intestinal wall permeability, intestinal microbial flora imbalance, intestinal bacterial overgrowth, and intestinal bacterial translocation. When intestinal bacteria and endotoxins enter

the blood, leading to multiple organ failure (multiple organ dysfunction syndrome), the end result is death[4].

The results of animal experiments have shown that when intestinal ischemia occurs in SAP, the mechanical barrier of the intestinal tract is damaged, the permeability of small intestine and colon mucosa is increased, and damage in the small intestine becomes more obvious; at that time, small intestine peristalsis decreases or even disappears, and the number of bacteria in the small intestine and colon rapidly increases, leading to small intestinal bacterial overgrowth (SIBO)[5]. Cen *et al*[4] established a model of intestinal motility disorder in AP rats, indicating that gastrointestinal motility disorder is the early cause of SIBO. Intestinal motility disorder is caused by neurohormone-mediated intestinal motility reduction induced by AP[6]. *Escherichia coli* and other bacteria grow excessively due to stagnation of substances in the cavity.

The gold standard for SIBO evaluation is quantitative culture of duodenal or jejunal aspiration under aerobic and anaerobic conditions, and a study found colony formation higher than 10^3 CFU/mL (colony-forming unit/mL)[7]. Because of the high cost and invasive nature of small intestinal fluid aspiration culture, the hydrogen breath test, as an alternative method for SIBO testing, has been suggested as an inexpensive, safe, and noninvasive alternative. It is widely used in the clinic as an important method to diagnose SIBO. However, the sensitivity range of the hydrogen breath test is 20%-90%, possibly because of the lack of uniform diagnostic criteria, which has been resolved by the recently published North American breath test consensus[8].

The lactose hydrogen respiration test is a noninvasive semi-qualitative test, originally designed to measure the oral-cecum passage time; it is currently used for many diseases of the digestive system, such as lactose intolerance, irritable bowel syndrome, dyspepsia, and non-alcoholic fatty liver[9,10]. The premise of the hydrogen breath test to detect SIBO is that the human metabolism does not produce hydrogen or methane. Methanogens are not bacteria, but belong to the category of archaea, mainly present in the colon, accounting for 10% of all anaerobic organisms[7]. Since the production of methane requires hydrogen metabolism, the hydrogen and methane concentrations can be simultaneously detected with the carbohydrate breath test. The lactulose hydrogen respiration test involves oral lactulose (non-absorbent sugar) to the cecum, fermentation with coliform bacteria to produce hydrogen and methane, absorption into the systemic circulation, and then exhalation to detect the concentration of hydrogen/methane in the breath; when the concentration of hydrogen/methane in the breath exceeds the baseline, a SIBO diagnosis can be reached[11].

This study aimed to examine the change in intestinal bacteria measured using the hydrogen breath test in the early stage of AP to elucidate the relationship between intestinal bacteria and ALI/ARDS. Early clinical intervention and maintenance of intestinal barrier function will be very beneficial in controlling the development of SAP.

MATERIALS AND METHODS

Research object and grouping

We retrospectively analyzed the data of 456 patients with AP who met the diagnostic criteria of the 2012 "Consensus on New Atlanta Classification Standards for Acute Pancreatitis" and who were admitted to the intensive care unit (ICU) of the Department of Digestive Department, Xuanwu Hospital of Capital Medical University from 2016 to 2019. A total of 152 patients were admitted with SIRS or organ failure and could cooperate with the hydrogen breath test within 1 wk of admission. According to the 2012 "Consensus on New Atlanta Classification Standards for Acute Pancreatitis" improved Marshall scoring standard[1], 1 patient had circulatory failure, 2 patients had renal failure, 66 patients had no organ failure, and 83 patients had respiratory failure. One patient with circulatory failure and 2 patients with renal failure were excluded. A total of 149 patients were finally included. General data including age, sex, body mass index (BMI), Acute Physiology and Chronic Health Evaluation (APACHE)-II score, and mediators of inflammation were collected.

Inclusion criteria: (1) admission within 48 h after onset; (2) admission to the hospital with SIRS; (3) organ failure within 1 wk after admission; (4) age older than 18 years; and (5) clear mind, understanding ability normal, ability to communicate with the

physician.

Exclusion criteria: (1) chronic pancreatitis; (2) history of immune-related diseases, immune deficiency, and AIDS; (3) malignant tumors; (4) inability to cooperate and complete the hydrogen breath test; (5) incomplete case data; or (6) circulatory failure or renal failure.

Grouping

According to the improved Marshall score standard in the 2012 "Consensus on New Atlanta Classification Standards for Acute Pancreatitis"[1]: $PaO_2/FiO_2 \geq 301$ mmHg was scored as 1 point, 201 mmHg ≤ $PaO_2/FiO_2 \leq 300$ mmHg was scored as 2 points, 101 mmHg ≤ $PaO_2/FiO_2 \leq 200$ mmHg was scored as 3 points, and $PaO_2/FiO_2 \leq 101$ mmHg was scored as 4 points.

Methods: 66 patients with a PaO_2/FiO_2 score ≤ 1 were included in the mild AP (MAP) group, 18 patients with a PaO_2/FiO_2 score ≥ 2 and duration < 48 h were included in the moderately SAP (MSAP) group, and 65 patients with a PaO_2/FiO_2 score ≥ 2 and duration > 48 h were included in the SAP group (Figure 1).

ALI and ARDS: ALI and ARDS were defined according to the European Union Conference[12]. The simplified criteria recommended by the meeting included defining arterial hypoxia $PaO_2/FiO_2 < 300$ mmHg as ALI and $PaO_2/FiO_2 < 200$ mmHg as ARDS, excluding cardiogenic pulmonary edema. In this study, a PaO_2/FiO_2 score = 2 was used to define ALI, and a PaO_2/FiO_2 score > 2 was used to define ARDS.

Methods: The MSAP group was divided into two groups according to the PaO_2/FiO_2 score: 15 patients with PaO_2/FiO_2 score = 2 were included in group A, and 3 patients with score > 2 were included in group B. Similarly, the SAP group was divided into two groups: 28 patients with score = 2 were included in group C, and 37 patients with score > 2 were included in group D (Figure 1).

Treatment

MAP: (1) abrosia, gastrointestinal decompression, saline enema; (2) fluid resuscitation, water maintenance, electrolyte and acid-base balance; and (3) lactulose to improve intestinal function, proton pump inhibitors for gastric acid secretion, ulinastatin and/or gabexate to improve pancreatic microcirculation.

MSAP/SAP: (1) abrosia, gastrointestinal decompression, saline enema; (2) fluid resuscitation, water maintenance, electrolyte and acid-base balance; and (3) lactulose to improve intestinal function, proton pump inhibitors for gastric acid secretion, ulinastatin and/or gabexate to improve pancreatic microcirculation; octreotide to inhibit pancreatic exocrine function, and parenteral nutrition support.

When ARDS appeared it was treated with a mechanical ventilator. When renal failure appeared it was treated with CRRT (continuous renal replacement therapy). Vasoactive drugs were used to increase blood pressure when circulatory failure could not be corrected. Peri-pancreatic and extra-pancreatic infections were treated with anti-infection therapy.

Outcome: After treatment, when there was no abdominal pain, abdominal distension, or fever, and when there were active bowel sounds, spontaneous defecation, and the abdominal computed tomography re-examination showed marked improvement, the patient could be discharged from the ICU (Figure 2).

Hydrogen breath test

Test methods: The patient remained awake and quiet during the examination and did not exercise vigorously. For the detection process, the fasting breath was collected twice, and the highest value was used as the basic exhaled hydrogen concentration (H_0). The subjects were asked to quickly consume a lactulose solution including 10 g of lactulose in 100 mL of room-temperature pure water. Subjects then exhaled every 20 min 9 times (H_1-H_9). The hydrogen concentration in the exhalation was measured and recorded at each time point.

Data processing: Hydrogen production at admission, 72 h and 96 h was measured. The average hydrogen production rate was calculated as follows: $[H_n\text{-}H_{(n-1)}]/H_0$, ($0< n \leq 9$).

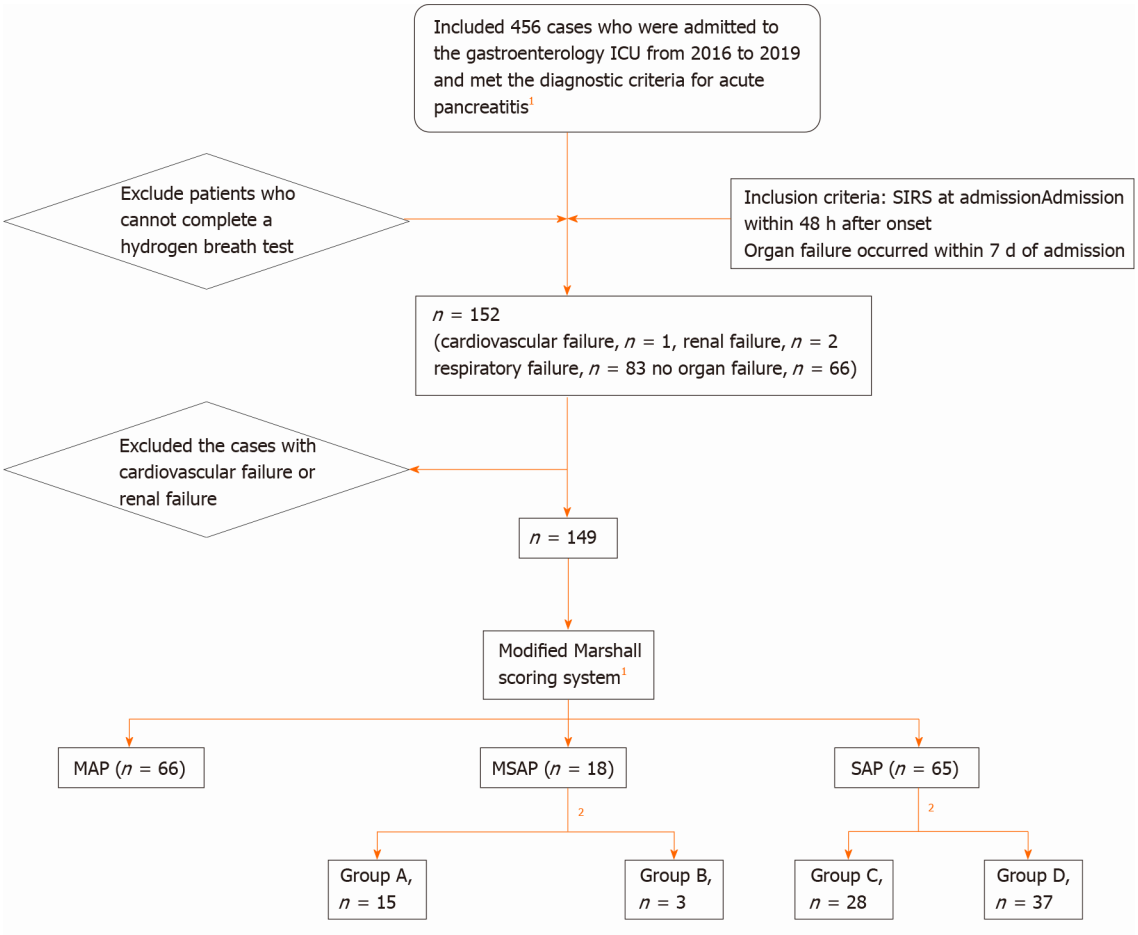


Figure 1 Research sample screening and grouping process. [1]Classification of acute pancreatitis 2012: revision of the Atlanta classification and definitions by international consensus. [2]Report of the American-European Consensus conference on acute respiratory distress syndrome in 1994. Simplified standard: PaO_2/FiO_2 < 300 mmHg is defined as acute lung injury and PaO_2/FiO_2 < 200 mmHg is defined as acute respiratory distress syndrome, and excludes cardiogenic pulmonary edema. Group A: PaO_2/FiO_2 = 2; Group B: PaO_2/FiO_2 > 2; Group C: PaO_2/FiO_2 = 2; Group D: PaO_2/FiO_2 > 2; ICU: Intensive care unit; SIRS: Systemic inflammatory response syndrome; MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis.

Statistical analysis

SPSS 25.0 (IBM Corp., Armonk, NY, United States) was used for data analysis, $P < 0.05$ was considered statistically significant. Age, BMI, APACHE-II score, and mediators of inflammation were measured, and the data are expressed as mean ± SD; and one-way analysis of variance (ANOVA) was used to compare the two groups. The hydrogen breath test data are expressed as the average hydrogen production rate ± SD, and the repeated-measures ANOVA was used in the comparison between the two groups. The number and percentage of counting data were expressed, and the chi-square test was used for comparison between the groups.

RESULTS

Clinical characteristics

A total of 149 patients were included: 66 patients in the MAP group, of whom, 53 patients were male (80.3%) and 13 patients were female (19.7%); 18 patients in the MSAP group, of whom 13 patients were male (72.2%) and 5 patients were female (27.8%); 65 patients in the SAP group, of whom 48 patients were male (73.8%) and 17 patients were female (26.2%) (Table 1). There was a significant difference in age, sex, and APACHE-II score among the MAP, MSAP, and SAP groups (both, $P < 0.001$) (Table 1).

Correlation between inflammation indicators and ALI/ARDS

There was no significant difference in interleukin (IL)-6 and procalcitonin among the MAP, MSAP, and SAP groups ($P = 0.445$ and $P = 0.399$, respectively) (Table 2). There

Table 1 Clinical characteristics

Variable	Total	MAP group	MSAP group	SAP group
Number of patients	149	66	18	65
Age, yr, (mean ± SD)	42.26 ± 12.95	36.62 ± 9.74	47.17 ± 15.17	43.00 ± 13.95
Sex (male/female)	114/35	53/13	13/5	48/17
BMI (kg/m^2)	28.21 ± 3.85	27.19 ± 3.77	27.98 ± 4.60	29.47 ± 3.77
APACHE-II score	5.92 ± 3.02	4.00 ± 3.23	6.50 ± 4.75	7.26 ± 4.07
Mortality (%)	0	0	0	0

MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis; BMI: Body mass index; APACHE: Acute Physiology and Chronic Health Evaluation.

Table 2 Correlation between inflammation indicators and acute lung injury/acute respiratory distress syndrome

Variable	Time	MAP group	MSAP group	SAP group	*P* value
IL-6	On admission	118.62 ± 137.66	98.36 ± 106.36	143.74 ± 174.40	0.445
PCT	On admission	0.46 ± 1.29	0.46 ± 0.74	0.67 ± 1.01	0.399
	72 h	0.35 ± 0.57	0.39 ± 0.49	0.66 ± 0.83	
	96 h	0.54 ± 2.51	0.19 ± 0.12	0.51 ± 0.64	

MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis; IL: Interleukin; PCT: Procalcitonin.

was no significant difference in IL-6 and procalcitonin among the MAP and MSAP groups (P = 0.306 and P = 0.683, respectively) (Table 3). There was no significant difference in IL-6 and procalcitonin among the MAP and SAP groups (P = 0.879 and P = 0.311, respectively) (Table 4). There was no significant difference in IL-6 and procalcitonin among the MSAP and SAP groups (P = 0.455 and P = 0.150, respectively) (Table 5).

Correlation between changes in the intestinal bacteria and ALI/ARDS

There was no significant difference in the number of intestinal bacteria among the MAP, MSAP, and SAP groups (P = 0.649) (Table 6). There was no significant difference in the number of intestinal bacteria among the MAP and MSAP groups (P = 0.196) (Table 7). There was no significant difference in the number of intestinal bacteria among the MAP and SAP groups (P = 0.494) (Table 8). There was no significant difference in the number of intestinal bacteria among the MSAP and SAP groups (P = 0.784) (Table 9). There was no significance difference in the number of intestinal bacteria and ALI/ARDS between group A and group B (P = 0.353) (Table 10). There was a significant difference in the number of intestinal bacteria and ALI/ARDS between group C and group D (P = 0.038) (Table 11, Figure 3). The changes in intestinal bacteria in the early stage of SAP were correlated with ARDS.

DISCUSSION

In this study, we used the hydrogen breath test to detect changes in intestinal bacteria in the early stage of AP and demonstrated that there was a correlation between ARDS and SIBO in the early stage of SAP.

This study also had some limitations. The results of the hydrogen breath test are easily affected by the number of aerogenes and the enzymatic activity of fructase. The number of gas-producing bacteria in the intestinal tract is affected by individual factors, including the environment and dietary habits, resulting in varying numbers of gas-producing bacteria among individuals. Abrosia and the use of antibiotics[13] also affect the number of gas-producing bacteria. The higher the enzymatic activity of fructase, the more gas is produced[11]. Routine lactulose treatment can improve the intestinal acid-base environment, reduce intestinal bacteria, and inhibitory activity[14];

Table 3 Correlation between the mild acute pancreatitis and moderately severe acute pancreatitis groups inflammation indicators and acute lung injury/acute respiratory distress syndrome

Variable	Time	MAP group	MSAP group	*P* value
IL-6	On admission	118.62 ± 137.66	98.36 ± 106.36	0.306
PCT	On admission	0.46 ± 1.29	0.46 ± 0.74	0.683
	72 h	0.35 ± 0.57	0.39 ± 0.49	
	96 h	0.54 ± 2.51	0.19 ± 0.12	

MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; IL: Interleukin; PCT: Procalcitonin.

Table 4 Correlation between the mild acute pancreatitis and severe acute pancreatitis groups inflammation indicators and acute lung injury/acute respiratory distress syndrome

Variable	Time	MAP group	SAP group	*P* value
IL-6	On admission	118.62 ± 137.66	143.74 ± 174.40	0.879
PCT	On admission	0.46 ± 1.29	0.67 ± 1.01	0.311
	72 h	0.35 ± 0.57	0.66 ± 0.83	
	96 h	0.54 ± 2.51	0.51 ± 0.64	

MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis; IL: Interleukin; PCT: Procalcitonin.

Table 5 Correlation between the moderately severe acute pancreatitis and severe acute pancreatitis groups inflammation indicators and acute lung injury/acute respiratory distress syndrome

Variable	Time	MSAP group	SAP group	*P* value
IL-6	On admission	98.36 ± 106.36	143.74 ± 174.40	0.455
PCT	On admission	0.46 ± 0.74	0.67 ± 1.01	0.150
	72 h	0.39 ± 0.49	0.66 ± 0.83	
	96 h	0.19 ± 0.12	0.51 ± 0.64	

MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis; IL: Interleukin; PCT: Procalcitonin.

Table 6 Correlation between changes in intestinal bacteria and acute lung injury/acute respiratory distress syndrome

Hydrogen breath test	MAP group	MSAP group	SAP group	*P* value
On admission	1.63 ± 1.28	2.35 ± 2.14	1.68 ± 1.85	0.649
72 h	1.58 ± 1.29	1.83 ± 2.58	2.13 ± 3.27	
96 h	1.60 ± 1.22	1.58 ± 0.93	1.54 ± 1.35	

MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis.

there is also the catharsis effect of reduced intestinal bacteria; long-term lactulose treatment is equivalent to increasing the dose of lactulose in the hydrogen breath test, resulting in higher or lower results. In this study, quantitative analysis and the growth ratio were used to neutralize the influence of the basic value on the qualitative results.

As early as 2017, Zhang *et al*[15] and others were using the qualitative standard of the hydrogen breath test to examine the relationship between SAP and SIBO. The results showed that damage of intestinal barrier function is already present in the early stage of SAP; the positive SIBO rate was higher in patients with SAP, and SIBO mainly occurred within 72 h from onset. Finally, we found that there was a correlation

Table 7 Correlation between changes in intestinal bacteria and acute lung injury/acute respiratory distress syndrome

Hydrogen breath test	MAP group	MSAP group	*P* value
On admission	1.63 ± 1.28	2.35 ± 2.14	0.196
72 h	1.58 ± 1.29	1.83 ± 2.58	
96 h	1.60 ± 1.22	1.58 ± 0.93	

MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis.

Table 8 Correlation between changes in intestinal bacteria and acute lung injury/acute respiratory distress syndrome

Hydrogen breath test	MAP group	SAP group	*P* value
On admission	1.63 ± 1.28	1.68 ± 1.85	0.494
72 h	1.58 ± 1.29	2.13 ± 3.27	
96 h	1.60 ± 1.22	1.54 ± 1.35	

MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis.

Table 9 Correlation between changes in intestinal bacteria and acute lung injury/acute respiratory distress syndrome

Hydrogen breath test	MSAP group	SAP group	*P* value
On admission	2.35 ± 2.14	1.68 ± 1.85	0.784
72 h	1.83 ± 2.58	2.13 ± 3.27	
96 h	1.58 ± 0.93	1.54 ± 1.35	

MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis.

Table 10 Correlation between changes in intestinal bacteria and acute lung injury/acute respiratory distress syndrome in the moderately severe acute pancreatitis group

Hydrogen breath test	*P* value	MSAP group (*n* = 18)	
		Group A (*n* = 15)	Group B (*n* = 3)
On admission	0.353	2.59 ± 2.28	1.12 ± 0.04
72 h		1.97 ± 2.81	1.08 ± 0.43
96 h		1.54 ± 0.95	1.82 ± 0.95

MSAP: Moderately severe acute pancreatitis; Group A: $PaO_2/FiO_2 = 2$; Group B: $PaO_2/FiO_2 > 2$.

between ARDS and SIBO in the early stage of SAP ($P < 0.05$) in line with the results of previous studies. ALI/ARDS are the earliest organ-dysfunction symptoms in SAP. In this study, it was concluded that the occurrence of ARDS was related to SIBO within 72 h of admission. Studies have shown that SAP intestinal failure appears early, consistent with the results of our study. SAP-induced ALI/ARDS is mainly due to lung injury mediated by inflammatory factors[16]. Both infectious and non-infectious factors can lead to a large number of inflammatory factors. In the early stage of AP, inflammatory factors produced by pancreatic digestion damage intestinal barrier function, leading to persistent SIRS and SIBO, with SIBO leading to harmful metabolite accumulation in the intestinal tract, especially endotoxins and pathogenic intestinal bacteria[17]; subsequently, a large number of endotoxins and bacteria enter the bloodstream, flow through the pulmonary capillaries, activate neutrophils, damage the pulmonary vascular endothelium, leading to diffuse alveolar injury and type I alveolar necrosis, and eventually to ALI/ARDS[18,19]. Zhang *et al*[20] found that serum endotoxins

Table 11 Correlation between changes in intestinal bacteria and acute lung injury/acute respiratory distress syndrome in the severe acute pancreatitis group

Hydrogen breath test	*P* value	SAP group (*n* = 65)	
		Group C (*n* = 28)	Group D (*n* = 37)
On admission	0.038	2.39 ± 2.61	1.15 ± 0.58
72 h		1.68 ± 1.85	2.85 ± 4.79
96 h		1.84 ± 1.86	1.31 ± 0.71

SAP: Severe acute pancreatitis; Group C: $PaO_2/FiO_2 = 2$; Group D: $PaO_2/FiO_2 > 2$.

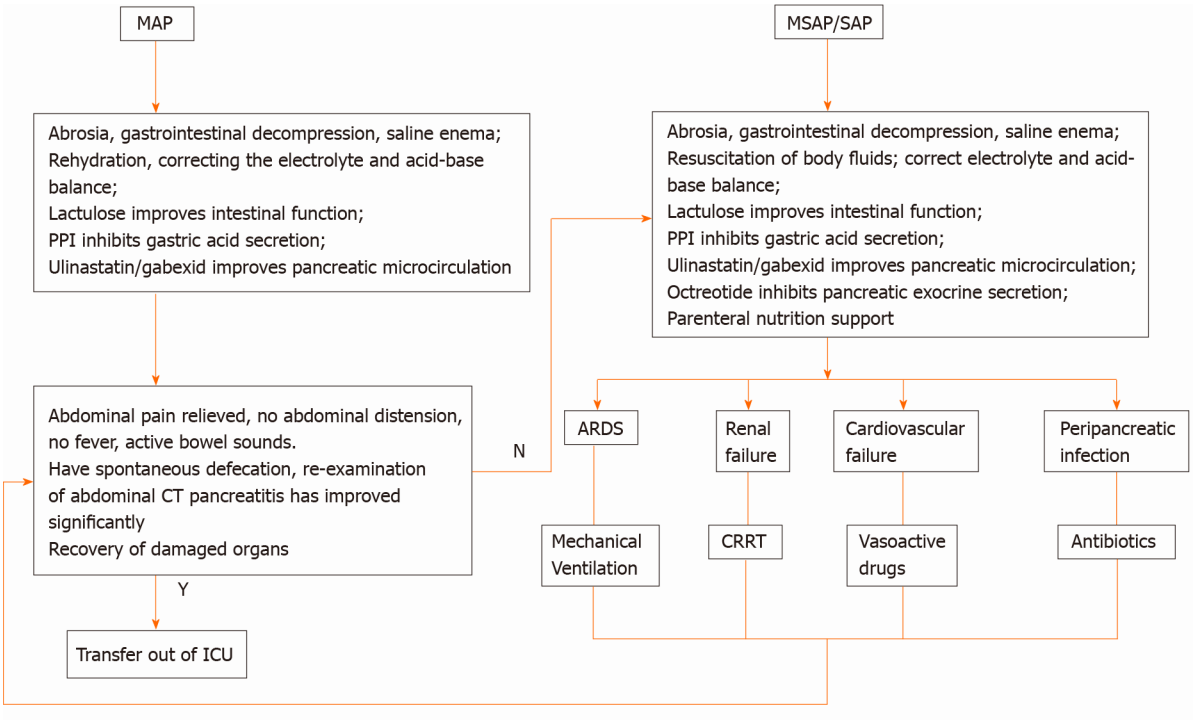


Figure 2 Treatment process. MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis; PPI: Proton pump inhibitor; CT: Computed tomography; ICU: Intensive care unit; ARDS: Acute respiratory distress syndrome; CRRT: Continuous renal replacement therapy; Y: Yes; N: No.

and intestinal mucosal permeability in patients with SAP were higher than in normal controls. Wang *et al*[3] found that lung bacteria were rich in intestine-related bacteria in mice with AP, sepsis, and ARDS. This study only showed the translocation of intestinal bacteria when sepsis and ARDS occurred simultaneously but did not explain the relationship between intestinal bacteria and ARDS when only ARDS occurred. Unfortunately, we could not locate animal experimental data on the correlation between ARDS and intestinal bacteria in the early stage of AP. The results of this clinical study showed that ARDS was correlated with intestinal bacteria, which should be further explored using animal models. However, although animal test support is lacking, in a clinical study on the relationship between the onset time and treatment time window of AP[21], the researchers concluded that the key time window for AP treatment is within 1 wk after the onset of AP; otherwise, the disease will worsen, the clinical course will be prolonged, and the mortality rate will increase; in terms of time, this study completely agrees with the previous research results. Therefore, the results of this study are of high guiding significance for clinicians who manage patients with AP. Early attention to the changes in intestinal bacteria can prevent the occurrence of SAP and high mortality.

This study also explored the correlation between IL-6 and ALI/ARDS. At present, the research results show that IL-6 is related to SAP. IL-6 can be used as an early predictor of complications[22]. The specificity of serum IL-6 > 160 pg/mL in the

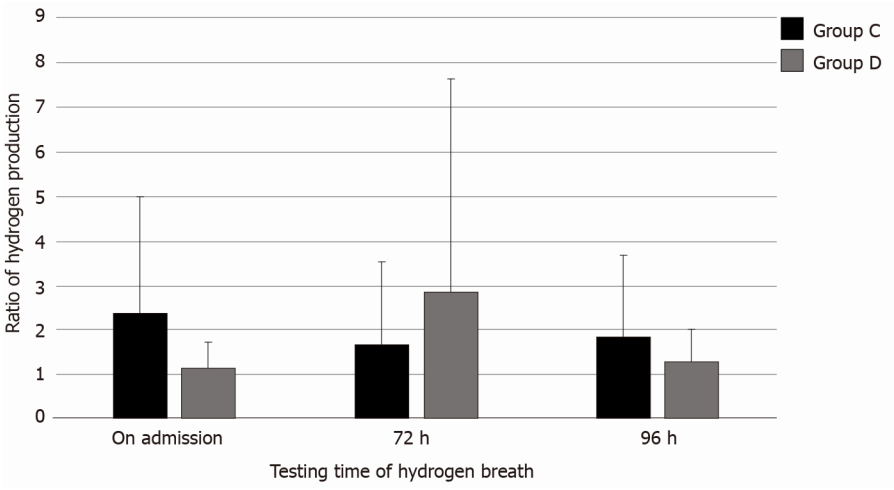


Figure 3 The correlation between changes in intestinal bacteria and acute lung injury/acute respiratory distress syndrome in the severe acute pancreatitis group. Group C: $PaO_2/FiO_2 = 2$; Group D: $PaO_2/FiO_2 > 2$.

diagnosis of SAP was 95%[23]. Relevant animal experiments have shown that when pancreatic injury occurs, IL-6 is rapidly synthesized to play an anti-inflammatory and protective role. When IL-6 continues to be synthesized and accumulates in large quantities, it mainly plays a pro-inflammatory role, leading to the occurrence and development of SAP[22]. In a clinical study conducted by Rezaie *et al*[13], it was found that the content of IL-6 in the blood was positively correlated with the abundance of *Enterobacteriaceae* and *Enterococci*, but negatively correlated with the abundance of *Bifidobacteria*. That study also compared the characteristics of intestinal bacteria between patients with AP and healthy controls. The results showed that the intestinal bacteria of patients with AP and healthy controls differed, and the abundance of *Enterobacteriaceae* and *Enterococci* and other potentially pathogenic bacteria differed from the abundance of beneficial bacteria such as *Bifidobacteria*, which decreased significantly. Although the final results of this study did not show a correlation between IL-6 and SAP, it is considered that this may be related to the treatments administered after admission.

CONCLUSION

Early organ failure in SAP was found to be closely related to the intestinal bacteria. Importantly, this study also determined the timing of SIBO, which is of great guiding significance for the clinical treatment of AP.

ARTICLE HIGHLIGHTS

Research background

In the early stage of acute pancreatitis (AP), a large number of cytokines induced by local pancreatic inflammation seriously destroy the intestinal barrier function, resulting in intestinal bacteria and endotoxins in the blood, causing inflammatory storms, and leading to multiple organ failure. Hydrogen breath testing is currently used for many diseases of the digestive system, such as lactose intolerance, irritable bowel syndrome, dyspepsia, and non-alcoholic fatty liver. This test, as an alternative method to detect small intestinal bacterial overgrowth (SIBO), was used to indirectly reflect the number of intestinal bacteria in AP. This study aimed to examine the change in intestinal bacteria measured using the hydrogen breath test in the early stage of AP to elucidate the relationship between intestinal bacteria and acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). Early clinical intervention and maintenance of intestinal barrier function will be very beneficial in controlling the development of severe AP (SAP).

Research motivation

Clinical trials showed that intestinal bacteria in AP patients were different to those in healthy people and the abundance of potential pathogenic bacteria such as *Enterobacteriaceae* and *Enterococcus* increased significantly while the abundance of beneficial bacteria such as *Bifidobacterium* decreased significantly. Animal experiments showed that not only the changes in intestinal bacteria in patients with AP were verified, but also there was a correlation between the changes in intestinal bacteria and organ failure. However, neither of them showed a relationship between intestinal bacteria and organ failure in the early stage of AP. If we can understand the change in intestinal bacteria using the hydrogen breath test in the early stage of AP, in order to understand the relationship between intestinal bacteria and ALI/ARDS, we can intervene and maintain the stability of intestinal barrier function as soon as possible, which will be very beneficial in controlling the development of SAP.

Research objectives

Maintaining intestinal barrier function and reducing and preventing intestinal bacteria translocation have become the key to controlling the occurrence and development of SAP. This reduces complications in the early stage of the disease. We performed the present study to investigate the changes in organ function and intestinal bacteria in AP, and to explore the correlation between SIBO and AP organ function.

Research methods

Principle of the hydrogen breath test: after taking lactulose (nonabsorbable sugar), it reaches the cecum and is fermented with coliform bacteria to produce hydrogen and methane, which are absorbed into the systemic circulation and exhaled through the lung. When the hydrogen concentration in the exhaled breath exceeds the baseline, it is diagnosed as SIBO. In this study, a portable hydrogen expiratory detector was used to detect the patients' expired hydrogen concentration to diagnose SIBO. The change in exhaled hydrogen concentration can reflect the number of bacteria in the small intestine.

Research results

The results showed that in 37 SAP patients with ARDS, the average hydrogen production rate at admission was 1.15 ± 0.58, at 72 h was 2.85 ± 4.79 and at 96 h was 1.31 ± 0.71. In 28 SAP patients without ARDS, the average hydrogen production rate at admission was 2.39 ± 2.61, at 72 h was 1.68 ± 1.85, and at 96 h was 1.84 ± 1.86. The increase in intestinal bacteria in patients with SAP within 72 h after admission was related to the occurrence of ARDS. How to effectively maintain the stability of intestinal barrier function in the early stage of the disease and prevent the overgrowth of intestinal bacteria is a problem to be solved.

Research conclusions

In this study, intestinal bacterial overgrowth in the early stage of SAP was associated with ARDS. Moreover, the occurrence of ARDS was related to SIBO within 72 h of admission. It is of guiding significance to maintain the stability of intestinal barrier function in the early stage in order to reduce the complications of early organ failure and late infection.

Research perspectives

How to effectively maintain the stability of intestinal barrier function and prevent the excessive growth of intestinal bacteria in the early stage of disease, in order to reduce the occurrence and development of SAP, is the future research direction.

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CASE REPORT

Gastrointestinal cytomegalovirus disease secondary to measles in an immunocompetent infant: A case report

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Abstract

BACKGROUND

Gastrointestinal cytomegalovirus (CMV) disease occurs commonly in immunocompromised/immunodeficient patients with advanced human immunodeficiency virus infection, neoplasm, solid organ transplantation, hematopoietic stem cell transplantation, or treatment with immunosuppressants, but is rarely reported in association with measles infection.

CASE SUMMARY

We describe a case of extensive gastrointestinal CMV disease secondary to measles infection in a 9-mo-old boy who presented with persistent fever and bloody diarrhea. His condition was improved after ganciclovir treatment. Serological analysis of CMV showed negative immunoglobulin (Ig) M and positive IgG. Blood CMV-DNA was 9.26×10^3 copies/mL. The diagnosis of gastrointestinal CMV disease was confirmed by histopathological findings of intranuclear and intracytoplasmic inclusions and Owl's eye inclusion. This case highlights the differential diagnosis and histopathological characteristics of gastrointestinal CMV infection and laboratory tests.

CONCLUSION

Extensive gastrointestinal CMV lesions can be induced by the immune suppression secondary to measles infection. Rational, fast, and effective laboratory examinations are essential for suspected patients.

Key Words: Cytomegalovirus; Diarrhea; Gastrointestinal; Infant; Measles; Case report

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Core Tip: We report a case of gastrointestinal cytomegalovirus (CMV) disease secondary to measles infection in a 9-mo-old Chinese boy who had extensive gastrointestinal lesions; the diagnosis was confirmed by histopathological analysis. His condition was improved by ganciclovir treatment. This case highlights the differential diagnosis and histopathological characteristics of gastrointestinal CMV infection and laboratory tests and sheds light on the difficulty in diagnosing gastrointestinal CMV disease due to its nonspecific clinical presentation and the weak diagnostic value of serologic antibody detection.

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INTRODUCTION

Cytomegalovirus (CMV) is an intracellular virus, and CMV infection of the gastrointestinal tract is commonly documented in immunocompromised/immunodeficient patients. Measles infection induces immune suppression and is associated with an increased susceptibility to secondary infections[1], and this effect has typically been thought to last from a few weeks to a few months[2]. The Th2 response during convalescence of measles might inhibit Th1 responses, increasing susceptibility to other infections in children with measles[3]. The morbidity from diarrhea, one of the most common complications of measles, is reported to increase in the acute phase of measles[4]; however, gastrointestinal CMV infection is rarely reported as a causative etiology of persistent diarrhea secondary to measles infection.

CMV disease of the gastrointestinal tract is associated with significant mortality, mostly resulting from considerable bleeding and perforation when the patient is in an immunodepressed status[5]. However, it is not so common for the lesions to be very large. Limitations of the serologic assay of CMV and nonspecific clinical manifestations that mimic many other infectious diseases present a significant challenge to early and accurate diagnosis in clinical practice. Clinicians should consider CMV infection as a differential diagnosis even in immunocompetent patients with common alimentary symptoms, particularly intractable diarrhea, hematochezia, or vomiting in conjunction with fever. Here, we describe a case of gastrointestinal CMV disease secondary to measles infection that involved vast lesions, and we review the differential diagnosis, histopathological diagnosis, and treatment of gastrointes-tinal CMV infection.

CASE PRESENTATION

Chief complaints

A 9-mo-old Chinese boy was admitted to Shenzhen Children's hospital because of a persistent high fever and diarrhea that lasted for 17 d.

History of present illness

After 3 d of fever, he had a rash extending from his face to his trunk and limbs, and 5 d later, he suffered watery and bloody diarrhea. Positive immunoglobulin (Ig) M on the 10th day of fever confirmed a diagnosis of measles. Peripheral white blood cells, neutrophils, and C-reactive protein were elevated. Many leukocytes were found in his stool. Cefoperazone-sulbactam was administered intravenously for 4 d before admission, but the frequency of diarrhea increased, along with abdominal distension.

History of past illness

The patient had two episodes of pneumonia 40 d before this admission.

Personal and family history

The patient was born by cesarean section after full-term gestation, without complications during gestation or delivery. He had met physical and developmental milestones. Routine childhood immunizations were administered except for the measles vaccine.

Physical examination

On admission, the patient was weak but alert with a dehydrated appearance and typical measles skin (pigmentation and desquamation all over his body). The abdomen was soft and distended, with no tenderness or masses. The remainder of the physical examination was normal. Rapid intravenous rehydration was applied before further evaluation.

Laboratory examinations

Serological analysis for CMV showed negative IgM and positive IgG. PCR blood assay showed an elevation of CMV-DNA. The other laboratory results are shown in Tables 1 and 2.

Imaging examinations

Abdominal radiography (Figure 1) revealed upper gastrointestinal obstruction, while abdominal ultrasonography showed no obvious dilatation of the intestine or free effusion. Enhanced computed tomography demonstrated obstruction in the horizontal duodenum.

Further diagnostic work-up

On the 23rd day, gastroscopy (EG-99WR; Fujinon, Tokyo, Japan) and colonoscopy (GIF-XQ240; Olympus, Tokyo, Japan) were performed. Gastroscopy (Figure 2) revealed gastroduodenal mucosal edema and hyperemia, lymphoid hyperplasia, focal ulceration, pseudotumor formation, and stenosis in the horizontal duodenum. Incomplete colonoscopy (Figure 3) displayed diffuse mucosal edema and roughness, stiffness, friability, and white membranoid substances in the descending colon, sigmoid colon, and rectum. The histopathological examination revealed characteristic inclusions suggestive of CMV.

FINAL DIAGNOSIS

The final diagnosis of the presented case was gastrointestinal CMV disease.

TREATMENT

After admission to the hospital, suspected bacterial infection was treated with intravenous vancomycin and meropenem without improvement. On hospital day 12, diarrhea was aggravated with persistent high fever and episodes of vomiting and abdominal distension. Fasting and gastrointestinal decompression did not improve his condition. After the diagnosis of gastrointestinal CMV disease was made, intravenous ganciclovir was administered at 5 mg/kg every 12 h on the 30th hospital day for 2 wk and then reduced to 5 mg/kg each day for 1 wk.

OUTCOME AND FOLLOW-UP

The patient's condition improved after treatment with IV ganciclovir and he was discharged on the 54th hospital day. He was followed regularly to 4.5 years old, and he is doing well.

DISCUSSION

This 9-mo-old Chinese boy had persistent fever and diarrhea after measles infection; ensuing vomiting, abdominal distension, and hematochezia presented at a later stage.

Table 1 Laboratory results (international system of units)

Parameter	Reference range	5 d before admission	1 d before admission	On admission	Day 6	Day 12	Day 23	Day 37
Hemoglobin (g/L)	110-160	120	115	79	91	73	126	69
Erythrocytes (× 10^{12}/L)	3.5-5.5	4.70	4.56	2.93	3.21	2.50	3.93	2.34
Leucocytes (× 10^{9}/L)	5-12	14.96	9.36	17.87	8.37	20.42	38.68	10.35
Neutrophils (%)	50-70	54.9	32.6	55.1	62.7	61.6	80.4	59.9
Lymphocytes (%)	20-40	36.9	56.7	27	29.3	28.3	15.4	33.4
Atypical lymphocytes (%)	0-2			9	7	0	0	0
Eosinophils (%)	0.02-0.5	1.3	1.4	0.9	0.4	0	0	0
Platelets (× 10^{9}/L)	100-300	234	226	769	200	276	165	110
Sedimentation rate (mm/h)	0-15			78		1		
C-reactive protein (mg/L)	0-10	39.7		176	80	114	3.7	4.7
Procalcitonin (ng/L)	0-0.5			31.87	0.77		0.31	
Sodium (mmol/L)	135-146			116.3	138.4	131.2	139.4	135
Potassium (mmol/L)	3.5-5.5			6.15	4.53	3.9	4.2	3.61
Chloride (mmol/L)	101-111			91.1	102.7	97.8	116.9	110.5
Calcium (mmol/L)	2.25-2.75			1.79	2.46	1.87	2.04	1.66
Magnesium (mmol/L)	0.7-1.15			0.96	0.61	0.53	0.35	0.38
Albumin (g/L)	35-55			25.8	37.3	25.9	15.5	20.6
Globulin (g/L)	20-30			28.1	17.6	19.3	13.2	5.9
Aspartate amino transferase (IU/L)	0-40			70	28	48	85	22
Alanine amino transferase (IU/L)	0-40			61	12	41	53	6
Prothrombin time (s)	9.3-12.9			17.4	19.6	12.5	11.4	16.2
Activated partial thromboplastin time (s)	26.1-40.7			34.5	57.8	38	39.5	55.2
Fecal occult blood test	-			-	+	+	+	+
Fecal white blood cell	-	++	-	-	++	++++	++	-

All of these symptoms are indicative of digestive system diseases. Fever and diarrhea are common signs of different diagnoses in infants and children, including gastrointestinal infectious and noninfectious causes. In complicated cases with a prolonged course, there are difficulties in distinguishing pathogens; thus, detailed clinical data, exposure history, and pertinent laboratory tests can provide valuable assessment information.

Bacteria are reported as predominant pathogens of measles associated with diarrhea. However, this patient failed to respond to various antibiotics. Some distinct pathogens were considered. Enteroaggregative *Escherichia coli* (*E. coli*) and enteropatho -genic *E. coli* are the most commonly implicated bacterial pathogens in persistent infections in developing countries, especially among children[6]. The patient was a breastfed infant who had no ingestion of contaminated food, water, or unpasteurized milk, no exposure to sick contacts, no contact with infected pets or fowl, no travel history, and a negative result from stool culture, all of which indicated a low possibility of *shigella, salmonella, campylobacter* or other enteric infections[7].

While asymptomatic colonization of *Clostridium difficile* (*C. difficile*) is prevalent in infants, for patients with recent exposure to hospitals and antibiotics, microbiological evaluation should focus on the diagnosis of *C. difficile* infection[8]; however, *C. difficile* should be sensitive to vancomycin, together with repeated negative bacterial separation and culture and enzyme immunoassay tests for toxins A and B and glutamate dehydrogenase. There was no support for the diagnosis of *C. difficile* infection in this patient.

The patient lives in China, a country that ranks 3rd in the world for high tuberculosis

Table 2 Microbiology results

	Peripheral blood	Feces
Day 1	HBsAg (-); HBsAb (+); HBeAg (-); HBeAb (-); HBcAb (-); TP antibody (-); HIV antibody (-); HCV antibody (-); EBNA IgG (+); EBV-CA IgG (+); EBV-CA IgM (±); EBV-EA IgM (-); EBV DNA < 5.00×10^2 copies/mL (LD)	Norovirus (-); Rotavirus (-); Astrovirus (-); Enteral adenovirus (-); Shigella NA (-); Salmonella NA (-); Widal's test (-)
Day 6	G test (-); EBV DNA < 5.00×10^2 copies/mL (LD)	Norovirus (-); Rotavirus (-); Astrovirus (-); Enteral adenovirus (-)
Day 12	G test (-); Interferon-γ release assay of tuberculosis (-)	Clostridium difficile GDH (-); Clostridium difficile toxin A/B (-)
Day 16	CMV-IgM 11.5 U/mL (ref 0-22); CMV-IgG 18.3 U/mL (ref 0-14); CMV DNA quantitation 9.26×10^3 copies/mL	Clostridium difficile GDH (-); Clostridium difficile toxin A/B (-)
Day 36	CMV DNA quantitation < 5.00×10^2 copies/mL (LD); CMV-IgG 82.2 U/mL (ref 0-14)	

HBsAg: Hepatitis B virus surface antigen; HBsAb: Hepatitis B virus surface antibody; HBcAg: Hepatitis B virus core antigen; HBcAb: Hepatitis B virus core antibody; HBeAg: Hepatitis B virus e antigen; TP: Treponemal; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; EBV-CA: Epstein-Barr virus capsid antigen; EBNA: Epstein-Barr nuclear antigen; EBV-EA: Epstein-Barr virus early antigen; MV: Measles virus; RV: Rubella virus; GDH: Glutamic acid dehydrogenase; LD: Limit of detection; NA: Nucleic acid.

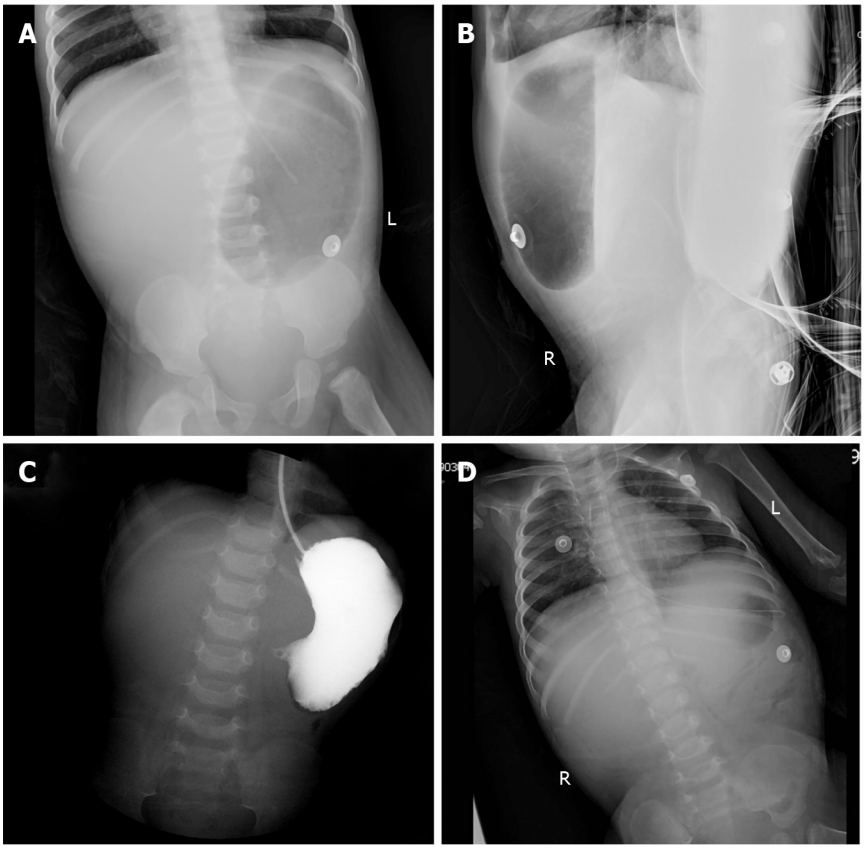


Figure 1 Abdominal plain. A and B: Supine (A) and lateral (B) abdominal plain films showed a large stomach, and no free gas was found in the small intestine, colon, or rectum. A nasogastric tube was coiled in the stomach; C: The iopromide administered by gastric tube did not enter the duodenum; D: Re-examination of abdominal film revealed decreased gas in the stomach and a few inflatable intestines.

(TB) morbidity; thus, intestinal TB (ITB) should be considered even though it is more likely to cause miliary TB in infants. ITB is characterized by low-grade fever, abdominal pain, loss of weight, altered bowel habits, and night sweats[9]. The absence of exposure to *Mycobacterium tuberculosis* and negative chest X-ray and interferon-γ release assay for TB revealed no direct evidence of TB in this patient.

As a noninfectious disease, inflammatory bowel disease (IBD) should be included in the differential diagnosis when lacking evidence of clear pathogenic microorganism infection[7]. The typical clinical presentations of infantile-onset IBD are intermittent fever and bloody diarrhea[10]; some cases are also associated with oral ulcers and

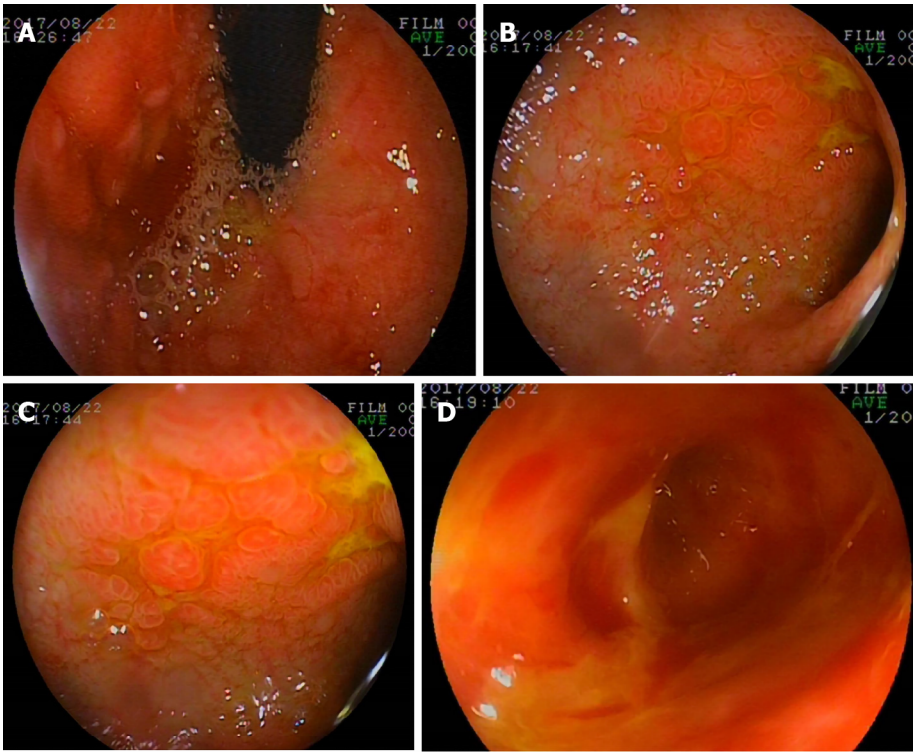


Figure 2 Gastroscopic findings. A gastroscope was inserted into the horizontal part of the duodenum. Hyperemia, edema, lymphoid hyperplasia, focal ulceration, and pseudotumor formation were seen in the stomach and duodenum. A: Gastric fundus; B and C: Duodenal bulb; D: Dilatation of proximal duodenum.

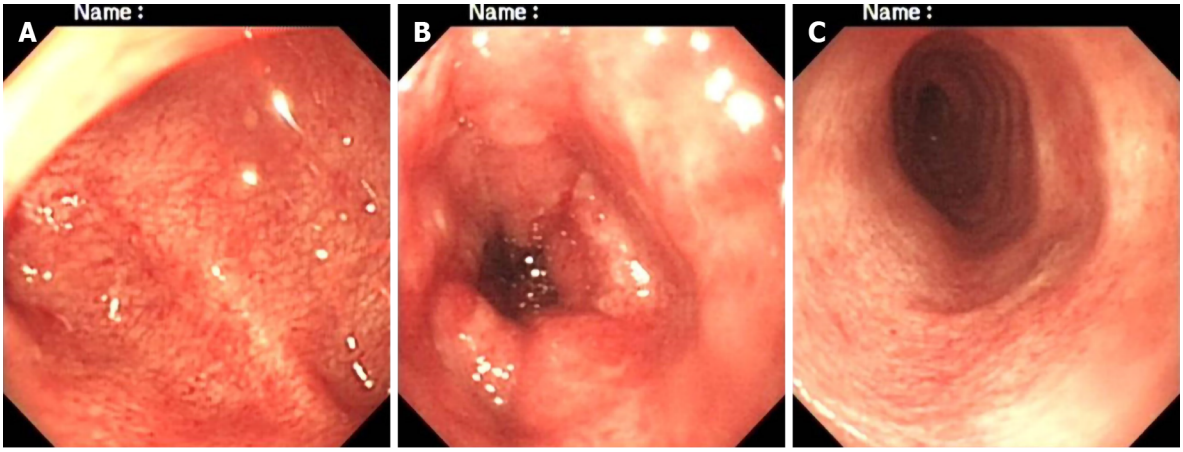


Figure 3 Colonoscopic findings. A colonoscope reached the splenic flexure of the colon. It showed hyperemia, roughness, stiffness, friability, and easy bleeding of the mucosa. A: Rectum; B: Sigmoid colon; C: Descending colon.

perianal abscess and fistula. The diagnosis relies on endoscopic evaluation and histopathological findings of mucosal biopsy other than on clinical characteristics and history. Macroscopic features revealed no contiguous or skip linear ulceration, aphtha ulceration, cobblestoning, fistula, abscesses, or other changes, and microscopic features showed no crypt architectural changes or noncaseating granuloma, so the evidence supporting IBD was insufficient[11]. Granting that very-early onset IBD may have heterogeneous or atypical symptoms and signs, endoscopy and mucosal biopsy were of great benefit to exclude IBD in this patient. There was no evidence of indigestion, malabsorption, autoimmune enteropathy, hemolytic uremic syndrome, allergic or neoplastic disorders, drug- or poison-induced diarrhea, structural diseases, or functional enteropathy in this case (Table 3).

Multiple factors may contribute to several weeks of measles-induced immune suppression[1]. There is an epidemiologically detectable long-term increase in susceptibility to infectious diseases in those who survive measles[12]. Measles infection contributes to transient lymphopenia with decreased numbers of T cells and B cells in

Table 3 Some noninfectious factors in diarrhea

Gastrointestinal diseases	Digestion and absorption disorder
(1) Inflammatory bowel disease; (2) Gastroenteric tumor; (3) Intussusception; (4) Enteric cyst; (5) Duplication of digestive tract; (6) Diverticulosis; (7) Polyposis coli; and (8) Ischemic enteropathy	(1) Short bowel syndrome; (2) Exocrine pancreatic insufficiency; (3) Bile acid-losing syndrome; and (4) Lactose intolerance
Systemic disease: (1) Anaphylactoid purpura; (2) Hyperthyroidism; and (3) Primary chronic adrenocortical hypofunction	Neoplastic diseases: (1) Gastrin adenoma; (2) Carcinoid syndrome; (3) Vasoactive intestinal peptide tumor; and (4) Pheochromocytoma
Allergic diarrhea: (1) Food protein-induced enterocolitis syndrome; (2) food protein-induced proctocolitis; and (3) eosinophilic gastroenteritis	Primary immunodeficiency diseases: (1) Chronic granulomatous disease; (2) Common variable immunodeficiency; and (3) X linked agammaglobulinemia
	Secondary immunodeficiency diseases
	Functional diarrhea
	Drugs/toxicants factor

circulation during the acute phase of infection. As viral RNA persists, suppression of lymphocyte proliferation is induced. During recovery, the later Th 2 CD4+ T-cell response may suppress macrophage activation and Th 1 responses to new infections. Moreover, measles-infected dendritic cells induce lymphocyte unresponsiveness. The suppression of interleukin (IL)-12 production, lymphocyte expression of CD30, and elevation of IL-4, IL-10, and IL-13 might contribute to immune suppression. Deficiencies of both innate and adaptive immune responses can render individuals with measles more susceptible to secondary bacterial and viral infections[13]. The susceptibility to CMV is considered to be due to impaired cell-mediated immunity and a reduction in the levels of IgM[14].

Any part of the alimentary tract can be impacted by CMV infection but the typical CMV lesions are limited to either the upper or the lower gastrointestinal tract, while both the upper and the lower gastrointestinal tracts are rarely involved simultaneously[15]. Gastrointestinal CMV disease with complex and diverse but nonspecific symptoms is a remediable disease with a high mortality; delayed diagnosis, misdiagnosis, and missed diagnosis greatly increase fatality. To the best of our knowledge, in both immunocompetent and immunodeficient patients, intractable diarrhea and fever seem to be the most common presentations of gastrointestinal CMV disease, so differentiating CMV infection from infectious diarrhea as early as possible is vital in clinical practice.

In addition to broad clinical presentations and signs, confirmation of the virus *via* laboratory methods is indispensable in diagnosing CMV disease. In this patient, CMV-IgM was negative, and the absence of evidence of active infection led to an uncertain causal relationship between CMV infection and various symptoms, which demanded histopathology detection for further verification. Nonetheless, the positive CMV-IgG titre was more than 4-fold in 2 wk, and elevated CMV DNA copies provided clues for diagnosis. Nevertheless, false negative IgM results can be obtained in immunocompromised patients and infants. Positive measles IgM was detected in this patient because the immune status was not affected by measles infection at the early stage. However, due to an immunocompromised status secondary to measles infection later, the negative IgM significantly limits its clinical application in the early diagnosis of CMV infection, which may delay diagnosis and treatment[16].

Negative results of CMV PCR do not rule out CMV disease[17]. As a marker of early diagnosis, CMV pp65 antigen is not able to differentiate between latent infection and active disease. Viral culture is the diagnostic gold standard, but its poor sensitivity and slow turn-around time limit its clinical utility, while histopathology assays can provide confirmatory information for invasive disease which is highly specific[18] (Figure 4). Therefore, when gastrointestinal CMV infection is suspected, endoscopy and biopsy must be performed to obtain positive evidence for diagnosis.

The macroscopic features of gastrointestinal CMV disease vary from normal mucosa, focal erythematous, erosion, and pseudotumor formation to deep ulceration, lacking specificity[19]. Based on the patchy distributions and diverse endoscopic findings, biopsy location and number are important to assess CMV[20]. Gastroduodenoscopy and incomplete colonoscopy were performed in this patient due to the risk of intestinal perforation and massive hemorrhage. The outcome of the histopathology assay confirmed invasive CMV disease.

CMV disease requires evidence of parenchymal organ damage before it can be diagnosed and treated with antiviral therapy. Anti-CMV treatment in immunocom-

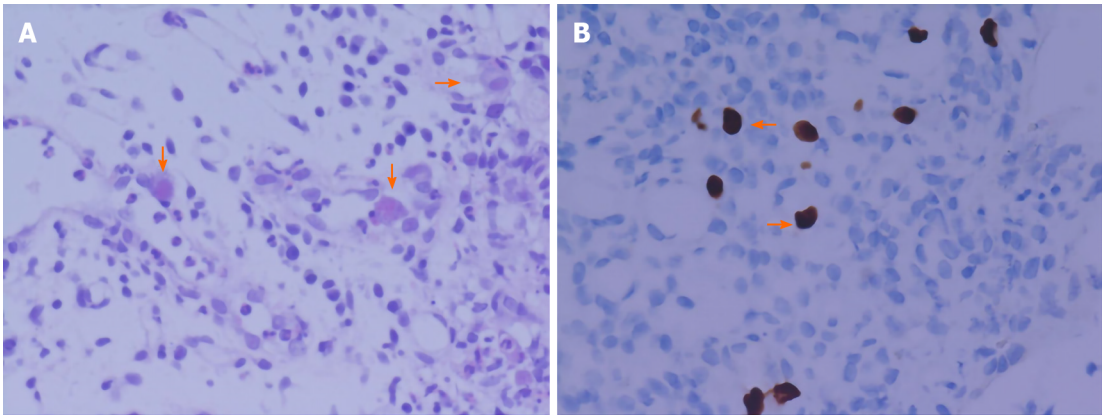


Figure 4 Gastrointestinal histopathologic findings. Histopathologic photomicrographs of gastric, duodenal, descending colony-like, sigmoid colony-like, and rectal specimens. A: Inflammatory cell infiltration and characterized cytomegalic cells (arrows) containing eosinophilic intranuclear inclusions (hematoxylin and eosin: × 400); B: Owl's eye inclusions (arrows) in cytomegalovirus-infected cells (immunohistochemical staining: × 400).

promised patients is of vital importance to improve prognosis. Ganciclovir is recommended as the first-line pharmacological treatment for CMV infection, which comprises induction and maintenance. The initial dose must be adjusted for renal function and it is commonly advised to be 5 mg/kg every 12 h for at least 2 wk to 3 wk in the induction stage[21]. The course of treatment varies with therapeutic reactions, including symptoms, quantitative detection of viral load, whole blood counts, hepatic function and renal function. When patients cannot tolerate side effects or diseases worsen with the use of ganciclovir, foscarnet is generally used as a substitution drug, whereas which can result in renal toxicity and electrolyte disturbances[22]. After the diagnosis of CMV disease, the auditory, ophthalmic, and neurodevelopmental assessments of the patient were normal. His temperature decreased, vomiting disappeared, and the bowls recovered after intravenous ganciclovir administration. Inflammation biomarkers and CMV DNA copies decreased. An obvious improvement in imaging was shown on re-examination (Figure 1). The amelioration after antiviral therapy helped exclude IBD completely. Undoubtedly, this patient was fortunate. For immunocompromised patients with severe CMV infection, surgical treatment is required when medical treatment is ineffective.

In conclusion, we sought to review the differential diagnosis for similar clinical outcomes in a young child with diarrhea, hematochezia, and fevers, and to clarify endoscopic and histopathological findings as well as outline treatment options for CMV colitis. We highlighted the diagnostic challenges of gastrointestinal CMV disease due to its nonspecific clinical presentation and the weak diagnostic value of serologic antibody detection. We hope to help clinicians improve the understanding of the importance of considering this disease in immunocompromised patients, as well as to consider it in otherwise healthy patients with a prolonged course characterized by fever, diarrhea, and hematochezia who may become immunocompromised due to measles infection.

CONCLUSION

This is the first case of gastrointestinal CMV infection due to transient immunosuppression secondary to measles infection in a presumably immunocompetent child. Gastrointestinal CMV disease should be taken into considera-tion in patients with persistent fever and diarrhea followed by measles infection. Rational, fast, and effective laboratory examinations are essential for a timely diagnosis, avoiding inappropriate treatment and reducing mortality in suspected patients.

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REVIEW

Artificial intelligence in gastroenterology and hepatology: Status and challenges

Jia-Sheng Cao, Zi-Yi Lu, Ming-Yu Chen, Bin Zhang, Sarun Juengpanich, Jia-Hao Hu, Shi-Jie Li, Win Topatana, Xue-Yin Zhou, Xu Feng, Ji-Liang Shen, Yu Liu, Xiu-Jun Cai

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Abstract

Originally proposed by John McCarthy in 1955, artificial intelligence (AI) has achieved a breakthrough and revolutionized the processing methods of clinical medicine with the increasing workloads of medical records and digital images. Doctors are paying attention to AI technologies for various diseases in the fields of gastroenterology and hepatology. This review will illustrate AI technology procedures for medical image analysis, including data processing, model establishment, and model validation. Furthermore, we will summarize AI applications in endoscopy, radiology, and pathology, such as detecting and evaluating lesions, facilitating treatment, and predicting treatment response and prognosis with excellent model performance. The current challenges for AI in clinical application include potential inherent bias in retrospective studies that requires larger samples for validation, ethics and legal concerns, and the incomprehensibility of the output results. Therefore, doctors and researchers should cooperate to address the current challenges and carry out further investigations to develop more accurate AI tools for improved clinical applications.

Key Words: Artificial intelligence; Gastroenterology; Hepatology; Status; Challenges

interest.

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Core Tip: Artificial intelligence (AI) technologies are widely used for medical image analysis in the gastroenterology and hepatology fields. Several AI models have been developed for accurate diagnosis, treatment, and prognosis based on images of endoscopy, radiology, pathology, achieving high performance comparable to experts. However, we should be aware of the certain constraints that limit the acceptance and utilization of AI tools in clinical practice. To use AI wisely, doctors and researchers should work together to address the current challenges and develop more accurate AI tools to improve patient care.

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INTRODUCTION

Originally proposed by John McCarthy in 1955, artificial intelligence (AI) which involves machine learning (ML) and problem solving, has achieved a breakthrough and revolutionized the processing methods of clinical medicine with the increasing workloads of medical records and digital images. In clinical practice, AI consists of several overlapping technologies such as ML, artificial neural networks (ANNs), deep learning (DL), convolutional neural networks (CNNs), and recurrent neural networks[1,2] (Figure 1). Since the 1980s, ML has been performed to construct a mathematical model and predict outcomes based on input data, and it is roughly divided into supervised (labeled data), unsupervised (unlabeled data), and semi-supervised (both labeled and unlabeled data) learning techniques[3]. Recently, as a subset of ML, ANNs have received increased interest because they can identify and learn input data by themselves instead of being labeled by experts[4]. In the last decade, DL, a new model of ML, holds great promise in clinical medicine. DL is particularly suitable for enormous complex or highly dimensional medical image analysis and predictive modeling tasks using the multilayers of ANNs, including CNNs and recurrent neural networks[5,6]. Notably, given that convolutional and pooling layers can extract distinct features and fully connected layers can make a final classification, CNNs have demonstrated excellent performance in image recognition such as endoscopy, radiology, and pathology[7,8].

In the fields of gastroenterology and hepatology, doctors are paying attention to AI technologies for the diagnosis, treatment, and prognosis of various diseases due to the heterogeneous expertise levels of doctors (majoring in endoscopy, radiology, and pathology), time-consuming procedures, and increasing workloads. Specifically, doctors usually assess medical images visually to detect and diagnose diseases based on personal expertise and experience. As the maturity of digitalization increases, a quantitative assessment of imaging information has become the reality instead of relatively inaccurate qualitative reasoning[9,10]. Although a lot of time is necessary to review and check image analysis traditionally, little information can be obtained. For example, using AI technologies to process pathology images can assess the histopathological classification and predict gene mutations in liver cancer[11], while only the mass nature can be identified by conventional pathology assessment. As a country with a high population, China has produced rapidly increasing medical records, which result in the high workloads[12]. Despite the progression of AI, gastroenterologists and hepatologists should always be aware of its limitations such as the retrospective manner of included studies and the utilization of not particularly suitable databases. In addition, it demands that doctors prepare for the effects and changes of AI on clinical practice in the real world.

In this review, we aim to (1) introduce how AI technologies process input data, learn from input data, validate the established model; (2) summarize the AI applications in endoscopy, radiology, pathology for accurate diagnosis, treatment, and prognosis; and (3) discuss the current limitations and future considerations of AI

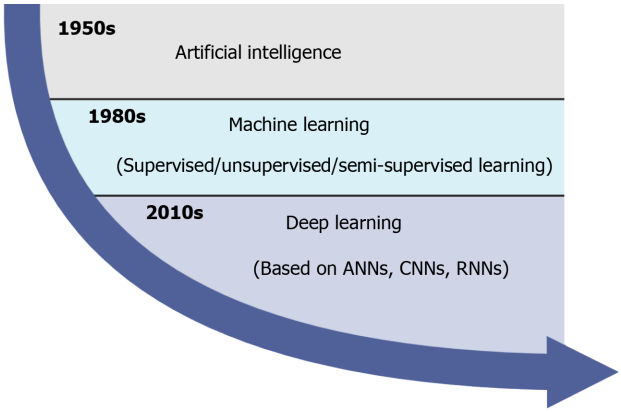


Figure 1 Timeline and related technologies of artificial intelligence. AI: Artificial intelligence; ANN: Artificial neural network; CNN: Convolutional neural network; RNN: Recurrent neural network.

applications in the fields of gastroenterology and hepatology (Figure 2).

METHODS IN DEEP LEARNING

As the most suitable approach for medical image analysis, the DL approach does not require shaped regions of interest on images to complete feature selection and extraction based on a neural network structure[13,14]. After data collection and processing, the correct neural network is chosen to establish a model, followed by model validation to assess its true generalizability.

Data processing

Raw data are collected and analyzed, and corrupt data are identified and cleaned in the processing phase. Data selection methods are provided in Scikit-Learn[15], a Python machine learning library, which consist of univariate selection, feature importance, correlation matrix, and recursive feature elimination or addition. Other programming languages such as R Studio (http://www.r-project.org) or MATLAB software (University of New Mexico, New Mexico, United States) also offer a successful environment for AI, and they provide similar approaches to address specific tasks. Useful data and relevant variables from multiple data sources, which are applied to predict outcomes, are selected and divided into an initial training set and a testing set that allow training and internal validation of the model. Data in the training set should be different and nonredundant from that in the testing set. Notably, for small datasets, a higher proportion of data should be included in the testing set to measure the performance of the trained model accurately through cross-validation or in a bootstrapping procedure.

Modeling

After transforming the data into an appropriate format, different tools are developed for implementing ML. Although several programming tools such as Python, R Studio, and MATLAB vary among themselves, they provide similar options and algorithms to adjust the parameters based on specific tasks. The major classification algorithms for testing are Naive Bayes, Decision Trees, Support Vector Machine, K-Nearest Neighbor, and Ensemble Classifiers. Oversampling or undersampling of the unbalanced training data can be utilized to improve the representation of classes and prevent model bias during the modeling stage. Currently, as the calculation workload of the batch learning process is heavy, minibatch learning is more popularized with repeating epochs, which usually decreases errors for the training and testing phases. However, an early stopping technique would be adopted to address the overfitting problem if repeating epochs cannot ensure error reduction. Based on the evaluations of model performance, developers conduct feature engineering again to manipulate the features and approve the predictive values of the model. After the optimization phase, selection for the model is primarily based on trial-and-error and the best performance for specific problem-solving. Finally, model optimization is performed with adjusted parameters by testing different configurations.

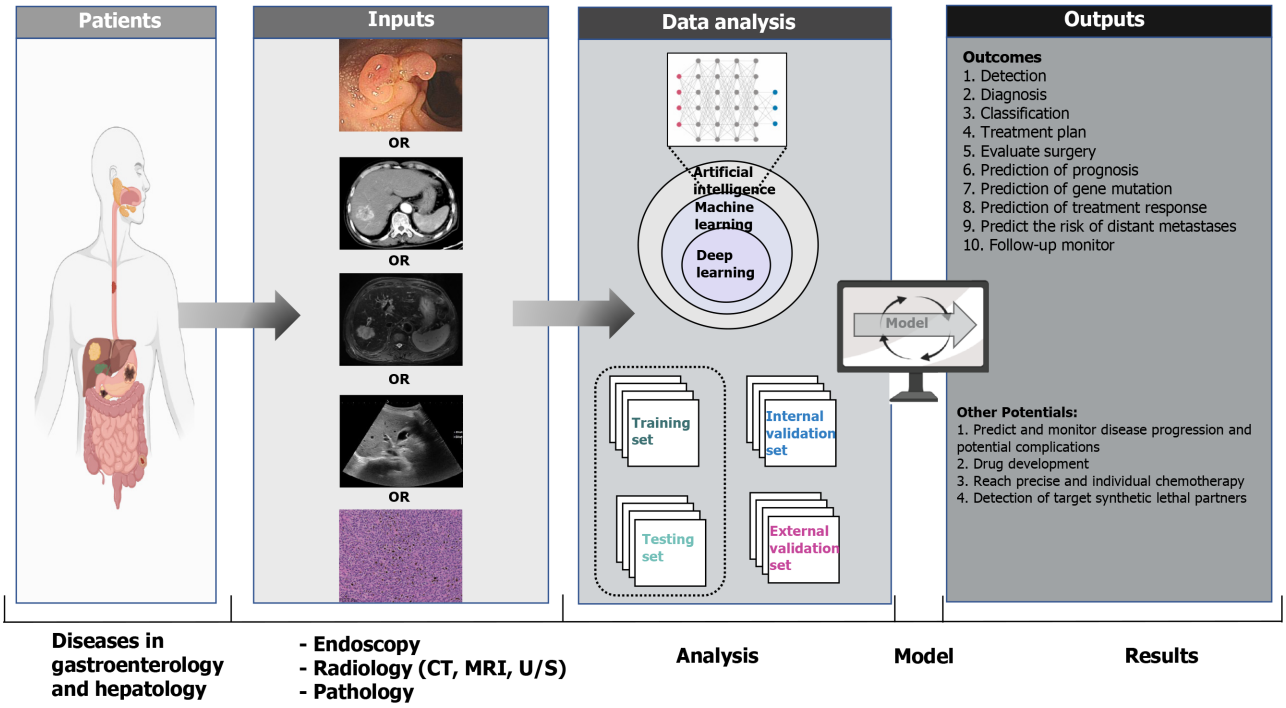


Figure 2 Artificial intelligence-assisted endoscopy, radiology, and pathology applications for medical image analysis in the fields of gastroenterology and hepatology, including detecting and evaluating lesions, facilitating treatment, and predicting treatment response and prognosis, and other potentials, using several deep learning models. CT: Computed tomography; MRI: Magnetic resonance imaging.

Model validation

To evaluate the AI approaches, one of the most significant requirements is external validation, which is called the blind test. Any model developed within one dataset will merely reflect its idiosyncrasies, and will have poor performance in analyzing new settings. In addition, models can also be validated by internal data testing (*e.g.*, *k*-fold cross-validation). In *k*-fold cross-validation, the dataset is separated into *k* subsets, including one subset for testing and the remaining (*k*-1) subsets for training a model. With all data used in both training and testing sets, the cross-validation process is repeated *k* times. The model performance is finally calculated as the average value of all *k* iterations. The *k* varies depending on the size of the dataset. For example, leave-one-out validation may be used in a small training set (< 200 data points), which means that *k* is equivalent to the dataset size. The appropriate and robust predictive model should have consistent performance between training and testing sets, preventing overfitting discrepancies.

ARTIFICIAL INTELLIGENCE IN ENDOSCOPY

With the advent and continuous improvement of fiberoptics, endoscopy has been playing a significant role in the diagnosis and treatment of gastrointestinal diseases. However, gastrointestinal diseases remain an enormous economic burden and lead to high mortality worldwide. AI is applicable in the gastroenterology fields within endoscopy[16-18], such as identification of esophageal and gastric neoplasia in esophago-gastroduodenoscopy (EGD), detection of gastrointestinal bleeding in wireless capsule endoscopy (WCE), and polyp detection and characterization in colonoscopy, *etc*[19-63] (Table 1).

EGD

Inadequate examination of the upper gastrointestinal tract is one of the reasons for misdiagnosing several EGD diseases. Based on AI-assisted EGD, the upper gastrointestinal tract can be divided into the pharynx, esophagus, stomach (upper, middle, lower), and duodenum with high values of the area under the curve (AUC)[19]. Furthermore, several AI technologies have classified the images of the stomach within EGD to significantly monitor the blind spots, and their accuracy has reached the ability

Table 1 Summary of key studies on artificial intelligence-assisted endoscopy in gastroenterology fields

Ref.	Country	Disease studied	Design of study	Application	Number of cases	Type of machine learning algorithm	Outcomes (%)	
							Accuracy	Sensitivity/Specificity
Esophagogastroduodenoscopy								
Takiyama *et al*[19], 2018	Japan	Anatomical location of upper gastrointestinal tract	Retrospective	Recognition of the anatomical location of upper gastrointestinal tract	Training: 27335 images: 663 larynx, 3252 esophagus, 5479 upper stomach, 7184 middle stomach, 7539 lower stomach, and 3218 duodenum; Testing: 17081 images: 363 larynx, 2142 esophagus, 3532 upper stomach, 6379 middle stomach, 3137 lower stomach, and 1528 duodenum	CNNs	Larynx: 100; Esophagus: 100; Stomach: 99; Duodenum: 99	Larynx: 93.9/100; Esophagus: 95.8/99.7; Stomach: 98.9/93; Duodenum: 87/99.2
Wu *et al*[20], 2019	China	Diseases of upper gastrointestinal tract	Prospective	Monitor blind spots of upper gastrointestinal tract	Training: 1.28 million images from 1000 object classes; Testing: 3000 images for DCNN1, and 2160 images for DCNN2	CNNs	90.4	87.57/95.02
van der Sommen *et al*[21], 2016	Netherlands	EN-BE	Retrospective	Detection of EN in BE	21 patients with EN-BE (60 images), 23 patients without EN-BE (40 images)	SVM	NA	86/87
Swager *et al*[22], 2017	Netherlands	EN-BE	Retrospective	Detection of EN in BE	60 images: 40 with EN-BE and 30 without EN-BE	SVM	95	90/93
Hashimoto *et al*[23], 2020	United States	EN-BE	Retrospective	Detection of EN in BE	Training: 916 images with EN-BE; Testing: 458 images: 225 dysplasia and 233 non-dysplasia	CNNs	95.4	96.4/94.2
Ebigbo *et al*[24], 2020	Germany	EAC-BE	Retrospective	Detection of EAC in BE	Training: 129 images; Testing: 62 images: 36 EAC and 26 normal BE	CNNs	89.9	83.7/100
Horie *et al*[25], 2019	Japan	EAC and ESCC	Retrospective	Detection of EAC and ESCC	Training: 384 patients with 32 EAC and 397 ESCC (8428 images); Testing: 47 patients with 8 EAC and 41 ESCC (1118 images)	CNNs	98	98/79
Kumagai *et al*[26], 2019	Japan	ESCC	Retrospective	Detection of ESCC	Training: 240 patients (4715 images: 1141 ESCC and 3574 benign lesions); Testing: 55 patients (1520 images: 467 ESCC and 1053 benign)	CNNs	90.9	92.6/89.3
Zhao *et al*[27], 2019	China	ESCC	Retrospective	Detection of ESCC	165 patients with ESCC and 54 patients without ESCC (1383 images)	CNNs	89.2	87.0/84.1
Cai *et al*[28], 2019	China	ESCC	Retrospective	Detection of ESCC	Training: 746 patients (2438 images: 1332 abnormal and 1096 normal); Testing: 52 patients (187 images)	CNNs	91.4	97.8/85.4
Nakagawa *et al*[29], 2019	Japan	ESCC	Retrospective	Determination of invasion depth	Training: 804 patients with ESCC (14338 images: 8660 non-ME and 5678 ME); Testing: 155 patients with ESCC (914 images: 405 non-ME and 509 ME)	CNNs	SM1/SM2, 3: 91.0; Invasion depth: 89.6	SM1/SM2, 3: 90.1/95.8; Invasion depth: 89.8/88.3
Tokai *et al*[30], 2020	Japan	ESCC	Retrospective	Determination of invasion depth	Training: 1751 images with ESCC; Testing: 42 patients with ESCC (293 images)	CNNs	80.9	84.1/80.9

Ali *et al*[31], 2018	Pakistan	EGC	Retrospective	Detection of EGC	56 patients with EGC, 120 patients without EGC	SVM	87	91.0/82.0
Sakai *et al*[32], 2018	Japan	EGC	Retrospective	Detection of EGC	Training: 58 patients (348943 images: 172555 EGC and 176388 normal); Testing: 58 patients (9650 images: 4653 EGC and 4997 normal)	CNNs	87.6	80.0/94.8
Kanesaka *et al*[33], 2018	Japan	EGC	Retrospective	Detection of EGC	Training: 126 images: 66 EGC and 60 normal; Testing: 81 images: 61 EGC and 20 normal	SVM	96.3	96.7/95.0
Wu *et al*[34], 2019	China	EGC	Retrospective	Detection of EGC	Training: 9691 images: 3710 EGC and 5981 normal; Testing: 100 patients: 50 EGC and 50 normal	CNNs	92.5	94.0/91.0
Horiuchi *et al*[35], 2020	Japan	EGC	Retrospective	Detection of EGC	Training: 2570 images: 1492 EGC and 1078 gastritis; Testing: 285 images: 151 EGC and 107 gastritis	CNNs	85.3	95.4/71.0
Zhu *et al*[36], 2019	China	Invasive GC	Retrospective	Determination of invasion depth	Training: 245 patients with GC and 545 patients without GC (5056 images); Testing: 203 images: 68 GC and 135 normal	CNNs	89.2	76.5/95.6
Luo *et al*[37], 2019	China	EAC, ESCC, and GC	Prospective	Detection of upper gastrointestinal cancers	Training: 15040 individuals (125898 images: 31633 cancer and 94265 control); Testing: 1886 individuals (15637 images: 3931 cancer and 11706 control)	CNNs	91.5-97.7	94.2/85.8
Nagao *et al*[38], 2020	Japan	GC	Retrospective	Determination of invasion depth	1084 patients with GC (16557 images); Training: Testing = 4:1	CNNs	94.5	84.4/99.4
Wireless capsule endoscopy								
Ayaru *et al*[39], 2015	United Kingdom	Small bowel bleeding	Retrospective	Prediction of outcomes	Training: 170 patients with small bowel bleeding; Testing: 130 patients with small bowel bleeding	ANNs	Recurrent bleeding 88; Therapeutic intervention: 88; Severe bleeding: 78	Recurrent bleeding: 67/91; Therapeutic intervention: 80/89; Severe bleeding: 73/80
Xiao *et al*[40], 2016	China	Small bowel bleeding	Retrospective	Detection of bleeding in GI tract	Training: 8200 images: 2050 bleeding and 6150 non-bleeding; Testing: 1800 images: 800 bleeding and 1000 non-bleeding	CNNs	99.6	99.2/99.9
Usman *et al*[41], 2016	South Korea	Small bowel bleeding	Retrospective	Detection of bleeding in GI tract	Training: 75000 pixels: 25000 bleeding and 50000 non-bleeding; Testing: 8000 pixels: 3000 bleeding and 5000 non-bleeding	SVM	91.8	93.7/90.7
Sengupta *et al*[42], 2017	United States	Small bowel bleeding	Retrospective	Prediction of 30-d mortality	Training: 4044 patients with small bowel bleeding; Testing: 2060 patients with small bowel bleeding	ANNs	81	87.8/90/9
Leenhardt *et al*[43], 2019	France	Small bowel bleeding	Retrospective	Detection of GIA	Training: 600 images: 300 hemorrhagic GIA and 300 non-hemorrhagic GIA; Testing: 600 images: 300 hemorrhagic GIA and 300 non-hemorrhagic GIA	CNNs	98	100.0/96.0
Aoki *et al*[44], 2020	Japan	Small bowel bleeding	Retrospective	Detection of small bowel bleeding	Training: 41 patients (27847 images: 6503 bleeding and 21344 normal); Testing: 25 patients (10208 images: 208 bleeding and 10000 non-bleeding)	CNNs	99.89	96.63/99.96
Yang *et al*[45],	China	Small bowel polyps	Retrospective	Detection of small bowel	1000 images: 500 polyps and 500 non-polyps	SVM	96.00	95.80/96.20

2020				polyps				
Vieira *et al*[46], 2020	Portugal	Small bowel tumors	Retrospective	Detection of small bowel tumors	39 patients (3936 images: 936 tumors and 3000 normal)	SVM	97.6	96.1/98.3
Colonoscopy								
Fernández-Esparrach *et al*[47], 2016	Spain	Colorectal polyps	Retrospective	Detection of polyps	24 videos containing 31 different polyps	Energy maps	79	70.4/72.4
Komeda *et al*[48], 2017	Japan	Colorectal polyps	Retrospective	Detection of polyps	Training: 1800 images: 1200 adenoma and 600 non-adenoma; Testing: 10 cases	CNNs	70.0	83.3/50.0
Misawa *et al*[49], 2017	Japan	Colorectal polyps	Retrospective	Detection of polyps	Training: 1661 images: 1213 neoplasm and 448 non-neoplasm; Testing: 173 images: 124 neoplasm and 49 non-neoplasm	SVM	87.8	94.3/71.4
Misawa *et al*[50], 2018	Japan	Colorectal polyps	Retrospective	Detection of polyps	196631 frames: 63135 polyps and 133496 non-polyps	CNNs	76.5	90.0/63.3
Chen *et al*[51], 2018	China	Colorectal polyps	Retrospective	Detection of diminutive colorectal polyps	Training: 2157 images: 681 hyperplastic and 1476 adenomas; Testing: 284 images: 96 hyperplastic and 188 adenomas	DNNs	90.1	96.3/78.1
Urban *et al*[52], 2018	United States	Colorectal polyps	Retrospective	Detection of polyps	Training: 8561 images: 4008 polyps and 4553 non-polyps; Testing: 1330 images: 672 polyps and 658 non-polyps	CNNs	96.4	96.9/95.0
Renner *et al*[53], 2018	Germany	Colorectal polyps	Retrospective	Differentiation of neoplastic from non-neoplastic polyps	Training: 788 images: 602 adenomas and 186 non-adenomatous polyps; Testing: 186 images: 52 adenomas and 48 hyperplastic lesions	DNNs	78.0	92.3/62.5
Wang *et al*[54], 2018	United States	Colorectal polyps	Retrospective	Detection of polyps	Training: 5545 images: 3634 polyps and 1911 non-polyps; Testing: 27113 images: 5541 polyps and 21572 non-polyps	CNNs	98	94.4/95.9
Mori *et al*[55], 2018	Japan	Colorectal polyps	Prospective	A diagnose-and-leave strategy for diminutive, non-neoplastic rectosigmoid polyps	Training: 61925 images; Testing: 466 cases (287 neoplastic polyps, 175 nonneoplastic polyps, and 4 missing specimens)	SVM	96.5	93.8/91.0
Byrne *et al*[56], 2019	Canada	Colorectal polyps	Retrospective	Detection and classification of polyps	Training: 60089 frames of 223 videos (29% NICE type 1, 53% NICE type 2 and 18% of normal mucosa with no polyp); Testing: 125 videos: 51 hyperplastic polyps and 74 adenoma	CNNs	94.0	98.0/83.0
Blanes-Vidal *et al*[57], 2019	Denmark	Colorectal polyps	Retrospective	Detection of polyps	131 patients with polyps and 124 patients without polyps	CNNs	96.4	97.1/93.3
Lee *et al*[58], 2020	South Korea	Colorectal polyps	Retrospective	Detection of polyps	Training: 306 patients (8593 images: 8495 polyp and 98 normal); Testing: 15 patients (15 polyps videos)	CNNs	93.4	89.9/93.7
Gohari *et al*[59], 2011	Iran	CRC	Retrospective	Determination of prognostic factors of CRC	1219 patients with CRC	ANNs	Colon cancer: 89; Rectum cancer: 82.7	NA/NA

Biglarian *et al*[60], 2012	Iran	CRC	Retrospective	Prediction of distant metastasis in CRC	1219 patients with CRC	ANNs	82	NA/NA
Takeda *et al*[61], 2017	Japan	CRC	Retrospective	Diagnosis of invasive CRC	Training: 5543 images: 2506 non-neoplasms, 2667 adenomas, and 370 invasive cancers; Testing: 200 images: 100 adenomas and 100 invasive cancers	SVM	94.1	89.4/98.9
Ito *et al*[62], 2019	Japan	CRC	Retrospective	Diagnosis of cT1b CRC	Training: 9942 images: 5124 cTis + cT1a, 4818 cT1b, and 2604 cTis + cT1a; Testing: 5022 images: 2604 cTis + cT1a, and 2418 cT1b	CNNs	81.2	67.5/89.0
Zhou *et al*[63], 2020	China	CRC	Retrospective	Diagnosis of CRC	Training: 3176 patients with CRC and 9003 patients without CRC (464105 images: 28071 CRC and 436034 non-CRC); Testing: 307 patients with CRC and 1956 patients without CRC (84615 images: 11675 CRC and 72940 non-CRC)	CNNs	96.3	91.4/98.0

AI: Artificial intelligence; CNN: Convolutional neural network; EN: Early-stage neoplasia; BE: Barrett's esophagus; SVM: Support vector machine; NA: Not available; EAC: Esophageal adenocarcinoma; ESCC: Esophageal squamous cell carcinoma; EGC: Early-stage gastric cancer; GC: Gastric cancer; ANN: Artificial neural network; GI: Gastrointestinal; GIA: Gastrointestinal angioectasia; DNN: Deep neural network; CRC: Colorectal cancer.

of experienced endoscopists[20,34].

Endoscopic surveillance for Barrett's esophagus (BE) is the potential risk factor for esophageal adenocarcinoma (EAC), of which the prognosis is related to disease staging[64,65]. However, accurate detection of esophageal neoplasia and early EAC remains difficult for experienced endoscopists[66]. An AI system developed by Ebigbo *et al*[24] enabled early detection of EAC with high sensitivity and specificity, they subsequently designed a real-time system for neoplasia classification in magnification. Both accurate detection of early EAC is important in BE images and the novel system of high invasion accuracy also deserves clinical attention[23]. In esophageal squamous cell carcinoma (ESCC), these tumors are often diagnosed at advanced stages, while early ESCC seems to be detected based on endoscopists' experience because they are almost impossible to visualize with white light endoscopy. Fortunately, with AI technologies, small esophageal lesions (< 10 mm) are recognized, and there is an AI system showing diagnostic accuracy of 91.4%, which is higher than that of high-level (with experience of > 15 years, 88.8%), mid-level (with experience of 5-15 years, 81.6%), and junior-level (with experience of < 5 years, 77.2%) endoscopists[28]. In addition, the prognosis of ESCC can be proved by differentiating tumor invasion depth[29,30].

The prognosis of gastric cancer (GC) mainly depends on the early detection and invasion depth of the disease. It is extremely difficult for endoscopists to recognize early gastric cancer (EGC), which is often accompanied by gastric mucosal inflammation, and the false-negative rate of EGC in EGD has reached nearly 25.0%[67,68]. AI-assisted EGD has the potential to address tough tissues. However, the first reported CNNs-based AI system for detection of EGC had a low positive predictive value of 30.6%, leading to misdiagnosis of gastritis and misinterpretation of the gastric angle as GC[69]. In 2019, Wu *et al*[34] examined the detection of GC by AI and

validated 200 endoscopic images, with increased accuracy, sensitivity, and specificity values (92.5%, 94%, and 91%, respectively). Furthermore, the AI system, named GRAIDS, has achieved diagnostic sensitivity close to that of expert endoscopists (94.2% *vs* 94.5%), and it demonstrated a robust performance showing high diagnostic accuracy in a multicenter study[37]. Besides detection, one of the most important criteria for curative resection is the invasion depth. The invasion depth prediction of GC by AI was first developed by Kubota *et al*[70], and the model showed the accuracy of T-stages (T1 = 77%, T2 = 49%, T3 = 51%, T4 = 55%, respectively). Considering that endoscopic mucosal resection is appropriate for intramucosal cancers (M) and submucosal cancers (invasion < 500 μm) (SM1), a more detailed classification is urgently needed. Therefore, an AI system was developed to differentiate the depths of M or SM1 and SM2 (submucosal invasion ≥ 500 μm) for GC with significantly higher sensitivity, specificity, and accuracy than those of skilled endoscopists[38].

WCE

AI-assisted WCE enables endoscopists to highlight suspicious regions on examination of the digestive tract noninvasively, including detection of small bowel bleeding, ulcers, and polyps, celiac disease, *etc*. Based on specific AI classifiers and validation techniques (mainly *k*-fold cross-validation), these models utilized still frames, pixels, or real-time videos to identify patients with small bowel bleeding with accuracy above 90% for most studies[40,41,43,44]. A CNNs-based algorithm, established in a retrospective analysis of 10000 WCE images (8200 and 1800 in the training and testing set, respectively) and validated by 10-fold cross-validation, was proposed for automatic detection of small bowel bleeding. The model was performed with a high *F*-1 score of 99.6% and precision of 99.9% for both active and inactive bleeding frames[40]. Besides detection, several emerging AI tools have been developed to stratify patients for the possibility of recurrent bleeding, treatment requirement, and mortality estimate to prevent repeated endoscopies in a significant proportion of patients with potential recurrent upper or lower gastrointestinal bleeding[39,42].

Colonoscopy

Colorectal polyp detection and appropriate polypectomy during colonoscopy is the standard way to prevent colorectal cancer (CRC). Since missed colorectal polyps can potentially progress into CRC, AI-assisted colonoscopy has been developed for polyp detection and characterization, and predicting the prognosis of CRC. In terms of polyp detection, an automated AI system using an energy map was developed in 2016, and it showed barely satisfactory performance[47]. Urban *et al*[52] used 8641 labeled images and 20 colonoscopy videos as the training and testing set to establish a CNNs model to identify colonic polyps, and the model had an accuracy of 96.4%[52]. Notably, the models should be validated to improve accuracy. After validating the model developed by Wang *et al*[54] with 27113 newly collected images from 1138 patients, the model showed acceptable performance (sensitivity = 94.38%, specificity = 95.2%, and AUC = 0.984). In addition, polyp characterization with magnifying endoscopic images is useful for identifying pit or vascular patterns to improve performance. AI tools with narrow-band imaging[51] or endoscopic videos[56] can be used to differentiate diminutive hyperplastic polyps and adenomas with high accuracy. Specifically, diminutive polyps (≤ 5 mm) may also be identified during colonoscopy[55]. In addition, AI may assist doctors in predicting the prognosis of CRC. An ANNs model, which was developed from a dataset of 1219 CRC patients, may predict patient survival and influential factors more accurately than a Cox regression model[59], and it also enables doctors to predict the risk of distant metastases[60].

ARTIFICIAL INTELLIGENCE IN RADIOLOGY

There is a disproportionate growing rate between radiological imaging data and the number of trained radiologists, and it has forced radiologists to compensate by increasing productivity[71]. The emergence of AI technologies has eased the current dilemma and dramatically advanced radiology image analysis, including ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) in the fields of gastroenterology and hepatology. In addition, the rise of radiomics, which is a new technology in radiology and cancer, can extract abundant quantifiable objective data to evaluate surgical resection and predict treatment response[72-112] (Table 2).

Table 2 Summary of key studies on artificial intelligence-assisted radiology in hepatology fields

Ref.	Country	Disease studied	Design of study	Application	Number of cases	Type of machine learning algorithm	Outcomes (%)	
							Accuracy	Sensitivity/Specificity
Ultrasound-based medical image recognition								
Gatos *et al*[72], 2016	United States	Hepatic fibrosis	Retrospective	Classification of CLD	85 images: 54 healthy and 31 CLD	SVM	87	83.3/89.1
Gatos *et al*[73], 2017	United States	Hepatic fibrosis	Retrospective	Classification of CLD	124 images: 54 healthy and 70 CLD	SVM	87.3	93.5/81.2
Chen *et al*[74], 2017	China	Hepatic fibrosis	Retrospective	Classification of the stages of hepatic fibrosis in HBV patients	513 HBV patients with different hepatic fibrosis (119 S0, 164 S1, 88 S2, 72 S3, and 70 S4)	SVM, Naive Bayes, RF, KNN	82.87	92.97/82.50
Li *et al*[75], 2019	China	Hepatic fibrosis	Prospective	Classification of the stages of hepatic fibrosis in HBV patients	144 HBV patients	Adaptive boosting, decision tree, RF, SVM	85	93.8/76.9
Gatos *et al*[76], 2019	United States	Hepatic fibrosis	Retrospective	Classification of CLD	88 healthy individuals (88 F0 fibrosis stage images) and 112 CLD patients (112 images: 46 F1, 16 F2, 22 F3, and 28 F4)	CNNs	82.5	NA/NA
Wang *et al*[77], 2019	China	Hepatic fibrosis	Prospective	Classification of the stages of hepatic fibrosis in HBV patients	Training: 266 HBV patients (1330 images); Testing: 132 HBV patients (660 images)	CNNs	F4: 100; ≥ F3: 99; ≥ F2: 99	F4: 100.0/100.0; ≥ F3: 97.4/95.7; ≥ F2: 100.0/97.7
Kuppili *et al*[78], 2017	United States	MAFLD	Retrospective	Detection and characterization of FLD	63 patients: 27 healthy and 36 MAFLD	ELM, SVM	ELM: 96.75; SVM: 89.01	NA/NA
Byra *et al*[79], 2018	Poland	MAFLD	Retrospective	Diagnosis of the amount of fat in the liver	55 severely obese patients	CNNs, SVM	96.3	100/88.2
Biswas *et al*[80], 2018	United States	MAFLD	Retrospective	Detection and risk stratification of FLD	63 patients: 27 healthy and 36 MAFLD	CNNs, SVM, ELM	CNNs: 100; SVM: 82; ELM: 92	NA/NA
Cao *et al*[81], 2020	China	MAFLD	Retrospective	Detection and classification of MAFLD	240 patients: 106 healthy, 57 mild MAFLD, 67 moderate MAFLD, and 10 severe MAFLD	CNNs	95.8	NA/NA
Guo *et al*[82], 2018	China	Liver tumors	Retrospective	Diagnosis of liver tumors	93 patients with liver tumors: 47 malignant lesions (22 HCC, 5 CC, and 10 RCLM), and 46 benign lesions	DNNs	90.41	93.56/86.89
Schmauch *et al*[83], 2019	France	FLL	Retrospective	Detection and characterization of FLL	Training: 367 patients (367 images); Testing: 177 patients	CNNs	Detection: 93.5; Characterization: 91.6	NA/NA
Yang *et al*[84], 2020	China	FLL	Retrospective	Detection of FLL	Training: 1815 patients with FLL (18000 images); Testing: 328 patients with FLL (3718 images)	CNNs	84.7	86.5/85.5

CT/MRI-based medical image recognition								
Choi *et al*[85], 2018	South Korea	Hepatic fibrosis	Retrospective	Staging liver fibrosis by using CT images	Training: 7461 patients: 3357 F0, 113 F1, 284 F2, 460 F3, 3247 F4; Testing: 891 patients: 118 F0, 109 F1, 161 F2, 173 F3, 330 F4	CNNs	92.1–95.0	84.6–95.5/89.9–96.6
He *et al*[86], 2019	United States	Hepatic fibrosis	Retrospective	Staging liver fibrosis by using MRI images	Training: 225 CLD patients; Testing: 84 patients	SVM	81.8	72.2/87.0
Ahmed *et al*[87], 2020	Egypt	Hepatic fibrosis	Retrospective	Detection and staging of liver fibrosis by using MRI images	37 patients: 15 healthy and 22 CLD	SVM	83.7	81.8/86.6
Hectors *et al*[88], 2020	United States	Liver fibrosis	Retrospective	Staging liver fibrosis by using MRI images	Training: 178 patients with liver fibrosis; Testing: 54 patients with liver fibrosis	CNNs	F1-F4: 85; F2-F4: 89; F3-F4: 91; F4: 83	F1-F4: 84/90; F2-F4: 87/93; F3-F4: 97/83; F4: 68/94
Vivanti *et al*[89], 2017	Israel	Liver tumors	Retrospective	Detection and segmentation of new tumors in follow-up by using CT images	246 liver tumors (97 new tumors)	CNNs	86	70/NA
Yasaka *et al*[90], 2018	Japan	Liver masses	Retrospective	Detection and differentiation of liver masses by using CT images	Training: 460 patients with liver masses (1068 images: 240 Category A, 121 Category B, 320 Category C, 207 Category D, 180 Category E); Testing: 100 images with liver masses: 21 Category A, 9 Category B, 35 Category C, 20 Category D, 15 Category E	CNNs	84	Category A: 71/NA; Category B: 33/NA; Category C: 94/NA; Category D: 90/NA; Category E: 100/NA
Ibragimov *et al*[91], 2018	United States	Liver diseases requiring SBRT	Retrospective	Prediction of hepatotoxicity after liver SBRT by using CT images	125 patients undergone liver SBRT: 58 liver metastases, 36 HCC, 27 cholangiocarcinoma, and 4 other histopathologies	CNNs	85	NA/NA
Abajian *et al*[92], 2018	United States	HCC	Retrospective	Prediction of HCC response to TACE by using MRI images	36 HCC patients treated with TACE	RF	78	62.5/82.1
Zhang *et al*[93], 2018	United States	HCC	Retrospective	Classification of HCC by using MRI images	20 patients with HCC	CNNs	80	NA/NA
Morshid *et al*[94], 2019	United States	HCC	Retrospective	Prediction of HCC response to TACE by using CT images	105 HCC patients received first-line treatment with TACE	CNNs	74.2	NA/NA
Nayak *et al*[95], 2019	India	Cirrhosis; HCC	Retrospective	Detection of cirrhosis and HCC by using CT images	40 patients: 14 healthy, 12 cirrhosis, 14 cirrhosis with HCC	SVM	86.9	100/95
Hamm *et al*[96], 2019	United States	Common hepatic lesions	Retrospective	Classification of common hepatic lesions by using MRI images	Training: 434 patients with common hepatic lesions; Testing: 60 patients with common hepatic lesions	CNNs	92	92/98
Wang *et al*[97], 2019	United States	Common hepatic lesions	Retrospective	Demonstration of a proof-of-concept interpretable DL system by using MRI images	60 common hepatic lesions patients	CNNs	NA	82.9/NA

Jansen *et al*[98], 2019	Netherlands	FLL	Retrospective	Classification of FLL by using MRI images	95 patients with FLL (125 benign lesions: 40 adenomas, 29 cysts, and 56 hemangiomas; and 88 malignant lesions: 30 HCC and 58 metastases)	RF	77	Adenoma: 80/78; Cyst: 93/93; Hemangioma: 84/82; HCC: 73/56; Metastasis: 62/77
Mokrane *et al*[99], 2020	France	HCC	Retrospective	Diagnosis of HCC in patients with cirrhosis by using CT images	Training: 106 patients: 85 HCC and 21 non-HCC; Testing: 36 patients: 23 HCC and 13 non-HCC	SVM, KNN, RF	70	70/54
Shi *et al*[100], 2020	China	HCC	Retrospective	Detection of HCC from FLL by using CT images	Training: 359 lesions: 155 HCC and 204 non-HCC; Testing: 90 lesions: 39 HCC and 51 non-HCC	CNNs	85.6	74.4/94.1
Alirr *et al*[101], 2020	Kuwait	Liver tumors	Retrospective	Segmentation of liver tumors	Training: 100 images with liver tumors;Testing: 31 images with liver tumors	CNNs	95.2	NA/NA
Zheng *et al*[102], 2020	China	Pancreatic cancer	Retrospective	Pancreas segmentation by using MRI images	20 patients with PDAC	CNNs	99.86	NA/NA
Radiomics								
Liang *et al*[103], 2014	China	HCC	Retrospective	Prediction of recurrence for HCC patients who received RFA	83 patients with HCC receiving RFA as first treatment (18 recurrence and 65 non-recurrence)	SVM	82	67/86
Zhou *et al*[104], 2017	China	HCC	Retrospective	Characterization of HCC	46 patients with HCC: 21 low-grade (Edmondson grades I and II) and 25 high-grade (Edmondson grades III and IV)	Free-form curve-fitting	86.95	76.00/100.00
Abajian *et al*[105], 2018	United States	HCC	Retrospective	Prediction of response to intra-arterial treatment	36 patients undergone trans-arterial treatment	RF	78	62.5/82.1
Ibragimov *et al*[91], 2018	United States	Liver tumors	Retrospective	Prediction of hepatobiliary toxicity of SBRT	125 patients undergone liver SBRT: 58 metapatients, 36 HCC, 27 cholangiocarcinoma, and 4 other primary liver tumor histopathologies	CNNs	85	NA/NA
Morshid *et al*[94], 2019	United States	HCC	Retrospective	Prediction of HCC response to TACE	105 patients with HCC: 11 BCLC stage A, 24 BCLC stage B, 67 BCLC stage C, and 3 BCLC stage D	CNNs	74.2	NA/NA
Ma *et al*[106], 2019	China	HCC	Retrospective	Prediction of MVI in HCC	Training: 110 patients with HCC: 37 with MVI and 73 without MVI; Testing: 47 patients with HCC: 18 with MVI and 29 without MVI	SVM	76.6	65.6/94.4
Dong *et al*[107], 2020	China	HCC	Retrospective	Prediction and differentiation of MVI in HCC	Prediction: 322 patients with HCC: 144 with MVI and 178 without MVI; Differentiation: 144 patients with HCC and MVI	RF, mRMR	Prediction: 63.4; Differentiation: 73.0	Prediction: 89.2/48.4; Differentiation: 33.3/80.0
He *et al*[108], 2020	China	HCC	Prospective	Prediction of MVI in HCC	Training: 101 patients with HCC; Testing: 18 patients with HCC	LASSO	84.4	NA/NA
Schoenberg *et al*[109], 2020	Germany	HCC	Prospective	Prediction of disease-free survival after HCC resection	Training: 127 patients with HCC; Testing: 53 patients with HCC	RF	78.8	NA/NA
Zhao *et al*[110],	China	HCC	Retrospective	Prediction of ER of HCC	Training: 78 patients with HCC: 40 with ER and	LASSO	80.8	80.0/81.6

2020				after partial hepatectomy	38 without ER; Testing: 35 patients with HCC: 18 with ER and 17 without ER			
Liu *et al*[111], 2020	China	HCC	Retrospective	Prediction of progression-free survival of HCC patients after RFA and SR	RFA: Training: 149 HCC patients undergone RFA Testing: 65 HCC patients undergone RFA; SR: Training: 144 HCC patients undergone SR Testing: 61 HCC patients undergone SR	Cox-CNNs	RFA: 82.0; SR: 86.3	NA/NA
Chen *et al*[112], 2021	China	HCC	Retrospective	Prediction of HCC response to first TACE by using CT images	Training: 355 patients with HCC; Testing: 118 patients with HCC	LASSO	81	85.2/77.2

AI: Artificial intelligence; CLD: Chronic liver disease; SVM: Support vector machine; HBV: Hepatitis-B virus; RF: Random forests; KNN: K-nearest neighbor; CNN: Convolutional neural network; NA: Not available; MAFLD: Metabolic associated fatty liver disease; FLD: Fatty liver disease; ELM: Extreme learning machine; HCC: Hepatocellular carcinoma; CC: Cholangiocarcinoma; RCLM: Colorectal cancer liver metastases; DNN: deep neural network; FLL: Focal liver lesions; CLD: Chronic liver disease; SBRT: Stereotactic body radiation therapy; TACE: Transarterial chemotherapy; PDAC: Pancreatic ductal adenocarcinoma; RFA: Radiofrequency ablation; BCLC: Barcelona clinic liver cancer staging; MVI: Microvascular invasion; mRMR: Minimum redundancy maximum relevance; LASSO: Least absolute shrinkage and selection operator; ER: Early recurrence; SR: Surgical resection.

Abdominal ultrasound

AI technologies have been applied to abdominal ultrasound-based medical images for the assessment of liver diseases, such as hepatic fibrosis and mass lesions. A support vector machine-derived approach was developed by Gatos *et al*[72] to detect and classify chronic liver disease (CLD) based on abdominal ultrasound. After quantifying 85 ultrasound images (54 healthy and 31 with CLD), the proposed model showed superior results (accuracy = 87.0%, sensitivity = 83.3%, and specificity = 89.1%), which greatly improved the diagnostic and classification accuracy of CLD. Furthermore, CNNs are employed to identify and isolate regions of different stiffness temporal stability under ultrasound to explore the impact on CLD diagnosis. The updated detection algorithm has augmented the accuracy to 95.5% after excluding unreliable areas and reducing interobserver variability[76]. Detecting and classifying hepatic mass lesions as benign or malignant is equally important. Schmauch *et al*[83] performed supervised training (367 ultrasonic images together with the radiological reports) to build a DL model, and the resulting algorithm had high receiver operating characteristic curves of 0.93 and 0.916 in lesion detection and characterization, respectively. Although the model could increase the diagnostic accuracy and detect potential malignant mass lesions, it should be further validated. In addition, combining AI technologies with contrast-enhanced ultrasound may improve the performance to identify and characterize liver cancer. For example, after AI-assisted contrast-enhanced ultrasound was applied to detect liver lesions in the arterial, portal, and late phases, the accuracy, sensitivity and specificity of the examination were markedly increased[82]. Due to the misty demonstration of gastroenterology within ultrasound, an AI-assisted ultrasound tool was limited.

CT/MRI

Liver diseases often present indeterminate behaviors on abdominal CT, and a biopsy is

recommended according to the European Association for the Study of the Liver guidelines[113]. Based on a large dataset of CT images (7461 patients diagnosed with liver fibrosis), a CNNs model was developed and it outperformed the radiologists' interpretation[85]. Furthermore, depending on ANNs-based contrast-enhanced CT images from 460 patients, Yasaka *et al*[90] conducted a retrospective study to classify liver masses into five categories with high accuracy, including (1) primary hepatocellular carcinoma (HCC); (2) malignant tumors apart from HCC; (3) early HCC, indeterminate masses, or dysplastic nodules; (4) hemangiomas; and (5) cysts. For patients diagnosed with liver tumors or pancreatic cancer, it is crucial to complete the liver or pancreas segmentation to assess the lesions and make the ideal treatment plan. Instead of conventional manual segmentation, a CNNs model was proposed to segment liver tumors based on CT images, with an accuracy of more than 80.0%, favoring suitable decision-making[101]. Additionally, a CNNs model was also developed for pancreas localization and segmentation using CT images[102]. Furthermore, monitoring tumor recurrence plays an important role in follow-up CT. Vivanti *et al*[89] collected and integrated the initial appearance of tumors, CT behaviors, and quantification of the tumor loads throughout the disease course, and then they designed an automated detection model of tumor recurrence with an accuracy of 86%.

Besides depending on CT images, a DL approach for pancreas segmentation can also be designed from MRI images. Several AI-assisted studies have shown promising results in classifying MRI liver lesions with/without risk factors and patients' clinical data, improving the accuracy and yields of reference models[93,96,98,102].

Radiomics

Currently, radiomics has received great interest from doctors because this AI-assisted technology can extract indiscoverable quantifiable objective data of the radiological images and reveal the association with potential biological processes[114,115]. Preoperative stratification of patients at different risk of recurrence and prediction of survival after resection is fundamental to improve prognosis. As an independent risk factor of recurrence, microvascular invasion (MVI) cannot be provided in conventional radiological techniques[116]. Several studies have managed to use radiomic algorithms based on ultrasound, CT, or MRI to elaborate radiomic signatures for preoperative prediction of MVI[106-108]. Besides the prediction of recurrence, radiomics may also be utilized to predict survival after surgical resection. However, compared to the excellent AI models based on pathologic images, radiomics-based predictive models merely attain a low value of 0.78[109].

Beyond recurrence and survival prediction purposes, radiomics can also be utilized for prediction of patients' response to transarterial chemoembolization (TACE) and radiofrequency ablation (RFA), and post-radiotherapy hepatotoxicity. A CNNs model developed from 105 HCC patients' CT images had higher accuracy in predicting response to TACE than the Barcelona Clinic Liver Cancer stages[94]. In addition, Chen *et al*[112] designed an excellent clinical-radiomic model to predict objective response to first TACE based on 595 HCC patients' CT images, which could assist the selection of HCC patients for TACE. Another study used radiomics of MRI images with clinical data to perform prediction of TACE response[105]. For HCC in the early stages, RFA is a recommended option. Based on radiomics, Liang *et al*[103] designed a model to predict the RFA response and HCC recurrence after RFA, obtaining high AUC, sensitivity, and specificity. Additionally, post-radiotherapy hepatotoxicity should be monitored to adjust the position and dose of radiotherapy. A CNNs model not only identified that irradiation of the proximal portal vein was associated with poor prognosis, it also predicted post-radiotherapy hepatotoxicity with an AUC of 0.85[91]. Ibragimov *et al*[91] applied a CNNs model to determine the consistent patterns in toxicity-related dose plans, and the AUC of the model for dose planned analysis was increased from 0.79 to 0.85 after the combination with some pre-treatment clinical features, showing that the combined framework can indicate the accurate position and dose of radiotherapy.

ARTIFICIAL INTELLIGENCE IN PATHOLOGY

Pathological analysis is considered the gold standard for the diagnosis of diseases in the fields of gastroenterology and hepatology. Currently, there is a shortage of pathologists around the world, which has become an obstruction for maintaining the accuracy of pathological analysis[117]. With the development of the whole-slide imaging (WSI) scanner and AI technologies, a combination of both technologies can ease the medical burden, improve the diagnosis accuracy, and even predict gene mutations and

prognosis[118-147] (Table 3).

Basic AI-assisted pathology: diagnosis

The basic role of pathology is disease diagnosis. In the fields of gastroenterology, there is an increasing need for automatic pathological analysis and diagnosis of GC. Based on the virtual version of pathological slices, several studies were performed to identify and classify GC automatically with high AUCs[120-122,126]. For example, a CNNs model was developed to distinguish gastric mass lesions including gastric adenocarcinoma, adenoma and non-neoplastic lesions, and it has achieved the highest AUC of 0.97 for the identification of gastric adenocarcinoma[126]. With regard to colorectal lesions, Wei *et al*[128] trained an AI-assisted model to classify colorectal polyps on WSIs, and notably, the performance of the model was similar to that of local pathologists whether in a single institution or other institutions. Besides diagnosis, a model based on more than 400 WSIs was developed to differentiate five common subtypes of colorectal polyps with accuracy of 93%[127]. In CRC, Shapcott *et al*[129] performed a retrospective study to develop a CNNs model for diagnosis based on 853 hand-marked images with an accuracy of 84%.

In the fields of hepatology, AI-assisted pathology is applied in patients with hepatitis B virus (HBV), metabolic associated fatty liver disease, HCC, *etc*. An automated, stain-free AI system can quantify the amount of fibrillar collagen to evaluate the degree of HBV-related fibrosis with the AUC > 0.82[132]. For patients with metabolic associated fatty liver disease, AI-assisted pathology tools were used to identify and quantify pathological changes including steatosis, macrosteatosis, lobular inflammation, ballooning, and fibrosis[133], and the algorithm output scores for quantitative comparison with experienced pathologists achieved good agreement. However, limited AI-assisted pathology tools have been built for HCC diagnosis. Notably, the MFC-CNN-ELM program was designed for nuclei grading of biopsy specimens from HCC patients, which revealed high performance in classifying tumor cells of different differentiation stages[134].

Advanced AI-assisted pathology: prediction of gene mutations and prognosis

Apart from AI-assisted pathology tools in diagnosis, it is no surprise that many tools have been developed for the prediction of gene mutations and prognosis in the fields of gastroenterology and hepatology. In CRC, AI tools have shown great effectiveness in predicting prognosis across all tumor stages based on WSIs[139,140], and several prospective multicenter studies have further validated the high prognosis performance[142]. Notably, a subset of genetic defects occurring in gastroenterology is related to some morphological features detected on WSIs. Among screened genetic defects, microsatellite instability and mismatch-repair deficiency are associated with the survival of gastrointestinal and colorectal cancer patients receiving immunotherapy. Therefore, an AI tool was designed to predict microsatellite instability and mismatch-repair deficiency directly from pathology, and it finally showed reasonably good performance in assisting immunotherapy[138,141]. Notably, Kather *et al*[140] further validated the above model's performance in predicting overall survival from CRC pathology slides with a hazard ratio of 2.29 in CRC-specific overall survival (OS) and an hazard ratio of 1.63 in OS, respectively. However, besides the above-mentioned studies that have focused on tumor detection of CRC, few studies were designed to predict gene mutations and prognosis due to the more complicated and heterogeneous histomorphology in gastric diseases than that in the colon[136,137].

In the fields of hepatology, AI tools are mainly used to predict gene mutations and prognosis in HCC. For example, a model has higher accuracy in predicting survival postoperatively than using a composite score of clinical and pathological factors in HCC. In addition, the model may generalize well after validating the performance in an external dataset with different staining and scanning methods[146]. Chen *et al*[11] investigated a CNN (Inception V3) for automatic classification (benign/malignant classification with 96.0% accuracy, and differentiation degree with 89.6% accuracy) and gene mutation prediction from WSIs after resection of HCC. It was found that *CTNNB1, FMN2, TP53,* and *ZFX4* could be predicted from WSIs with external AUCs from 0.71 to 0.89. Currently, after integrating clinical data, biological data, genetic data, and pathological data, the novel model may also be a promising approach. The first multi-omics model combined ribonucleic acid (RNA) sequencing, miRNA sequencing and methylation data from The Cancer Genome Atlas, and then employed AI technologies to predict and differentiate survival of HCC patients[145]. Other attempts have been made to develop models that can predict gene mutations directly based on WSIs of HCC. Using AI-assisted pathology, some approaches can predict gene expression and RNA sequencing, which may have the potential for clinical

Table 3 Summary of key studies on artificial intelligence-assisted pathology in the gastroenterology and hepatology fields

Ref.	Country	Disease studied	Design of study	Application	Number of cases	Type of machine learning algorithm	Outcomes (%)	
							Accuracy	Sensitivity/Specificity
Basic AI-based pathology: diagnosis								
Tomita *et al*[118], 2019	United States	BE and EAC	Retrospective	Detection and classification of cancerous and precancerous esophagus tissue	Training: 379 images with 4 classes: normal, BE-no-dysplasia, BE-with-dysplasia, and adenocarcinoma; Testing: 123 images with 4 classes: normal, BE-no-dysplasia, BE-with-dysplasia, and adenocarcinoma	CNNs	Mean: 83; BE-no-dysplasia: 85; BE-with-dysplasia: 89; Adenocarcinoma: 88	Normal: 69/71 BE-no-dysplasia: 77/88; BE-with-dysplasia: 21/97; Adenocarcinoma: 71/91
Sharma *et al*[119], 2017	Germany	GC	Retrospective	Classification and necrosis detection of GC	454 patients (6810 WSIs: 4994 for cancer classification and 1816 for necrosis detection) (HER2 immunohistochemical stain and HE stained)	CNNs	Cancer classification: 69.90; Necrosis detection: 81.44	NA/NA
Li *et al*[120], 2018	China	GC	Retrospective	Detection of GC	700 images: 560 GC and 140 normal (HE stained)	CNNs	100	NA/NA
Leon *et al*[121], 2019	Colombia	GC	Retrospective	Detection of GC	40 images: 20 benign and 20 malignant	CNNs	89.72	NA/NA
Sun *et al*[122], 2019	China	GC	Retrospective	Diagnosis of GC	500 WSIs of gastric areas with typical cancerous regions	DNNs	91.6	NA/NA
Ma *et al*[123], 2020	China	GC	Retrospective	Classification of lesions in the gastric mucosa	Training: 534 WSIs (1616713 images: 544925 normal, 544624 chronic gastritis, and 527164 cancer) (HE stained) Testing: 153 WSIs (399240 images: 135446 normal, 125783 chronic gastritis, and 138011 cancer) (HE stained)	CNNs, RF	Benign and cancer: 98.4; Normal, chronic gastritis, and GC: 94.5	Benign and cancer: 98.0/98.9; Normal, chronic gastritis, and GC: NA/NA
Yoshida *et al*[124], 2018	Japan	Gastric lesions	Retrospective	Classification of gastric biopsy specimens	3062 gastric biopsy specimens (HE stained)	CNNs	55.6	89.5/50.7
Qu *et al*[125], 2018	Japan	Gastric lesions	Retrospective	Classification of gastric pathology images	Training: 1080 patches: 540 benign and 540 malignant; Testing: 5400 patches: 2700 benign and 2700 malignant	CNNs	96.5	NA/NA
Iizuka *et al*[126], 2020	Japan	Gastric and colonic epithelial tumors	Retrospective	Classification of gastric and colonic epithelial tumors	4128 cases of human gastric epithelial lesions and 4036 of colonic epithelial lesions (HE stained)	CNNs, RNNs	Gastric adenocarcinoma: 97; Gastric adenoma: 99; Colonic adenocarcinoma: 96; Colonic adenoma: 99	NA/NA
Korbar *et al*[127], 2017	United States	Colorectal polyps	Retrospective	Classification of different types of colorectal polyps on WSIs	Training: 458 WSIs; Testing: 239 WSIs	A modified version of a residual network	93	88.3/NA
Wei *et al*[128], 2020	United States	Colorectal polyps	Retrospective	Classification of colorectal polyps on WSIs	Training: 326 slides with colorectal polyps: 37 tubular, 30 tubulovillous or villous, 111 hyperplastic, 140 sessile serrated, and 8 normal;	CNNs	Tubular: 84.5; Tubulovillous or villous: 89.5; Hyperplastic: 85.3;	Tubular: 73.7/91.6; Tubulovillous or villous: 97.6/87.8; Hyperplastic: 60.3/97.5; Sessile

					Testing: 238 slides with colorectal polyps: 95 tubular, 78 tubulovillous or villous, 41 hyperplastic, and 24 sessile serrated		Sessile serrated: 88.7	serrated: 79.2/89.7
Shapcott *et al*[129], 2018	UnitedKingdom	CRC	Retrospective	Diagnosis of CRC	853 hand-marked images	CNNs	84	NA/NA
Geessink *et al*[130], 2019	Netherlands	CRC	Retrospective	Quantification of intratumoral stroma in CRC	129 patients with CRC	CNNs	94.6	91.1/99.4
Song *et al*[131], 2020	China	CRC	Retrospective	Diagnosis of CRC	Training: 177 slides: 156 adenoma and 21 non-neoplasm; Testing: 362 slides: 167 adenoma and 195 non-neoplasm	CNNs	90.4	89.3/79.0
Wang *et al*[132], 2015	China	Hepatic fibrosis	Retrospective	Assessment of HBV-related liver fibrosis and detection of liver cirrhosis	Training: 105 HBV patients; Testing: 70 HBV patients	SVM	82	NA/NA
Forlano *et al*[133], 2020	UnitedKingdom	MAFLD	Retrospective	Detection and quantification of histological features of MAFLD	Training: 100 MAFLD patients; Testing: 146 MAFLD patients	K-means	Steatosis: 97; Inflammation: 96; Ballooning: 94; Fibrosis: 92	NA/NA
Li *et al*[134], 2017	China	HCC	Retrospective	Nuclei grading of HCC	4017 HCC nuclei patches	CNNs	96.7	G1: 94.3/97.5; G2: 96.0/97.0;G3: 97.1/96.6; G4: 99.5/95.8
Kiani *et al*[135], 2020	United States	Liver cancer (HCC and CC)	Retrospective	Histopathologic classification of liver cancer	Training: 70 WSIs: 35 HCC and 35 CC Testing: 80 WSIs: 40 HCC and 40 CC	SVM	84.2	72/95
Advanced AI-based pathology: prediction of gene mutations and prognosis								
Steinbuss *et al*[136], 2020	Germany	Gastritis	Retrospective	Identification of gastritis subtypes	Training: 92 patients (825 images: 398 low inflammation, 305 severe inflammation, and 122 A gastritis) (HE stained) Testing: 22 patients (209 images: 122 low inflammation, 38 severe inflammation, and 49 A gastritis) (HE stained)	CNNs	84	A gastritis: 88/89; B gastritis: 100/93; C gastritis: 83/100
Liu *et al*[137], 2020	China	Gastrointestinal neuroendocrine tumor	Retrospective	Prediction of Ki-67 positive cells	12 patients (18762 images: 5900 positive cells, 6086 positive cells, and 6776 background from ROIs) (HE and IHC stained)	CNNs	97.8	97.8/NA
Kather *et al*[138], 2019	Germany	GC and CRC	Retrospective	Prediction of MSI in GC and CRC	Training: 360 patients (93408 tiles); Testing: 378 patients (896530 tiles)	CNNs	84	NA/NA
Bychkov *et al*[139], 2018	Finland	CRC	Retrospective	Prediction of CRC outcome	420 CRC tumor tissue microarray samples	CNNs, RNNs	69	NA/NA
Kather *et al*[140], 2019	Germany	CRC	Retrospective	Prediction of survival from CRC histology slides	Training: 86 CRC tissue slides (> 100000 HE image patches); Testing: 25 CRC patients (7180 images)	CNNs	98.7	NA/NA

Echle *et al*[141], 2020	Germany	CRC	Retrospective	Detection of dMMR or MSI in CRC	Training: 5500 patients; Testing: 906 patients	A modified shufflenet DL system	92	98/52
Skrede *et al*[142], 2020	3R23 Song 2020	CRC	Retrospective	Prediction of CRC outcome after resection	Training: 828 patients (> 12000000 image tiles); Testing: 920 patients	CNNs	76	52/78
Sirinukunwattana *et al*[143], 2020	UnitedKingdom	CRC	Retrospective	Identification of consensus molecular subtypes of CRC	Training: 278 patients with CRC; Testing: 574 patients with CRC: 144 biopsies and 430 TCGA	Neural networks with domain-adversarial learning	Biopsies: 85; TCGA: 84	NA/NA
Jang *et al*[144], 2020	South Korea	CRC	Retrospective	Prediction of gene mutations in CRC	Training: 629 WSIs with CRC (HE stained) Testing: 142 WSIs with CRC (HE stained)	CNNs	64.8-88.0	NA/NA
Chaudhary *et al*[145], 2018	United States	HCC	Retrospective	Identification of survival subgroups of HCC	Training: 360 HCC patients' data using RNA-seq, miRNA-seq and methylation data from TCGA; Testing: 684 HCC patients' data (LIRI-JP cohort: 230; NCI cohort: 221; Chinese cohort: 166, E-TABM-36 cohort: 40, and Hawaiian cohort: 27)	DL	LIRI-JP cohort: 75; NCI cohort: 67; Chinese cohort: 69; E-TABM-36 cohort: 77; Hawaiian cohort: 82	NA/NA
Saillard *et al*[146], 2020	France	HCC	Retrospective	Prediction of the survival of HCC patients treated by surgical resection	Training: 206 HCC (390 WSIs); Testing: 328 HCC (342 WSIs)	CNNs (SCHMOWDER and CHOWDER)	SCHMOWDER: 78; CHOWDER: 75	NA/NA
Chen *et al*[11], 2020	China	HCC	Retrospective	Classification and gene mutation prediction of HCC	Training: 472 WSIs: 383 HCC and 89 normal liver tissue; Testing: 101 WSIs: 67 HCC and 34 normal liver tissue	CNNs	Classification: 96.0; Tumor differentiation: 89.6; Gene mutation: 71-89	NA/NA
Fu *et al*[147], 2020	UnitedKingdom	EAC, GC, CRC, and liver cancers	Retrospective	Prediction of mutations, tumor composition and prognosis	17335 HE-stained images of 28 cancer types	CNNs	Variable across tumors/gene alterations	NA/NA

AI: Artificial intelligence; BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; CNN: Convolutional neural network; GC: Gastric cancer; WSI: Whole-slide image; NA: Not available; DNN: Deep neural network; RF: Random forests; RNN: Recurrent neural network; CRC: Colorectal cancer; HBV: Hepatitis-B virus; SVM: Support vector machine; MAFLD: Metabolic associated fatty liver disease; HCC: Hepatocellular carcinoma; CC: Cholangiocarcinoma; ROI: Region of interest; IHC: Immunohistochemistry; MSI: Microsatellite instability; dMMR: Mismatch-repair deficiency; TCGA: The Cancer Genome Atlas; DL: Deep learning.

translation[147]. Interestingly, some gene expression such as PD-1 and PD-L1 expression, inflammatory gene signatures, and biomarkers of inflammation did trend with improved survival and response in HCC patients[148].

LIMITATIONS AND FUTURE CONSIDERATIONS

This review retrospectively summarized some key and representative articles with the possibility of missing some publications in AI-related journals. Although various studies have shown promising results in the fields of AI-assisted gastroenterology and

hepatology, there are still several limitations to be discussed and resolved. One of the major criticisms is the lack of high-quality training, testing, and validation datasets for the development and validation of AI models. Due to the retrospective manner of most studies, selection bias must be considered at the training stage, meanwhile, overfitting and spectrum bias may result in overestimation of the model accuracy and generalization. According to the rigorous "six-steps" translation pipelines[149], doctors and AI researchers should join the calls that advocate for developing interconnected networks of collecting raw acquisition data which was shifted from processed medical images over the world and training AI on a large scale to obtain robust and generalizable models. Furthermore, the black-box nature of AI technologies has become a barrier to clinical practice, because developers and users do not know the details about how computers output the conclusion. Explainable AI for reliable healthcare is worth investigating to reach clinical interpretability and transparency. In addition, from the perspective of ethics and legal liabilities, AI models may potentially cause errors and challenge the patient-doctor relationship despite the fact that they improve the clinical workflow with enhanced precision. Especially in the fields of gastroenterology and hepatology, cancer discrimination may mean a completely different treatment. If misdiagnosis occurs during AI application, who should take responsibility- the doctor, the programmer, the company providing the system, or the patient? Issues such as ethics and legal liabilities should be demonstrated in the early phase to maintain the balance between minimal error rates and maximal patient benefits[150,151].

There have been an increasing number of studies applying AI to gastroenterology and hepatology over the past decade. In the future, the trend will continue and larger studies will be carried out to compare the performance of medical professionals with AI *vs* professionals without AI to highlight the importance of AI assistance. AI technologies will be utilized to develop more accurate models to predict and monitor disease progression and potential complications, and these models may ameliorate the insufficiency of medical resources in the remote underserved or developing regions. Besides, AI-assisted personalized imaging protocols and immediate three-dimensional reconstruction may further improve the diagnostic efficiency and accuracy. Researchers will be able to realize the mechanism of disease progression and treatment response through the combination of multi-modality images or multi-omics data. In addition, there is an emerging trend applying AI to drug development, such as prediction of compound toxicity, physical properties, and biological activities, which may assist chemotherapy for digestive system malignancy. Furthermore, AI could be used to process the data generated from the tissue-on-a-chip platform which could better summarize the tumor microenvironment, thus reach precise and individual chemotherapy in gastroenterology and hepatology. As synthetic lethality becomes a promising genetically targeted cancer therapy[152,153], AI could also be used for the detection of target synthetic lethal partners of overexpressed or mutated genes in tumor cells to kill cancers. Finally, AI tools could not replace endoscopists, radiologists, and pathologists in the near and even distant future. Computers would make predictions and doctors would make the final decision, in other words, they would always work together to benefit patients.

CONCLUSION

AI is rapidly developing and becoming a promising tool in medical image analysis of endoscopy, radiology, and pathology to improve disease diagnosis and treatment in the fields of gastroenterology and hepatology. Nevertheless, we should be aware of the constraints that limit the acceptance and utilization of AI tools in clinical practice. To use AI wisely, doctors and researchers should cooperate to address the current challenges and develop more accurate AI tools to improve patient care.

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REVIEW

From hepatitis A to E: A critical review of viral hepatitis

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Abstract

Viral infections affecting the liver have had an important impact on humanity, as they have led to significant morbidity and mortality in patients with acute and chronic infections. Once an unknown etiology, the discovery of the viral agents triggered interest of the scientific community to establish the pathogenesis and diagnostic modalities to identify the affected population. With the rapid scientific and technological advances in the last centuries, controlling and even curing the infections became a possibility, with a large focus on preventive medicine through vaccination. Hence, a comprehensive understanding of hepatitis A, B, C, D and E is required by primary care physicians and gastroenterologists to provide care to these patients. The review article describes the epidemiology, pathogenesis, clinical presentation, diagnostic tools and current medication regimens, with a focus on upcoming treatment options and the role of liver transplantation.

Key Words: Hepatitis A; Hepatitis B; Hepatitis C; Hepatitis D; Hepatitis E; Treatment

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Core Tip: Viral hepatitis (A, B, C, D and E) diagnosis and treatment have evolved through the last decades, with recent investigations aiming to cure and prevent them pharmacologically or through liver transplantation. This state-of-art review focuses on the epidemiology, pathogenesis, clinical presentation, with a special focus on upcoming diagnostic tools and treatments.

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INTRODUCTION

Viral hepatitis has been a formidable challenge eliciting epidemic dating back to ancient times, with documented outbreaks 5000 years ago in China and similar jaundice descriptions by Hippocrates in the fifth century BC in the island of Thassos[1,2]. Technological advancements over the modern era allowed for the viruses to be identified and subsequent scientific inquiry revolutionized the morbidity and mortality caused by these viral pathogens. We aim to provide an overview of viral hepatitis with discussion of current and prospective therapies. Though most remain dominant in certain parts of the world, globalization demands a fundamental understanding of each virus as we all have the daily potential of encountering any of them at our doorstep.

HEPATITIS A

Epidemiology

First discovered in 1973 by Feinstone, a spherical 27 nanometer particle was seen on immune electron microscopy in the fecal sample of hepatitis A patients[3]. A member of the picornavirus family, the hepatitis A virus (HAV) is an RNA virus responsible for 1.4 million cases per year globally[4], with an estimated 7134 deaths in 2016; almost half of these cases were reported in Asia[5]. In the United States, the annual incidence rate was reported to be 2 cases per 100000 people, in 2006. Recent outbreaks of the disease have shown a 294% increase in infections between 2016-2018 compared to 2013-2015[6,7].

Recent outbreaks

In 1988, about 300000 people in Shanghai reported symptoms of HAV after consumption of raw clams, described as one of the largest outbreaks in the modern era[8,9]. Several outbreaks related to specific food products affecting over 300 patients from multiple states in the United States have been reported in the recent years (2013-2019). The outbreaks have been related to consumption of fresh blackberries, frozen strawberries, raw scallops, and pomegranate seeds. A recent study in 2017 conducted over 4 states (California, Kentucky, Michigan, and Utah) has shown evidence for shift towards large community outbreaks with person-to-person transmission. The majority of these infections have been reported among persons with injection or non-injection drug use and/or persons experiencing homelessness[10].

Current data from state health departments shows, over 37000 new outbreak related cases with greater than 22600 hospital admissions and approximately 350 HAV deaths reported between July 2016 and December 2020[11].

On the contrary, 19947 cases of HAV from 24 European Union countries have been reported between January and December 2017, which is four times higher when compared to similar time periods between 2012 and 2015[12]. The number of outbreak related cases from 22 European Union countries was 4475 in 2016 and most outbreaks were related to men who have sex with men.

Pathogenesis

The transmission of HAV occurs *via* fecal-oral route, which includes consumption of contaminated food or water and person to person contact. Polymerase chain reaction testing for blood donors is performed as transmission through blood transfusion is noted on rare occasions[13]. The dissemination of the HAV into the liver occurs *via* the portal vein after the virus traverses the mucosa of the small intestinal wall. The virus particles subsequently replicate and are secreted into the biliary canaliculi, reaching back to the small intestine through the bile ducts and being re-excreted in the feces. Until the body responds with appropriate immune reaction in antibodies, the HAV enterohepatic cycle continues. Human leukocyte antigen-restricted, HAV-specific CD8+ T lymphocytes and natural killer cells have been implicated in the damage and destruction of infected hepatocytes[14-16].

Clinical presentation

The usual HAV incubation period is about 2-4 wk[17]. Fever, malaise, jaundice have been described as the most common presenting symptoms for HAV infection[18]. Other common symptoms include weakness, fatigue, nausea, vomiting, abdominal pain, arthralgias, myalgias, diarrhea and anorexia[12]. Patients rarely enter a prolonged cholestatic phase through recovery, while relapsing infections have been described as well[19].

About 10%-15% of patients present with a relapsing course within a 6-mo period of the initial infection[20]. The symptoms during the relapse are usually less severe than the initial infection. Notably, on extremely rare occasions a type 1 autoimmune hepatitis has been observed in genetically predisposed patients[21]. The spectrum of infections can range from asymptomatic patients without jaundice, symptomatic patients with jaundice, cholestasis with prolonged jaundice, to relapsing infections or acute liver failure[19].

Serum aminotransferases above 1000 U/dL are usually noted, with total bilirubin typically ≤ 10 mg/dL, and alkaline phosphatase below 400 U/L. Usually the serum alanine aminotransferase (ALT) is higher than the aspartate aminotransferase (AST)[22,23]. In general, older patients are more likely to have severe hepatocellular derangements, hospital admissions and higher mortality[24]. These findings can be attributed to an impaired regeneration capacity of the liver and a relatively weaker immune system in the older population[25]. In addition to old age, higher mortality has been reported in males[26]. Old age, underlying liver pathology and chronic viral hepatitis are reported risk factors for acute liver failure. In patients who develop acute liver failure, higher mortality has been associated with creatinine > 2 mg/dL (strongest predictor) total bilirubin > 9.6 mg/dL and albumin < 2.5 g/L[18].

Diagnosis

Specific antibodies against HAV (anti-HAV) in the serum can be detected. The diagnosis is confirmed by the presence of immunoglobulin (Ig) M anti-HAV. The antibodies can be detected at the time of onset of symptoms. Serum IgM levels peak during the acute infection and remain positive for up to 4 mo on an average from the onset of symptoms[27]. Immunity is usually tested with HAV total antibody to determine HAV natural exposure or secondary to vaccination[28]. The presence of IgM antibodies without any clinical symptoms is indicative of HAV infection in the past with persistent antibodies, asymptomatic infection or false positive test[29].

Liver biopsy or imaging studies are not required to make a diagnosis. If performed, a liver biopsy may show marked portal inflammation with typically a lesser degree of necrosis, Kupffer cell proliferation, acidophil bodies, or ballooning when compared to non-HAV viral hepatitis[30].

Management

No specific treatment is available for HAV and the management is mainly symptomatic. The primary focus remains on improving sanitary conditions to minimize the transmission in the community. Historically, immunoglobulins have been used in the prevention of HAV infections. With the availability of an effective vaccine, the use of immunoglobulins has been largely abandoned except in infants below the age of 12 mo. Post exposure prophylaxis with hepatitis A vaccine has been approved since 2007 for immunocompetent patients without chronic liver disease, who are between the ages of 12 mo and 40 years of age[11].

An inactivated HAV vaccine has been licensed in Europe since 1991, while a live attenuated hepatitis A vaccine has been in use in China since 1992[31]. The inactivated virus vaccine was first approved for use in United States in 1995. The vaccine is given in 2 to 3 dose series at least 6 mo apart, depending on the vaccine formulation used. In 1996, the Advisory Committee on Immunization Practices (ACIP) recommended vaccination for people at high risk for HAV infection or adverse consequences of the infection[32]. In 2006, ACIP recommended routine vaccination of all children 12-23 mo of age; and in 2019, the committee recommended routine HAV vaccination of people experiencing homelessness[33,34]. Other recommendations for groups that would benefit from vaccination are described in Table 1[35].

The vaccine has had significant effect on the decrease in HAV infections, although coverage rates remain lower when compared to other childhood vaccines. These rates were noted to be around 87% for first dose and 57% for the second dose. Long term immune response up to 40 years in noted in over 90% patients who receive both doses of the 2-vaccine series[36].

Table 1 Centers for Disease Control and Prevention recommendations for hepatitis A vaccination

	Recommendations
Children	Children age 12-23 mo
	Age 2-18 yr who have not received the vaccine (catch up vaccination)
High risk population	International travelers
	Men who have sex with men
	Illicit drug users
	People at occupational risk of exposure
	People anticipating close personal contact with international adoptee
	People experiencing Homeless
Population at high risk of severe hepatitis A	People with chronic liver disease
	HIV+ people
Others	Pregnant women at high risk or at risk of severe hepatitis A
	Any person requesting the vaccine

HIV: Human immunodeficiency virus.

Role of liver transplant

Acute liver failure occurs in less than 1% of acute HAV infections[6]. From these patients, only 31% require emergent liver transplant for treatment of fulminant disease, while the remaining patients recover spontaneously with symptomatic management[37]. In a study comparing liver transplant outcomes in patients with HAV *vs* hepatitis B infection, the patients with HAV were found to have lower 1- and 5-year survival rates. Presence of acute pancreatitis and HAV recurrence in this population was identified as risk factors for shorter graft and patient survival. Following transplant, patients should be carefully monitored for HAV recurrence as it is common and is associated with poor outcomes[38].

HEPATITIS B

Epidemiology

Chronic hepatitis B virus (HBV) infection had an estimated prevalence of 257 million people in 2015 worldwide, with the highest proportion of cases in Western Pacific and African regions (68% of the cases) and with the lowest prevalence in North America[39]. An estimated 29% of cirrhosis-related deaths worldwide were due to HBV in 2017[40], while also contributing to the majority of liver cancer-deaths the same year. There are ten identified HBV genotypes (A-J) collectively, with 35 identified sub-genotypes; the distribution of the genotypes varies broadly worldwide (Figure 1)[41]. The variation in the genotype distribution and risk factors relies on common factors in high-risk populations, such as vertical transmission which is associated to higher risk of chronic disease and hepatocellular carcinoma (HCC)[42]. Risk factors for HBV transmission include a history of blood transfusion, intravenous drug or paraphernalia use[43], contaminated piercing instruments[44], sexual intercourse with an infected person and organ transplantation from HBV positive donors[45].

Pathogenesis

The HBV is a double-stranded DNA virus belonging to the *Hepadnaviridae* family, first identified in 1963 and named the Australian Antigen due to the protein reacting to antibodies from a hemophiliac patient. The virus has a special trophism for hepatocytes, to which it adheres and integrates upon initial infection[46]. After viral uncoating, the DNA integrates to the host nucleus as a covalently closed circular DNA (cccDNA) that can persist indefinitely in the hepatocytes; this explains the possibility of HBV reactivation in chronic inactive disease[47]. Ultimately, through a process driven by reverse transcriptase, new viral molecules are assembled from the cccDNA and are released by exocytosis. This process leads to immune-mediated liver injury as antigens

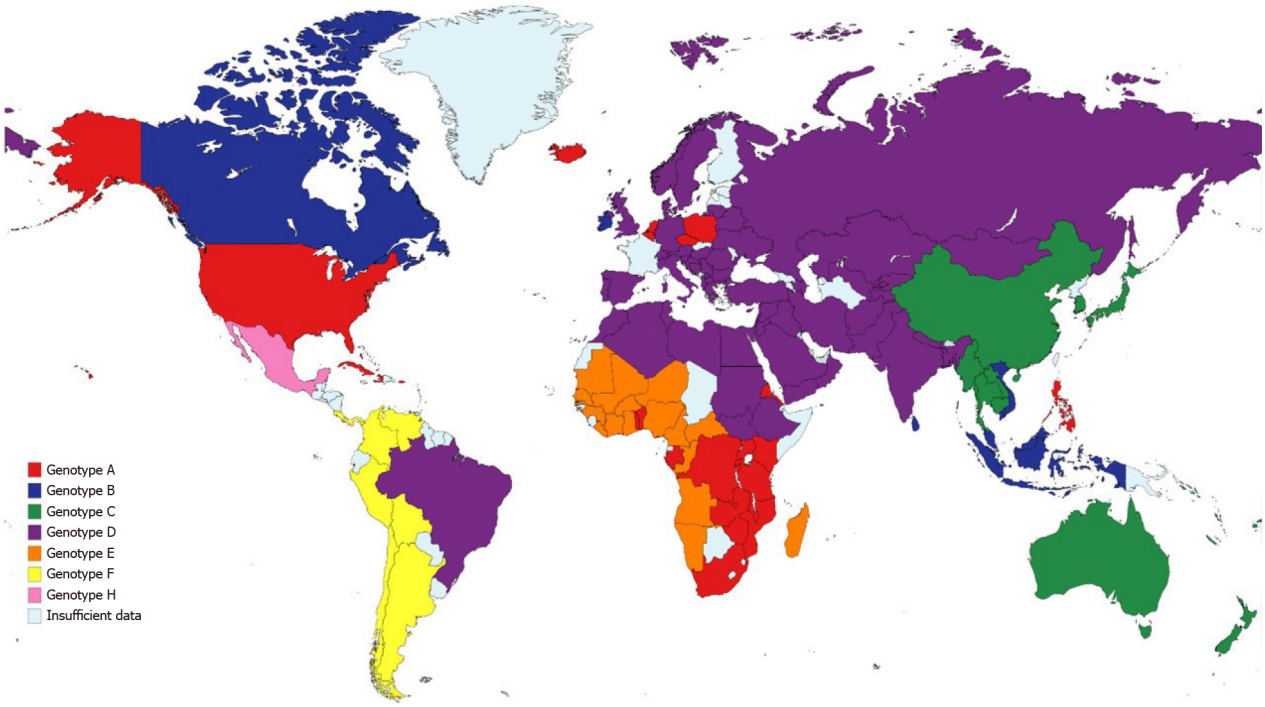


Figure 1 Global geographic distribution of most common hepatitis B virus genotypes per country.

are recognized by the immunologic system, with a lesser effect from direct viral cytotoxicity. Importantly, the DNA expresses important proteins for its survival including two capsid core proteins—core antigen and e antigen (hepatitis B e antigen, HBeAg), and the surface antigen (hepatitis B surface antigen, HBsAg), all of which are relevant for diagnosis and surveillance. Variations in genotypes have been described, with differences clinically relevant for pegylated (PEG)-interferon response and HCC risk[48].

Clinical presentation

Acute HBV infection can range from a subclinical disease to an icteric hepatitis, the former being the most common presentation[49]. Patients may present fatigue, nausea, vomiting and right upper quadrant pain before or during jaundice onset. Notably, less than 1% of cases may present fulminant hepatitis. During the acute phase, there is a rapid HBV viral load increase in the 10000-100000 ng/mL range, along with ALT and AST elevation in the 1000-2000 IU/L range, and total bilirubin elevation[50]. At this point, HBsAg and IgM core antibody (HBcAb) become positive, supporting an acute HBV diagnosis. Although a subset of acute HBV can have resolution of the infection with liver enzyme normalization, if the ALT remains elevated after 6 mo from initial presentation, a chronic HBV phase is established.

Chronic HBV may develop in patients exposed at a younger age[51], with genetic predisposition or in those that did not develop symptoms when acute HBV infection occurred[52]. These patients are usually asymptomatic for years, unless there is HBV exacerbation[53] or development of complications[54]. The occurrence of HBV exacerbations is best explained by four phases reflecting the disease activity[55]. The first phase (known as immune tolerant) shows markedly elevated HBV titers and positive HBeAg, without ALT elevation or liver inflammation for which patients are asymptomatic. The second phase (immune clearance) is characterized by activation of the immune system, leading to elevation of ALT at least five-fold the upper limit, HBV DNA decrease and histological evidence of inflammation, possibly leading to fatigue, jaundice, and right upper quadrant pain. The third phase (immune control) shows negative HBeAg, as there is seroconversion to positive HBeAb (hepatitis B e antibody) and undetectable to low HBV DNA titers. The fourth phase (immune active) is characterized by ALT and HBV DNA elevation due to triggers such as hepatitis D virus (HDV) superinfection or immunosuppression, leading to similar symptoms seen in the immune clearance phase with potential risk for acute liver failure. Importantly, the disease can fluctuate between the third and fourth phases.

Diagnosis

Patients that present with acute HBV present with serologies as previously discussed. However, some patients will be in the "window period", where the immune system has cleared the HBsAg, but no HBV surface antibody (HBsAb) is present; in this scenario, the only positive marker is an elevated IgM HBcAb. Ultimately, once the patient clears the infection, both positive IgG HBcAb and HBsAb will be present. On the other hand, if the patient develops chronic HBV infection, no HBsAb will be detected and HBsAg will persist, even if IgG HBcAb is present. Also, the HBeAg status, HBV DNA and aminotransferases level help determining the phase of the disease and tailor treatment considerations. Finally, the population that has been vaccinated and has never been exposed to HBV will only show positive HBsAb[56].

Complications

Due to parenchymal inflammation and fibrosis with long-term disease, patients with chronic HBV are predisposed to develop liver cirrhosis. Also, patients with HBV have an increased risk to develop HCC, regardless of the presence of cirrhosis; this risk is well established, but the mechanisms of carcinogenicity are hypothesized to be multifactorial including length[57] and severity[58] of HBV DNA elevation, ethnicity of the patients and more recently has been associated to the actual genome strain of the virus, especially patients with genotype C[59].

Current treatment options

Based on consensus from multiple societies, treatment with viral suppression therapy is recommended if patients have extrahepatic manifestations, pregnancy, family history of HCC, hepatitis C virus (HCV)/HDV co-infection, immunosuppression prophylaxis, compensated/decompensated liver cirrhosis[60], or if in the immune active phase and meeting specific criteria established by liver society guidelines (Table 2)[61-63].

Current HBV treatments include nucleoside/nucleotide analogs and PEG-interferon. Overall, the HBsAg seroconversion is higher with PEG-interferon regimen compared to other medications, but its efficacy is limited due to poor patient tolerance[64]. Available nucleoside/nucleotide analogs include lamivudine, adefovir, telvidudine, entecavir, tenofovir fumarate and tenofovir alafenamide[61,63]. From these medications, the first line regimen for treatment consists of entecavir, tenofovir fumarate or tenofovir alafenamide monotherapy due to high genetic resistance barrier, while the other medications can be considered as options based on medication availability and poor access to first line agents.

Role for liver transplant

Patients with HBV that develop acute liver failure, decompensated liver cirrhosis or HCC can potentially undergo liver transplantation as ultimate therapy. In the setting of posttransplant immunosuppression, the rate of HBV recurrence is high with survival rates < 50% after 2 years if no preventive measures are taken[65]. Hence, based on donor and recipient serologies different strategies have been developed including the use of combination of HBV immunoglobulin during the anhepatic and postoperative phase when indicated, with indefinite use of high-genetic barrier nucleoside/nucleotide analogs demonstrating the lowest rates of HBV recurrence and post-transplant decompensation[66].

Ongoing research and future directions

Current treatment options have changed the prognosis for HBV patients, but have been limited by its low cure rates. Hence, other targets in the viral life cycle have been evaluated to improve HBsAg seroconversion and potential cure. For example, inhibition of the HBV entry into the uninfected hepatocytes by blockage of the sodium taurocholate co-transporting peptide (NTCP) receptor in the HBV capsid has been studied with three drugs in different trial stages. Bulevirtide is a NTCP antagonist found to inhibit infection in mice injected with HBV[67]; in a phase 1 study it showed to be safe without occurrence of serious side effects in 36 healthy subjects at a max dose of 20 mg[68]. Subsequently, a multicenter phase 2b randomized trial in 60 HBV/HDV patients receiving PEG-interferon, bulevirtide 2 mg or both with bulevirtide at 2 mg and 5 mg dosing for 48 wk showed a higher proportion of HBsAg decline or loss in patients with combination therapy[69]. Other potential NTCP inhibitor are the cyclosporin derivatives such as SCY450 and SCY995[70], which have been found to inhibit hepatocyte HBV entry *in vitro* without affecting bile acid uptake, opening a new therapeutic window. Other experimental medications from the cyclophilin inhibitor family like alisporivir[71] and CRV431[72], have shown reduction of HBV DNA and

Table 2 Treatment criteria in patients with chronic hepatitis B virus and immune active phase per society guidelines

Criteria	American Association for the Study of Liver Disease[61]	European Association for the Study of the Liver[63]	Asian Pacific Association for the study of Liver Diseases[62]
ALT	≥ 2 times ULN—males 35 U/L, females 25 U/L	> 1 time ULN—40 U/L	> 2 times ULN—40 U/L
HBV DNA viral load	> 2000 IU/mL if HBeAg negative or > 20000 IU/mL if HBeAg positive	> 2000 IU/mL, regardless of HBeAg status	> 2000 IU/mL if HBeAg negative or > 20000 IU/mL if HBeAg positive
Degree of liver fibrosis/inflammation	Liver biopsy with moderate-severe inflammation or advanced liver fibrosis (F3-F4). Liver elastography or serum markers showing advanced liver fibrosis (F3-F4)	Liver biopsy, liver elastography or non-invasive testing consistent with moderate-severe fibrosis (F3-F4)	Liver biopsy with moderate-severe inflammation or advanced liver fibrosis (F3-F4). Fibroscan or serum markers showing advanced liver fibrosis (F3-F4)

ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; ULN: Upper limit of normal.

HBsAg in lab models possibly through a similar mechanism with promising results.

Another potential target is at the level of capsid assembly, where core assembly modulators have been found to disrupt the assembly by producing abnormal capsid proteins (Type I) or virions without DNA (Type II)[73]. *In vitro* evaluation of infected hepatocytes showed a decrease in HBV DNA with different investigational molecules such as JNJ-6379, RO7049389, EDP 514, GLS4 and BAY 41-4109. From these, JNJ-6379, R07019389 and GLS4 have advanced to phase 2 and 3 clinical studies[64].

High interest has focused on the use of interfering RNA molecules. These are small molecules administered intravenously, with the capacity of covalently binding to RNA before assembly and leading to RNA degradation, which could decrease the HBV DNA, HBeAg, HBsAg and HBcAg titers while increasing the seroconversion rates. A few molecules have advanced to phase 2 studies with no delivery method concern, with most of the studies presented at major hepatology conferences. The use of GSK3389404 in a phase IIa study showed a dose-dependent reduction in HBsAg, with an adequate safety profile in 66 patients[74]. Similarly, JNJ-3989 Led to sustained HBsAg reduction in 56% of 39 patients in a phase II study[75]. Also, a phase 2a randomized study evaluating GSK3228836 showed several log HBsAg reduction among 17 HBV patients[76]. Further phase 3 studies are needed to evaluate the seroconversion and functional cure rates in larger populations.

Other relevant target has been the inhibition of HBsAg release from infected hepatocytes to decrease the immunogenic tolerance induced by this molecule. A phase 2 trial study evaluating the combination of PEG-interferon and REP 2139 in 12 patients with HDV coinfection showed a titer reduction to < 50 IU/mL in 6 subjects and HBV DNA suppression in 10 subjects[77]. A second study on REP 2139 and a derivative (REP 2165) in combination with tenofovir and PEG-interferon showed functional cure rates up to 40% in HBeAg negative HBV patients[78].

Lastly, newer nucleoside/nucleotide analogs affecting the HBV life cycle have been developed. Besifovir is a molecule with similar chemical structure to tenofovir fumarate. These two medications were compared in a phase 3 randomized trial including 197 HBV patients receiving the medications for 48 wk. There was no difference in HBV DNA suppression rate with the use of besifovir (80.9%) compared to tenofovir (84.9%), showing equivalence in treatment rates between both arms[79].

On another hand, modulation of the immune system to increase HBV clearance has been a hot topic since the introduction of interferon-based treatments. Recently, the use of immune checkpoint inhibitor *via* programmed death receptor 1 (PD-1) blockage has been explored as a mechanism to overcome the T-cell anergy seen in chronic HBV. Nivolimumab is a PD-1 inhibitor that was studied in a phase I trial along with and without a therapeutic vaccine in 24 patients with HBeAg negative chronic HBV, showing a significant HBV DNA log reduction but no significant HBsAg seroconversion rates (only seen in one patient)[80]. Other studied mechanisms include the innate immunity stimulation through stimulation of toll-like receptors (TLR). However, in phase 1 studies neither TLR-7 nor TLR-8 agonists have shown to decrease significantly the HBsAg titers in the short term[81]; further phase 2 studies with longer treatment duration may help elucidate its role in HBV treatment.

Finally, HBV vaccination has become revolutionary in public health infection prevention by reducing vertical transmission and decreasing HCC incidence in younger populations[82]. While the currently widespread regimen with recombinant HBsAg (Engerix-B/Recombivax HB) includes three doses for immunization, a recent regimen utilizing an adjuvant in conjunction with recombinant HBsAg (Heplisav) has

shown to be as efficacious after two doses, with consideration to administer in non-compliant patients or patients with HBsAb titers < 10 IU/mL. Special interest has been directed to therapeutic vaccines to achieve functional HBV cure. Even though different therapeutic vaccines have been studied, the HeberNavac HBsAg-HBcAg is the only vaccine that has shown significant HBeAg seroconversion rates compared to PEG-interferon in a randomized phase III trial[83]. Although promising, immunotherapy remains a territory to be explored and similar to HCV the goal of viral eradication remains at the core of research efforts.

HEPATITIS C

Epidemiology

Greater than 71 million persons worldwide are infected with HCV[84]. Prevalence has increased from 1990 to 2010[85]. The Eastern Mediterranean region has an HCV prevalence greater than 2%, considered to be the highest in the world. The continents of Africa and Europe have a prevalence of 1%-2%. North and South America and the Western Pacific region have a prevalence of less than 1% (Figure 2)[84].

There are seven main genotypes and 67 subtypes. HCV genotype 1 represents about half of all HCV infections, making it the most prevalent genotype in the world. The second most common is HCV genotype 3, involving approximately one-third of HCV infections; it is found more commonly in south Asia, Australia, and several European countries. Genotypes 2 is found in Asia and West Africa, while genotype 4 is found in central and eastern sub-Saharan Africa, North Africa, and the Middle East. Genotypes 2 and 4 represent about 9%-13% of all HCV cases. Genotypes 5, 6, and 7 are restricted to South Africa, South-east Asia, and Democratic Republic of Congo, respectively[86-88] (Figure 3).

Pathogenesis

Hepatitis C is a small, enveloped, positive single-stranded RNA virus that was first discovered in 1989 as a member of the *Flaviridae* family and is the only member of genus *Hepacivirus*. It enters the body percutaneously through needlestick injuries (including healthcare or intravenous drug use) containing contaminated blood. It may also enter non-percutaneously through organ transplantation, blood transfusions, sexual intercourse, perinatal transmission, hemodialysis, religious scarification, body piercings, tattoos, and immunoglobulin injection[89]. There are 4 host-derived factors that facilitate the entry of HCV into the bloodstream: Scavenger receptor class B type I, Occludin, Claudin-I and CD81[90]. Additionally, CD81 binds with the viral particle, either through viral envelope protein (E)2 or other molecules and facilitates its entry into the hepatocyte[91].

Although it is considered a non-cytopathic virus, once HCV enters the hepatocyte, it replicates and causes cell necrosis *via* immune-mediated cytolysis (innate immunity and adaptive immunity) and metabolism-mediated inflammation (hepatic steatosis, oxidative stress, and insulin resistance)[90].

Non-structural protein 5A (NS5A) and E2 regions of HCV also play an important role in the pathogenesis. NS5A inactivates RNA dependent protein kinase (PKR) in hepatocytes, inhibiting the apoptotic pathway and inducing anti-inflammatory interleukin secretion, allowing for viral replication. E2 protein inhibits PKR, allowing the evasion of HCV. The role that different HCV genotypes play in the progression of liver disease is still controversial, but genotype 1b seems to have a more aggressive course, as it has been more associated to cirrhosis progression and liver disease decompensation requiring transplantation[92].

HCV core proteins leads to insulin resistance by direct receptor effect or through increased secretion of tumor necrosis factor-alpha; this leads to further hepatic steatosis, inflammation, and fibrosis. HCV associated oxidative stress is mainly caused by HCV-core protein which induces oxidation of glutathione and increase in reactive oxygen species[93]. NS5A promotes production of reactive oxygen species in the membrane of the endoplasmic reticulum[94]. NS3 is also thought to directly induce oxidative stress[95]. Finally, HCV causes steatosis through impaired secretion of lipids from liver cells, increased production of free fatty acids, and impaired fatty acid breakdown[90].

Clinical presentation

Chronic HCV infection can occur in 50%-85% of patients, while 15%-45% of patients may present spontaneous clearance of the virus[96]. Most patients with HCV infection

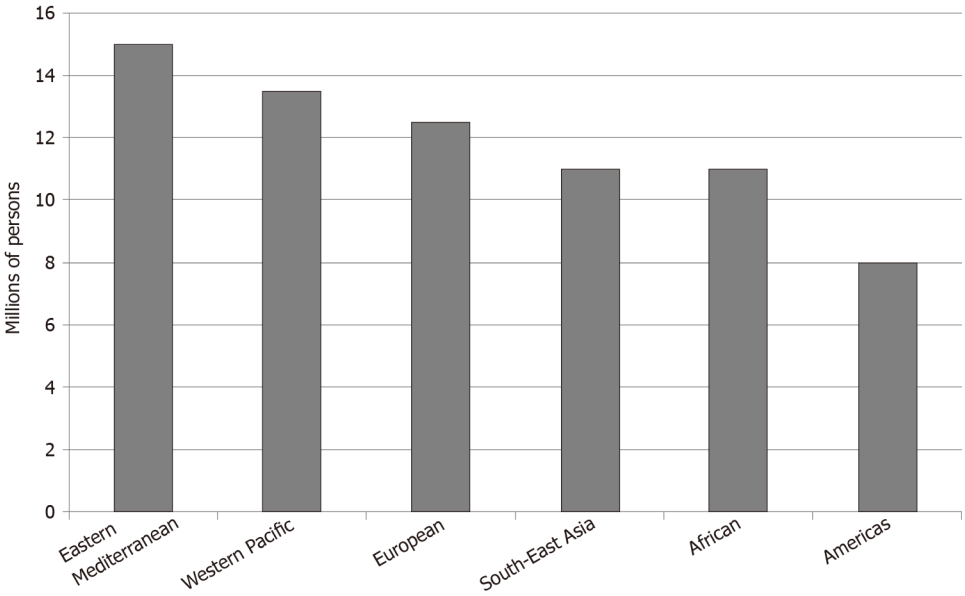


Figure 2 World Health Organization estimated chronic hepatitis C infection prevalence worldwide per region in 2015.

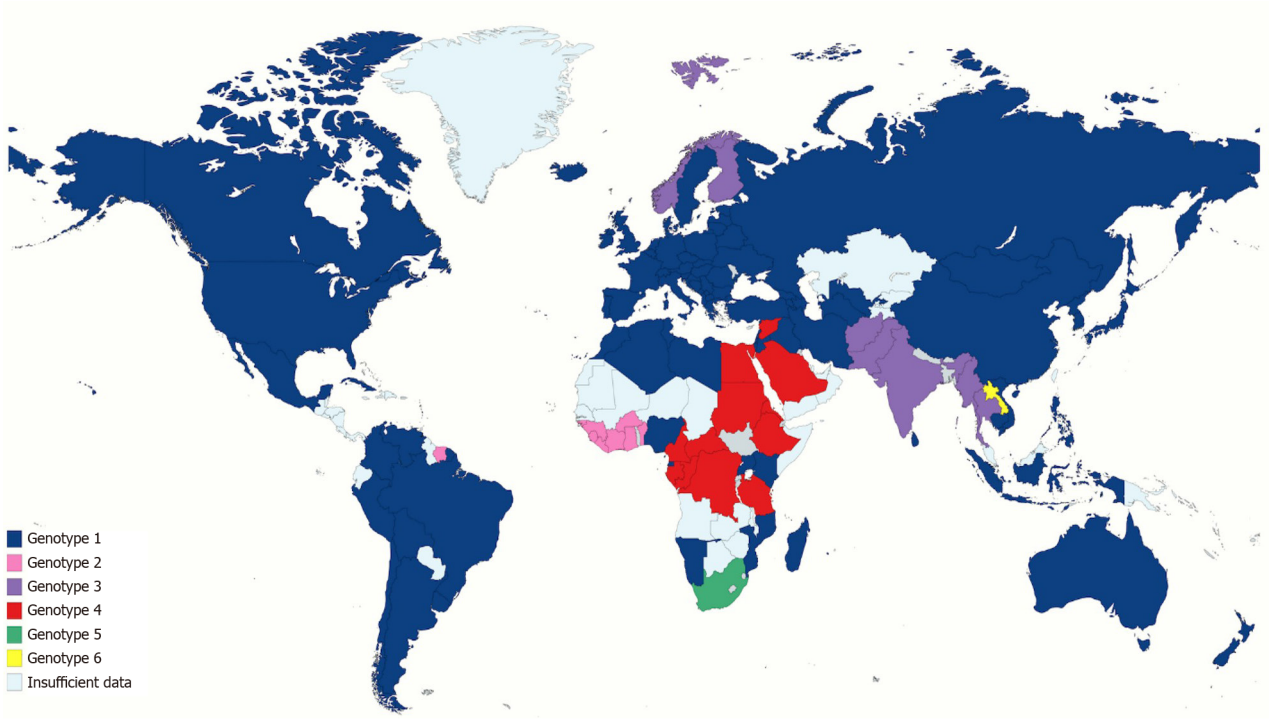


Figure 3 Global geographic distribution of most common hepatitis C virus genotype per country.

are asymptomatic or have nonspecific symptoms. These may include fatigue, sleep disturbances, nausea, diarrhea, abdominal pain, anorexia, myalgia, arthralgia, weakness, depression, anxiety, and weight loss[97]. Patients who develop cirrhosis may develop jaundice, ascites, and other stigmata of cirrhosis.

Serum aminotransferase levels remain relatively stable over time in chronic HCV patients. One in three patients have a normal ALT; only one fourth have a serum ALT more than twice the upper limit of normal; the rest of patients have slight enzyme elevations, usually less than twice the upper limit of normal. Rarely, ALT elevations more than 10-fold the upper limit of normal may occur[98]. There is little correlation between aminotransferase levels and liver histologic findings[99].

On the other hand, it is estimated that acute HCV accounts for 15%-20% of cases of acute hepatitis[100]. More than two thirds of acute HCV infections are usually asymptomatic[101]. Symptomatic patients present similarly to other acute hepatitis, with

jaundice, nausea, dark urine, and right upper quadrant pain. These symptoms develop 2-26 wk after exposure and lasts 2-12 wk[102]. Aminotransferase levels tend to be greater than 10 times to 20 times the upper limit of normal but fluctuate widely and may even normalize[103]. Acute liver failure due to acute HCV infection is extremely rare, but patients with chronic HBV[104] or other underlying chronic liver conditions may be predisposed to this outcome.

Extrahepatic manifestations are found in up to 38% of patients with chronic HCV[105]. These may include hematologic abnormalities, such as essential mixed cryoglobulinemia and lymphoma; glomerular renal disease, most notably membranoproliferative glomerulonephritis and cryoglobulinemia; dermatologic disorders such as porphyria cutanea tarda and lichen planus; rheumatologic complaints such as arthralgias/myalgias; neurological involvement with sensory or motor neuropathy; and autoimmune conditions such as thyroiditis. Resolution of these manifestations can be expected with treatment.

Complications

Spontaneous clearance of HCV occurs in about one fourth to one half of patients[106]. This usually occurs within 12 wk of exposure. A C/C type allele polymorphism in the chromosomal locus close to interleukin-28B has been associated with higher spontaneous clearance (50%) compared to the 15% rate of spontaneous viral clearance seen with the T/T allele[107]. Other favorable factors associated with higher rates of clearance include HLA-DRB1 and DQB1 alleles, white race, female sex, childhood infection, symptomatic acute infection, and high titers of neutralizing antibodies[106].

Rate of progression of chronic HCV infection to cirrhosis varies widely among the literature, but pooled data estimates that 16% of patients develop cirrhosis within 20 years[108]. It estimated that an HCV infected liver develops cirrhosis after approximately 20-30 years[109,110] with approximately 700000 deaths annually worldwide[111]. Risk factors for progression of hepatic fibrosis in patients with chronic HCV infection include age > 40 years[110], alcohol consumption, HBV coinfection human immunodeficiency virus coinfection, immunosuppressed stage, marijuana use, obesity, diabetes mellitus[112], schistosomiasis, severe hepatic necroinflammation, smoking, male sex[113], and white race[114]. The viral load has not correlated with risk of progression to cirrhosis. Studies have described a 3.9% risk of decompensation per year with ascites presence representing the most common form of decompensation[115]. Once patients develop cirrhosis, there is a 1.4%-4.9% yearly incidence rate of HCC[115-117]. It is estimated that HCV accounts for 25% of HCC cases worldwide[118].

Diagnosis

Third generation enzyme immunoassays (EIAs) detect antibodies against different HCV antigens, including HCV core, NS3, NS4, and NS5 as early as 8 wk, with sensitivity and specificity of 99% after 2-6 mo after exposure[119]. False negatives may arise in patients on hemodialysis and immunocompromised patients. These are considered indirect assays.

Quantitative, HCV RNA tests are a type of direct immunoassays that may detect HCV viremia in patients with at last 10-15 IU/mL[120]. HCV core antigen immunoassay is an alternative to HCV RNA testing but cannot be used to monitor response to antiviral therapy as it has limitations in sensitivity[121].

In patients with low pre-test probability for HCV infection, a negative EIA for anti-HCV antibody effectively excludes HCV infection. However, in patients suspected acute HCV infection, HCV RNA viral load by polymerase chain reaction (PCR) must also be obtained as antibodies may not become positive until 2-6 mo on average. A positive HCV antibody and positive HCV RNA confirms HCV infection but cannot distinguish between acute and chronic infection. Acute infection can be confirmed if a patient previously tested negative in the 6 mo prior to infection and has not tested positive for both RNA and antibody, or if the RNA and antibody become negative within 6 mo. Chronic HCV infection is defined as positive HCV RNA of at least 6 mo duration.

For patients with suspected exposure to HCV, HCV RNA, anti-HCV antibody, and serum aminotransferases are obtained within 48 h to obtain baseline values. If HCV RNA is positive, infection is confirmed, and acuity will depend on whether HCV antibody is positive (chronic) or negative (acute). However, if HCV RNA is negative, it should be rechecked in 4 wk and if positive it is considered an acute HCV infection. If HCV RNA is negative 4 wk after exposure, it should be re-checked at 12-16 wk. If negative again, HCV should be checked once more at 6 mo.

Currently available treatments

Antiviral therapy should be contemplated for almost all patients with HCV. Clinicians should consider withholding treatment in patients with decompensated cirrhosis listed for liver transplant with a high Model for End-Stage Liver Disease (MELD) score, pregnant patients, and patients with less than 12 mo of life expectancy.

The intent of HCV treatment is to eradicate the virus to mitigate cirrhosis and associated complications, such as portal hypertension, HCC, need for liver transplantation, and death. The goal is to obtain sustained viral remission (SVR), which is the absence of virus in the blood 12 wk after treatment completion. SVR has been associated with improvement in elastography fibrosis scores[122], while decreasing hepatic decompensation[123], HCC[124], and liver-related deaths[125].

The major groups of therapy include interferon-alpha, ribavirin, and direct acting antivirals (DAAs). Interferon was the first treatment available for HCV in the 1980s. More recently, interferon has been bound to PEG, which increases its half-life and has increased SVR rates[126]. Ribavirin, an oral guanosine analog, may be used in a synergistic combination interferon to increase response and reduce relapse. Ribavirin is usually well tolerated but may produce hemolytic anemia, especially in patients with renal disease given its excretion in the kidney. It should be avoided in patients with a creatinine clearance less than 50 mL/min. In addition, ribavirin is teratogenic[127].

DAAs inhibit replication of HCV by targeting the NS3/NS4 protease, NS5A protein, and the NS5B polymerase. NS3/NS4 protease inhibitors end in the suffix "-previr" and include boceprevir, telaprevir, simeprevir, paritaprevir, grazoprevir, glecaprevir, and voxilaprevir. NS5A inhibitors end in suffix "-asvirs" and include daclatasvir, ledipasvir, ombitasvir, and elbasvir, velpatasvir, and pibrentasvir. NS5B polymerase inhibitors end in "-buvirs" and are either nucleoside, nucleotide (sofosbuvir), or non-nucleoside (dasabuvir).

The classes of DAAs are typically used in combination: sofosbuvir/lepidasvir, elbasvir/grazoprevir, sofosbuvir/velpatasvir, sofosbuvir/velpatasvir/voxilaprevir, and glecaprevir/pribrentasvir. The choice of regimen and duration depend on the patient's genotype, renal function, and concurrent medications. The genotypic coverage provided by each regimen is outside of the scope of this review, but importantly sofusbuvir/velpatasvir and glecaprevir/pibrentasvir are considered pangenotypic regimens. Protease inhibitors are primarily hepatically excreted and thus are contraindicated in Child-Pugh B and C cirrhosis. Nucleoside NS5B inhibitor sofosbuvir is primarily renally excreted and should be avoided in patients an estimated glomerular filtration rate less than 30 mL/min per 1.73 m^2.

Role of liver transplant

Liver transplantation frequently provides destination treatment for HCV decompensated cirrhosis or HCC in candidates who are not deemed appropriate for surgical resection. HCV infection remains one of the leading etiologies for liver transplantation worldwide[128]. Since 2012, the percentage of liver transplants allocated for chronic HCV infection has steadily decreased in United States[129] due to the use of DAAs. The use of liver transplant for acute liver failure secondary to HCV infection has not been a common phenomenon.

Virtually all recipients who are serologically positive for HCV at the time of transplant develop recurrent HCV infection during reperfusion of the transplanted liver graft[130]. The main consequence is the development of post-transplant liver injury in the form of fibrosis in up to 40%[130], recurrent cirrhosis in up to 30% after 5 years[131], and fibrosing cholestatic HCV in up to 10%[132]. This has been associated with lower overall survival in HCV infected recipients in comparison to HCV negative recipients[133]. To counteract this, therapy with DAAs have been used and have increased post-transplant one-year survival rates in HCV viremic recipients from 90%-92%[129]. DAAs may be used to cure HCV prior to transplant, eliminating the chance of HCV recurrence[134], or can be used post-transplant. Reasons why HCV treatment with DAAs may be delayed until after transplant include: improvement of MELD score leading to de-listing or increased wait time on transplant list, lower likelihood of SVR, and limited access to available regimens for advanced cirrhosis[135].

Utilization of HCV infected organs has been one of the major advancements aimed at increasing the donor organ pool and decreasing waitlist time. Initially, this was primarily performed in recipients already infected with chronic HCV. Between 2010 to 2015, the proportion of HCV positive livers transplanted into HCV seropositive recipients increased 2.4-fold[136]. More recently, the transplantation of HCV infected organs including livers into HCV seronegative recipients has been utilized with

similarly good outcomes[137,138]. Donors positive for HCV RNA have a high risk of transmitting infection to HCV negative recipients but this may also occur with HCV antibody positive HCV RNA negative donors in up to 16% of cases[139].

HCV negative recipients who receive HCV positive organs may receive DAA therapy prophylactically or reactively after they are found to be positive for HCV. In liver transplantation, DAA therapy is typically given for at least 12 wk to recipients once they are found to have positive HCV RNA, usually within 3 mo. Thus far, studies have shown SVR of up to 100%[140,141]. Questions are easing surrounding the concern whether the risk of rejection is increased in recipients of HCV organs, either from HCV or DAA therapy[140]. Initial 2-year outcomes of HCV positive organ transplantation in HCV negative recipients have shown promising similar patient and graft survival[138]. Short duration of DAA has been considered but not extensively studied in HCV positive liver transplantation.

In non-hepatic transplantation, prophylactic DAA therapy is favored before viremia to reduce the chances of complications and the duration of DAA therapy[142]. Some authors have even limited duration of DAA therapy to as few as 8 d, but this is not currently recommended outside a clinical trial setting[143]. A pangenotypic regimen is typically utilized if prophylactic DAA therapy is used. Thus far, outcomes of transplantation of HCV positive nonhepatic organs in HCV negative patients have been promising, but data is limited. There is still some concern regarding risks such as DAA treatment failure with evolution of resistance associated substitutions and fibrosing cholestatic hepatitis[144].

Ongoing research and future directions

Much of the future research and guidelines will aim to answer questions regarding the safety, ethical, and practical concerns regarding the transplantation of HCV viremic organs into HCV negative recipients. Several factors related to the HCV donor organ remain nebulous. It is unclear whether duration of HCV infection, viral load, genotype, and failed prior treatment of the donor will affect SVR rates post-transplant in this patient population as studies have not controlled for these factors[145]. In addition, several optimal donor factors, specifically age and fibrosis grade, are not clear. Previous studies showed that HCV infected livers of donors 50 years or older have been found to have increased fibrosis progression and worse graft survival[146], but this data is prior to the advent of DAAs and should be re-examined. Similarly, data prior to the widespread use of DAAs showed that HCV positive donor kidneys had worse outcomes; because of this, the kidney donor profile index includes HCV status as a relevant factor but more recent data from the DAA era shows similar outcomes in these HCV positive donor kidneys[147]. Further studies are needed to establish its effects in the outcomes and limit the number of discarded HCV infected organs.

Recipient selection to match donor characteristics when utilizing HCV organs need further investigation to establish best practice guidelines for HCV organ utilization. For now, HCV infected donor allografts are used mainly in patients whose waitlist duration is expected to exceed their duration of transplant-free survival, but no studies have explicitly examined this. Another scarcely studied but relevant risk is how healthy the recipient's liver condition factors to receive an HCV infected non-liver organ, and what the long-term outcomes of this are. Timing of DAA therapy, optimal regimen, duration, cost effectiveness of different strategies, interactions between DAAs and calcineurin inhibitors, and other drug interactions with DAAs will also need to be investigated. Finally, data on long-term outcomes, specifically efficacy and safety, following transplantation of HCV viremic organs into HCV negative recipients is lacking, as current data is limited to 1-2 years.

The ongoing pursuit for a HCV vaccine remains prudent, especially since DAAs do not prevent against reinfection. To date, there have been a few clinical trials studying different methods for active immunity, including viral vectors[148], DNA-plasmid, recombinant proteins, and prime-boost vaccination strategies[149]. However, thus far, many of these trials are still in the early phases or have not conferred consistent long-term immunity.

In 2020, the United States Center for Disease Control and Prevention increased the previous screening expanse by recommending one-time universal screening for all adults at least 18 years of age. Similarly, the United States Preventive Services Task Force recommended one-time universal screening for patients 18-79 years old. The World Health Organization (WHO), the American Association for the Study of Liver Diseases/Infectious Diseases Society of America joint guideline group, the European Association for the Study of the Liver, the National Health Service in the United Kingdom, the Canadian Task Force on Preventive Health Care, the Canadian

Association for the Study of the Liver, and other groups have yet to recommend universal screening.

Given the vast prevalence of HCV infections worldwide, there has been an increased effort in the last five years to encourage primary care physicians to treat HCV. This has been aided by the ease of using DAAs and the advent of pangenotypic drugs. However, studies have shown that up to 59% of primary care physicians still refer all of their HCV patients to specialists, citing needing additional training to feel comfortable treating the infection[150]. Thus, further studies investigating the best methods in educating and involving primary care physicians to treat HCV are needed.

HEPATITIS D

Epidemiology

In a recent large meta-analysis by Chen *et al*[58] with 182 articles included from 61 countries, it was estimated that 62-72 million individuals around the globe are infected with HDV. Approximately 10.58% of HBsAg carriers were coinfected with HDV, which is two-fold higher than what has been estimated previously[150,151]. Areas of highest HDV infection carriage include western and middle Africa, the Amazon Basin, eastern and Mediterranean Europe, the Middle East, and parts of Asia. Notably, a recent epidemiological study examining a cohort of HBV-positive intravenous drug users revealed an alarming increase in HDV seroprevalence from 29% in 1988-1989 to 50% in 2005-2006[152]. Nonetheless, the lack of HDV RNA validation in many of these studies hinders a true estimation of HDV prevalence.

Pathogenesis

HDV is a single-stranded RNA virus, considered a defective virus that requires the existence of HBV for full expression and replication. It was first described in 1977 by Rizzetto *et al*[153] in Italy, when they detected a new antigen-antibody system associated with but immunologically distinct from HBV by direct immunofluorescence. Although there are 8 different described HDV genotypes, HDV-1 is responsible for most cases in North America, Europe, and the Middle East[154].

Like HBV, HDV is transmitted *via* the parenteral route through exposure to infected blood or body fluids with an exceedingly small inoculum needed to transmit infection[155].

Clinical presentation

The clinical spectrum of HDV infection ranges from inactive asymptomatic carrier to acute liver failure[156]. Concomitant infection of HBV and HDV typically leads to mild self-limited disease or less likely severe acute hepatitis with spontaneous resolution of both infections[157]. In contrast, HDV superinfection in chronic HBV carriers usually results in a protracted clinical course[158]. Nevertheless, patients exhibit heterogeneous clinical presentations with more severe disease reported in genotypes 1 and 3[159], intravenous drug users, and older patients in European cohorts. This could be due to the variable direct cytotoxic effect and immunogenic response to HDV on the host hepatocytes[160-162].

Screening for HDV should be considered in all HBsAg-positive patients especially those presenting with worsening liver disease. Initially, testing for total anti-HDV antibody (IgM and IgG) should be attempted by EIAs or radioimmunoassays. A positive test warrants confirmation by serum reverse transcriptase-PCR assays for HDV RNA. Quantification of serum HDV RNA is important in evaluating the need for and efficacy of antiviral therapy.

Currently available treatments

Current evidence supports treating chronic HDV infection in those with detectable viral RNA and evidence of biochemical or histological active liver disease, particularly if significant fibrosis exists[61]. On the contrary, asymptomatic patients with normal liver enzymes do not require therapy.

With nearly 34 years lapsed since the introduction of interferon for the treatment of chronic HDV infection, it remains the only available treatment option of proven benefit[163]. Nonetheless, sustained virologic response is low even with the newer PEG-interferon and rarely exceeds 25%[164]. A meta-analysis of five trials comparing standard 48-wk PEG-interferon with no treatment confirmed not only a modest benefit to PEG-interferon but such benefit was not sustained[165].

Ongoing research and future directions

The past decade has witnessed the evolution of multiple novel antiviral agents that target various critical steps of HDV life cycle. Three new drugs have emerged for the treatment of chronic HDV infection. Among them is the specific inhibitor of HDV prenylation lonafarnib which showed promising results as a monotherapy or when combined with ritonavir or PEG-interferon. Reduction in viral RNA load and improvement in liver enzymes were observed at variable endpoints[166]. Although observed therapeutic benefits correlated with lonafarnib dose used, that was challenged by its universal limiting adverse effects like nausea, diarrhea, abdominal bloating, and weight loss.

Other new therapies included HDV entry inhibitors such as bulevirtide. As mentioned above, this medication was evaluated in patients with HBV coinfection for 24 wk as a monotherapy or in combination with PEG-interferon or tenofovir. The majority of bulevirtide treated patients experienced a > 1 log10 reduction in HDV RNA after 24 wk of therapy with combination therapy being more effective than monotherapy. Finally, as mentioned in the hepatitis B section, REP 2139 was introduced as an inhibitor of virion secretion with potential for viral load suppression in patients with HBV-HDV coinfection. However, marked ALT elevations were noted in these studies[77,78]. Combination of inhibitors over an enduring treatment regimen are likely required. These novel agents are being considered in combination with PEG-interferon, which carries known side effects that may be prohibitive in its use with HDV cirrhosis.

Animal studies suggest that vaccine strategies for preventing HDV superinfection may be feasible[167,168]. Those studies used groundhogs as animal models and focused on inducing active immunity by injecting the animal with a full-sized HDV antigen or synthetic peptides. These investigations, although promising, were limited by their small size and heterogenous results. As of today, vaccination against HBV remains the most cost-effective means to prevent HDV infection.

Role of liver transplant

Liver transplantation is indicated in patients with fulminant liver failure. Communication and arrangement for transfer to a transplant facility are paramount in the ability to rescue these individuals. Post liver transplantation recurrence of HDV infection despite appropriate medical therapy was estimated to be 13.4%[169] with comparable outcomes between patients with and without recurrence.

HEPATITIS E

Epidemiology

The WHO reported an estimated 20 million new hepatitis E virus (HEV) infections every year globally, leading to 3.3 million symptomatic cases of acute hepatitis and in another report to over 44000 deaths in 2015[39]. Although the use of current antibody assays in seroprevalence studies remains debatable, the overall seroprevalence of the HEV in the United States between 1988 and 1994 was projected to be 21%[170]. Other more recent analyses reported a lower but rising overall seroprevalence in the United States born individuals from 4.5% in 2013-2014 to 8.1% in 2015-2016, with increasing age, female sex, and Asian ethnicity being significant predictors of HEV seropositivity[171].

Pathogenesis

The HEV is a small, single-stranded RNA virus that is considered one of the most common, yet underdiagnosed etiologies of acute viral hepatitis with the earliest epidemic going back to 1955 in New Delhi[172]. While it stands as the only member of the genus *Hepevirus* in the family *Hepeviridae*a, a plethora of genotypes were identified to date with four of particular clinical significance; genotypes 1 and 2 being confined to humans, while genotypes 3 and 4 can infect humans and animals largely *via* the fecal-oral route[173,174].

Clinical presentation

Most HEV infected patients are either asymptomatic or may develop mild, self-limited, HAV-like illness. Typically preceded by an incubation period ranging between 2 wk to 10 wk[175]. This is essentially true of HEV genotypes 1 and 2. However, the zoonotic genotypes HEV 3 and 4 have been increasingly reported as causes of

chronic hepatitis almost exclusively in those who are immunocompromised[176,177]. Less than 5% of acutely infected HEV patients will progress to acute liver failure and those patients are more likely to be pregnant, malnourished, or have a preexisting liver condition[178,179].

Although a uniform diagnostic approach for HEV infection is limited by the lack of standardized antibody assays, patients with consistent clinical presentation should be tested for anti-HEV IgM antibody assay with a confirmatory HEV RNA viral load if positive.

Currently available treatments

Similar to HAV infection, the majority of patients with acute HEV infection require no specific treatment and the management is mostly supportive. However, patients who develop acute liver failure may warrant liver transplantation[180]. A rising body of literature investigated the role of ribavirin in treating chronic HEV infection in immunosuppressed patients with variable success[181]. Whenever possible, treatment should be accompanied by reducing immunosuppressive therapy and followed by testing for clearance to confirm eradication[182]. A 3-mo course of ribavirin has been proposed for the above indication, while its use in acute fulminant hepatitis appear promising[183], more robust evidence is yet awaited.

Prevention remains the most effective approach to combat HEV-related disease. This can be achieved by maintaining quality standards for public water supplies and maintaining hygienic practices as per the WHO recommendation[175].

In 2011, a recombinant vaccine to prevent HEV infection was registered in China. Although it is not approved yet in other countries, few studies have emerged to evaluate efficacy and safety of this vaccine. In a large, randomized trial from China including 112604 healthy adults that evaluated different doses of HEV vaccine compared to placebo, a protective effect of 100% was reported at 1 year following 30 d from the last dose received. Another trial revealed a protective efficacy of the vaccine against HEV to be 86.8% after 4.5 years of the first vaccination[184]. Other preventive options such as immune globulin remain experimental[185].

Ongoing research and future directions

For chronic HEV patients who fail ribavirin therapy, some alternatives such as PEG-interferon[186] and sofosbuvir are being considered pending more evidence[187]. Their routine use is being hindered by the lack of safety and efficacy profile in this setting besides the perceived higher risk of acute rejection with PEG-interferon due to its immunostimulatory effect in organ transplant recipients.

Role of liver transplant

Although liver transplant recipients may develop chronic HEV in the setting of prolonged immunosuppression, acute HEV infection may rarely result in acute liver failure necessitating liver transplant. Accumulating epidemiologic evidence described significant heterogeneity in HEV infection prevalence and outcomes. For example, HEV remains the most common cause of acute viral hepatitis and acute liver failure (up to 44%) in some Asian countries such as India and Bangladesh[187,188]. This contrasts with Western Europe and the United States where it is a rare cause of acute liver failure[189].

CONCLUSION

The recognition of the epidemiological and clinical features of hepatitis A, B, C, D and E is crucial to guide the diagnosis of these conditions. Fortunately, the medical advances in the last centuries have allowed to effectively diagnose them and comprehensively establish its multisystemic impact in this population. With the advent of new therapies, the possibility of achieving control of the disease is a reality, while reaching the cure and eradication of chronic hepatitis B, C and D is around the corner.

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MINIREVIEWS

Paediatric gastrointestinal disorders in SARS-CoV-2 infection: Epidemiological and clinical implications

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic is a threat worldwide for individuals of all ages, including children. Gastrointestinal manifestations could be the initial presenting manifestation in many patients, especially in children. These symptoms are more common in patients with severe disease than in patients with non-severe disease. Approximately 48.1% of patients had a stool sample that was positive for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral RNA. Children typically form 1%-8% of all laboratory-confirmed cases of SARS-CoV-2. Gastrointestinal manifestations of COVID-19 in children are not rare, with a prevalence between 0 and 88%, and a wide variety of presentations, including diarrhoea, vomiting, and abdominal pain, can develop before, with or after the development of respiratory symptoms. Atypical

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manifestations such as appendicitis or liver injury could also appear, especially in the presence of multisystem inflammatory disease. In this review, we discussed the epidemiology of COVID-19 gastrointestinal diseases in children as well as their implications on the diagnosis, misdiagnosis, prognosis, and faecal-oral transmission route of COVID-19 and the impact of gastrointestinal diseases on the gut microbiome, child nutrition, and disease management.

Key Words: COVID-19; SARS-CoV-2; Gastrointestinal diseases; Children; Dysbiosis; Faecal-oral transmission

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Core Tip: Coronavirus disease 2019 (COVID-19) presents with different manifestations, including gastrointestinal inflammation, especially in children. Gastrointestinal effects of COVID-19 have a significant impact on the diagnosis, misdiagnosis, prognosis, faecal-oral transmission route, gut microbiota, and child nutrition. The presence of gastrointestinal symptoms in children with COVID-19 should not be ignored.

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INTRODUCTION

The rise of coronavirus disease 2019 (COVID-19) at the end of 2019 became a real and significant challenge to humanity, especially as the outbreak escalated to a global pandemic in March 2020. The challenge was related not only to the rapid transmission of COVID-19 but also to the dilemma and conflict in the clinical presentations, rate of mutation, laboratory diagnosis, and management of the disease. COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a virus that is related to a large group of enveloped, positive-stranded RNA viruses called coronaviruses because of the crown-like spikes on their surface. There are four subgroups (genera) of coronaviruses, named alpha, beta, gamma, and delta; the alpha and beta subgroups can cause disease in humans, causing respiratory infections, gastrointestinal infections, and hepatic and central nervous system diseases[1]. The most recent outbreaks of coronavirus belong to beta coronaviruses and include the SARS-CoV outbreak, the Middle East respiratory syndrome coronavirus outbreak, and the recent SARS-CoV-2 pandemic. In humans, coronaviruses primarily affect the upper respiratory tract, causing different manifestations of respiratory infections. However, gastrointestinal manifestations could be the initial presenting manifestation in many patients, especially in children. These manifestations could also be the sole manifestations in a small percentage of patients. This article will review the different gastrointestinal disorders in association with SARS-CoV-2 infection (COVID-19) and discuss their epidemiological and clinical implications[2].

EPIDEMIOLOGY OF COVID-19 AND GASTROINTESTINAL DISORDERS IN CHILDREN

Although it predominantly affects adults of higher age groups, SARS-CoV-2 can infect children at any age, even during the neonatal period; intrauterine or perinatal transmission of the virus is uncertain, and vertical transmission is not yet proven[3]. Children typically form 1%-8% of all laboratory-confirmed cases of COVID-19: 7% in neonates, 29% in infants less than 1 year old, 10% in the 2nd year of life, 11% between 2 and 5 years old, 16% between 5 and 10 years old, and 34% between 10 and 18 years of

age[4]. However, the percentage of the disease in children may differ from one country to another and from one ethnic group to another according to the underlying health, social, and economic status of individuals[5]. As the number of children infected is currently increasing, studies have shown that most infected children have mild disease. However, approximately 4.4% of infected children have severe disease, with a mortality rate of 0.2%, with no deaths reported below the age of 9 years. Infants less than 1 year old and children who have other underlying comorbidities are more prone to severe COVID-19. There was no sex difference in the infected children, unlike that observed in adults with a male to female ratio of 1.1:1. The source of infection was usually an infected family member as a part of a family cluster outbreak[6].

The low prevalence of COVID-19 in children is multifactorial. Children have few outdoor activities, especially with school shutdowns, lower travelling rates than adults, and less exposure to smoking and air pollution; hence, children are less likely to be exposed to infection. Their immune status plays an important role in modifying their risk of infection with SARS-CoV-2. Children have a more active innate immune response and a healthier respiratory system than adults, especially children less than 10 years of age. They also respond with a normal or high lymphocytic count in response to infection with SARS-CoV-2, in contrast to adults, who respond with a decreased lymphocytic count. Coinfection with other viruses may limit the replication of SARS-CoV-2 *via* direct virus-to-virus interactions and competitive inhibition and may be a possible mechanism like what is observed with the common cold and influenza. However, the main factor for the low incidence of COVID-19 in children is related to the age-dependent immaturity of angiotensin-converting enzyme 2 (ACE2) receptors in children and their different distribution and function compared to adults. These receptors serve as a binding site for SARS-CoV-2 and a portal of entry to the inside of the cell by the binding of the viral spike proteins to ACE2 receptors. The degree of cell susceptibility to viral infection is correlated with the number of ACE2 receptors on those cells. However, ACE2 has dual effects on cell infection by the virus. In addition to ACE2 receptors acting as a binding site for the virus, they have a very important pulmonary protective role, as they protect against severe lung injury caused by the virus. Therefore, this dual role of ACE2 pushes us to perform more research to confirm its implication in COVID-19 pathogenesis[7-10]. The low number of children infected with SARS-CoV-2 does not necessarily mean a high resistance to the infection. Another possible theoretical reason is related to Bacillus Calmette-Guérin (BCG) vaccination in children. BCG may provide some protection against COVID-19 as it modulates cell-mediated immunity, including innate cells such as macrophages, monocytes, and epithelia, and many children are vaccinated with BCG[11]. However, the protective effects of BCG against COVID-19 are still unknown. As more than 95% of children infected with SARS-CoV-2 are asymptomatic and less likely to be tested, epidemiological surveillance may be inadequate, and children may still contribute to viral transmission.

Although fever and respiratory manifestations are the most common features of COVID-19, approximately 17.6% of patients present with gastrointestinal symptoms that usually appear 1-2 d before respiratory symptoms. Verified cases of COVID-19 with the sole gastrointestinal manifestation have been described in both adults and children. Gastrointestinal manifestations were more common in patients with severe disease (17.1%) than in patients with non-severe disease (11.8%). Approximately 48.1% of the patients had stool that was positive for viral RNA, even in stool samples collected after the respiratory samples turned negative for the virus. The exact rate of gastrointestinal symptoms is a matter of debate, with various incidences among the different studies. Anorexia and poor appetite were the most common gastrointestinal symptoms (a range between 347% and 67% and a mean of 47%), followed by diarrhoea (range between 2.1% and 32.5% and a mean of 11.6%), abdominal pain and discomfort (range between 1% and 11.9% and a mean of 5.2%), and nausea and vomiting (range between 1% and 11.7%, and a mean of 5.1%), while the incidence of liver damage ranged from 15% to 53%[12-18]. Liver damage is doubled in the presence of gastrointestinal manifestations compared to in the absence of gastrointestinal manifestations. Gastrointestinal manifestations usually deteriorate with disease progression, and severe gastrointestinal bleeding has been reported in some cases[19].

Gastrointestinal manifestations of COVID-19 in children are not rare, with a prevalence between 0 and 88%, and a wide variety of presentations, including diarrhoea, vomiting, and abdominal pain, that can develop before, with or after the development of respiratory symptoms. Atypical manifestations such as acute appendicitis or liver injury could also appear, especially in the presence of multisystem inflammatory disease[20]. An American study including 44 children showed that gastrointestinal manifestations were present in 84.1% of children

admitted to the hospital and were most often associated with fever and rash[21]. In a meta-analysis including 280 children from 9 studies, the pooled prevalence of gastrointestinal manifestations was 22.8%. Diarrhoea was the most common presentation (12.4%), followed by vomiting (10.3%) and abdominal pain (5.4%)[22]. In a prospective study including 992 healthy children of healthcare workers in the United Kingdom, approximately 7% tested positive for SARS-CoV-2 antibodies, half of them were asymptomatic, and 19% had gastrointestinal symptoms, including diarrhoea, vomiting, and abdominal cramps[23]. Another study in Wuhan, China showed that gastrointestinal manifestations were observed in 15.2% of children infected with SARS-CoV-2 (diarrhoea 8.8% and vomiting 6.4%). In contrast to adults, gastrointestinal manifestations in children were associated with less severe disease and less need for oxygen therapy[24].

Pathogenesis of gastrointestinal infection in COVID-19

The exact mechanism of gastrointestinal symptoms caused by SARS-CoV-2 infection is still unclear. The detection of SARS-CoV-2 RNA in the stool suggests faecal-oral transmission. The presence of gastrointestinal manifestations before respiratory symptoms in a group of patients, along with the presence of viral shedding from the gastrointestinal tract in cases with a more aggressive clinical course, may indicate the role of the gastrointestinal tract in modifying the course of COVID-19 and help in predicting prognosis[25]. Several studies suggest that the virus actively infects the cells of the gastrointestinal tract, replicating itself in the epithelium of the small and large intestine and producing an excessive immunological reaction in the host[26]. ACE2 is highly expressed in lung alveolar type II cells and the upper oesophagus, in absorptive enterocytes from theileum and colon, and in hepatocytes and cholangiocytes. The ACE2 receptor is an important receptor on the cell membrane of host cells. The interaction between the S protein of SARS-CoV-2 and ACE2 promotes the invasion of host cells by SARS-CoV-2. Abundant expression of ACE2 throughout the endothelial surface of the gastrointestinal tract, especially in enterocytes, may act as a secondary entry site for SARS-CoV-2 infection. The magnitude of ACE2 expression in the gut may ameliorate or worsen gut dysbiosis and gastrointestinal leakage[27]. Dysbiosis and the consequent leaky gut further impair the gut-lung axis and are related to the onset of pulmonary hypertension as well as the hyperactivation of the ACE/Ang II/AT1R (angiotensin II type 1 receptor) axis from ACE2 Loss. At the same time, infection with SARS-CoV-2 causes infiltration with many plasma cells and lymphocytes and potentially induces interstitial oedema and the degeneration of the gut-blood barrier, leading to the spread of viruses, bacteria, endotoxins, and microbial metabolites into the systemic circulation, affecting the host's response to SARS-CoV-2 infection and inducing multisystem dysfunction and septic shock[28]. Persistent viral faecal shedding alters the gut into a base for sustained viral replication and consequently may explain the associated poor prognosis. On the other side of the gut-lung axis, SARS-CoV-2-infection-induced respiratory lesions impair the respiratory tract microbiota, which adversely compromises the digestive tract through immune dysregulation[29].

Failure to detect viral RNA in the stool in some patients infected with SARS-CoV-2 that have gastrointestinal manifestations suggests the presence of other mechanisms of indirect gastrointestinal injury in COVID-19 patients. Disturbed cellular immunity has a significant impact on gastrointestinal health in patients with COVID-19. Lung-derived C-C chemokine receptor type 9-CD4- T cells were significantly increased in patients infected with SARS-CoV-2 compared to healthy individuals. These cells are important for mucosal immunity and have a significant role in the development of chronic enteritis by intestinal immune damage and the disruption of the intestinal flora[30]. The resulting dysbiosis will further promote the polarization of Th17 cells in the small intestine, and the production of excessive interleukin (IL)-17A leads to the recruitment of neutrophils, causing more intestinal immune damage, and inducing diarrhoea and other gastrointestinal symptoms[31]. The induced intestinal inflammation impairs the intestinal mucosal barrier, allowing easy access of the bacteria and its toxin to circulate in the blood and affect other organs, including the liver (gut-liver axis). Intestinal inflammation-induced host and microbial metabolites can reach the liver through the portal vein, impairing liver function[32]. Consequently, the inflamed liver releases bile acids and other bioactive materials into the biliary and systemic circulation, reaching the intestines and causing more intestinal lesions (bidirectional effect). The liver could also be affected by the adverse effects of various drugs used to treat COVID-19 (Figure 1).

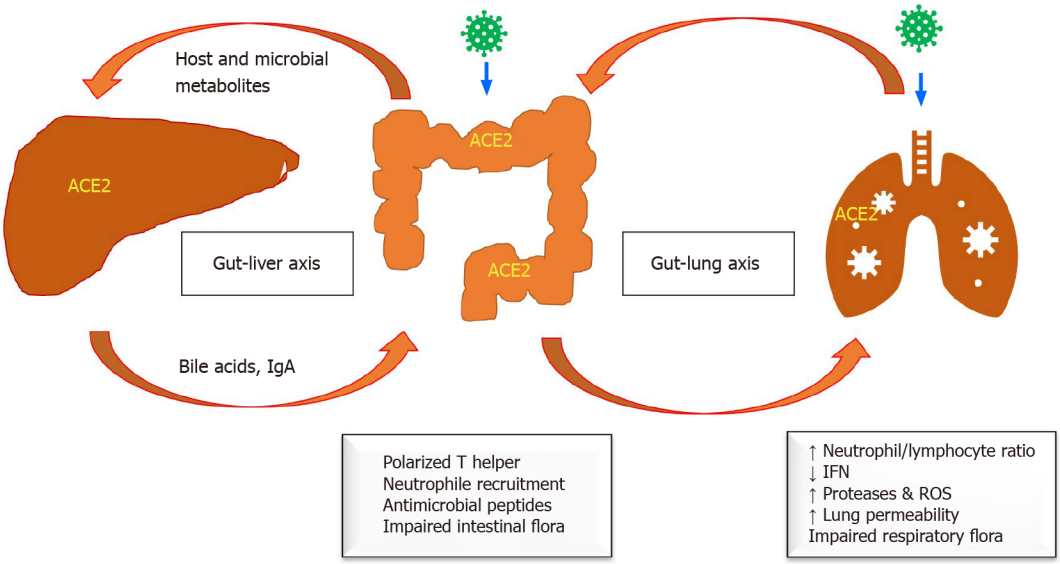


Figure 1 Pathogenesis of gastrointestinal infection in coronavirus disease 2019. ACE2: Angiotensin-converting enzyme 2; IFN: Interferon; IgA: Immunoglobulin A; ROS: Reactive oxygen species.

Diagnosis

Gastrointestinal manifestations are present in more than half (57%) of the patients infected with commonly circulating coronaviruses, especially in children. These manifestations are considered a warning in the presence of other medical comorbidities[33]. Children infected with SARS-CoV-2 usually have milder clinical manifestations of the disease than adults, and occasionally, they act as asymptomatic carriers. Gastrointestinal symptoms are common in children with COVID-19; approximately 25% of them exhibit at least one gastrointestinal symptom. These symptoms may occur in the absence of respiratory symptoms. The most common gastrointestinal symptoms in children are diarrhoea, followed by vomiting and abdominal pain. The manifestations usually come in combination. Gastrointestinal bleeding was reported in adults, but no data were reported in children. Abdominal pain is sometimes severe enough to be mistaken with acute abdomen such as acute appendicitis or systemic sepsis[20].

Infants less than one year of age may present with food intolerance or feeding difficulties. These atypical presentations, especially as initial symptoms, may be misleading and lead to delayed diagnosis of COVID-19. Diagnosis is usually based on the detection of viral nucleic acids in respiratory tract specimens, while faecal nucleic acid detection is often neglected. Therefore, children with gastrointestinal symptoms as the predominant manifestation of COVID-19 are often misdiagnosed[34]. Gastrointestinal manifestations are a prominent presenting feature of multisystem inflammatory syndrome in children (MIS-C). When presenting with MIS-C, COVID-19 could be confused with gastrointestinal infections or even inflammatory bowel disease. Therefore, any child with a history of recent SARS-CoV-2 exposure or infection and prominent gastrointestinal symptoms should be considered to have MIS-C, especially in the presence of other clinical comorbidities and extremely high inflammatory markers[21].

Diagnostic tools

The initial diagnostic tools to monitor infected children are the same as those used for adults, *i.e.*, tracing the history of contact with any infected individuals. Real-time reverse transcription-polymerase chain reaction (RT-PCR) testing of nose and throat swabs for the detection of SARS-CoV-2 nucleic acid is the gold-standard confirmatory test for COVID-19. The virus can be detected from the stool until 12 d after disease onset. Stool can still give positive results despite negative respiratory tests. Patients who present with gastrointestinal symptoms may have a longer duration between symptom onset and viral clearance and may have increased faecal virus positivity compared with those who present with respiratory symptoms[35]. Children have a higher nucleic acid positivity rate of faeces and prolonged faecal viral shedding compared to adults[34]. SARS-CoV-2 RNA can be found in stool specimens and anal/rectal swabs, often with more positivity than oral samples, in the late phase of

the disease. Next-generation sequencing is utilized to identify SARS-CoV-2 strains and mutations for epidemiological and research purposes. Rapid antigen detection tests depend on the detection of the viral antigen in the nose and throat swab in cases with high viral load and in pre-symptomatic and early symptomatic cases up to five days from symptom onset. These tests offer multiple benefits in comparison to RT-PCR tests, including shorter turnaround times, easier techniques, and reduced costs, especially in situations with limited RT-PCR testing capacity. However, there is a high chance of cross-reactivity with other coronavirus families. Additionally, rapid antigen detection tests have lower sensitivity and specificity than RT-PCR. Therefore, positive results should be confirmed with RT-PCR. On the other hand, antibody detection tests are usually negative during the first 7–10 d of infection, with a high risk of missing cases of SARS-CoV-2 infection[36,37].

There are relatively lower rates of lymphopenia and higher inflammatory markers in children than in adults, and thrombocytopenia may occur. Monitoring the lymphocyte count and C-reactive protein level as signs of severe infection while using procalcitonin levels to detect potential bacterial coinfection is encouraged. Patients with severe infection may have high plasma levels of IL-2, IL-7, IL-10, granulocyte colony-stimulating factor, interferon-gamma-inducible protein 10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1-alpha and tumour necrosis factor alpha. Levels of bilirubin and hepatic enzymes are excellent markers for the severity of infections. Elevated lactate dehydrogenase levels, abnormal coagulation, elevated D-dimers, and progressively decreased lymphocytic count may also be observed in severe cases[6,38,39].

Positive faecal occult blood testing indicates upper gastrointestinal bleeding. Endoscopy may show mucosal damage to the oesophageal, gastric, duodenal, and colonic mucosa. Biopsy could show numerous infiltrating plasma cells and lymphocytes, as well as interstitial oedema in the lamina propria of the stomach, duodenum, and rectum. Viral-induced gastrointestinal inflammation causes elevated serum IL-6 levels and elevated levels of faecal calprotectin with significantly higher concentrations in patients with COVID-19 suffering from diarrhoea[26]. The ACE2 protein, used by the virus as a receptor for entry, could be stained mainly in the cytoplasm of gastrointestinal epithelial cells. Immunofluorescence testing may show abundant expression of ACE2 in the glandular cells of the gastric, duodenal, and rectal epithelia[40]. Lung imaging examination has been considered a complimentary confirmation method. Chest X-ray findings in children appear to be nonspecific. However, children with mild disease should not routinely have computed tomography chest imaging in view of high radiation exposure[13].

IMPLICATIONS OF GASTROINTESTINAL INFECTION IN COVID-19

Faecal-oral transmission

Oral gastrointestinal infection with SARS-CoV-2 alters the gastrointestinal tract into a reservoir for viral replication, leading to virus spread to other organs, as well as viral shedding in the stool. Faecal viral shedding could continue for 1 wk after clearance from the respiratory tract. This was observed to be more prevalent in children than in adults, with possible long-term faecal-oral transmission, increasing the potential risk for stools to become a source of the contamination of airdrops and several environmental surfaces and may play an important role in viral spread. Therefore, negative nasopharyngeal or oral samples for the virus may not be sufficient to confirm "non-infectivity", as the virus might still be intermittently shed in body fluids and excreta, including stool. Adding one negative stool sample may increase the negativity yield, especially in patients with gastrointestinal symptoms. However, intermittent shedding should also be considered. The risk of faecal-oral transmission of SARS-CoV-2 is suspected to increase, especially with studies reporting that viral particles in environmental settings may remain viable in aerosols for up to 3 h and for 72 h on solid surfaces. When combined with poor hygiene, including poor handwashing techniques, children have a high risk of transmitting the disease to their contacts. Children should be taught the importance of proper hygiene in an easily understandable language. They should also avoid contact with elderly people if they test positive for COVID-19[41-43].

Impact on the gut microbiome

The gut microbiome is the collection of microorganisms (bacteria, yeast, fungi, archaea, protozoa, and viruses) found in the gastrointestinal tract, mostly in the large bowel,

and it performs many beneficial functions, including immune modulation, with pro-/anti-inflammatory effects. The relation between infection with SARS-CoV-2 and the microbiota is bidirectional. Infection with SARS-CoV-2 is well known to cause the dysbiosis of both the respiratory and gastrointestinal microbiota, which negatively impacts gastrointestinal health and contributes to the development of gastrointestinal disease. The dysbiosis of the gut microbiota creates a suitable environment for SARS-CoV-2 replication and subsequent effects. The inflammatory proteins, cytokines and other mediators that are released because of gut dysbiosis will be augmented with coronavirus infection, which may induce a "cytokine storm" causing more damage than the virus alone, which can ultimately result in multiorgan injury[44]. Zuo *et al*[45] examined the effect of infection with SARS-CoV-2 on the gut microbiome. They found that the virus induces dysbiosis, an effect that begins even before the start of antiviral therapy. They observed the enrichment of opportunistic pathogens and the depletion of beneficial commensals, an effect that could persist even after the clearance of SARS-CoV-2 from the body, suggesting a more long-lasting detrimental effect on the gut microbiome. They observed a decline in beneficial microbes such as *Lactobacillus*, *Bifidobacterium* and *Faecalibacterium prausnitzii*, which are inversely correlated with the severity of the disease. They also observed the enrichment of *Clostridium hathewayi, Clostridium ramosum,* and *Coprobacillus*, which are positively correlated with disease severity[45]. Zuo *et al*[46] also found heterogeneous configurations of the faecal mycobiome, with the enrichment of fungal pathogens from the genera *Candida* and *Aspergillus,* during the hospitalization of patients with COVID-19 compared with controls. This effect persisted even after nasopharyngeal clearance of SARS-CoV-2[46]. Other factors could also play a role in inducing dysbiosis in patients with COVID-19, including the presence of other comorbidities that negatively affect the gut microbiota, such as diabetes mellitus, hypertension, and old age; the use of antibiotics, antivirals, antifungals, and steroids; and other intensive care units milia that could negatively affect and alter the gut microbiome[47].

Impact on patient nutrition

COVID-19 affects the nutrition of patients in many aspects. The poor appetite found in a significant number of patients with COVID-19 makes it difficult to achieve the desired nutrition goals with an oral diet alone, causing undernutrition and sarcopenia. Diarrhoea and vomiting, which are common presentations in children with COVID-19, play an important role in undernutrition development. Anosmia may impair taste perception and interfere with nutritional supplementation or food intake. At the same time, the disruption of normal intestinal mucosal integrity by SARS-CoV-2 could compromise the digestion and absorption of nutrients and may even prevent trophic enteral nutrition[48]. Mechanical ventilation that may be needed in the management of children with severe respiratory disease causes excessive swallowing of air and further gastric distention, which predisposes them to gastroesophageal reflux. Pneumonia and respiratory distress may also cause delayed gastric emptying and intestinal hypomotility, leading to constipation, which is an important factor able to interfere with proper nutritional therapy[49].

Impact on patient management

Gut dysbiosis is an important cause of/and results from gastrointestinal inflammation in patients with COVID-19. Gut microbiota diversity and the presence of beneficial microorganisms in the gut may play an important role in determining the course of the disease. The restoration of gut microbiota diversity could help ameliorate the severity of the disease. Since the gut microbiota is malleable and can be modulated by dietary modification, the addition of specialized pre-/probiotics such as fructooligosaccharides, galactooligosaccharides and various *Lactobacillus* strains to the diet could help to improve gut dysbiosis, especially for patients presenting with diarrhoea, thereby improving the overall immune response in these patients[50]. Fermented food with probiotics can produce bioactive peptides able to inhibit ACE enzymes by blocking the active sites. Furthermore, fragments of dead probiotic cells can also serve as ACE inhibitors. Consequently, probiotics could have the potential to block the ACE receptor, which acts as an entryway for SARS-CoV-2 to attack gastro-intestinal cells[51]. At the same time, prebiotics, probiotics, or symbiotics could be supplied as a prophylactic measure to high-risk groups, such as front-line caregivers and healthy contacts with a suspected case of COVID-19[50]. However, microbiota modulation as a method of treatment of patients with COVID-19 is based on indirect evidence and needs further study.

Dietary management plays an important role in patients with COVID-19. Oral intake is the preferred method of nutrition if the patient condition allows. Oral intake is of paramount importance, as a lack of nutrient contact with intestinal mucosa could lead to the atrophy of lymphoid tissue and functional decline of the immune system, as well as the intensification of bacterial translocation. The patients are advised to take a high-calorie and high-protein diet (1-2.5 g/kg/d) to maintain adequate metabolic functions and proper body weight. If the nutrition targets are not met by the oral diet, oral nutrition supplements are recommended and should be started within 1-2 d of hospitalization. Supplementation with digestive enzymes can be given for patients with poor general conditions and impaired digestion. Breast milk is a true immune nutrient, and breastfeeding should be encouraged[52]. If oral intake fails or is not tolerated, enteral nutrition is still the preferred method of nutrition therapy, as it stimulates the gut. Gastric feeding is preferred to optimize digestive activity, and the post-pyloric route (preferably jejunal) is recommended in the presence of a high risk of reflux aspiration, intolerance, or the failure of nasogastric tube feeding. Prokinetic agents may be used to enhance motility prior to this intervention. As enteral feeding (especially when using post-pyloric tubes) carries a high risk of aerosol generation, healthcare professionals should use proper personal protective equipment during insertion of the tubes. Enteral nutrition ensures adequate anthropometric progression and decreases the length of hospital stay, costs, mortality rate, and septic complications compared with parenteral nutrition. Overfeeding should be avoided in patients with COVID-19 and severe respiratory disease, as this can increase the risk of hypercapnia. The prone position during enteral feeding is permitted and may lead to a better prognosis. Parenteral nutrition is indicated when nutrition targets cannot be met by enteral nutrition or gastrointestinal intolerance is present despite different measures to address intolerance[53-55].

Occasionally, children with severe gastrointestinal symptoms may need endoscopy. This procedure could stimulate excessive saliva and other digestive secretions that increase the risk of choking, vomiting, or diarrhoea with a greater risk of transmission of infection during digestive endoscopy, biopsy, and treatment *via* an airborne route, including the aspiration of oral and faecal material *via* endoscopes. Therefore, these procedures should be performed in a specialized endoscopic operation room, if possible, with negative pressure with standard level-three protection and strict disinfection procedures[56,57].

Impact on misdiagnosis and prognosis

Children with COVID-19 have been reported to present as asymptomatic or with only mild symptoms of the respiratory or gastrointestinal system. Infants are more likely to present with atypical clinical symptoms than older children. A study reported five infants diagnosed with COVID-19 who presented with isolated fever and reduced oral intake without associated respiratory symptoms[58,59]. The characteristics of gastrointestinal symptoms in COVID-19 are more insidious than the characteristics of respiratory symptoms, making them easy to overlook. However, some patients might have only gastrointestinal symptoms during the whole course of the disease, and some continue to shed the virus in faeces, despite respiratory samples testing negative[60]. An awareness of these insidious or atypical presentations will allow appropriate investigation and help to decrease the risk of misdiagnosis and consequently decrease the risk of the spread of the disease in the community.

Compared to patients without digestive symptoms, those presenting with digestive symptoms have a longer time from onset to admission and a worse prognosis[61]. A study by Chen *et al*[62] showed that the presence of gastrointestinal symptoms was associated with a high risk of adult respiratory distress syndrome, non-invasive mechanical ventilation, and tracheal intubation, but not mortality, in patients with COVID-19[62]. Another study by Zhou *et al*[63] showed that patients with gastrointestinal symptoms have a similar rate of complications, treatment, and clinical prognosis as patients without gastrointestinal symptom in medical and nonmedical staff[63]. However, these studies were performed in the adult population and cannot be extrapolated in children.

CONCLUSION

COVID-19 has a wide variety of clinical presentations, and gastrointestinal effects are not rare. Gastrointestinal manifestations could be the initial presenting manifestation in many patients, especially in children, and are more common in patients with severe

disease. Gastrointestinal inflammation associated with COVID-19 greatly affects diagnosis and may increase the rate of misdiagnosis, with a significant impact on prognosis, faecal-oral transmission, the gut microbiome, and patient nutrition and management.

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MINIREVIEWS

Hepatitis C virus micro-elimination: Where do we stand?

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Abstract

Hepatitis C virus (HCV) elimination by 2030, using direct-acting antiviral treatments, has been promoted by the World Health Organization. This achievement is not attainable, however, particularly after the 2020 pandemic of the coronavirus disease 2019. Consequently, the more realistic objective of eliminating HCV from population segments for which targeted strategies of prevention and treatment are easily attained has been promoted in Europe, as a valid alternative. The underlying idea is that micro-elimination will ultimately lead to macro-elimination. The micro-elimination strategy may target different specific populations and at-risk groups. Different settings, including prisons and hospitals, have also been identified as micro-elimination scenarios. In addition, dedicated micro-elimination strategies have been designed that are tailored at the geographical level according to HCV epidemiology and individual country's income. The main elements of a valid and successful micro-elimination project are reliable epidemiological data and active involvement of all the stakeholders. Community involvement represents another essential component for a successful program.

Key Words: Hepatitis C virus antibodies; Hepatitis C virus elimination; Hepatitis C virus epidemiology; Hepatitis C virus RNA; Hepatitis C virus diagnosis; Hepatitis C virus infection

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Core Tip: Achievement of hepatitis C virus (HCV) elimination using direct-acting antiviral drugs treatment by 2030 promoted by World Health Organization is hardly attainable especially amidst the coronavirus disease 2019 pandemic. The smaller goal of eliminating HCV from population segments for which tailored strategies of prevention and treatment can be easily implemented appears more realistic. Different

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specific populations and at-risk groups, as well as different settings including prisons and hospitals have been selected for micro-elimination campaigns. Dedicated micro-elimination strategies have also been designed at geographical level according to the countries income and HCV epidemiology. The success of micro-elimination depends on reliable epidemiological data and active involvement of all the different stakeholders.

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INTRODUCTION

Chronic hepatitis C is a major public health problem, affecting approximately 71 million people worldwide[1]. In 2016, the World Health Organization (WHO) published a document highlighting their global strategy aiming to achieve viral hepatitis elimination by 2030[2,3]. The objectives of this strategy for hepatitis C virus (HCV) infection-as a public health problem-are 90% reduction of new infections, 65% reduction in mortality, 90% increase in HCV diagnosis, and 80% increase in treatment rates.

The availability of low cost and highly efficient pangenotypic regimens for HCV treatment has led to the treatment of 5 million infected persons across the world. However, all the steps along the HCV care cascade need to be enhanced, starting with the proportion of infected people screened, followed by the number of patients testing positive for HCV antibodies and diagnosed with HCV RNA, and finishing with the consequent proportion of patients linked to care and treatment[4]. While safety improvements and harm reductions can be achieved and will ensure a reduction in incidence, HCV diagnoses remain largely suboptimal, with less than 20% of the estimated 71 million chronic HCV-infected individuals identified globally. Razavi *et al*[5]. reported that, among high-income countries, only 11 are on track to eliminate HCV by 2030 and 5 more countries are on track for elimination by 2040; all the remaining are expected to eliminate HCV by 2050 or later. This is mostly due to an insufficient number of patients diagnosed, linked to care and treated, across the majority of countries.

A low level of community awareness as well as stigma and fears related to special populations, such as people who use intravenous drugs (PWID), prevent an expanded screening. Referral of patients already diagnosed represents another major challenge. Even more critical are geographical and transportation barriers. Long wait times before the start of treatment and reimbursement policies in some countries account for additional obstacles. Although pre-treatment evaluations, including viral load confirmation and liver disease staging, can be simplified using the pangenotypic and panfibrotic regimens currently available[4], treatment restrictions related to the stage of fibrosis remain a limiting factor in several countries.

More recently it has become evident that a key aspect in improving the results of treatment strategies is patient engagement. In addition, the coronavirus disease 2019 (COVID-19) pandemic is having a deep impact on management of chronic liver diseases in general. Screening campaigns appear more difficult to implement, and testing and access to treatment have been reduced[6]. Moreover, modeling the global impact of COVID-19 on global HCV elimination efforts has shown that a 1-year delay scenario could result in an excess of incidence of HCV cases and liver-related events among such cases, as well as an excess of cases with the infection's consequent hepatocellular carcinoma development[7].

Thus, it is clear that a one-size-fits-all strategy would be unsuccessful to achieve global elimination and that different populations with chronic HCV infection require dedicated programs[8,9]. Achievement of elimination in a well-defined group or context currently appears more feasible and checkable than a macro-elimination plan[10]. The inclusion of key interventions and the adoption of test-and-treat strategies and simplified treatment regimens were then considered more tangible and achievable milestones. In particular, since 2017, the European Association for Study of the Liver

adopted a micro-elimination strategy as a stepwise, savvy approach in the fight against HCV. The goal of macro-elimination was accordingly adjusted in order to pursue micro-elimination in defined subgroups of patients, such as patients with human immunodeficiency virus (HIV)/HCV coinfection or hemophilia, and prisoners or PWID[10].

Micro-elimination requires involvement of all the different stakeholders, including administrative local representatives, health providers, and patients representatives. This strategy is based on different criteria that allow stakeholders to adapt to different situations. The key aspect is to individualize access to the services on the basis of the patients' needs in order to overcome barriers and to achieve higher rates of diagnosis and treatment in a given population of interest during an established period of time. It is important that the progress attained is publicly announced and analyzed using adequate performance indicators[11]. One of the advantages of this strategy is that once results are achieved in a given context, the success translates into leverage for new initiatives. Of course, stakeholders' engagement is key to successful micro-elimination, together with funding, advocacy efforts and ability to scale, and most importantly involvement of the community. The approaches to micro-elimination may differ and they might be based on different locations, different settings, or different populations[11].

AVAILABLE HCV TREATMENT REGIMENS

Current developments in the treatment of chronic hepatitis C are dramatic. Indeed, several direct-acting antiviral drugs (DAA) have been approved from 2013 to 2017 for treatment of HCV-infected patients, with high rates of sustained virological response (Table 1). The arrival of these highly effective treatment regimens has improved prospects for the eradication of HCV worldwide. DAA are well tolerated. The first approved DAA was sofosbuvir a once-daily pangenotypic oral nucleotide analog polymerase inhibitor as a component of combination antiviral regimens. It was approved on December 2013[4]. The protease inhibitor simeprevir was used as a two pills combination with sofosbuvir for all the different HCV genotypes but 2 and 3 on 2014[4]. Sofosbuvir was later in the same year used as a single pill fixed dose combination with the NS5A inhibitor, ledipasvir for genotype 1 and 4 treatment[4]. Simultaneously, on 2014 the three compounds combination of ombitasvir, paritaprevir /ritonavir boosted and dasabuvir not including polymerase inhibitor was approved for genotype 1 treatment[4]. Daclatasvir another NS5A inhibitor was approved for genotype 2 and 3 on 2015[4]. The combination of NS5 and NS3 protease inhibitor elbasvir and gazoprevir with or without ribavirin for GT1 and 4 infection was approved on 2016[4]. However, the true revolution was the approval on January 2016 of sofosbuvir/velpatasvir (SOF/VEL). Velpatasvir is a second generation NS5A inhibitor administered with sofosbuvir as single pill fixed combination[4]. This was the first pangenotypic regimen to be used without ribavirin for 12 wk regardless of severity of liver disease even in patients with compensated cirrhosis[4]. The other pangenotypic regimen based on glecaprevir and pibrentasvir without the inclusion of a polymerase inhibitor to be administered as three pills daily initially for a variable duration of treatment of 8 or 12 wk and later for only 8 wk in patients with or without cirrhosis infected with genotypes 1, 2, 4 and for no cirrhotic genotype 3 and for 12 wk in patients with cirrhosis of genotype 3 was approved on 2017. The use of easy to manage and safe pangenotypic regimens provide not only effective treatment options but also the most powerful opportunity to achieve HCV elimination.

MICRO-ELIMINATION IN DIFFERENT GEOGRAPHICAL LOCATIONS

Egypt has a very high burden of HCV infections and severe HCV-related liver diseases. In 2015, the reported prevalence of HCV chronic infection in the general population was 7%[12]. The national plan involved testing of 35 million Egyptians for HCV antibodies, over a 6-mo period. As a result, over 2.4 million Egyptians had been treated by 2019. This is an example of strong political commitment and industry support, allowing barriers to be removed. However, implementation of systems to track patients who were treated and cured, and to monitor the achievement of HCV elimination have not been properly developed. Pilot projects conducted at community level were recently completed[12]. From 2018, a test-and-treat strategy was started, targeting each individual aged 18-80 years in 73 villages under the auspices of 7

Table 1 Treatment compounds available for hepatitis C virus infection

	No cirrhosis		Cirrhosis (Child-Pugh class A)	
	Naïve	Experienced	Naïve	Experienced
HCV-1a, 1b, 2, 4, 5	SOF/VEL 12 wk	SOF/VEL 12 wk	SOF/VEL 12 wk	SOF/VEL 12 wk
	GLE/PIB 8 wk	GLE/PIB 8 wk	GLE/PIB 8 wk	GLE/PIB 12 wk
	GZR/ELB (GT 1b or 4 only) 12 wk	GZR/ELB (GT 1b or 4 only) 12 wk	GZR/ELB (GT 1b or 4 only) 12 wk	GZR/ELB (GT 1b or 4 only) 12 wk
	SOF/VEL/VOX 8 wk	SOF/VEL/VOX 8 wk	SOF/VEL/VOX 12 wk	SOF/VEL/VOX 12 wk
HCV-3	SOF/VEL 12 wk	SOF/VEL 12 wk	SOF/VEL 12 wk	SOF/VEL 12 wk
	GLE/PIB 8 wk	GLE/PIB 12 wk	GLE/PIB 8-12 wk	GLE/PIB 16 wk
	SOF/VEL/VOX 8 wk	SOF/VEL/VOX 8 wk	SOF/VEL/VOX 8 wk	SOF/VEL/VOX 8 wk

SOF: Sofosbuvir; VEL: Velpatasvir; GLE: Glecaprevir; PIB: Pribentasvir; EBR: Elbasvir; GZR: Grazoprevir; VOX: Voxilaprevir; HCV: Hepatitis C virus.

governates. Free testing was offered, and patients were linked to care and offered free treatment during an educational and prevention campaign. Of the 200000 early tested individuals, 34000 tested positive for HCV antibodies and 14500 were treated. Of those, 99.9% completed the assigned treatment and 97% achieved viral clearance[13].

Another example of a well-conducted HCV micro-elimination campaign comes from Iceland, where the HCV prevalence before the direct-acting antivirals revolution was low. Of 1000 persons infected with HCV in the country, nearly all have been diagnosed within a test-and-treat campaign started in 2016. Treatment was supported by pharmaceutical companies. So far, the island is on track to eliminate HCV by 2021[14].

Georgia was the first European country that started an active national hepatitis elimination program tailored to local needs, based on the 5.4% HCV prevalence in the country. A nationwide case-finding integrated program was designed according to previous experiences in HIV prevention and a control program was initiated for HCV[15]. The HCV testing was integrated in the country's healthcare services for substance users, patients with mental health disorders, and HIV-coinfected subjects. The initial program expanded its scope in 2016, aiming for complete HCV elimination. Of the 150000 estimated HCV-infected subjects, 58% were diagnosed and 80% initiated the HCV treatment; the cure rate was 98.8% for those who completed it. Identification of HCV infection among the remaining population appears to be challenging. According to a recent model based on the Georgia data, in 2019, mortality was reduced by 14% and both prevalence and incidence were reduced by 37%[16].

Among the other European countries, Scotland raised awareness on the impact of HCV through strong campaigns. This led to political support in scaling up the issue, with consequent reduction in HCV incidence and prevalence as well as in mortality. The improvements in harm reduction settings were mainly driven by the use of rapid and accurate diagnosis through dried blood spot testing. Given that there remain undiagnosed subgroups, the next challenge will be involving primary care physicians to increase the diagnosis rate and treatment access[17]. In Spain, with 46.9 million inhabitants and an HCV prevalence of 0.22%, a nationwide plan was initiated in 2016. According to the national data, while HCV testing has been performed in 90% of the general population, testing and linkage to care in at-risk groups remain suboptimal[18].

In other countries, including some from Europe and the United States, the lack of comprehensive data on local situations in terms of prevalence and incidence, or the lack of good quality data in given high-risk groups-other than the lack of affordable tests or harm reductions programs-seem to be affecting achievement of the WHO's goal.

In particular, HCV elimination in low- and middle-income countries has been considered prohibitive until recently. Low-cost screening and drop of drug cost attained only recently are the cornerstone of a published experience involving Cambodia, India, Indonesia, Myanmar, Nigeria, Rwanda and Vietnam by intensive screening and decentralized programs this program has led to cure over 120 people with cure rate higher than 90%. This initiative-supported by Clinton Health Access-proves that the combination of political will, modest financial investment and adequate training pave the way for viral elimination even in the presence of limited

infrastructures[19].

Exploring worldwide progresses in the path to achieve a testing cure protocol for HCV, special attention needed to be reserved to Pakistan. Pakistan has the second highest global burden of HCV infection worldwide with very low level of infection awareness[20]. The Government launched a National Hepatitis Framework for 2017-2021 planning to cure 95% of infected people with DAA (generic sofosbuvir in combination with ribavirin). This initiative resulted in a doubled number of treatments. Despite this success, the country should develop an extensive monitoring and evaluation system and should further implement the national plan[21].

Moving to other countries, low testing rate and poor linkage to care are barriers to treatment in China. Due to its large population a considerable number of infected patients have not yet been discovered. In order to curb the spread of HCV infection and relate morbidity hospitals conduct blood-borne virus screening for inpatients[22]. In addition, several projects are exploring the impact of social media in increasing HCV infection awareness. Among them an interesting randomized controlled study based on crowdsourcing is ongoing in Shenzhen in the setting of primary care. This study aims at enrolling more than 1000 subjects older than 30 years. Subjects randomized into the active arm will receive promotional material by social media campaigns and their HCV testing uptake will be compared to those of the control group just attending the primary care departments and not involved in crowdsourcing activities[23]. A model of targeted HCV screening in past PWID has been adopted in Hong Kong since 2012. Although based on a limited number of subjects, this project represents the first example of targeted screening in Asia[24].

MICRO-ELIMINATION IN DIFFERENT SETTINGS

Prisons' and hospitals' emergency departments are considered appropriate settings for universal screening programs. HCV is common in prisons and guidelines recommend that HCV treatment should be offered to all HCV-infected prisoners. However, a significant proportion of incarcerated patients remain untreated and, once released, contribute to the spread of HCV through the local community. The incidence of HCV infection among prisoners is consequent to drug use and has been estimated globally as 16 *per* 100 person-years (1-34 years)[25]. Prisoners are difficult to engage, due to established high-risk behaviors and psychological disorders[26]. On the other hand, the specific context of prisons allows, after a rapid testing, a quick treatment plan implementation and completion, with the only limiting factor being a short permeance in the same prison[27]. A study conducted in Australia showed that of 562 HCV RNA-positive patients, 416 started treatment with different direct-acting antiviral agents. A sustained virological response (SVR) at 12 wk of 72% was attained, with patients lost to treatment after release accounting for this particular SVR rate. For patients able to complete treatment, per protocol analysis revealed higher SVR at 12 wk (SVR12) rates, up to 96% and comparable to those for the general population[28]. This study was based on a nurse-centered strategy.

Other models based on multidisciplinary networks and telemedicine have been explored in the United Kingdom[29]. Initiatives based on telemedicine were promoted and associated with a high number of treatments and treatment responders in Spain, in a large experiment conducted in "El Dueso" prison between May 2016 and July 2017. Overall, 847 inmates agreed to participate. Among them, 110 were HCV antibodies-positive and 86 HCV RNA-positive. Only 69 patients were treated and 66 of those competed the treatment, yielding a 96.9% SVR rate by intention-to-treat analysis[30]. In the United Kingdom, 128 of 266 individuals who started treatment based on telemedicine had available follow-up data. Of them, 87% achieved SVR; however, the proportion of patients who experienced reinfection was high[31].

The feasibility of a test-and-treat strategy in incarcerated subjects has been highlighted recently in a study by Wong *et al*[32] This study was based on a pangenotypic regimen, with minimal monitoring required. Of the 526 incarcerated adults treated with SOF/VEL in different countries across the world, SVR12 was attained by 437, representing 98.9% of those who completed treatment. A non-virological failure was reported in 82 individuals.

Another example of micro-elimination in a dedicated setting is provided by various interventions implemented in hospital emergency or surgical departments. Examples of these strategies are provided by studies conducted in Spain, in Italy and, more recently, in the United States. In a Spanish study conducted at the emergency department of Hospital Val d'Hebron on 5000 subjects, it was shown that the rate of

HCV antibodies positivity was 4%, higher than that in the general population; in contrast, the rate of active infections was low, with only 16% being HCV RNA-positive. Interestingly, the study showed that in 40% of cases, patients were unaware of their infectious condition[33].

An Italian survey evaluated 11000 patients from the Venetian area. Each patient was tested upon admission to the surgery department[34]. Overall, 2% showed HCV antibodies positivity. Again, the rate was slightly higher than in the general population. Unfortunately, in this study, the results from HCV RNA data were not available for all and estimating active infections was not possible.

In the United States, an automated emergency department cohort screening for viral hepatitis was started in 2018 in New Jersey. The strategy was based on testing Baby Boomers until 2019 and was implemented by extending screening to at-risk in-patients from 2019 to 2020, with further expansion of the screening according to the Centers for Disease Control (CDC) screening guidelines form 2020 onwards. With the aid of a patient navigator, subjects testing HCV RNA-positive were linked to treatment. Universal screening results showed a 3.4% rate for HCV antibodies positivity and 0.9% of HCV RNA positivity among missed Baby Boomers subjects; the rates among non-Baby Boomers were 0.8% and 0.2%, respectively. Rates were higher for subjects aged 55-75 years. The combined cohort and universal screening led to identification of 195 infected individuals among the 37000 subjects who were screened to be linked to care[35].

MICRO-ELIMINATION IN DIFFERENT POPULATIONS

As reported by Degenhardt *et al*[36], the estimated prevalence of PWID by country is variable, with the highest rates reported in United States, Russia, Australia, Brazil, and the European Union. In the United States, PWID account for 60% of new HCV infections (60%) (CDC 2018), while in the European Union, 53.2% of PWID are reportedly HCV antibodies-positive. In the United Kingdom, 80% of individuals infected with HCV are PWID. Therefore, PWID are a key population for targeted micro-elimination strategies. In this population, pangenotypic regimens are associated with high rates of SVR, regardless of the recent use of substances[37].

A large number of studies have investigated different strategies to eliminate HCV in this target population. Integrated approaches based on harm reduction in community settings to enable scale-up and use of simplified models based on low-cost diagnostics have been shown successful in improving screening, linkage to care, treatment access, and response. Studies have suggested that scale-up treatment in this population is mandatory[38]. A study conducted in Scotland in Tayside, a region with a population of 400000 and with an HCV prevalence of 0.55%-0.56%, explored enhanced testing and treatment service focusing on the HCV/HIV coinfection subpopulation. Of the total 2700 subjects, 2300 were diagnosed. In comparison to the pegylated-interferon treatment era, higher rates of patients were linked to treatment and cured. Multidisciplinary involvement included pharmacies, drug treatment centers and prisons, and was based on dedicated educational programs. Of the positive patients, 76% were treated[39].

Multidisciplinary involvement has been pursued by other groups, including ours. In late 2019, our center launched a program promoting ad hoc transportation from "SERDS" (*i.e.*, Centers Taking Care of People with Substance Disorders) to prescribing centers, fast-track baseline evaluation at our center, and a close on-treatment monitoring at 15 different SERDS by the SERDS physician. Of 1500 patients screened, 239 (16%) were HCV RNA-positive, and all were linked to care and started treatment. Of them, 30% were active drug users and 70% were past drug users. High SVR12 rates were reported overall, being 98%. No reinfection was experienced during a short follow-up[40].

Other initiatives, within substance users disorders programs, have targeted their efforts to decentralization. Among them, one-within a continuum program adopted in Philadelphia, United States to compare navigator *vs* embedded treatment models-recently showed that navigators are not sufficient to link HCV PWID to HCV cure[41]. By contrast, embedding HCV treatment within a substance use disorders (SUD) treatment program increases the number of patients linked to care.

The feasibility of HCV eradication in intravenous drug use in Italy has been evaluated within the San Patrignano Therapeutic community. Results showed that one-third of the resident patients were unaware of their HCV infection status. The rate of HCV antibodies-positive patients was 65%, and 238 of the 293 patients who tested

HCV RNA-positive started treatment. As in our study, no case of reinfection was experienced after a short follow-up[42]. The SVR rate was 96.6%. In both these studies, patients were treated with a pangenotypic regimen of SOF/VEL for 12 wk.

Decentralization of the confirmatory testing in harm-reduction sites has been recently implemented. Test-and-treat in point-of care at harm-reduction and addiction centers was proposed as a strategy to treat patients monitored at these centers or at addiction centers in Catalonia, Italy[43]. In both cases, an increase in the number of treatments was registered (+ 57% and + 19%, respectively), although SVR remained suboptimal and associated with a reinfection rate of 6% in harm-reduction centers[43]. In Georgia, a decentralizing screening strategy was shown to be associated with an increase in every step of the HCV care cascade[44]. In the United States, decentralization was implemented with an internist addiction medicine specialist evaluating opiate-dependent patients in a hepatology clinic. This co-localization model was associated with an improvement in the number of patients taking treatment, with an adherence rate of 87.4% and SVR of 98.9%[45].

Among the higher risk groups, MSM cannot be forgotten. Data from population based on systems evaluating the progress through the cascade of care in this group are limited. In France, incidence continue to rise. Among 21.519 HIV positive, HCV negative patients followed between 2012 and 2016 in 16 centers, 218 first HCV infections occurred. Simultaneously, among 3392 patients with cured HCV infection, 74 reinfections were registered[46]. This data show why this is a target population for micro-elimination even despite the high uptake of DAA. As shown in a study from Switzerland, DAA use was able to halve HCV incidence in HIV positive MSM[47].

Very recently a study was designed to compare progress in care and treatment in MSM and non-MSM with HCV infection living in British Columbia[48]. Slightly more MSM *vs* non-MSM received HCV RNA confirmatory testing (83% *vs* 82%) and initiated treatment. In 2019 as compared to 2012, treatment uptake among MSM increased significantly (63% *vs* 37%). SVR rates of 91% and 90% were registered among MSM and non-MSM, respectively. These results suggest that DAAs were associated with substantial improvement in progression across the different HCV care cascade steps.

COVID-19 CHALLENGES CAN HELP HCV SCALE-UP

COVID-19 emergency is demonstrating a greater impact in HCV testing and diagnosis. In United States, at Boston Medical Center, testing decreased by 50, while new diagnoses decreased by more than 60%[49]. Consequently, nontraditional methods of preventive healthcare delivery as telemedicine and technology need to be used in order to emphasize the importance of prevention.

In African Countries integration of diagnostics platforms and implementation of digital technologies were rapidly promoted during the pandemic. Testing and treatment services are facilitating the continuation of essential prevention and are ensuring that people are not further marginalized. Although prevention interventions that require mass gathering are suspended, internet and postal systems may ensuring treatment distribution and help is assisting patients with self-testing[50].

CONCLUSION

The simplification of treatment access, and treatments tailored to the different settings are key aspects towards achieving HCV elimination. All the described interventions have demonstrated an ability to increase in one or more of the HCV cascade steps. The success of micro-elimination initiatives relies on correct targeting of a patient population or appropriate geographical context identification and patient setting.

The COVID-19 pandemic is affecting the results of some of these initiatives, requiring an extension of the timelines and further adjustments to reduce interpersonal contacts. However, it may also result in opportunities to spread awareness of the risks related to infectious disease and of the chances of combined screening programs for HCV and COVID-19.

As the WHO goal for HCV elimination seems hard to reach in the proposed timeframe, micro-elimination is a key alternative strategy. Provided that community involvement represents a free pass for a successful program, micro-elimination will ultimately lead to macro-elimination.

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MINIREVIEWS

Coronavirus disease 2019 severity in obesity: Metabolic dysfunction-associated fatty liver disease in the spotlight

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Abstract

The coronavirus disease 2019 (COVID-19) outbreak has drawn the scientific community's attention to pre-existing metabolic conditions that could aggravate the infection, causing extended viral shedding, prolonged hospitalization, and high death rates. Metabolic dysfunction-associated fatty liver disease (MAFLD) emerges as a surrogate for COVID-19 severity due to the constellation of metabolic alterations it entails. This review outlines the impact MAFLD exerts on COVID-19 severity in obese subjects, besides the possible mechanistic links to the poor outcomes. The data collected showed that MAFLD patients had poorer COVID-19 outcomes than non-MAFLD obese subjects. MAFLD is generally accompanied by impaired glycemic control and systemic arterial hypertension, both of which can decompensate during the COVID-19 clinical course. Also, MAFLD subjects had higher plasma inflammatory marker concentrations than non-MAFLD subjects, which might be related to an intensified cytokine storm syndrome frequently associated with the need for mechanical ventilation and death. In conclusion, MAFLD represents a higher risk than obesity for COVID-19 severity, resulting in poor outcomes and even progression to non-alcoholic steatohepatitis. Hepatologists should include MAFLD subjects in the high-risk group, intensify preventive measurements, and prioritize their vaccination.

Key Words: Metabolic dysfunction-associated fatty liver disease; Obesity; COVID-19; Severity; Cytokine storm syndrome

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Core Tip: It is notorious that obesity represents a risk for coronavirus disease 2019 (COVID-19) severity. However, COVID-19 often causes liver alterations or provokes the progression of pre-existing liver diseases. This review outlines the role of metabolic dysfunction-associated fatty liver disease in COVID-19 severity. The evidence available thus far supports the notion that metabolic dysfunction-associated fatty liver disease represents a more intense risk than obesity for hospitalization, extended viral shedding, and death. A pro-inflammatory state with inflammasome activation, implying increased susceptibility to cytokine storm syndrome, underlies these findings and emerges as, in addition to massive vaccination of subjects with liver diseases, potential targets for therapeutic strategies.

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INTRODUCTION

The infection caused by the coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [coronavirus disease 2019 (COVID-19), *Beta-coronavirus* genus, *Coronaviridae* family] was declared as a pandemic in March 2020 by the World Health Organization and caused an unprecedented burden to health systems worldwide because of its harmful evolution to severe acute respiratory syndrome[1,2]. Although most cases are asymptomatic, this fact facilitated the rapid viral spread worldwide, resulting in overloaded hospitals due to the extended hospitalizations and the frequent need for mechanical ventilation that severe COVID-19 requires[3,4]. From December 2019 to February 14, 2021, COVID-19 caused almost 2.4 million deaths, and more than 108 million confirmed cases in 222 countries or territories[1].

The SARS-CoV-2 relies on binding to the angiotensin-converting enzyme 2 (ACE2) receptor to enter target cells and start viral shedding[5]. Much as the infection frequently happens through the nasopharyngeal epithelium and has the lungs as the chief target with respiratory commitment, COVID-19 represents a systemic disease with adverse cardiac, circulatory, and cerebral outcomes after hematogenic propagation and virus neurotropism for the vagus nerve that links lungs to the central nervous system[6-8]. Of note, the presence of ACE2 receptors in the brush border of intestinal enterocytes and cholangiocytes makes possible the occurrence of gastrointestinal symptoms and aggravation of pre-existing metabolic diseases[9,10]. Figure 1 summarizes the most common infection and propagation pathways of SARS-CoV-2 with adverse effects in the human body.

In this regard, overweight emerged as a high-risk factor to hospitalization due to COVID-19, surpassed only by age ≥ 65 in the New York population[11], emphasizing the high risk of severe COVID-19. Body mass gain yields metabolic alterations that start in the adipose tissue, with hypertrophy and hyperplasia as attempts to buffer excessive energy intake by fat storage[12]. In the long run, adipogenesis capacity exhausts, and the existing enlarged adipocytes trigger lipolysis to manage a continuous lipid accumulation through lipogenesis[13,14]. At this stage, excessive non-esterified fatty acids (NEFAs) are diverted to other organs like the liver and drive steatosis[15].

Hepatic steatosis is known as the hepatic manifestation of metabolic syndrome. Hence, metabolic disturbances such as insulin resistance, systemic arterial hypertension, and meta-inflammation, typical from obesity, coexist with this hepatic impairment[15,16]. Therefore, it was recently renamed metabolic dysfunction-associated fatty liver disease (MAFLD) as it represents better the constellation of metabolic impairments involved in its etiology than the former acronym non-alcoholic fatty liver disease[17].

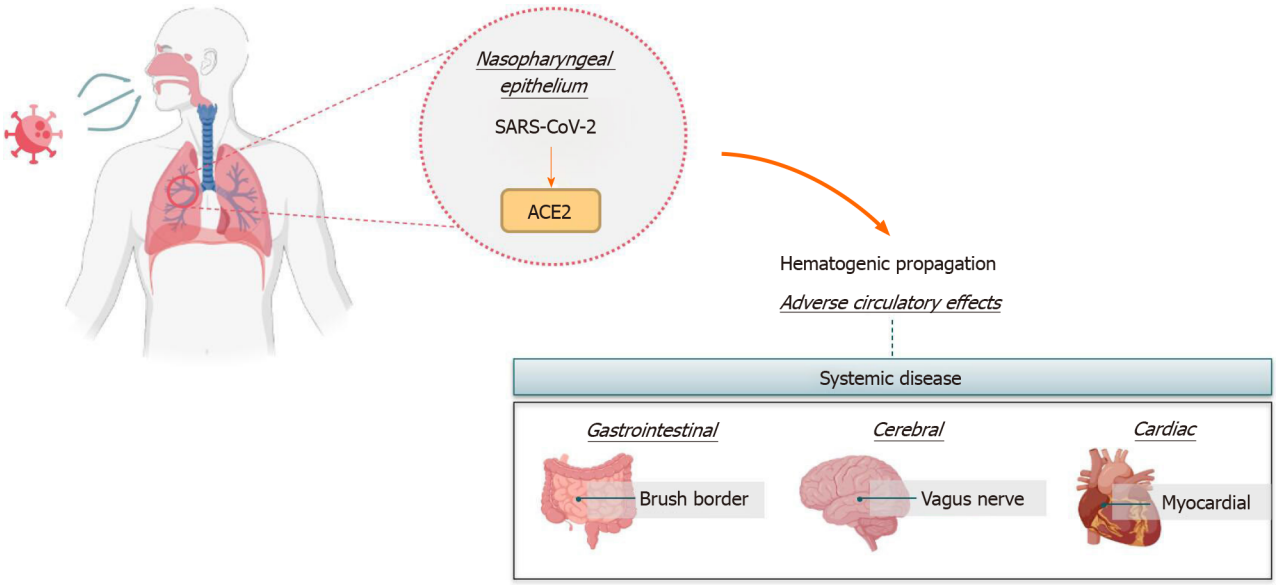


Figure 1 Most common infection and propagation pathways of severe acute respiratory syndrome coronavirus 2 in the human body.
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2.

MAFLD in obesity comprises lipotoxic effects on the liver by proinflammatory adipokines that influence hepatic energy metabolism in an endocrine fashion[18]. The inflamed adipose tissue has been linked to enhanced viral systemic spread, entry, and viral shedding in COVID-19 obese subjects[19]. This hypothesis complies with an intensified cytokine storm in obese patients with COVID-19, explaining the poor outcomes[20].

Among the metabolic impairments caused by excessive body fat, pre-existing MAFLD can result in COVID-19 patients being more prone to hospitalization and worsening metabolic conditions[21]. This review outlined the impact MAFLD exerts on COVID-19 severity in obese subjects in addition to the possible mechanistic links to poor outcomes. The information gathered relating to COVID-19 was that available on PubMed from the beginning of the pandemic until the end of 2020.

OBESITY AND FATTY LIVER: EPIDEMIOLOGY AND PATHOGENESIS

MAFLD is the most common chronic hepatic disease worldwide. Its global prevalence is around 25%, with the highest prevalence in the Middle East (31.79%) and South America (30.45%)[22]. MAFLD is not a disease exclusive of obesity, but obese subjects fall victim to fatty liver more frequently, as their insulin-resistant and pro-inflammatory adipokine profile converge to the accumulation of ectopic fat[18,23]. Recent evidence shows the presence of obesity among 51.34% of MAFLD patients and 81.83% of patients with non-alcoholic steatohepatitis (NASH)[22]. Although MAFLD silently initiates without symptoms, its harmful progression towards NASH (with hepatic fibrosis), cirrhosis, or hepatocellular carcinoma challenges the scientific community to understand its metabolic basis.

The pathogenesis of fatty liver is multifactorial and linked to diet, metabolic factors, intestinal microorganisms, and genetics. Exposure of hepatocytes to high levels of lipids and carbohydrates, usually included in the diet of obese individuals, leads to glucolipotoxicity and predisposes to MAFLD. Excessive circulating NEFAs come from accelerated lipolysis and reduced fatty acid uptake in subcutaneous adipose tissue, triggering ectopic fat accumulation (in the liver, skeletal muscle) and, therefore, insulin resistance in multiple organs[24]. The release of NEFAs from dysfunctional and insulin-resistant adipocytes causes lipotoxicity, induced by the ectopic accumulation of toxic metabolites stemmed from triglycerides. Hence, inflammatory pathways are activated, and cellular dysfunction occurs with liver incapacity to eliminate excessive NEFAs, ending up in lipoapoptosis, an essential feature of NASH[18].

Hepatic steatosis comprises approximately 60% of hepatic triglycerides from adipose tissue, 25% from *de novo* lipogenesis and 15% from dietary lipids[25]. Moreover, visceral adipose tissue is more closely related to the fatty liver than subcutaneous

adipose tissue, possibly because the former is more resistant to insulin than the latter[26]. Increasing evidence shows that during adipose tissue expansion in obesity, there are changes in the expression and secretion of adipokines, favoring pro-inflammatory mediators for the development of MAFLD, progression to NASH, and possible development of liver cirrhosis[27]. Leptin and adiponectin are the adipokines widely described in the literature as influences on fatty liver development.

In animal models of MAFLD, leptin seems to have a double action: It can protect from hepatic steatosis, at least in the early stages of the disease, but it also can act as an inflammatory and fibrogenic factor when the disease persists or progresses, that is when the mechanisms of counterweight do not limit it[28-30]. In this sense, leptin can directly increase the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-12, and IL-18, the chemokines IL-8 and monocyte chemoattractant protein 1, and the lipid mediators prostaglandin E2, leukotrienes, and LTB4, being responsible for the noxious MAFLD progression[26]. Persisting hyperleptinemia promotes liver inflammation and fibrosis as the disease progresses to NASH.

On the contrary, adiponectin decreases as the fat mass increases. At physiological conditions, adiponectin exerts an anti-steatotic effect on hepatocytes by increasing beta-oxidation, while decreasing gluconeogenesis, fatty acid input to the liver, and *de novo* lipogenesis. Furthermore, high molecular weight adiponectin isoform has anti-inflammatory properties like TNF-α blockade and downregulation, resulting in insulin resistance alleviation[29]. In the case of positive energy balance and overweight onset, this critical balance is disrupted towards the side of TNF-α, contributing to chronic low-grade inflammation, insulin resistance, and MAFLD[26]. In humans, patients with fatty liver exhibit low adiponectin concentrations, whereas control subjects show high adiponectin concentrations. However, the adiponectin concentrations are even lower in patients with NASH than in MAFLD. It can be argued that, initially, adipokine actions can limit hepatic steatosis, but as adipose tissue expands, adipokine changes become harmful and contribute to MAFLD progression[18].

An innovative concept proposes that the gut-liver axis plays a crucial role in the pathogenesis and progression of MAFLD[31]. Although there are some controversies, animal and human studies show that gut microbiota from obese subjects comprises an increase in the relative abundance of firmicutes followed by a decrease of bacteroidetes[32]. Moreover, a higher prevalence of gram-negative bacteria, such as proteobacteria, and reduced phylum-level diversity are usual features in non-alcoholic fatty liver disease patients[33]. Changes in the gut microbiota in obesity disrupt the intestinal endothelial barrier, eliciting systemic bacterial translocation and hepatic inflammation by microbiota-related endotoxins and short-chain fatty acids that reach the liver through the portal vein[26].

Concerning a possible genetic predisposition to MAFLD, the adipose tissue of obese patients with fatty liver has upregulation of pro-inflammatory genes and macrophages-producing cytokines compared to obese subjects that do not develop MAFLD[34]. This observation raises the hypothesis of a genetic polymorphism that explains why some obese individuals develop MAFLD, while others do not[35]. Briefly, an allele in the *PNPLA3* gene is a surrogate for the MAFLD onset and hepatic inflammation[36]. Other genes like *FDFT1*, *TM6SF2*, *GCKR*, and *MBAT7* have already been linked to the progression of MAFLD[37]. However, the complete identity of "MAFLD related to genetics" still needs to be further investigated.

CORONAVIRUS DISEASE 2019 PROGNOSIS IN METABOLIC DYSFUNCTION-ASSOCIATED FATTY LIVER DISEASE PATIENTS

Hepatic outcomes of COVID-19

The clinical course of COVID-19 encompasses elevated aspartate aminotransferase and alanine aminotransferase combined with a mild rise in bilirubin levels[38]. A study in China found that 37.3% of patients had an abnormal liver function at hospital admission[39]. In Mexico, 96.8% of patients (n = 150) had a liver impairment but without an association between abnormal liver function tests and mortality[40]. A Chinese study has suggested aspartate aminotransferase as a promising marker to predict hospital stay, with higher values suggestive of prolonged periods. However, this information might not apply to different populations and should be better addressed[41].

Concerning histological liver changes, a post-mortem study in Belgium revealed histological alterations in all 14 deceased COVID-19 patients, with central lobular necrosis and portal inflammation as the most frequent hepatic histological damages[42].

In agreement with this, COVID-19 patients with acute respiratory distress syndrome showed mild microvesicular steatosis and lobular and portal activity in China[43]. The most common post-mortem histological findings in 40 North Americans that died from COVID-19 were fatty liver (75%), mild acute hepatitis (50%), and portal inflammation (50%). However, the fat distribution pattern was not typical of MAFLD and might stem from the administration of steroids during hospitalization[44].

In line with these previous findings, a Chinese study showed 96% of hepatocellular liver injury among COVID-19 hospitalized subjects, with 50% of them presenting liver injury at hospital admission and 75.2% showing liver injury during hospital stay[45]. This observation emphasizes that not only could the pre-existing hepatic disease produce a severe form of COVID-19, but the virus itself can trigger liver damage. Indeed, it is hard to distinguish whether liver injury during hospital stay stemmed from the COVID-19 infection or some drugs used as attempts to stop viral replication[45,46]. Hepatotoxic effects of paracetamol, lopinavir/ritonavir, remdesivir, chloroquine, tocilizumab, and uminefovir can cause microvesicular steatosis and mild hepatic inflammation[47]. These findings are depicted in Figure 2.

METABOLIC DYSFUNCTION-ASSOCIATED FATTY LIVER DISEASE IN CORONAVIRUS DISEASE 2019 PATIENTS: INCREASED SEVERITY WHEN COMPARED TO OBESITY?

MAFLD is considered a pre-existing condition that turns individuals more vulnerable to COVID-19 infection and virus-related complications. Furthermore, these patients may have an increased risk of MAFLD to NASH progression, a long-term consequence of viral infection[48]. As MAFLD is frequently associated with overweight/obesity, it is sometimes hard to separate the single effect each one exerts on COVID-19 severity. Herein, we outlined the most recent evidence after almost 1 year from the beginning of the pandemic.

The presence of MAFLD could predict severe COVID-19 (more than 2-fold prevalence) and critical illness in patients younger than 60 years old in China[49], in addition to longer viral shedding time (17.5 d *vs* 12.1 d on average)[45]. Furthermore, Chinese MAFLD patients without type 2 diabetes showed a 4-fold high risk for severe COVID-19, and a 12% increase in the risk for severe COVID-19 for each 1-unit augmentation in body mass index[50]. Obesity conferred a 6 times greater risk of severe infection in Chinese patients with MAFLD [odds ratio (OR) did not adapt 5.77, P = 0.029]. The association of obesity with COVID-19 severity remained after adjusting for many factors (age, sex, smoking habits, diabetes, hypertension, and dyslipidemia, OR compatible 6.32, P = 0.033)[51].

Conversely, a study considering a large sample in the United States pointed out MAFLD/NASH as a greater risk for hospitalization than obesity itself[52]. In line with this, increased rates of COVID-19 clinical severity among MAFLD patients have been observed even after controlling for obesity, suggesting that the effect of MAFLD on disease severity may be more expressive than increased body mass index[53]. MAFLD/NASH might have a significant role in maximizing chronic inflammation and hypercoagulability, emerging as a pivotal surrogate for severe COVID-19 cases[52].

A study in Brazil showed that hepatic steatosis was found in computed tomography (CT) among 31.9% of COVID-19 positive patients, while only 7.1% of COVID-19 negative subjects showed hepatic steatosis. The OR was 4.698 after adjusting for age and sex, but they did not evaluate the severity or poor clinical outcomes[54]. In this context, a meta-analysis carried out with six Chinese studies with 1293 participants revealed that patients with COVID-19 showed a high percentage of MAFLD and increased risk of COVID-19 severity, indicating a need for intensive monitoring of MAFLD patients with COVID-19[55].

In agreement with this, MAFLD entails a constellation of metabolic abnormalities that can be aggravated during viral infection. Through univariate analysis, it was shown that metabolic syndrome leads to a higher cumulative incidence of COVID-19, with liver damage being the most associated with this outcome[56]. Moreover, COVID-19 can cause hypoglycemia or hyperglycemia, compromising glycemic control due to sedentarism, anxiety, or viral tropism to pancreatic tissue[57]. Continuous monitoring of blood glucose is encouraged as hypoglycemia should be avoided, and glycemia should not exceed 180 mg/dL or 10 mmol/L in COVID-19 patients. MAFLD patients commonly show hypertension, and surveillance of medication and blood pressure levels can avoid shock in severe COVID-19 cases[58].

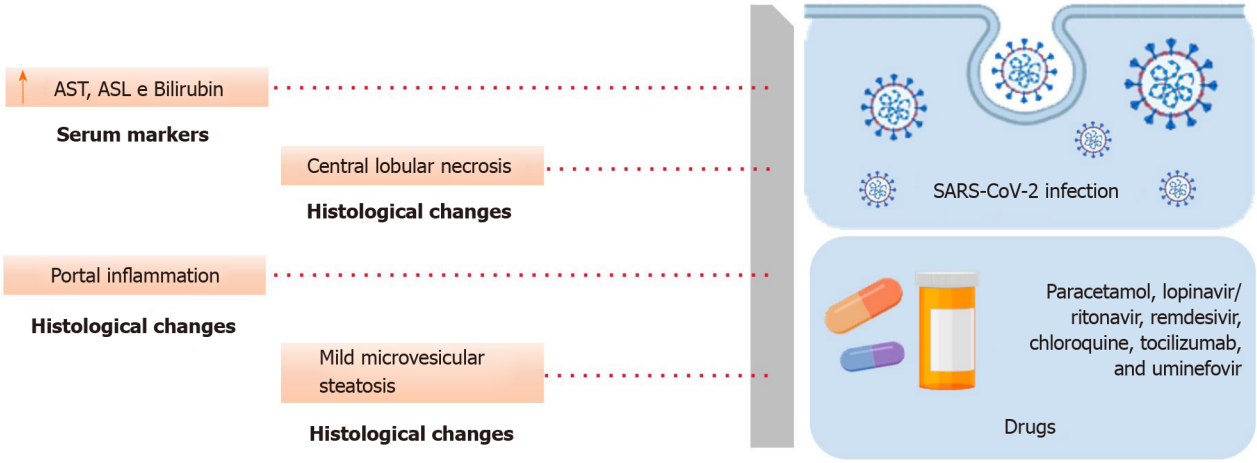


Figure 2 Clinical features and hepatological changes due to severe acute respiratory syndrome coronavirus 2 infection (upper panel) and hepatotoxic effects of some drugs used as an attempt to treat coronavirus disease 2019 (lower panel). SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; AST: Aspartate aminotransferase.

Despite showing similar COVID-19 symptoms to non-MAFLD, fever in 66.8% and cough in 55.7% of patients[39], MAFLD patients showed higher chances of intensive care unit admission (intensive care unit, OR: 1.60), intubation (2.51), need for vasopressors (1.22), and alanine aminotransferase > 5 times upper limit of normal (2.12)[59]. In univariate analyses, when compared with patients without chronic liver disease, subjects with MAFLD had significantly higher rates of intensive care unit admission (50.9% *vs* 35.2%, $P = 0.0095$) and need for mechanical ventilation (49.1% *vs* 30.4%, $P = 0.006$)[53].

A possible explanation for increased COVID-19 severity due to MAFLD includes higher C reactive protein (CRP) (107 *vs* 91.2 mg/L, $P = 0.05$) concentrations in COVID-19 patients with MAFLD than controls without MAFLD in London[60]. This favored pro-inflammatory state can result in high death rates, as MAFLD patients who died during hospitalization had higher concentrations of inflammatory markers like ferritin[61], prothrombin time, and lactate dehydrogenase than patients with MAFLD who survived hospitalization[60]. Notably, the combination of high CRP levels with low lymphocyte count predicted prolonged hospital stay (median 23 *vs* 18 d; $P = 0.037$) and a higher proportion of severe COVID-19 (33.3% *vs* 14.7%, $P = 0.007$) compared to non-obese patients[50]. Figure 3 outlines the mild symptoms and the factors that lead to severe forms of COVID-19.

Concerning imaging exams and COVID-19 prognosis in MAFLD patients, a CT study in the Mexican population proposed the liver to spleen ratio (CTL/S) as a surrogate for the diagnosis of MAFLD in COVID-19 patients, given that spleen does not store fat. Both organs are visualized in the CT performed to evaluate the degree of pulmonary commitment because it shows the upper abdomen, and the CTL/S ≤ 0.9 indicated the presence of fatty liver. Hepatic steatosis was associated with severe COVID-19 pneumonia, and CTL/S could be a substantial aid to estimate the prognosis of COVID-19 in MAFLD patients[62].

A recent systematic review evaluating clinical studies from China, Israel, and the United States showed that the presence of hepatic steatosis is a predictor for symptomatic and severe COVID-19, even after the adjustment for obesity presence (OR: 2.358, $P < 0.001$)[63]. Moreover, COVID-19 infection in pre-existing chronic liver disease patients from 13 different Asian countries caused decompensation in 20% of the cirrhotic patients with high mortality. Among MAFLD patients, the presence of comorbidities like obesity and type 2 diabetes entailed a risk of liver injuries due to COVID-19 infection[64]. In agreement, a recent Indian study using non-COVID-19 cirrhotic patients as controls showed that COVID-19 infection resulted in a 100% mortality rate in patients with acute-on-chronic liver failure in comparison with 53.3% in acute-on-chronic liver failure controls. The poor outcome that COVID-19 imposes on cirrhotic patients includes the need for mechanical ventilation and urges physicians and hepatologists to consider patients with chronic liver diseases as a high-risk group for COVID-19 and guarantee rigorous preventive measures[65].

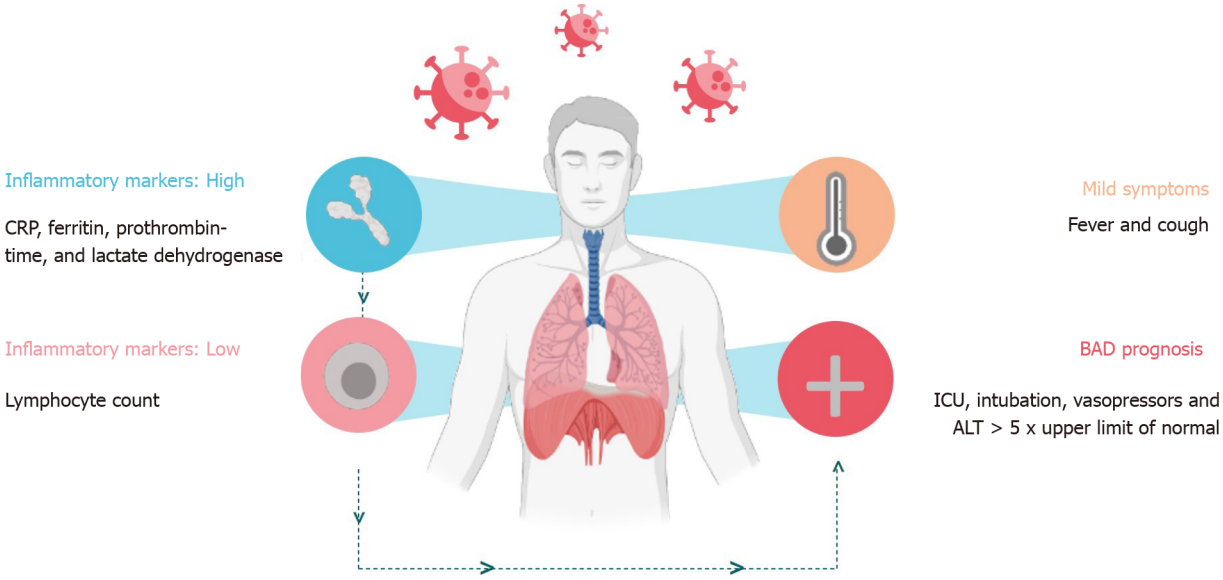


Figure 3 Mild symptoms, inflammatory markers, and serious developments of coronavirus disease 2019. ALT: Alanine aminotransferase; CRP: C reactive protein; ICU: Intensive care unit.

INVOLVED PATHWAYS IN INCREASED SEVERITY OF CORONAVIRUS DISEASE 2019 IN OBESE SUBJECTS WITH METABOLIC DYSFUNCTION-ASSOCIATED FATTY LIVER DISEASE

The post-mortem evaluation of the livers from people that died of COVID-19 often reveals that the virus causes inflammatory and immune-mediated liver damage rather than cytopathic harm[43]. The observation of different immune cell exudates near the portal area reinforces this hypothesis and links hepatic damage to the cytokine storm syndrome (CSS), which emerges as a promising explanation for the increased severity of COVID-19 in obese patients with MAFLD[66,67].

To put it simply, CSS comprises the activation of inflammasomes and the following release of pro-inflammatory cytokines like IL-1β and IL-6[68,69]. The inflammasomes are complexes of proteins that reside in macrophages, activated by tool-like receptors binding to a pathogen-associated molecular pattern or damage-associated molecular patterns[70,71]. The liver has the largest macrophage reservoir in the body (the Kupfer cells); and, therefore, the activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome can damage this organ and acts as a promising marker of COVID-19 severity in obese patients[43,72].

The adipose tissue expansion in obesity triggers fatty liver and inflammasome activity by lipotoxicity[73]. The dramatic changes that adipose tissue macrophages undergo during weight gain (polarization to M1 state) reflect in the Kupfer cells that produce inflammatory chemokines and cytokines, generating hepatic insulin resistance and inflammation[74,75]. In this context, IL-6 emerges as a pivotal marker of COVID-19 worsening as it can trigger the CSS[76], besides being generally high in obese and MAFLD subjects[77].

It can be supposed that the obese patient with MAFLD before catching COVID-19 could show a maximized CSS, which might be linked to the increased need for longer hospitalizations and intensive care. Consistent with this suggestion, inflammasome activation elicits massive augmentation of pro-inflammatory cytokine concentrations and induces pyroptosis[78]. Cell death mediated by pyroptosis in macrophages relies on NLRP3 activation and provokes severe acute respiratory syndrome in COVID-19 patients, picturing a frame of aggravated disease by the CSS in MAFLD patients[79]. Furthermore, severe obese subjects can show serious infections in the lower respiratory tract due to mechanical dysfunction, which also makes them more prone to secondary infections[80].

Additional mechanisms related to the intensification of COVID-19 in MAFLD obese patients include the hepatocellular hypoxia in chronic liver diseases that leads to ACE2 overexpression[81]. Hence, these changes can cope with MAFLD progression towards NASH by the overexpression of ACE2 in endothelial cells and the consequent

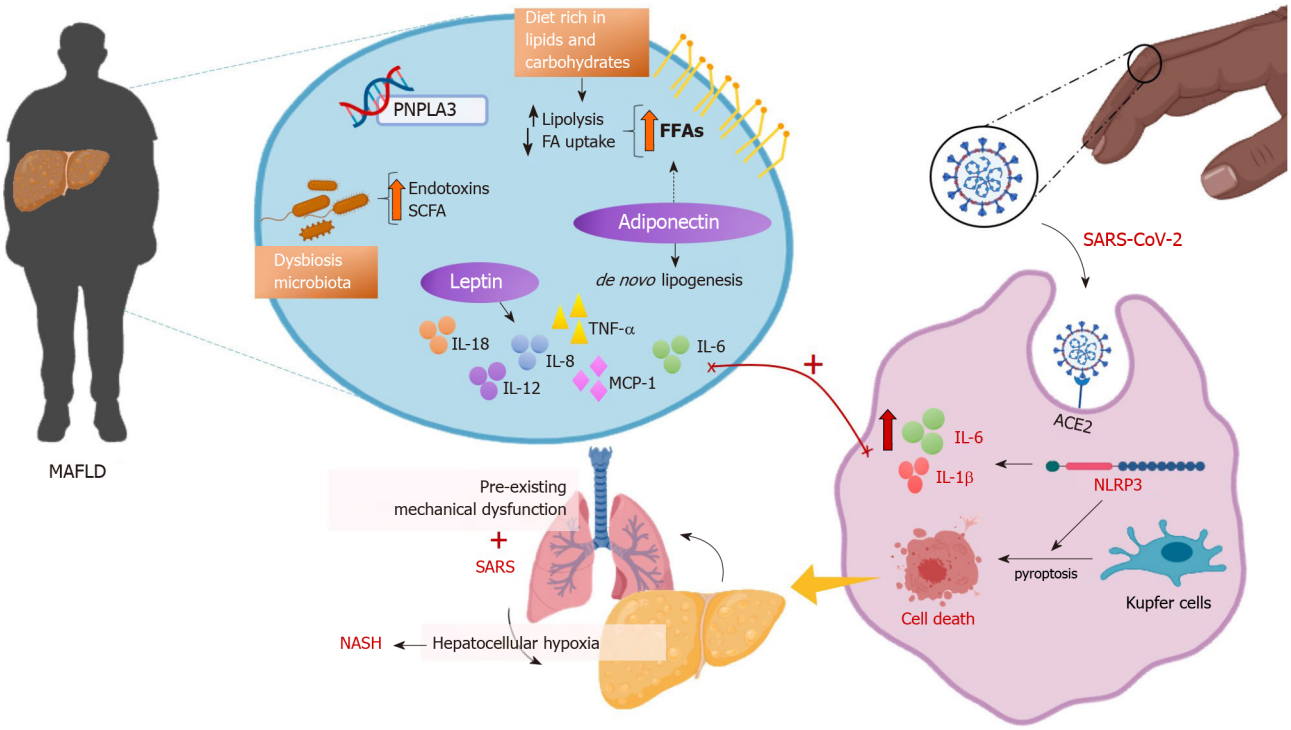


Figure 4 Graphical abstract. Metabolic dysfunction-associated fatty liver disease (MAFLD) patients usually show a constellation of metabolic impairments that include altered adipokine profile, glucolipotoxicity with ectopic lipid accumulation, and gut dysbiosis, besides a genetic background that favors these hepatic alterations and pre-existing mechanical lung dysfunction when obese. These clinical conditions before coronavirus disease 2019 (COVID-19) infection can aggravate the cytokine storm syndrome, with activation of macrophages that reside in the liver (Kupffer cells), resulting in inflammasome activation and cell death by pyroptosis. MAFLD patients are at high risk of complications and severe COVID-19 clinical course, with frequent mechanical ventilation, prolonged hospitalizations, and extended viral shedding. Upon survival, COVID-19 may act as a surrogate for MAFLD-non-alcoholic steatohepatitis progression, and hepatocellular hypoxia seems to underlie this process. ACE2: Angiotensin-converting enzyme 2; FFA: Free Fatty Acid; IL: Interleukin; MAFLD: Metabolic dysfunction-associated fatty liver disease; MCP1: Monocyte chemoattractant protein 1; NASH: Non-alcoholic steatohepatitis; NEFAs: Non-esterified fatty acids; NLRP: NLR family pyrin domain-containing; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; SCFA: Short-chain fatty acid; TNF-α: Tumor necrosis factor.

increased reactive oxygen species production, inflammatory pathways activated in the Kupfer cells, and peroxisome proliferator-activated receptor-alpha down-regulation with pro-lipogenic gene expression and steatosis maximization[82,83]. In this regard, the presence of hepatic steatosis before viral infection can amplify the symptoms of COVID-19 as the lipotoxicity sustains the activation of Kupfer cells and allows the progression to liver fibrosis. The main pathways involved with the severity of COVID-19 in MAFLD patients are depicted in Figure 4.

CONCLUSION

In conclusion, pre-existing MAFLD increases the risk of severe COVID-19 that requires hospitalization. MAFLD itself can represent an increased predisposition to CSS, and high CRP concentrations, NLRP3 activation, and IL-6 concentrations emerge as likely surrogates for the need for mechanical ventilation, with high death rates or evolution to NASH among survivors.

It is urgent to consider that MAFLD surpassed obesity as a risk factor to COVID-19 severity in populations with diverse genetic backgrounds. Therefore, MAFLD subjects should be ranked high-risk to catch COVID-19, submitted to rigorous preventive measurements, and classified as a prioritized group for vaccination.

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MINIREVIEWS

Preoperative physiological esophageal assessment for anti-reflux surgery: A guide for surgeons on high-resolution manometry and pH testing

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Abstract

Gastroesophageal reflux disease (GERD) is one of the most commonly encountered digestive diseases in the world, with the prevalence continuing to increase. Many patients are successfully treated with lifestyle modifications and proton pump inhibitor therapy, but a subset of patients require more aggressive intervention for control of their symptoms. Surgical treatment with fundoplication is a viable option for patients with GERD, as it attempts to improve the integrity of the lower esophageal sphincter (LES). While surgery can be as effective as medical treatment, it can also be associated with side effects such as dysphagia, bloating, and abdominal pain. Therefore, a thorough pre-operative assessment is crucial to select appropriate surgical candidates. Newer technologies are becoming increasingly available to help clinicians identify patients with true LES dysfunction, such as pH-impedance studies and high-resolution manometry (HRM). Pre-operative evaluation should be aimed at confirming the diagnosis of GERD, ruling out any major motility disorders, and selecting appropriate surgical candidates. HRM and pH testing are key tests to consider for patients with GERD like symptoms, and the addition of provocative measures such as straight leg raises and multiple rapid swallows to HRM protocol can assess the presence of underlying hiatal hernias and to test a patient's peristaltic reserve prior to surgery.

Key Words: Gastroesophageal reflux disease; Fundoplication; High resolution manometry; pH-impedance; Anti-reflux surgery; Pre-operative assessment

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Core Tip: The goal of this review is to discuss recent technological advancements that have utility for patients with gastroesophageal reflux disease (GERD) as a pre-operative assessment for anti-reflux surgery. Surgical treatment of GERD is centered around improving the integrity of the lower esophageal sphincter, therefore it is crucial to rule out other esophageal pathologies that may present with GERD-like symptoms. Advances in pH-impedance studies allow for assessment of patients with weak acid reflux of non-erosive reflux disease. High resolution manometry with the addition of provocative measures can uncover underlying esophageal motility disorders with GERD-like symptoms.

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INTRODUCTION

Gastroesophageal reflux disease (GERD), defined as an abnormal reflux of gastric contents with associated symptoms, is one of the most common digestive diseases in the world, and currently affects up to 30% of individuals in North America[1,2]. While the disease is commonly encountered in the outpatient setting, the true number of individuals with GERD could be even higher, as some patients may self-treat with over-the-counter medications[3]. Studies have also found the number of patients with GERD continues to rise, which may be due to the growing obesity epidemic[1]. Additionally, GERD is one of the costliest digestive diseases in the United States, with up to half of the cost attributed to the long term use of proton pump inhibitor (PPI) therapy[4].

The American College of Gastroenterology (ACG) guidelines state that a presumptive diagnosis of GERD can be made in patients with classical symptoms such as heartburn or regurgitation, with further diagnostic evaluation such as an upper endoscopy recommended for patients with alarm symptoms[5]. The ACG recommends management of these patients begin with lifestyle modifications, including weight loss and dietary changes, along with an 8-week course of PPI therapy[5]. While this approach is effective for most patients, a subset of patients may require further management including surgical options such as fundoplication. Surgical treatment of GERD is as effective as medical management in appropriate candidates and is currently recommended for reasons including complications from PPIs, medication non-compliance, and large hiatal hernias[3,5]. Additionally, the Society of American Gastrointestinal and Endoscopic Surgeons (SAGES) recommends surgical therapy for patients with GERD who: have failed medical management, request surgery due to quality of life issues related to long term medication use, have complications such as Barrett's esophagus, or have extra-esophageal symptoms related to their GERD[6].

While surgical treatment of GERD may be as effective as medical therapy, complications are also possible. The most commonly reported side effects of surgery include dysphagia, belching, and increased abdominal bloating, though it is also possible to develop more severe complications requiring repeat surgery[7]. A total 360° Nissen Fundoplication is the most common surgical treatment for GERD, but variations also exist involving differing degrees of wrapping. Studies have found partial fundoplication to be similarly effective for reducing reflux symptoms, and may also have less post-operative dysphagia and bloating[8]. This is supported by a level 1 recommendation from the SAGES that partial fundoplication is associated with less dysphagia and similar patient satisfaction[6]. Additionally, newer techniques such as the Linx procedure [which uses a circlet of magnets to augment the lower esophageal sphincter (LES)] have shown similar short-term outcomes for the treatment of GERD when compared to fundoplica-tion. More research is needed on the long-term outcomes[9].

While surgical treatment of GERD can successfully relieve symptoms in up to 90% of patients[10], a thorough pre-operative assessment is necessary to identify appropriate surgical candidates. The underlying mechanism of anti-reflux surgery is to improve the integrity of the LES. Therefore, in order to achieve the best post-operative outcomes, it is crucial to confirm a patient's symptoms are due to GERD and not another underlying pathology. Otherwise, a patient would likely continue experiencing symptoms post-operatively along with potentially developing complications such as dysphagia.

Newer technologies are becoming increasingly available for the assessment of patients with reflux symptoms and can help confirm the diagnosis of GERD. Improvements in pH studies with the inclusion of impedance measurements may help identify patients with non-erosive reflux disease (NERD). Additionally, advancements in high resolution manometry (HRM) help further classify esophageal pathologies and identify patients as having disorders with GERD like symptoms (Table 1). In this review, we aim to provide a guide for the pre-operative assessment and evaluation of patients with reflux. We will focus on newer technologies that can be used to identify appropriate candidates for anti-reflux surgery by confirming the diagnosis of GERD and ruling out other esophageal diseases.

The ACG guidelines state that a presumptive diagnosis of GERD can be made in patients with classical symptoms such as heartburn or regurgitation, with further diagnostic evaluation such as an upper endoscopy recommended for patients with alarm symptoms[5]. The ACG recommends management of these patients begin with lifestyle modifications, including weight loss and dietary changes, along with an 8-wk course of PPI therapy[5]. While this approach is effective for most patients, a subset of patients may require further management including surgical options such as fundoplication. Surgical treatment of GERD is as effective as medical management in appropriate candidates and is currently recommended for reasons including complications from PPIs, medication non-compliance, and large hiatal hernias[3,5]. Additionally, the SAGES recommends surgical therapy for patients with GERD who: have failed medical management, request surgery due to quality of life issues related to long term medication use, have complications such as Barrett's esophagus, or have extra-esophageal symptoms related to their GERD[6].

While surgical treatment of GERD may be as effective as medical therapy, complications are also possible. The most commonly reported side effects of surgery include dysphagia, belching, and increased abdominal bloating, though it is also possible to develop more severe complications requiring repeat surgery[7]. A total 360° Nissen fundoplication is the most common surgical treatment for GERD, but variations also exist involving differing degrees of wrapping. Studies have found partial fundoplication to be similarly effective for reducing reflux symptoms, and may also have less post-operative dysphagia and bloating[8]. This is supported by a level 1 recommendation from the SAGES that partial fundoplication is associated with less dysphagia and similar patient satisfaction[6]. Additionally, newer techniques such as the Linx procedure (which uses a circlet of magnets to augment the LES) have shown similar short-term outcomes for the treatment of GERD when compared to fundoplication. More research is needed on the long-term outcomes[9].

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Newer technologies are becoming increasingly available for the assessment of patients with reflux symptoms and can help confirm the diagnosis of GERD. Improvements in pH studies with the inclusion of impedance measurements may help identify patients with NERD. Additionally, advancements in HRM help further classify esophageal pathologies and identify patients as having disorders with GERD like symptoms (Table 1). In this review, we aim to provide a guide for the pre-operative assessment and evaluation of patients with reflux. We will focus on newer technologies that can be used to identify appropriate candidates for anti-reflux surgery by confirming the diagnosis of GERD and ruling out other esophageal diseases.

Table 1 Esophageal disorders with gastroesophageal reflux disease-like symptoms

Diagnosis	Definition	Clinical symptoms	Pathophysiology	Diagnostic evaluation
Structural disorders				
GERD	Symptoms and complications secondary to the reflux of gastic contents above the lower esophageal sphincter[5]	Regurgitation, reflux, dysphagia, retrosternal non-cardiac chest pain, globus sensation, extra esophageal symptoms	Abnormal transient LES relaxation, LES dysfunction secondary to anatomic abnormality such as hiatal hernia	Upper endoscopy, high resolution manometry, ambulatory pH testing, ambulatory impedance testing
Weak acid reflux	Symptoms secondary to reflux of gastric contents above the LES with pH ranging from 4-7[32]	Reflux, regurgitation, non-cardiac chest pain	Persistent reflux with pH from 4-7 due to transient LES relaxation	pH studies - on maximum PPI therapy
Eosinophilic esophagitis	Presence of symptoms of esophageal dysfunction such as reflux or dysphagia, eosinophilic inflammation on esophageal biopsy with ≥ 15 eosinophils per high power field, and exclusion of other disorders with similar presentations[81]	Dysphagia, reflux, non-cardiac chest pain	Eosinophil mediated inflammatory response in the esophagus secondary to allergenic antigens	Upper endoscopy with biopsy
Motility disorders				
Achalasia	Elevated IRP > 15 mmHg and absence of normal peristalsis[44]	Dysphagia, regurgitation, non-cardiac chest pain	Failure of LES relaxation and absence of normal peristalsis	High resolution manometry, upper endoscopy, barium studies
Absent peristalsis	Systemic symptoms with aperistalsis with failed peristalsis on 100% of swallows[49]	Reflux, dysphagia, non-cardiac chest pain	Lower esophageal collagen deposition leading to LES dysfunction	High resolution manometry, autoimmune antibody workup
Distal esophageal spasm	Normal IRP and ≥ 20% premature contractions with DCI > 450 mmHg[44]	Dysphagia, regurgitation, reflux, non-cardiac chest pain	Impaired inhibition and coordination of esophageal muscle contraction	High resolution manometry, Barium swallow "corkscrew esophagus"
Hypercontractile esophagus	Minimum of 2 swallows with DCI > 8000 mmHg[44]	Retrosternal non-cardiac chest pain, dysphagia, regurgitation	Increased contraction of esophageal smooth muscle	Upper endoscopy, barium studies, high resolution manometry
Esophagogastric junction outflow obstruction	Elevated median IRP > 15 mmHg with evidence of peristalsis on swallows[44]	Dysphagia, reflux, regurgitation	Impairment of esophagogastric junction relaxation with normal or weakened esophageal peristalsis	High resolution manometry, needs to be confirmed with further studies such as barium swallow or endoflip, must rule out artifact that can be seen with a hiatal hernia
Opioid induced esophageal dysfunction	Presence of symptoms of esophageal dysfunction with manometric evidence of esophageal dysmotility in the presence of chronic opioid use[55]	Regurgitation, dysphagia, reflux	Opioid induced blocking of esophageal inhibitory signals leading to increased spastic contraction and decreased LES relaxation	Clinical history, high resolution manometry
Gastroparesis	Presence of symptoms such as nausea, vomiting, and early satiety with mechanical obstruction ruled out and evidence of delayed gastric emptying on testing[82]	Nausea, reflux, regurgitation, early satiety, abdominal pain and bloating	Multiple etiologies caused slowed peristalsis and delayed gastric emptying	Gastric emptying study
Functional disorders				
Functional heartburn	Presence of burning retrosternal discomfort, no symptoms relief on optimal therapy, absence of GERD or EOE as cause of symptoms, and absence of major motility disorder[83]	Reflux, regurgitation, globus sensation	Potentially secondary to increased esophageal sensitivity	Upper endoscopy, high resolution manometry, pH-impedance studies
Reflux hypersensitivity	Presence of retrosternal chest pain, normal endoscopy and absence of EOE, absence of major motility disorder, and symptom association with reflux events with normal acid exposure on pH-impedance tests[83]	Reflux	Hypersensitization of esophageal nerve endings leading to pain secondary to physiologic esophageal stimuli	Upper endoscopy, high resolution manometry, pH-impedance studies
Rumination	Must include both persistent regurgitation of recently ingested food with subsequent spitting or re-mastication, and regurgitation that is not preceded by retching[83]	Regurgitation (frequently after meals), reflux	Behavioral contraction of abdominal muscles leading to increased intragastric pressure and reflux	Clinical history, high resolution manometry, pH-impedance studies
Supragastric	Presence of frequent repetitive	Frequent belching, reflux,	Behavioral swallowing of air	Clinical history, high

belching	belching, no established clinical correlate for gastric belching, and evidence of supragastric origin on impedance testing[83]	regurgitation, globus sensation	without LES relaxation	resolution manometry, pH-impedance studies

LES: Lower esophageal sphincter; GERD: Gastroesophageal reflux disease; IRP: Integrated relaxation pressure; DCI: Distal contractile integral; EOE: Eosinophilic esophagitis.

HISTORY TAKING

The most important step in evaluating a patient's candidacy for anti-reflux surgery is to confirm the diagnosis of GERD. This starts with careful history taking to assess the signs and symptoms a patient is suffering from. While the presence of typical GERD symptoms is enough for a presumptive diagnosis[5], these symptoms can also be present in a wide range of diseases and do not confirm the diagnosis of GERD. One recent systematic review found that classic symptoms such as heartburn and regurgitation had a varying degree of sensitivity, ranging from 30%-76%, for the presence of underlying GERD[11]. Additionally, the researchers found the studied symptoms were associated with a wide range of specificities (62%-96%)[11]. It is also important to identify if patients are suffering from extra-esophageal symptoms of GERD, as they are less likely to find symptom relief with surgery. One randomized control study compared patients with asthma on medical *vs* surgical therapy for reflux. While all patients experienced clinical improvement of their symptoms, researchers found no significant difference between the two modalities[12]. Overall, while a patient may display classic symptoms of GERD, further assessment must be completed to determine their appropriateness for surgery.

Another important aspect of the patient's history is to assess their response to PPI therapy, which may be a good predictor of outcomes after anti-reflux surgery[13]. One recent study involving 370 patients undergoing laparoscopic Nissan fundoplication found patients who responded well to PPI therapy had a significantly greater reduction in reflux symptoms after surgery[14]. Previous studies have also indicated that patients with symptoms refractory to PPI therapy may have a higher likelihood of poor surgical outcomes[15]. However, while PPI effectiveness may help predict surgical outcomes, it should not be used to confirm or rule out a diagnosis. One meta-analysis found that a successful response to PPI therapy was only 78% sensitive and 54% specific for an underlying diagnosis of GERD[16].

When assessing a patient for anti-reflux surgery, it is also important to understand the role of obesity in surgical outcomes. It is known that GERD is more common in obese patients, as greater central adipose tissue may lead to increased intraabdominal pressure stressing the LES[17]. While some studies indicated the morbidly obese are at a higher risk for surgical failure, more recent studies found similar outcomes of fundoplication in obese patients[18,19]. An alternative surgical option for obese patients includes Roux-en-Y gastric bypass surgery. The SAGES currently recommends bypass surgery for morbidly obese patients with GERD, as it can help decrease GERD symptoms in addition to the weight loss benefits for the patient[6]. However, evidence is still unclear regarding the optimal surgical treatment for patients with class I obesity, and while anti-reflux surgery is a safe option, these patients should be evaluated on a case-by-case basis. Additionally, it is still important to fully assess the reflux symptoms of patients undergoing weight loss surgery. While obese patients are more likely to have GERD, they may also have an underlying esophageal motility disorders, and the use of HRM can help distinguish between these conditions[20].

ESOPHAGEAL PH TESTING

After thorough history taking to identify patients with GERD like symptoms, a upper endoscopy can help make a diagnosis by directly visualizing esophageal lesions such as esophagitis. However, a subset of patients may still have gastric reflux without evidence of any erosions[5]. In these patients with NERD, further testing is required to accurately diagnose reflux and confirm if a patient might be a good candidate for surgical correction. Esophageal pH testing can be a useful modality to identify acidic reflux above the LES[21,22]. Standard ambulatory pH monitoring involves placement of a trans-nasal probe 5 cm above the LES[23]. The probe then measures acid exposure time (AET) by recording any drops in pH below 4 over a 24-h period (Figure 1). The

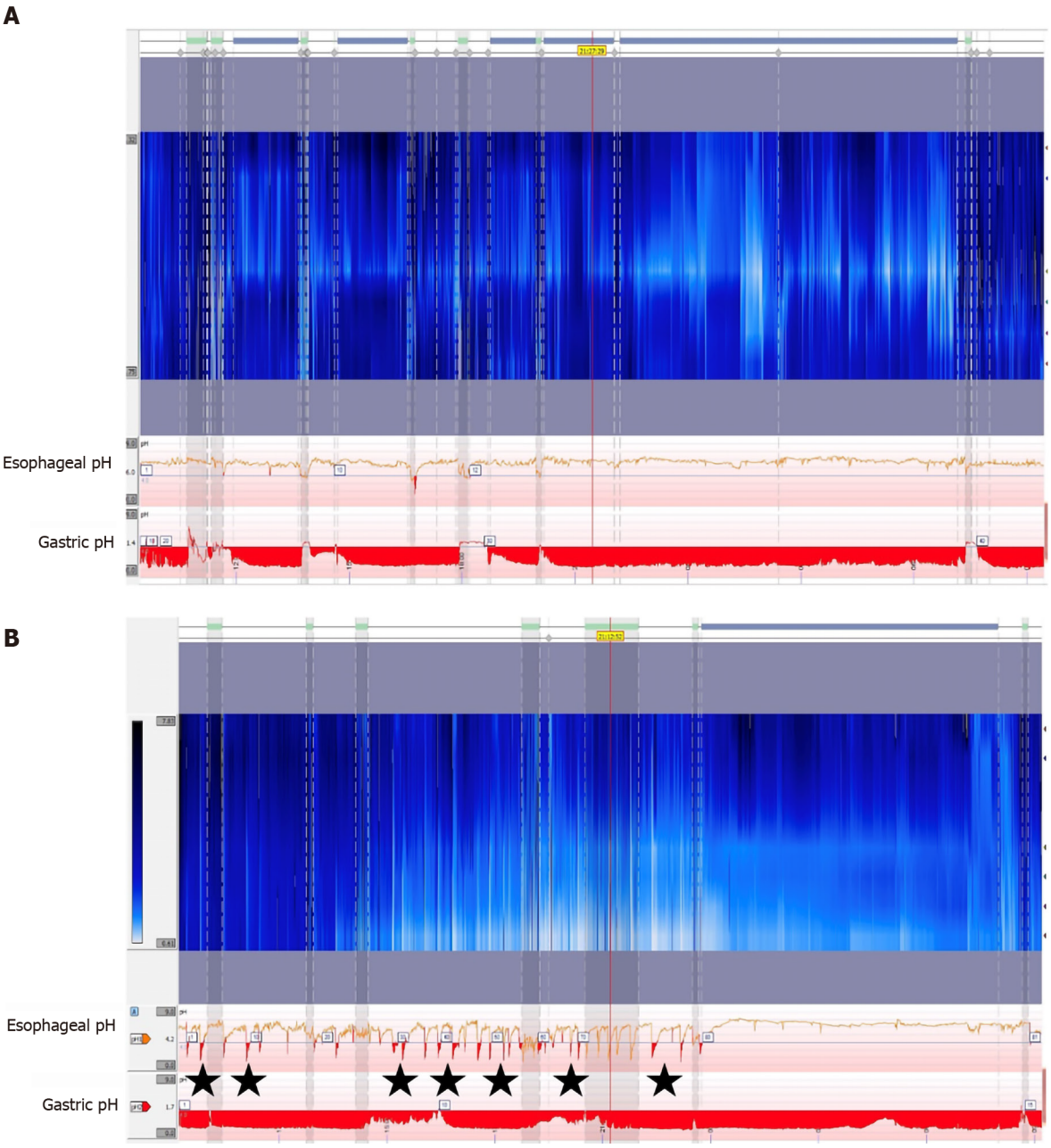


Figure 1 Twenty-four hours pH studies. A: Normal 24 h pH study showing acid in the stomach without acid reflux events in the esophagus; B: Abnormal 24 h pH monitoring test with multiple acid reflux events in the esophagus (star indicating reflux events).

DeMeester score was a system developed in the 1970's to categorize a patient's reflux based on parameters including the number and timing of reflux events as well as supine *vs* upright positioning during these periods[24]. More recently, the Lyon consensus recommends using only AET when determining GERD, and defined pathologic reflux as having greater than 6% AET[22]. Although there is debate regarding which method is more reliable, both have similar strengths and weaknesses, and there is no current evidence that one method is superior to the other[25]. In general, the ACG recommends pH monitoring for all patients with NERD as a pre-operative assessment, and more recent studies have also found utility in measuring AET for patients with LA grade A or B esophagitis prior to surgery[5,26]. Additionally, pH testing has utility for confirming reflux in patients with GERD or NERD who are unresponsive to PPI therapy[21].

One drawback of ambulatory pH monitoring is the discomfort patients may experience from the trans-nasal catheter, with some studies showing patients tend to decrease typical daily activities that might promote reflux after the probe is placed[27]. Wireless capsule pH monitoring is also available, and while it must be placed endoscopically, it is often better tolerated than the catheter probe and may provide

better measurements of physiologic reflux (Figure 2)[28]. In addition to improved patient tolerance, the capsule can record AET for 48-96 h depending on battery life, improving the sensitivity of the test[29]. However, both catheter and capsule pH monitoring are limited by the fact that they only measure acidic reflux. Studies have shown up to 30% of patients with pathologic reflux may have normal pH measurements on ambulatory monitoring[30]. This indicates up to 1/3 of patients with LES dysfunction who may benefit from surgery are missed with this form of testing (Table 2).

pH-impedance testing

Multichannel intraluminal impendence pH monitoring (MII-pH) is a newer technology that measures changes in electrical conductivity between multiple points on the probe[23]. This data is combined with the pH probe to measure reflux of any material above the LES, independent of pH (Figure 3)[30]. The Lyon consensus defines physiologic reflux as 40 or less reflux events, with pathologic defined as greater than 80 episodes of reflux[22]. This definition allows for patients to continue PPI therapy during testing, as pH does not factor into the diagnosis[31]. MII-pH testing also has utility in identifying patients with weak acid reflux (pH 4-7). Up to 30% of patients with GERD may continue to have symptoms refractory to acid suppression therapy[32]. While these symptoms could potentially be secondary to any of the diagnoses seen in Table 1, MII-pH testing can identify patients with weak reflux by independently measuring reflux and pH (Figure 3). Treatment of patients with weak acid reflux is controversial, especially because previous studies have shown failure of PPIs is an indicator for poor outcomes after anti-reflux surgery[15]. However, if clinicians can confirm refractory GERD is secondary to weak acid reflux and not alternative diagnoses such as functional heartburn, they can identify patients who would still be good surgical candidates[33].

Limitations of MII-pH testing include using a trans-nasal catheter and required manometry testing for all patients prior to placement[29]. Additionally, visual analysis of the data is time consuming, but computerized electronic review software can be imprecise. One study found that computer interpretation of results identified only 74% of reflux events confirmed by visual analysis[34]. The authors suggested visual review of the data may be necessary to confirm test accuracy[34]. There also remains a potential for patients to have increased AET and reflux events secondary to other esophageal pathologies not related to LES dysfunction or GERD. This is important to keep in mind during pre-operative assessment for anti-reflux surgery, as patients without underlying LES dysfunction would not be good candidates for surgical treatment. Patients with rumination syndrome can have false positives on pH testing due to frequent regurgitation, not transient LES relaxation[35]. Increased AET can also be secondary to a hypomotility disorders, such as in patients with achalasia or scleroderma. This is not limited to only pH testing, as impedance studies may also have inconclusive or misleading results in patients with achalasia, scleroderma, or rumination syndrome[29]. Due to these possibilities, the ACG recommends that all patients undergo manometry as part of their pre-operative assessment to rule out any motility disorders that may cause reflux[5].

Another important aspect of utilizing pH or pH-impedance testing is the measurement of any associated symptoms during testing (Figure 4). Since there is still the potential for false positives when looking at AET or number of reflux episodes during testing, it is also important for a patient to log any symptoms they experience during testing[36]. This also has utility with extra-esophageal symptoms and can help in identifying if symptoms are causing reflux or are secondary to reflux, such as coughing[36], The Symptoms Association Probability (SAP) is one example of a methods to assess the association of symptoms with reflux events. This involves using a 2 × 2 table assessing the presence of symptoms and reflux events in 2-min blocks over a 24 h period[37]. Analysis then allows the determination of whether the symptoms were more likely secondary to reflux events or caused by chance. While there are other methods to assess symptom association, a SAP can provide a probability of symptom association and is thought to have the best clinical utility[36].

MANOMETRY AND MOTILITY TESTING

Another important aspect of the pre-operative assessment is to rule out major esophageal motility disorders with HRM (Figure 5). Patients with GERD often have findings of decreased LES pressures on HRM, indicating an impaired ability for the

Table 2 Esophageal pH measurement options

	Overview	Benefits	Limitations
Twenty-four hours ambulatory catheter	Trans-nasal catheter placed 5 cm above the LES. Measures time of pH < 4	Can be placed in office	Catheter may cause discomfort; Patients may deviate from daily routine; Patients should refrain from taking PPI therapy during testing; False positives secondary eating/drinking acidic food
Wireless capsule	Small probe that is placed endoscopically in esophagus 5-6 cm above LES. Measures time of pH < 4	Little patient discomfort; Battery life of 48-96 h allows for better measurement of physiologic acid exposure	Must be placed endoscopically; Patients should refrain from taking PPI therapy during testing; False positives secondary eating/drinking acidic food
MII-pH catheter	Trans-nasal catheter placed 5 cm above LES. Contains pH probe along with electrodes to measure reflux episodes	Can be done on or off PPI; Measures pH and reflux independently; Patients can continue taking PPIs; Can identify patients with weak acid reflux	Catheter may cause discomfort; Patients must have prior manometry testing; False positive possible in patients with rumination, achalasia, and scleroderma

LES: Lower esophageal sphincter; PPI: Proton pump inhibitor.

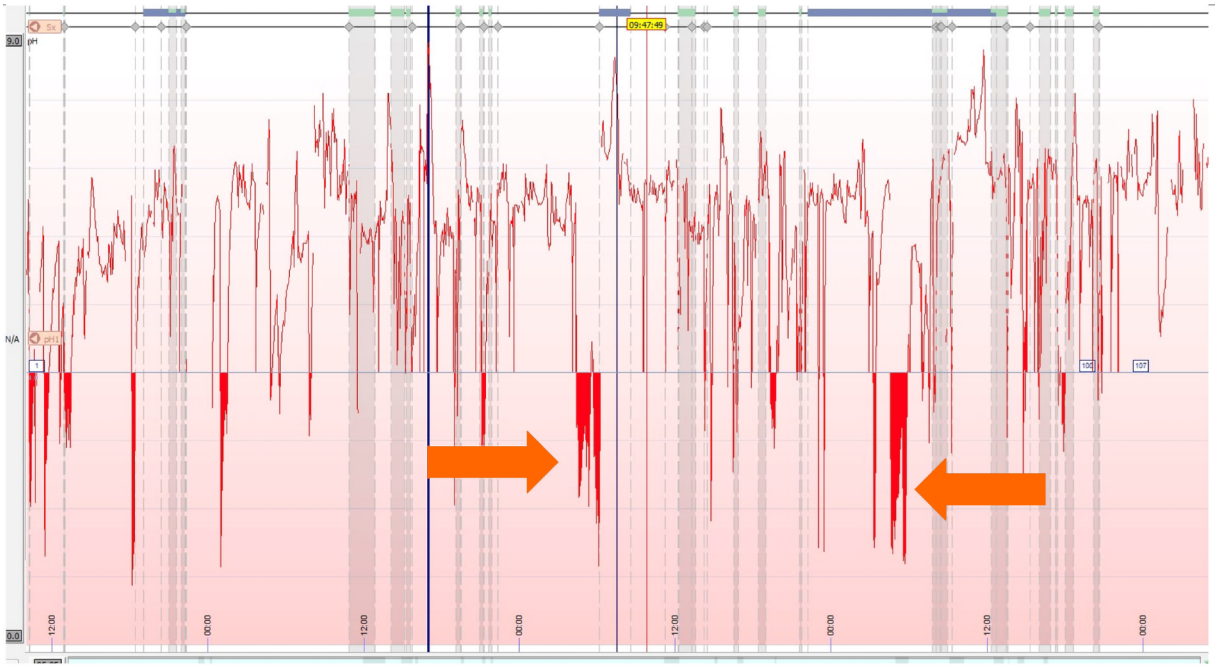


Figure 2 Abnormal wireless capsule pH study (arrows indicating prolonged reflux events).

LES to act as an anti-reflux barrier and prevent gastric contents from entering the esophagus[38]. The smooth muscle in the LES is also augmented by pressure generated from the crural diaphragm, and because HRM utilizes an increased number of sensors, these pressures can be differentiated when evaluating the LES strength[39]. The esophagogastric junction contractile integral can also be quantified on HRM and may identify patients with severe reflux, but further research is needed to create a standardized metric for evaluating patients[22]. Additionally, LES physiology can be altered in the presence of a hiatal hernia, and HRM can be used to assess and measure the size of a patient's hiatal hernia[40]. While esophageal manometry is not a confirmatory test for diagnosing GERD, HRM is useful in identifying any underlying motility issues (Figure 6)[41,42]. While there is limited data to support mandatory pre-operative manometry testing, the ACG still recommends manometry to specifically rule out achalasia and absent peristalsis seen in conditions such as scleroderma[5]. In the 2019 ICARUS guidelines, 94% of experts also strongly agreed with pre-op HRM testing, but this could only be supported with grade D evidence[42]. Additionally, HRM can be used to assess baseline esophageal physiology to predict the risk of post-operative dysphagia.

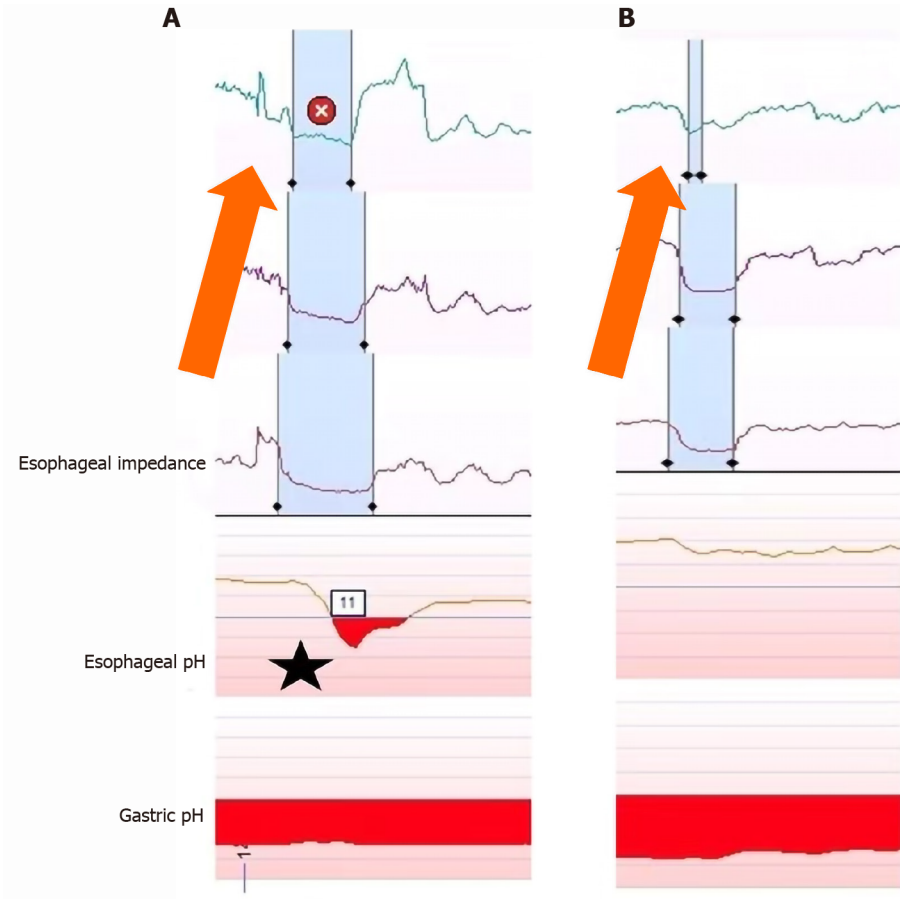


Figure 3 Examples of pH-impedance measurements. A: Reflux event recorded during 48 h pH-impedance study; B: Weak acid reflux event in the esophagus without acid exposure in the stomach detected on pH-impedance study (arrows indicating impedance events, star indicating pH drop and acid event).

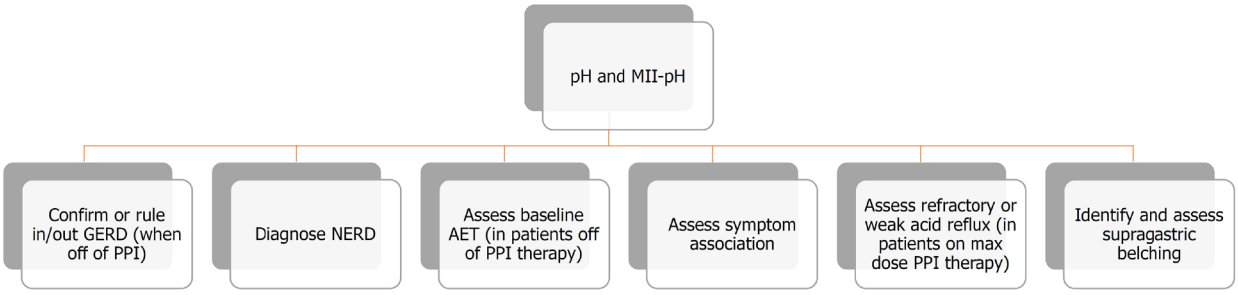


Figure 4 Utility of pH and multichannel intraluminal impendence pH testing for pre-operative assessment. GERD: Gastroesophageal reflux disease; NERD: Non-erosive reflux disease; PPI: Proton pump inhibitor; AET: Acid exposure time; MII-pH: Multichannel intraluminal impendence pH monitoring.

Ruling out major motility disorders

The main indication for HRM should be used to rule out major motility disorders, as these may be the underlying cause of a patient's reflux symptoms, and may also be a contraindication for fundoplication[6].

Achalasia: Abnormal esophageal peristalsis can manifest as GERD-like symptoms in patients with achalasia. The integrated relaxation pressure (IRP) is an important measurement during manometry that assesses esophageal pressures and transit across the esophagogastric junction[43]. According to the Chicago Classification (CC), type I achalasia is diagnosed in patients found to have an IRP > 15 mmHg and 100% failed peristalsis on HRM, with type II having the same characteristics as well as panesophageal pressurization in ≥ 20% of swallows[44]. Type III achalasia is defined as an elevated IRP > 15 mmHg, the absences of any normal peristalsis, and spastic contractions greater > 450 mmHg on ≥ 20% of swallows (Figure 7)[44]. As previously

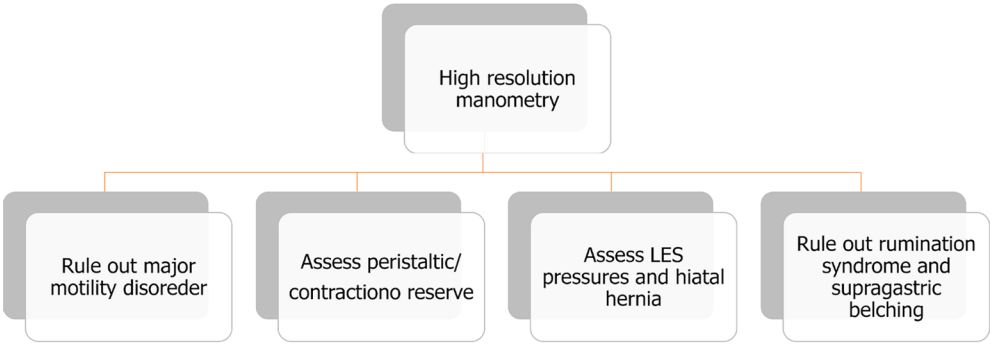


Figure 5 Utility of high-resolution manometry for pre-operative assessment. LES: Lower esophageal sphincter.

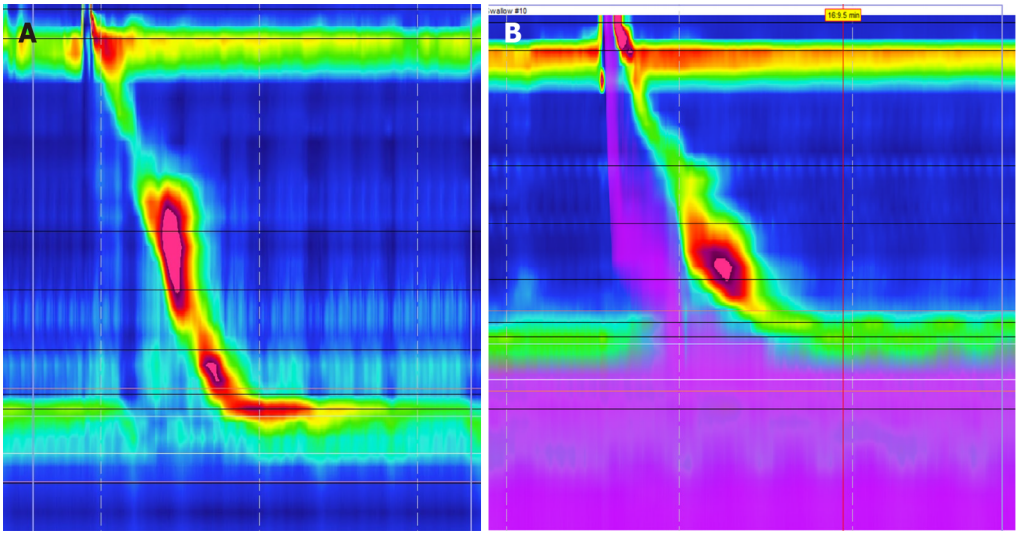


Figure 6 Normal peristalsis and lower esophageal sphincter relaxation on high-resolution manometry. A: Example of normal swallow on high-resolution manometry; B: Normal swallow with complete esophageal clearance by impedance.

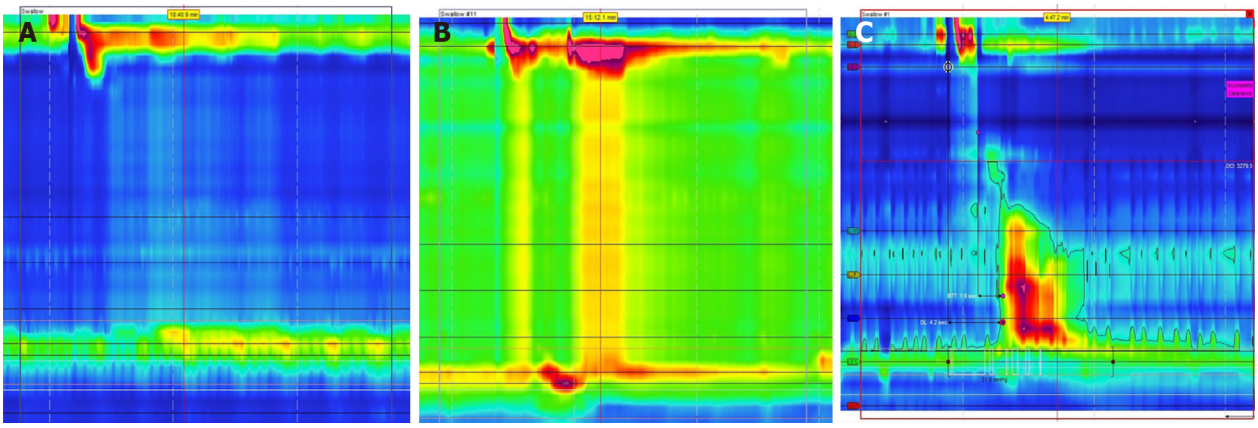


Figure 7 Examples of achalasia diagnosed on high-resolution manometry. A: Type I achalasia with failure of lower esophageal sphincter relaxation and absence of peristalsis; B: Type II achalasia with panesophageal pressurization; C: Type III achalasia with abnormal peristalsis (spastic/premature contractions).

discussed, the goal of fundoplication is to improve the integrity of the LES, but patients with achalasia suffer from impaired LES relaxation along with esophageal aperistalsis. While these patients may also have underlying GERD, surgical treatment revolves around addressing the LES with myotomy[42,45]. However, after myotomy weakens the LES, GERD can become a common side effect. While these patients would benefit from fundoplication, there is debate regarding the risk of post-operative

dysphagia[46]. One prospective randomized study compared outcomes of myotomy alone *vs* myotomy with fundoplication for 43 patients with achalasia. Researchers found that patients receiving myotomy with fundoplication had significantly less GERD and AET, with no significant difference in dysphagia between the two groups[47]. The group also followed patients long-term and concluded there was no significant differences in post-op dysphagia in patients who received myotomy with fundoplication[48]. Current guidelines from both the ACG and the SAGES recommend patients with achalasia undergo both myotomy and fundoplication for the best outcomes[45,46].

Absent peristalsis: Conflicting data also exists regarding fundoplication for patients with absent peristalsis. Evidence of aperistalsis, defined as failed peristalsis with 100% of swallows, on HRM can indicate patients with scleroderma-like esophagus (Figure 8)[49]. Older studies have confirmed anti-reflux surgery can lead to improved symptoms, but more recent studies indicated these patients might have a higher risk for post-operative dysphagia[42,50]. Additionally, studies have shown that alternative surgeries such as a Roux-en-Y bypass may lead to better outcomes. A 2018 study followed patients with systemic sclerosis undergoing surgical treatment for their GERD and compared fundoplication *vs* Roux-en-Y bypass. While the sample size was small, researchers found patients had better GERD symptom relief after Roux-en-Y compared to fundoplication[51].

Esophagogastric junction outflow obstruction: Esophagogastric junction outflow obstruction (EGJOO) is another esophageal motility disorder that can present with GERD like symptoms. In this disorder, patients have normal or weakened esophageal peristalsis on HRM with an elevated IRP[44]. While the current CC provides a guideline on making a diagnosis with HRM, it is important to remember that other factors can lead to an increased IRP. In some patients with hiatal hernias, the manometry catheter may be incorrectly positioned due to the underlying anatomical abnormality, which can contribute to increased IRP measurements[52]. These patients require confirmatory testing, and some centers have adopted the use of upright HRM measurements with an IRP > 12 mmHg as a cutoff[53]. Additionally, other esophageal abnormalities such as Schatzki's ring can lead to altered HRM results[54]. Data is currently limited regarding the outcomes of fundoplication in patients with EGJOO, but an accurate diagnosis is still important as it identifies esophageal dysmotility as the cause of a patient's GERD like symptoms.

Opioid induced esophageal dysfunction is increasingly being recognized as a potential cause of esophageal dysmotility. Just as chronic opioid use can lead to bowel hypomotility, it is theorized that opioids may block esophageal inhibitory signals leading to increased contractions and decreased LES sphincter relaxation[55]. This can in turn lead to findings of EGJOO, esophageal spasm, and hypercontractile esophagus on HRM (Figure 9)[56]. It is important to recognize the effect of chronic opioid use on esophageal motility, as cessation of opioid drugs may lead to resolution of GERD like symptoms.

Hypercontractile disorders: In addition to uncovering evidence of esophageal hypomotility, HRM can also identify hypercontractile patterns such as jackhammer esophagus and esophageal spasm (Figure 10). While the pathophysiology behind these diseases is still under investigation, both involve impaired inhibition and coordination of esophageal peristalsis and can present with dysphagia, non-cardiac chest pain, and reflux[57,58]. Jackhammer esophagus can be diagnosed on HRM if at least 20% of swallows have a distal contractile integral (DCI) of greater than 8000 mmHg[44]. According to the 2019 ICARUS guidelines, 64% of experts agreed that patients with jackhammer esophagus are still good candidates for anti-reflux surgery, but this assertion is only supported by grade D evidence[42]. Data is limited regarding outcomes of patients with jackhammer esophagus after anti-reflux surgery, but one retrospective study found no difference in outcomes when compared to patients with physiologic esophageal motility[59]. Distal esophageal spasm is diagnosed in patients with a normal IRP but ≥ 20% of premature contractions with a DCI of > 450 mmHg[44]. 64% of experts agreed that these patients were not good surgical candidates, but this again was only supported with grade D evidence[42]. Instead, the authors suggested specific therapeutic measures for esophageal spasm such as botulinum injections and myotomy instead of anti-reflux surgery[42].

Utility of provocative maneuvers during HRM

Provocative studies such as multiple rapid swallow (MRS) or rapid drink challenge

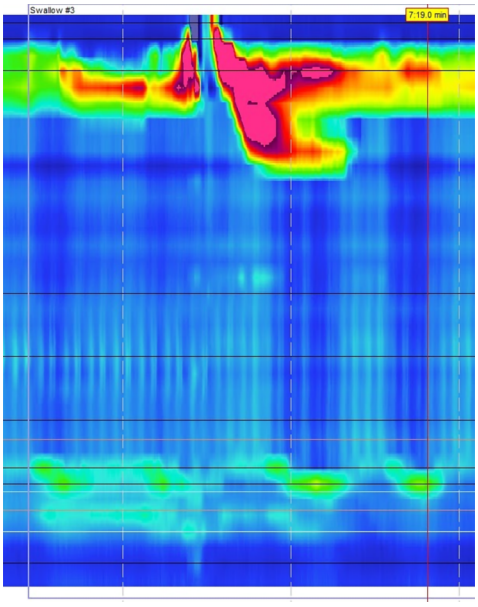


Figure 8 Scleroderma esophagus with absent peristalsis and hypotensive lower esophageal sphincter.

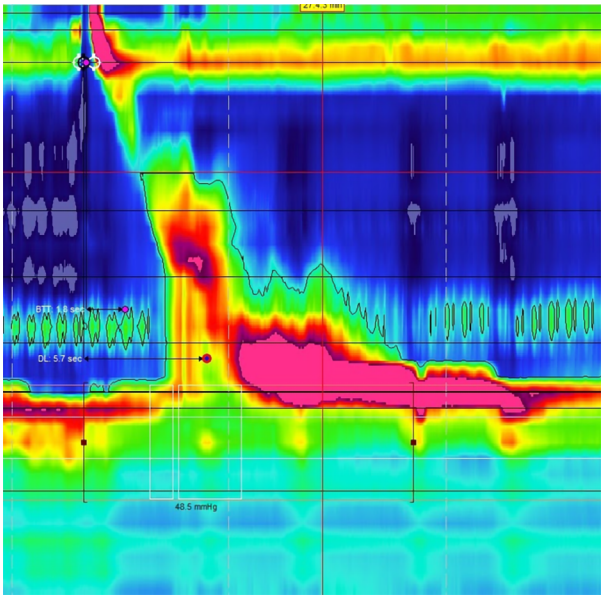


Figure 9 Outflow obstruction with elevated residual pressure and distal pressurization from chronic opioid use.

(RDC) tests can also be added to HRM to further evaluate esophageal physiology. MRS testing involves swallowing small amounts of water over a quick period of time which stresses the coordination of esophageal contraction and LES relaxation[60]. Recent studies have found the addition of MRS during HRM can help uncover pathologies that might have been missed on manometry alone such as distal esophageal spasm and achalasia variants[61]. MRS also allows for visualization of esophageal pressurization patterns which can help identify hypercontractility and EGJOO[62,63]. RDC is also a simple test that can be added to HRM to stress the esophagus and uncover motility disorders. RDC is a similar test to MRS, but involves drinking a larger total amount of water. A recent study in 2017 compared HRM and RDC results between healthy individuals and a cohort of patients with dysphagia and reflux. They found that the addition of RDC improved the sensitivity and specificity of HRM for identifying esophageal motility disorders to 85% and 95% respectively[64].

Another provocative test that can be added to HRM is apple viscous swallows (AVS). AVS involves patients swallowing apple sauce during manometry, and the increased viscosity may help better simulate physiologic swallowing of food. A study from 2011 Looked at patients with dysphagia and compared results of HRM with AVS

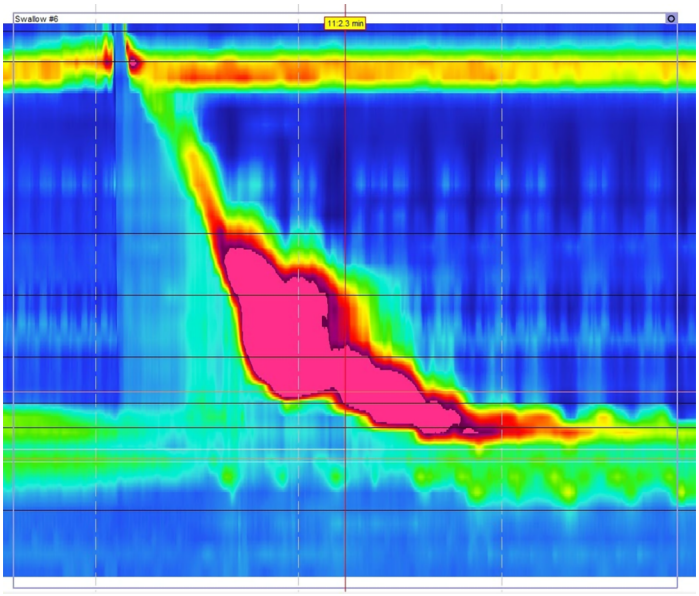


Figure 10 Example of hypercontractile esophagus with distal contractile integral > 8000 mmHg.

and standard water swallows. The researchers found that 4% of patients had abnormal results with water swallows, but this increased to 30% with AVS[65]. Overall, the additions of provocative maneuvers such as MRS, RDC, and AVS to HRM are quick and simple and can help identify esophageal motility disorders in patients with GERD like symptoms.

Provocative testing also has important utility as a pre-operative assessment by predicting the risk of post-operative dysphagia. The DCI is another measurement that can be used to assess the strength of esophageal contractions during HRM[63]. According to the CC, a DCI ratio can be calculated from standard manometry and MRS testing to assess peristaltic or contraction reserve (Figure 11)[44]. This can help indicate the ability of the esophageal body to augment contractions and may be predictive of multiple pathologies in patients with impaired esophageal motility[22,66]. Patients with weak contractions on HRM with MRS may have low peristaltic reserve and a DCI ratio cutoff of > 0.85 was found to be 67% sensitive and 64% specific for identifying late post-operative dysphagia in patients with GERD[67]. Additionally, a lack of manometric response can identify patients with esophageal involvement of their systemic sclerosis, as peristaltic reserve is typically absent in this population[68].

It is known from previous studies that impaired LES pressures are associated with increased esophageal acid exposure[69]. A 2012 retrospective study included over 2000 patients who underwent manometry and pH testing and found that patients with incompetent LES pressures had significantly higher DeMeester scores[70]. A hiatal hernia is one example of why the LES can be compromised in patients and lead to lower closing pressures[71]. The straight leg raise is another simple provocative maneuver that can be completed during HRM to assess patients with a hiatal hernia (Figure 12). Previous studies have indicated the size of a hiatal hernia is the best predictor for the severity of GERD[72]. Straight leg raises during HRM can increase intraabdominal pressure and stress the LES. A recent study measured trans-esophagogastric junction gradient pressures during straight leg raises on HRM and found a significant decrease in peak pressure gradient in patients with a hiatal hernia of 3 cm or greater[73]. Straight leg raises are a simple provocative maneuver to add during HRM and can identify patients that could benefit from surgical treatment for both their GERD and hiatal hernia with good outcomes[74] (Table 3).

Ruling out supragastric belching and rumination syndrome

Patients with GERD may also commonly complain of increased belching[75]. Supragastric belching is another disorder with many GERD-like symptoms, and is common in patients with GERD, NERD, reflux hypersensitivity, and functional dyspepsia[76,77]. Distinguishing between the two disorders is important, as both are due to different underlying pathologies requiring specific treatment approaches. Supragastric belching is seen as a learned behavioral disorder where air is pulled into the esophagus, but there is no transient relaxation of the LES as seen in physiologic

Table 3 Key measurements on high resolution manometry

Measurement	Utility
Integrated relaxation pressure	Measures esophageal pressures during transit and passage through esophagogastric junction. Can be used to diagnose achalasia and other hypomotility disorders
Distal contractile integral	Measures strength of esophageal contractions. Can diagnose hypercontractile disorders such as jackhammer esophagus
Distal latency	Measurement of esophageal transit and contraction time. Can indicate impaired or spastic peristalsis
DCI ratio	Ratio of DCI on normal swallows and MRS testing. Used to assess peristaltic reserve. This can be used to predict risk of post-operative dysphagia

DCI: Distal contractile integral; MRS: Multiple rapid swallow.

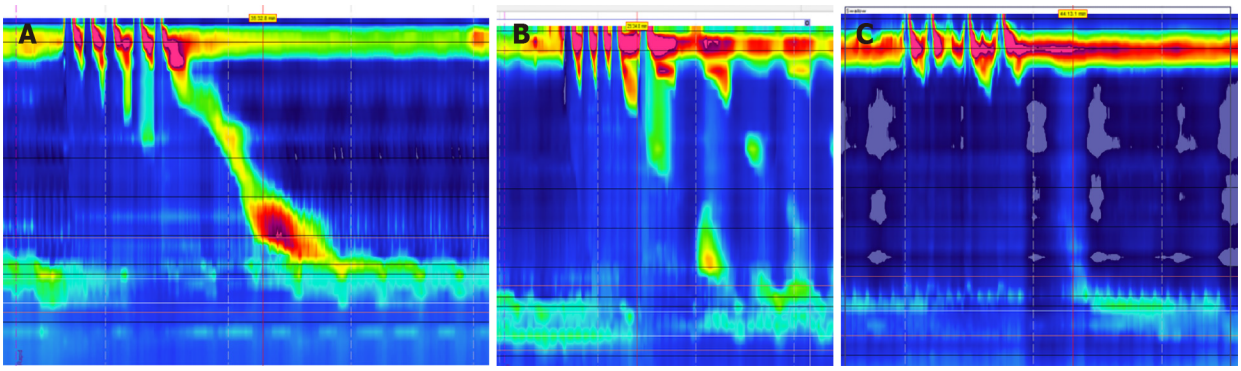


Figure 11 Findings on high-resolution manometry with multiple rapid swallow. A: Normal multiple rapid swallow (MRS) with good contraction distal contractile integral; B: Weak esophageal contractions with MRS; C: Failed esophageal contractions with MRS.

belching[78]. Even though these patients present with symptoms of reflux, the treatment is directed at behavioral therapy to decrease bringing air into the esophagus. These patients would not be good candidates for anti-reflux surgery. Recent improvements in high resolution impedance manometry (HRIM) allow for clinicians to measure both esophageal pressure and flow during swallows[79]. While supragastric belching can induce GERD, HRIM can identify episodes of supragastric belching with no LES relaxation and confirm only esophageal air is released instead of gastric air[80].

Rumination syndrome is another functional disorder involving patients suffering with GERD-like symptoms of frequent reflux and regurgitation. Like supragastric belching, HRM can be useful in identifying the underlying mechanism of the dysfunction. A recent study found that different subtypes of rumination can be identified on HRM, with some having pathophysiologies similar to supragastric belching, and others showing evidence of gastric contents refluxing above the LES[35]. While the first line treatment of rumination syndrome involves cognitive behavioral therapy, HRM can help identify patients with gastric rumination as the cause of their reflux symptoms, and these patients may benefit from further treatments such as anti-reflux surgery[35].

CONCLUSION

Fundoplication is a safe and effective treatment for individuals with GERD, but a thorough pre-operative assessment is critical to achieving good surgical outcomes. Newer technologies continue to become more widely available and can help clinicians in selecting appropriate surgical candidates. By accurately diagnosing GERD, assessing peristaltic reserve, and ruling out major motility disorders and diseases with GERD-like symptoms, clinicians can confidently identify patients with true LES dysfunction who would benefit from surgical intervention.

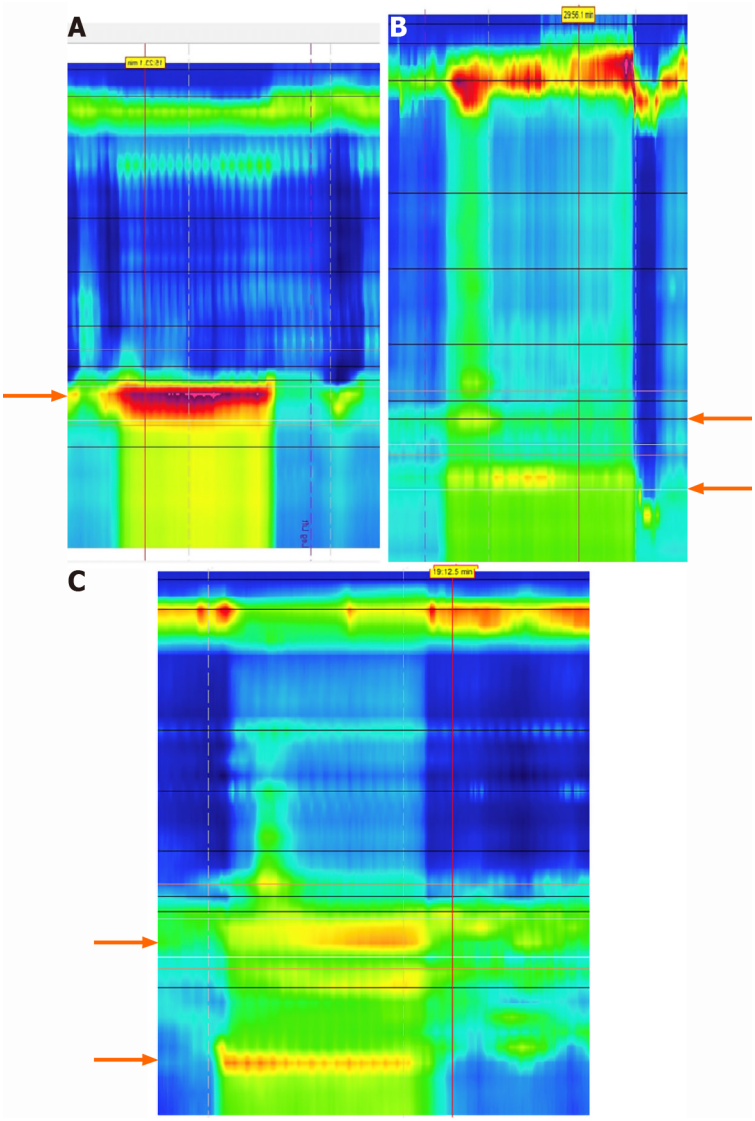


Figure 12 Examples of straight leg raise testing during high-resolution manometry. A: Normal straight leg raise test with single pressurization zone; B: Two pressurization zones after straight leg raise indicating presence of small hiatal hernia; C: Example of two pressurization zones after straight leg raise in patient with large hiatal hernia (Arrows indicate pressurization zones).

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ORIGINAL ARTICLE

Basic Study

Protective effect and mechanisms of action of Mongolian medicine Sulongga-4 on pyloric ligation-induced gastroduodenal ulcer in rats

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Abstract

BACKGROUND

Sulongga-4 (SL-4) is a herbal formula used in traditional Mongolian medical clinics for the treatment of peptic ulcers and gastroenteritis, even though its pharmacological mechanism has not been well characterized.

AIM

To evaluate the protective effect and identify the mechanisms of action of SL-4 on gastroduodenal ulcer induced by pyloric ligation (PL) in rats.

METHODS

PL was performed to induce gastric and duodenal ulcers in rats, which were then treated with oral SL-4 (1.3, 2.6, or 3.9 g/kg per day) for 15 d. PL-induced gastroduodenal ulceration. Therapeutic effects were characterized by pathological and histological evaluations and inflammatory indicators were analyzed by enzyme-linked immunosorbent assay. Microarray analyses were conducted to identify gene expression profiles of gastroduodenal tissue in PL rats with or without SL-4 treatment. The candidate target genes were selected and verified by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

RESULTS

SL-4 decreased histopathological features in the PL-induced ulcerated rats. SL-4

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significantly ($P < 0.05$) decreased expression of tumor necrosis factor-α, interleukin (IL)-1β, IL-6, endotoxin, platelet-activating factor, and increased prostaglandin E2 and epidermal growth factor in ulcer tissue. Microarray analysis was used to identify a panel of candidate target genes for SL-4 acting on PL-induced ulceration. Genes included some complement and coagulation cascade and retinol metabolism pathways that are closely associated with inflammatory responses and gastric mucosal protective mechanisms. qRT-PCR showed that altered expression of the selected genes, such as *CYP2b2*, *UGT2b1*, *A2m*, and *MASP1* was consistent with the microarray results.

CONCLUSION

SL-4 exerts protective effects against PL-induced gastroduodenal ulcers *via* reducing inflammatory cytokines and elevating expression of gastric acid inhibitory factors. Downregulation of *CYP2b2* and *UGT2b1* genes in retinol metabolism and upregulation of *A2m* and *MASP1* genes in the complement and coagulation cascades pathways are possibly involved in SL-4-mediated protection against gastroduodenal ulcer.

Key Words: Sulongga-4; Peptic ulcer; Pyloric ligation; Microarray analysis; Inflammatory reaction; Retinol metabolism

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Core Tip: Sulongga-4 (SL-4) is a classic herbal formula used in Mongolian medical clinics for the treatment of peptic ulcers and gastroenteritis. This study investigated the protective effects and molecular mechanisms of SL-4 in pyloric ligation (PL)-induced gastroduodenal ulcers in rats *via* microarray analysis. Our results suggest that SL-4 was strongly protective against PL-induced gastroduodenal ulcers *via* reducing inflammatory cytokines and elevating the expression of gastric acid inhibitory factors. Downregulation of *CYP2b2* and *UGT2b1*, and upregulation of *A2m* and *MASP1* may be involved in the gastroduodenal ulcer protection mechanism of SL-4.

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INTRODUCTION

Peptic ulcer disease is the most prevalent chronic disease of the digestive system, with complications resulting in high morbidity and mortality[1]. Many factors can lead to the formation of peptic ulcers, and one of the basic mechanisms is impairment of gastric mucosal barrier function by acidic gastric secretion[2]. Inflammation or injury of the digestive system cause visceral hypersensitivity that can drive the development of chronic pain and patient discomfort[3], while inflammation is a key driver of gastric ulcer formation[4]. For protection against inflammation, cells respond with attenuated production of proinflammatory cytokines such as interleukin (IL)-12, tumor necrosis factor (TNF)-α, IL1β, IL-6, IL-8, and interferon (IFN)[5,6]. These cytokines drive the aggregation and trigger activation of T cells in areas of inflammation. Mediators of inflammation protect against peptic ulcer formation. Although peptic ulcer disease can be treated with drugs such as proton pump inhibitors, H2 receptor antagonists and prostaglandin analogs, most of these drugs have adverse reactions and limitations when used for the long term[7]. Safer and more effective antiulcer agents are therefore needed.

The use of plant extracts as natural medications is well documented, with multifunctional effects reported such as immunostimulatory, anti-inflammatory, antiviral, anticancer, and radioprotective effects[8]. Plant or herbal extracts are also recognized as a source of therapeutics for gastric ulcers[9]. Traditional Mongolian

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medicine (TMM) has been used to treat peptic ulcers and is proven to be effective in clinical treatment. The use of TMM is widespread in North China, Mongolia, and the Buryat Republic. The use of TMM is spreading to more Asian countries because of its profound curative effects on some common diseases in the northern region. The Mongolian medicine Sulongga-4 (SL-4) is composed of four medicinal herbs: *Forsythia suspensa*, *Persicaria bistorta*, *Caulis clematidis* armandii, and *Ophiopogon japonicus*. According to ethnopharmacological records, SL-4 has shown good efficacy against Hododenbaoru, a term in TMM referring to peptic ulcer[10]. Modern clinical applications and preliminary studies have shown that SL-4 is generally used for abdominal pain, gastroenteritis, peptic ulcer, and diarrhea. For example, SL-4 has been found to have clinical therapeutic effects against infantile diarrhea and to improve intestinal health[11-13]. Wang *et al*[14,15] reported that SL-4 was protective against acute liver damage induced by pyloric ligation (PL) and CCl_4. To further evaluate the efficacy of SL-4, we investigated whether it had a protective effect on PL-induced gastroduodenal ulcer in a rat model. Additionally, combined microarray analysis and data mining were conducted to uncover the underlying molecular mechanisms of the gastroprotective effects of SL-4.

MATERIALS AND METHODS

Ethics statement

Male Sprague-Dawley rats (200 ± 20 g, 6 wk of age, SPF grade, batch no. SCXK 2015-0001) were purchased from Liaoning Chang-Sheng Biotechnology Co. The experimental procedures were approved by the Committee on the Ethics of Animal Experiments of Inner Mongolia University for Nationalities (approval no. NM-LL-2016-12-15-01), in accordance with the requirements and general guidelines of the Chinese Experimental Animals Administration Legislation. All surgery was conducted under sodium pentobarbital anesthesia, and every attempt was made to alleviate suffering.

Animal treatment

Sixty male rats were housed in environmentally controlled conditions (22 ± 2 °C, relative humidity of 50% ± 5%) with a 12-h light/dark cycle and had free access to food and water for 7 d acclimatization. SL-4 was provided by the Drug Manufacturing section of the Affiliated Hospital of Inner Mongolia University for Nationalities (batch No.: 20190312). Rats were randomly divided into five groups (n = 12) that were treated by a sham operation, PL, and 1.3, 2.6, or 3.9 g/kg SL-4. Rats in the sham operation and PL groups were given 0.5% carboxymethylcellulose sodium (CMC-Na, Sigma). Rats in the three SL-4 groups were given 1.3, 2.6, or 3.9 g/kg SL-4 in 0.5% CMC-Na suspension by gastric lavage once daily. The rats in all five groups were pretreated for 15 consecutive days.

Ulcer model induced by PL

One hour after the last drug administration, all rats were fasted for 24 h with free access to water prior to ulceration. Each rat was anesthetized by intraperitoneal injection of pentobarbital for induction of PL-induced gastric ulcer as previously described[16]. The abdominal cavity was opened and the pylorus was ligated, avoiding injury of the adjacent blood vessels. The stomach was gently replaced and the belly was sutured. After being deprived of food and water for 18 h, the animals were killed and the stomach and duodenum were dissected for subsequent analysis.

Pathological and histological examination

Rats were anesthetized intraperitoneally with sodium pentobarbital and killed. Gastric and duodenal sections were harvested for pathological examination. For histological examination, gastroduodenal ulcer tissue was fixed with 4% paraformaldehyde for 48 h; decalcified in 10% aqueous EDTA solution (Sigma-Aldrich, St. Louis, MO, United States) at room temperature and constant agitation, processed, and embedded in paraffin. The tissues were cut into 4 μm serial sections and dewaxed twice in xylene at 37 °C for 15 min; rehydrated through decreasing concentrations of ethanol, and washed in distilled water at room temperature for 5 min. The tissues were stained with hematoxylin and eosin (H&E, Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Measurement of tissue cytokine expression

Gastroduodenal tissues were homogenized in Tris buffer on ice and centrifuged at 12 000 *g* at 4 °C for 10 min. The supernatants were used to determine the activities of endotoxin (ET), IL-6, and platelet-activating factor (PAF) in the duodenal tissue and TNF-α, prostaglandin E2 (PGE2), epidermal growth factor (EGF) and IL-1β in the gastric tissue. Enzyme activity was determined with commercial assay kits (Nanjing Jiancheng Bioengineering Institute).

Total RNA extraction

Total RNA was extracted for microarray and qRT-PCR assay from gastric and duodenal tissue with TRIzol reagent (Invitrogen, Gaithersburg, MD, United States) and purified with mirVana miRNA Isolation Kits (Ambion, Austin, TX, United States). RNA was quantified with a Qubit fluorometer (Thermo Fisher Scientific, United States), and RNA integrity was assessed with a Bioanalyzer 2100 (Agilent, CA, United States). Only RNA extracts with an RNA integrity of > 6 were used in subsequent analyses.

Microarray analysis

Microarray hybridization, scanning, and analysis were performed by the Capital Biotechnology Corporation (Beijing, China). Using 100 ng total RNA, double-stranded cDNA containing the T7 RNA polymerase promoter sequence was prepared with CbcScript reverse transcriptase using a cDNA synthesis system (Capitalbio, Beijing, China) with the T7 Oligo(dT). cDNA labeled with a fluorescent dye (Cy5 or Cy3-dCTP) was produced by Eberwine's linear RNA amplification method and subsequent enzymatic reactions. The procedure was improved by using CapitalBio cRNA Amplification and Labeling Kits to produce higher yields of labeled cDNA. The microarray slides were read with an Agilent G2565CA Microarray Scanner to obtain the microarray hybridization images. The array data were analyzed for data summation, normalization, and quality control with GeneSpring V13 (Agilent).

Differentially expressed genes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

Chip signal intensities ≥ 400 were included for comparison. A two-class unpaired algorithm in the CapitalBio expression microarray analyzer software (CBC analyzer) was applied to identify differentially expressed genes in the sham operation and PL groups, and the PL and SL-4 groups in both gastric and duodenal tissues. Differentially expressed genes with fold-change ≥ 2.0 or ≤ 2 and P values ≤ 0.05 were evaluated. KEGG pathways were analyzed with a CaptitalBio Molecule Annotation System integrated with the KEGG database as previously described.

qRT-PCR validation

To validate the microarray results, qRT-PCR was used to validate the expression of the selected genes in the SL-4 (3.9 g/kg), PL and sham groups. The RNA samples used for qRT-PCR were the same samples from individual rats that made up the pools. One microgram of total RNA and an oligo(dT) primer were used for cDNA synthesis following the manufacturer's protocol for SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, United States). Primers for the four genes chosen for verification were designed based on sequences published in GeneBank (Table 1). The validated genes were *CYP2b2*, *UGT2b1*, *MASP1*, and *A2m*. qRT-PCR was performed with an ABI PRISM 7900 sequence detection system (PE Applied Biosystems, Foster City, CA, United States) under the following conditions: 50 °C for 10 min, 40 cycles of 95 °C for 1 min, and 60 °C for 1 min. The program was ended with a melting curve from 72 °C to 95 °C and cooling to 40 °C. The *GAPDH* gene was used as a housekeeping control. Relative gene expression (ΔΔct) was calculated and graphed as a fold-change.

Statistical analysis

Pharmacological data were analyzed using SPSS 17.0 and reported as means ± SD. Intergroup comparisons was conducted with *t*-tests, and $P < 0.05$ was taken to indicate a significant difference. Regarding the microarray, a CBC analyzer (expression profile analysis procedures based on R Bioconductor and PERL) was used for the analysis. To select the DEGs, the threshold values were ≥ 2 and ≤ 2 fold-change and a *t*-test P value of 0.05. The data were Log_2 transformed and median centered by genes using the adjust data function of CLUSTER 3.0 software and further analyzed by hierarchical

Table 1 Primers used for quantitative reverse transcription polymerase chain reaction analysis of gene expression in gastric and duodenal tissue

Gene	Accession No.	Forward primer	Reverse primer
MASP1	NM_022257.1	CAACTACATCGGCGGCTACTACTG	GCTGGTGATTGTGCCTGTCCTC
A2m	NM_012488.2	TCATCCAAGTCTGGTTCTTCTC	CCAGAACCATATACTGCGGT
CYP2b2	NM_001198676.1	GGAAGAACGGATTCAGGAGGAAGC	CTGTGATGCACTGGAAGAGGAAGG
UGT2b1	NM_173295.1	ATGTCATTCTCGCAGATGCTGTGG	ATAGGAAGGAGGCAGTGGAAGTCC

clustering with average linkage.

RESULTS

SL-4 decreased histopathological features in ulcerated rats

In the PL group, rats showed severe injury and inflammation of the gastroduodenal epithelium and edema of the submucosa. SL-4 improved these alterations dose-dependently, and showed less mucosal damage and milder inflammation than the PL group (Figure 1). H&E staining confirmed the pathological alterations of gastroduodenal tissues. The SL-4-treated groups showed a recovery trend toward normal histology and only superficial lesions. More precisely, the mucosal structure and the epithelium were intact, with normal glandular size, morphology, and distribution in the SL-4 3.9 g/kg group compared with PL untreated rats, which was essentially the same as the control sham group.

SL-4 modulated cytokine expression in the damaged gastroduodenal tissue

The levels of enzymes present in tissue homogenates were determined by enzyme-linked immunosorbent assays. SL-4 pretreatment significantly ameliorated elevation of the inflammatory factors TNF-α and IL-1β in rat gastric tissue and increased the expression of prostaglandin E_2 (PGE2) and EGF hormones that inhibit gastric acid secretion compared with the PL group (Figure 2). In comparison, SL-4 treatment attenuated the expression of IL-6, ET and PAF in ulcerated duodenal tissue compared with the PL group in (Figure 3).

SL-4 altered gene expression in rats with PL-induced gastroduodenal ulcers

DEGs were identified in the ulcerated gastroduodenal tissues by an mRNA fold-change between two experimental groups of ≥ 2 and a *P* value ≤ 0.05. Volcano plot analysis showed that 1415 genes (731 upregulated and 784 downregulated) and 1460 genes (792 upregulated and 668 downregulated) were differentially expressed in gastric tissue from sham and PL, and PL and SL-4 groups (Figure 4A and B), and 2610 (1344 upregulated and 1266 downregulated) and 209 (100 upregulated and 109 downregulated) DEGs were identified in sham *vs* PL and PL *vs* SL-4 in the duodenal tissue (Figure 4C and D).

KEGG pathways analysis

To define the biological pathways associated with PL and SL-4 treatment in rat gastroduodenal ulceration, we analyzed the microarray data using R software, which was based on the KEGG database. The top ten DEG-enriched pathways in the sham *vs* PL and PL *vs* SL-4 groups in gastric and duodenal tissue are summarized in Tables 2 and 3. In gastric tissue, four pathways were engaged in the crosstalk of sham *vs* PL and PL *vs* SL-4, including *Staphylococcus aureus* infection, retinol metabolism, cytokine-cytokine receptor interaction, and the hematopoietic cell lineage pathway. In duodenal tissue, among the top 10 KEGG pathways in the sham *vs* PL and PL *vs* SL-4, two were the same in both groups, which were the complement and coagulation cascade pathways and *S. aureus* infection pathway.

According to the rat phenotype, retinol metabolism in the gastric tissue and complement and coagulation cascade pathways in the duodenal tissue were both closely associated with inflammatory responses and gastric mucosal protection mechanisms. Twelve genes were found to have > 2-fold expression and *P* values ≤ 0.05 in the two pathways. Gene expression pattern variations had the same trends in the PL and SL-4 groups. More precisely, when gene expression associated with retinol

Table 2 Top 10 Kyoto Encyclopedia of Genes and Genomes pathways enriched with differentially expressed mRNAs in gastric tissue from rats the study groups

Treatment	KEGG pathway	*P* value
Sham *vs* PL	*Staphylococcus aureus* infection	1.83×10^{-5}
	Cytokine-cytokine receptor interaction	2.93×10^{-4}
	Renin-angiotensin system	7.29×10^{-4}
	Inflammatory bowel disease	8.59×10^{-4}
	Rheumatoid arthritis	1.52×10^{-3}
	Retinol metabolism	1.57×10^{-3}
	Amoebiasis	1.59×10^{-3}
	Mineral absorption	1.75×10^{-3}
	Hematopoietic cell lineage	2.35×10^{-3}
	Ascorbate and aldarate metabolism	2.51×10^{-3}
PL *vs* SL-4	Complement and coagulation cascades	2.59×10^{-11}
	Staphylococcus aureus infection	5.95×10^{-8}
	Cytokine-cytokine receptor interaction	2.06×10^{-7}
	Steroid hormone biosynthesis	8.49×10^{-6}
	Hematopoietic cell lineage	3.10×10^{-5}
	Malaria	6.08×10^{-5}
	Chemical carcinogenesis	1.22×10^{-4}
	Metabolism of xenobiotics by cytochrome P450	1.28×10^{-4}
	Pertussis	1.99×10^{-4}
	Retinol metabolism	2.25×10^{-4}

KEGG: Kyoto Encyclopedia of Genes and Genomes; PL: Pyloric ligation; Sham: Sham operation; SL-4: Sulongga-4 decoction.

metabolism was upregulated in the PL group, SL-4 downregulated the same genes relative to the PL group. In contrast, the pathways for certain genes in the complement and coagulation cascade were downregulated in the PL group and upregulated in the SL-4 group (Table 4).

qRT-PCR validation

The SL-4-mediated increase or decrease in *CYP2b2* and *UGT2b1* in the retinol metabolism pathway, and *A2m* and *MASP1* in the complement and coagulation cascades pathway were verified by qRT-PCR. Changes in the expression patterns of the four selected genes were consistent with the microarray results (Figure 5). Expression of *CYP2b2* and *UGT2b1* mRNA were increased in the PL group and decreased by SL-4 treatment. In contrast, expression of *A2m* and *MASP1* were decreased in the PL group and increased in the SL-4 group.

DISCUSSION

In Mongolian folk medicine, SL-4 is traditionally used to treat peptic ulcers and gastroenteritis. Previous studies have shown that SL-4 has therapeutic effects against infantile diarrhea and improves intestinal health[13-15]. However, the anti-ulcerogenic effect of SL-4 and its pharmacological mechanism have not been well investigated. Thus, we aimed to study whether SL-4 had a protective effect on PL-induced gastroduodenal ulcers in a rat model and examined its molecular mechanism by microarray analysis.

PL is a well-established methodology to develop gastric ulceration in rats[16]. PL induces accumulation of gastric acid and pepsin, resulting in breakdown of the gastric

Table 3 Top 10 Kyoto Encyclopedia of Genes and Genomes pathways enriched with differentially expressed mRNAs in duodenal tissue from rats the study groups

Treatment	KEGG pathway	*P* value
Shame *vs* PL	Complement and coagulation cascades	3.93×10^{-9}
	Staphylococcus aureus infection	4.53×10^{-9}
	Cytokine-cytokine receptor interaction	6.48×10^{-7}
	Hematopoietic cell lineage	9.84×10^{-6}
	Rheumatoid arthritis	2.78×10^{-5}
	Osteoclast differentiation	4.40×10^{-5}
	PI3K-Akt signalling pathway	2.13×10^{-4}
	Tuberculosis	2.89×10^{-4}
	ECM-receptor interaction	3.02×10^{-4}
	Amoebiasis	3.37×10^{-4}
PL *vs* SL-4	Maturity onset diabetes of the young	2.10×10^{-7}
	Complement and coagulation cascades	7.39×10^{-6}
	Staphylococcus aureus infection	7.99×10^{-5}
	Regulation of autophagy	2.26×10^{-3}
	Type II diabetes mellitus	8.47×10^{-3}
	Longevity regulating pathway – multiple species	1.51×10^{-2}
	Phototransduction	2.05×10^{-2}
	Mucin type O-Glycan biosynthesis	2.35×10^{-2}
	Porphyrin and chlorophyll metabolism	3.51×10^{-2}
	Longevity regulating pathway	4.01×10^{-2}

KEGG: Kyoto Encyclopedia of Genes and Genomes; Sham: PL: Pyloric ligation; Sham operation; SL-4: Sulongga-4 decoction.

mucosal barrier and causes inflammatory injury, which are key drivers of gastric ulcer formation[17]. To protect against inflammation, the cells respond with attenuated production of proinflammatory cytokines such as IL-12, TNF-α, IL-1, IL-6, and IFN[18]. Our findings show that SL-4 significantly improved the protective effects against gastroduodenal mucosal hemorrhage and inflammation, as confirmed by histopathology. Additionally, inflammatory factors such as IL-6, TNF-α and IL-1β were significantly reduced by SL-4 in contrast to the PL group, which indicates that the antiulcer effects of SL-4 can be attributed to anti-inflammatory activity. PAF, a potent phospholipid mediator of leukocyte activation, is a potent mediator of endogenous ulcer formation[19]. ET is a hormone secreted by the endothelial cells of blood vessels that can also promote ulceration[18]. Our data showed that SL-4 suppressed expression of PAF and ET in the gastroduodenal tissues in PL model mice in a dose-dependent manner, suggesting an inhibitory role for SL-4 in the activation of endothelial cells during ulcer maintenance. Additionally, SL-4 increased expression of PGE2 and EGF in gastroduodenal tissue, which indicates that it may effectively inhibit the secretion of gastric acid, enhance the gastric mucosal defensive function, promote the repair of gastric mucosal injury, and reduce recurrence rate of gastric ulcer[20]. A previous study reported that PGE2 was responsible for the production and maintenance of the cellular integrity of the gastric mucosa, and that depletion of PGE2 could cause gastric ulcers[21]. Histological evaluation showed that SL-4 reduced mean ulcer size and minimized ulcer formation, congestion, inflammation, hemorrhage, and necrosis of the gastric mucosa. Together, these findings demonstrated the antiulcer effect of SL-4 in PL rats.

Forsythia suspensa is a major herbal component of SL-4. It has been reported that its active ingredients forsythiaside and forsythin exert potent anti-inflammatory and antibacterial activities, which may have possible use as antiulcer agents[22]. A number of medicinal plants have been reported useful for the treatment of gastrointestinal

Table 4 Sequence of major genes involved in two main Kyoto Encyclopedia of Genes and Genomes pathways in gastric and duodenal tissue

Tissue pathway	Gene symbol	Gene name	Sham *vs* PL	PL *vs* SL-4	GeneBank accession No.
Gastric (retinol metabolism)	*UGT2b1*	UDP glucuronosyl transferase 2 family, polypeptide B1"	+13.71	-30.56	NM_173295
	SDR16c5	Short chain dehydrogenase/reductase family 16C, member 5	+7.16	+3.12	NM_001106634
	RDH7	Retinol dehydrogenase 7	+5.89	-45.38	NM_133543
	CYP2b2	Cytochrome P450, family 2, subfamily b, polypeptide 2	+5.57	-12.51	NM_001198676
	CYP4a1	Cytochrome P450, family 4, subfamily a, polypeptide 1	+3.93	-83.27	NM_175837
	CYP1a1	Cytochrome P450, family 1, subfamily a, polypeptide 1	-3.47	+1.86	NM_012540
Duodenal (complement and coagulation cascades)	*A2m*	Alpha-2-macroglobulin	-20.37	+33.00	NM_012488
	FGG	Fibrinogen gamma chain	-41.01	+15.50	NM_012559
	FGA	Fibrinogen alpha chain	-23.51	+6.49	NM_001008724
	MASP1	Mannan-binding lectin serine peptidase 1	-11.09	+3.19	AY149996
	VSIG4	V-set and immunoglobulin domain containing 4	-6.37	+3.26	NM_001025004
	CFB	Complement factor B	+3.08	-5.43	NM_212466

Genes involved in the retinol metabolism pathway in gastric tissue and in the complement and coagulation cascade pathways in duodenal tissue with > 2-fold change associated with different experimental treatments were determined by microarray analysis. Fold-induction was calculated as the normalized intensity in the study groups. Positive and negative numbers represent up- and downregulated gene expression. PL: Pyloric ligation; Sham: Sham operation; SL-4: Sulongga-4 decoction.

disorders. Wu *et al*[23] reported that a Chinese medicinal herb, *Pogostemon cablin*, and its main active compound β-patchoulene profoundly inhibited ulcer formation by reducing the inflammatory response and improving angiogenesis. Kumar *et al*[24] studied the antiulcer activity of an Indian medicine *Cedrus deodara* and found that its volatile oil had a potent gastroprotective effect on ulceration and inflammation in PL- and ethanol-treated rats. Al-Wajeeh *et al*[25] reported that a Malaysian medicine *Cibotium barometz* hair protected against ethanol-induced ulceration through regulation of antioxidant enzymes and gastric secretory actions. Consistent with those findings, our data confirmed that the gastroprotective effects of SL-4 components primarily work through modulation of inflammatory and gastric secretory mechanisms.

Microarray techniques are crucial for understanding and interpretating the activity of traditional medicines, particularly in revealing their mechanisms of action and drug targets[26]. To investigate the underlying molecular mechanisms of SL-4, we determined the gene expression profiles of gastric and duodenal tissues obtained from rats with PL-induced ulcers with or without SL-4 treatment. Retinol metabolism was one of the most significantly modified pathways in gastric tissue after SL-4 treatment. Retinoic acid (RA) intake and metabolism change dramatically during the acute-phase response (APR) to inflammation[27]. The APR is a metabolic system responding to infection, trauma, or tissue injury, and the PL method used in this study may have caused a strong APR in the intestinal tract. Inflammation drives decreased retinol usage by a reduction of retinol-binding protein synthesis[28]. RA increases the synthesis of inflammatory cytokines including transforming growth factor (TGF)-β, IL-10, IL-4, IL-5, and IL-6, and enhances white blood cell production[29]. Microarray analysis found that SL-4 decreased the expression of key transcriptomes associated with retinol metabo-lism. RA concentration is controlled by glucuronosyltransferases, including UGT2b1, and P450 cytochromes including CYP26A1, CYP26B1, and CYP26C1. These subfamilies catalyze RA oxidation to forms including 5,8-epoxy RA, 4-oxo RA, 4-hydroxy RA, and 18-hydroxy RA[30,31]. Thus, decreased enzyme activation blocks RA accumulation in the other areas and maintains a suitable physiological RA concentra-

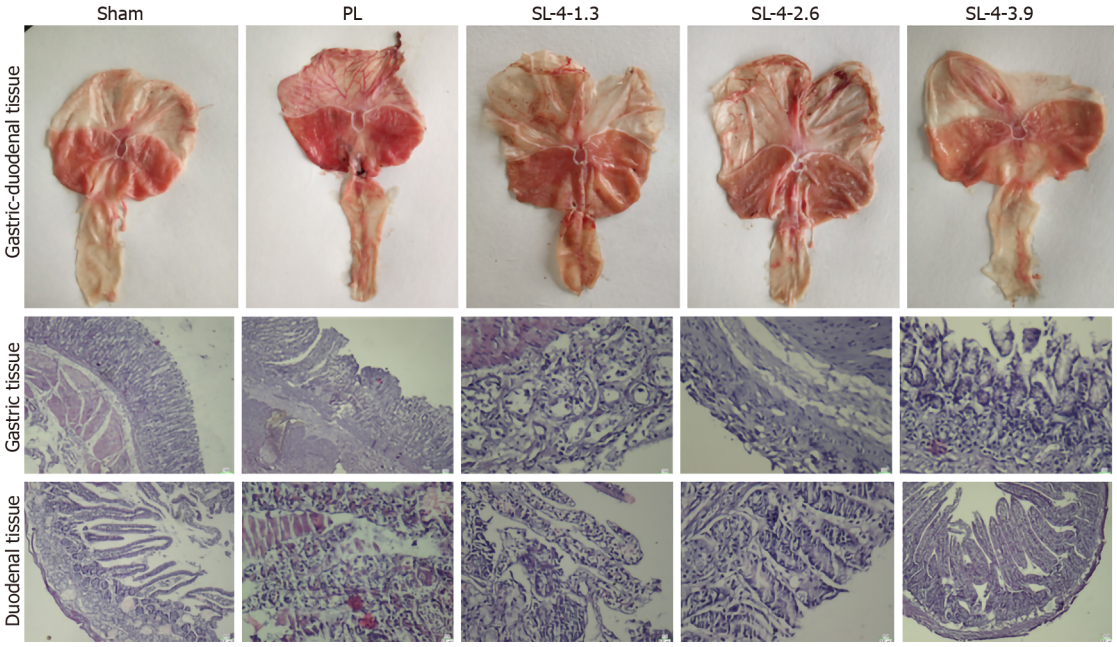


Figure 1 Histopathological assessment of the effects of Sulongga-4 on pyloric ligation-induced gastric and duodenal ulcers in rats. The gastroduodenal tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned. The tissue sections were dewaxed, rehydrated, and stained with hematoxylin and eosin before observation by light microscopy (10 × 10). PL: Pyloric ligation; Sham: Sham surgery; SL-4: Sulongga-4 decoction.

tion to achieve the anti-inflammatory function of the gastrointestinal tract. Therefore, SL-4 can enhance the role of RA in maintaining homeostasis at the intestinal barrier and equilibrating immunity and tolerance. Together with vitamin A supplementation, SL-4 treatment may improve the protective effect against gastric ulcers in a clinical setting. The current results also suggest that SL-4 accelerates the anti-inflammatory effects of retinol metabolism. This further indicates the therapeutic effect of SL-4 on inflammatory bowel disease and other clinical inflammatory symp-toms.

Additionally, KEGG analysis found significant differences in the complement and coagulation cascade pathways that could have been caused by an inflammatory response. Damage of the intestinal tract activates the coagulation cascade. Fibrin formation provides a physical barrier that can be a potential shield from inflammation[32]. Our microarray results indicated that SL-4 elevated the transcriptome expression of A2m, which acts as a carrier protein to bind numerous growth factors and cytokines, such as platelet-derived growth factor, basic fibroblast growth factor, TGF-β and IL-1β, thus inhibiting inflammation[33]. MASP1 is a serine protease that functions as a component of the lectin pathway of complement systems, which plays a pivotal role in the defense against infection[34]. Elevation of MASP1 expression by SL-4 may be responsible for improving the immune activity and defense against gastric infections. The above results suggest that SL-4 may have several active substances that promote anti-inflammatory responses that prevent peptic ulcer formation *via* retinol metabolism and the complement and coagulation systems.

S. aureus infection was found in both gastric and duodenal tissues, which indicated that PL activated the immune system against *S. aureus* infection. Although our KEGG analysis did not find a direct protective effect, antimicrobial activity for SL-4 has been reported[12]. The intestinal immune system plays a vital role in the prevention of and defense against inflammation caused by harmful pathogens. In our study, PL induced an increase in IL-1β secretion in gastric tissues. Although the IL-1β-mediated immune response to *S. aureus* has not been confirmed, IL-1β is a key cytokine in orchestrating host defenses against the organism[35]. Therefore, the effect of SL-4 on bacterial infection of the intestinal tract should be investigated in a future study.

CONCLUSION

Our data demonstrate that SL-4 protected against PL-induced gastroduodenal ulceration by reducing inflammatory cytokines and increasing the expression of gastric acid inhibitory factors. We propose that regulation of *CYP2b2* and *UGT2b1* gene

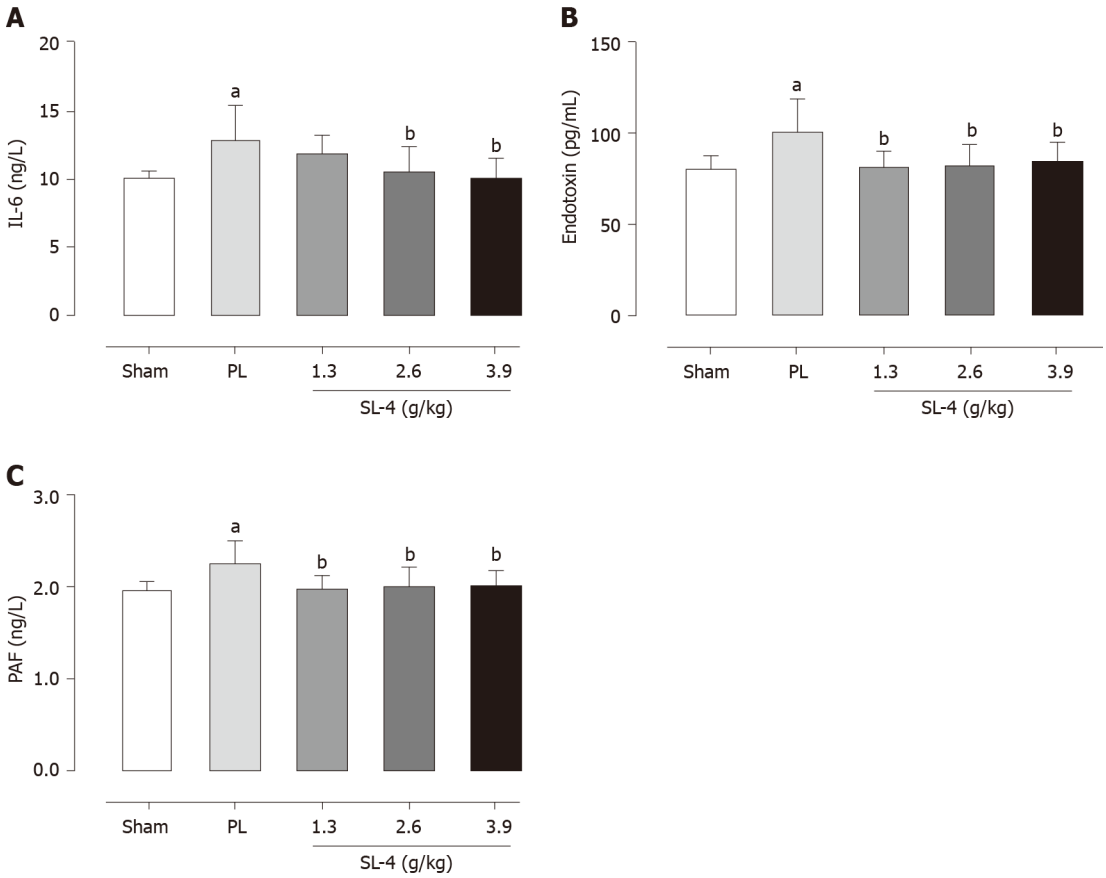


Figure 2 Expression of interleukin-6, endotoxin, and platelet-activating factor in duodenal tissue obtained from rats in different groups, and determined by enzyme-linked immunosorbent assay. Data are reported as means ± standard error (n = 10-12). A: Interleukin-6; B: Endotoxin; C: Platelet-activating factor. $^{a}P < 0.05$ *vs* the sham group; $^{b}P < 0.05$ *vs* the pyloric ligation (PL) group. Sham: Sham surgery; SL-4: Sulongga-4 decoction.

expression in the retinol metabolism pathway and *A2m* and *MASP1* genes in the complement and coagulation cascade pathways are may be involved in mechanism by which SL-4 protects against gastroduodenal ulcers. Most notable are the effects of SL-4 on the transcriptome involved in retinol metabolism, which has not been described previously. The Mongolian folk medicine SL-4 is a promising gastroprotective agent with potential use for treating gastric ulcers in clinical practice.

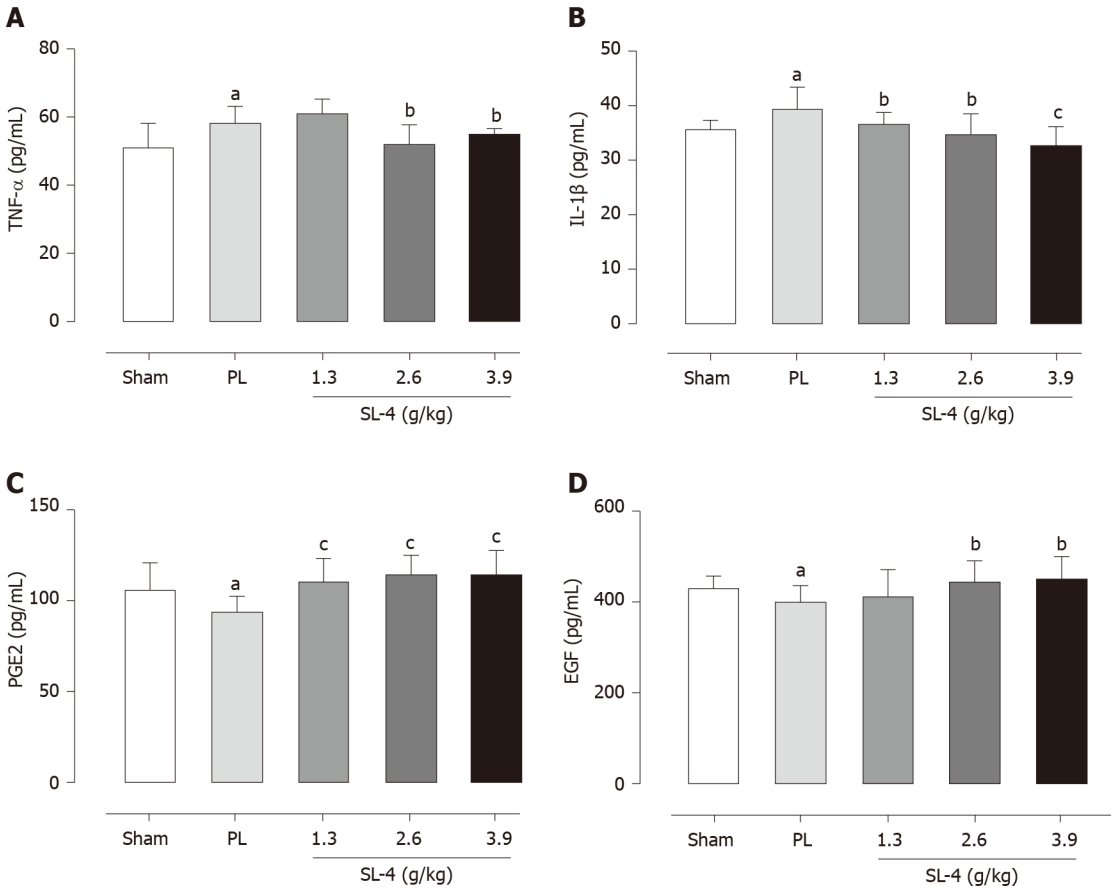


Figure 3 Expression of tumor necrosis factor-α, interleukin-1β, prostaglandin E2 and epidermal growth factor in the gastric tissue from rats in different groups, and determined by enzyme-linked immunosorbent assay. Data are means ± standard error (n = 10-12). A: Tumor necrosis factor-α; B: Interleukin-1β; C: Prostaglandin E2; D: Epidermal growth factor. $^{a}P < 0.05$ *vs* the sham group; $^{b}P < 0.05$ and $^{c}P < 0.01$ *vs* the pyloric ligation (PL) group, respectively. Sham: Sham surgery; SL-4: Sulongga-4 decoction.

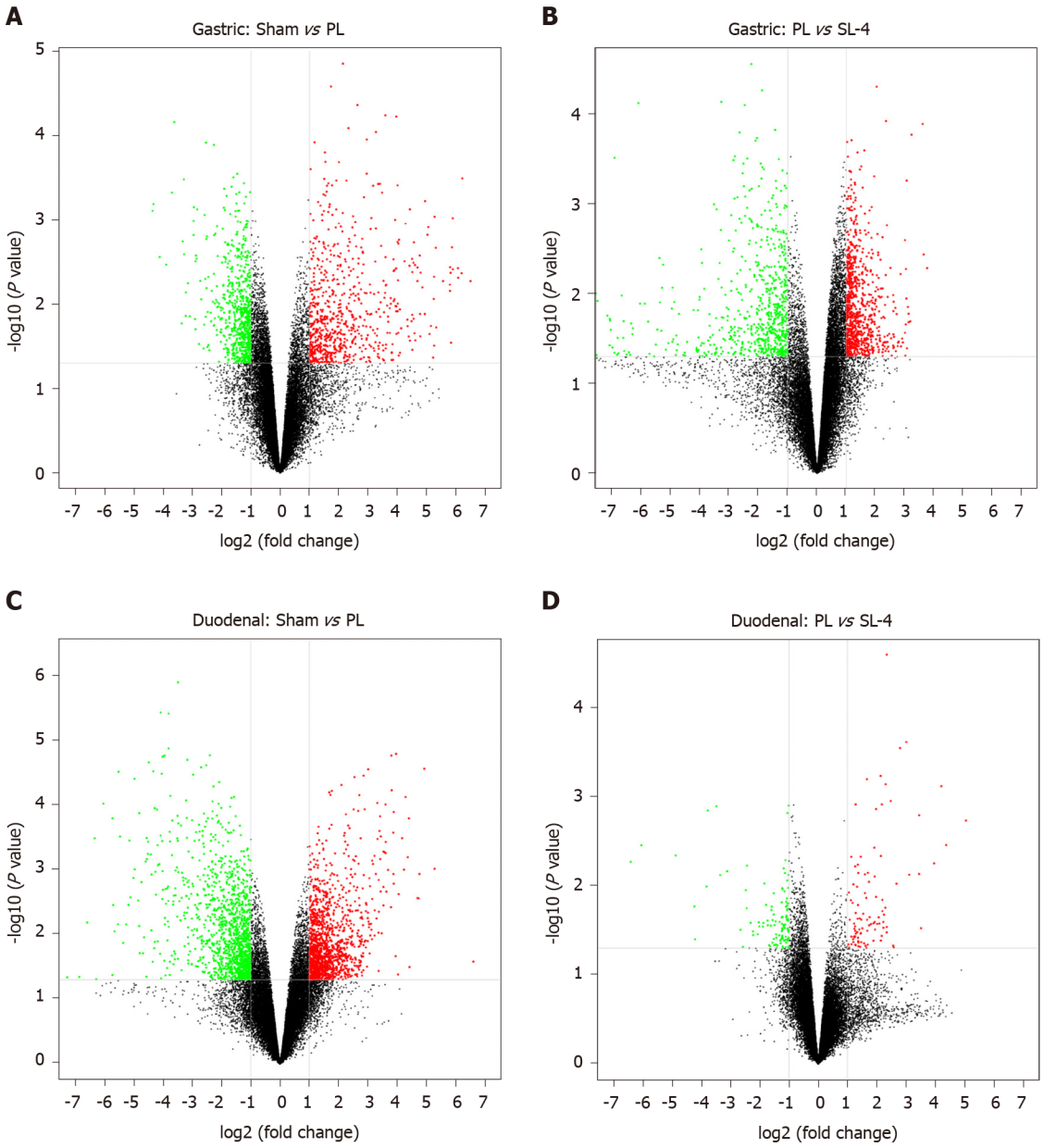


Figure 4 Volcano plots of microarray data on differentially expressed mRNA in gastric and duodenal tissue. A and B: There were 1415 and 1460 differentially expressed genes in the sham operation *vs* pyloric ligation (PL) (A) and PL *vs* SL-4 groups (B), respectively in gastric tissue; C and D: There were 2610 and 209 differentially expressed genes in the sham operation *vs* PL (C) and PL *vs* SL-4 groups (D), respectively in duodenal tissue. Vertical lines correspond to 2-fold up- and downregulation. The horizontal line represents a *P* value of 0.05. Significantly up- and downregulated genes are shown in red and green, respectively. Statistical significance was defined as a fold-change of 2.0 and *P* value of 0.05 between groups. Sham: Sham operation, SL-4: Sulongga-4 decoction.

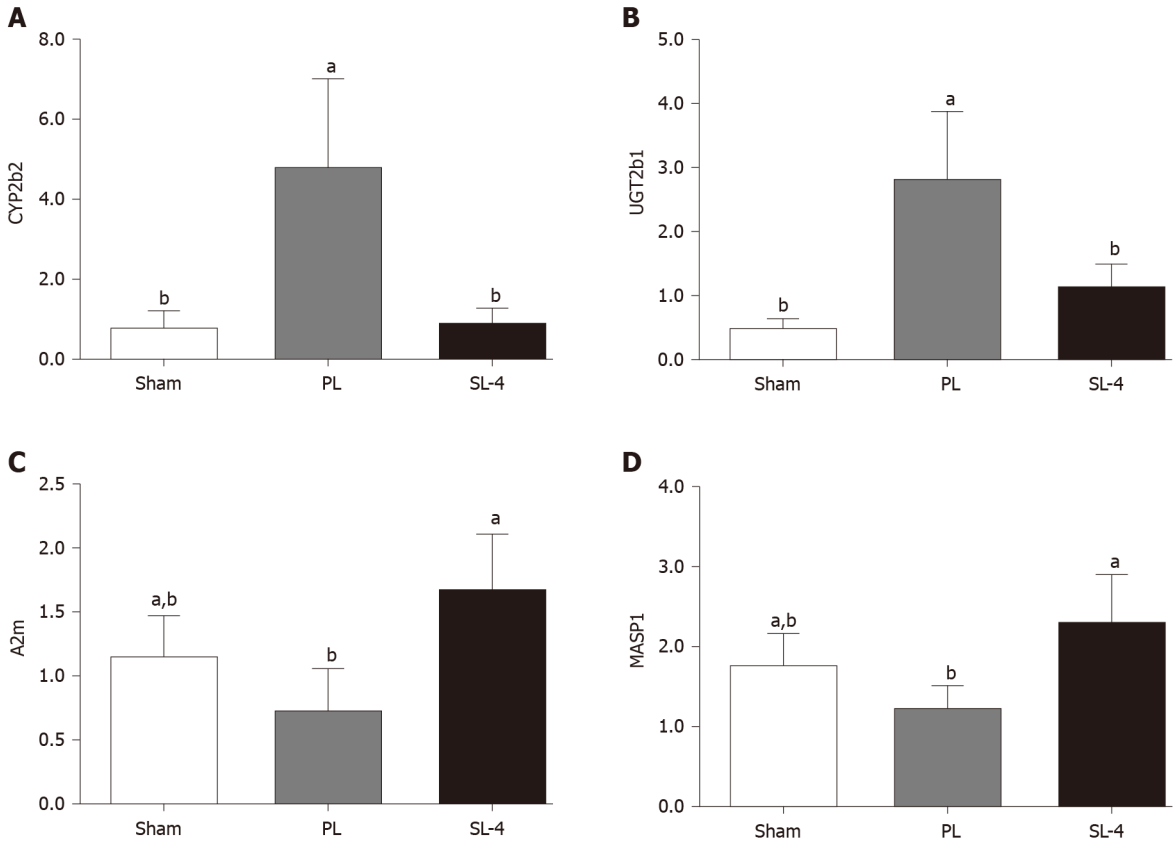


Figure 5 Relative expression of *CYP2b2* and *UGT2b1* mRNA in gastric tissue and *A2m* and *MASP1* mRNA in duodenal tissue by reverse transcription polymerase chain reaction assay. A: *CYP2b2*; B: *UGT2b1*; C: *A2m*; D: *MASP1*. *GAPDH* was used as the reference gene. Values are means ± standard error (n = 6). $^{a}P < 0.05$ *vs* sham operation; $^{b}P < 0.05$ *vs* pyloric ligation (PL). Sham: Sham operation; SL-4: Sulongga-4 decoction.

ARTICLE HIGHLIGHTS

Research background

Sulongga-4 (SL-4) is a classic herbal formula used in traditional Mongolian medical clinics for the treatment of peptic ulcers and gastroenteritis, even though its pharmacological mechanism has not been well characterized.

Research motivation

The study objective was to identify the protective effect and mechanism of SL-4 against peptic ulcer disease.

Research objectives

To evaluate the protective effect and identify the mechanisms of action of SL-4 on gastroduodenal ulcer induced by pyloric ligation (PL) in rats.

Research methods

PL was performed to induce gastric and duodenal ulcers in rats that were then treated with oral SL-4 (1.3, 2.6, or 3.9 g/kg per day) for 15 d. PL-induced gastroduodenal ulceration. Therapeutic effects were evaluated by pathological and histological evaluation. Inflammatory indicators were analyzed by enzyme-linked immunosorbent assay. Microarray analyses were conducted to determine the gene expression profiles of gastroduodenal tissue in PL rats with or without SL-4 treatment. The candidate target genes were selected and verified by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Research results

SL-4 improved the histopathology of the PL-induced ulcers. SL-4 significantly ($P <$ 0.05) decreased the expression of tumor necrosis factor-α, interleukin (IL)-1β, IL-6, endotoxin, PAF, and increased prostaglandin E2 and EGF in ulcer tissue. Microarray

analysis was used to identify a list of candidate target genes for SL-4 acting on PL-induced ulceration. The genes included some that modulate complement and coagulation cascade pathways, and retinol metabolism pathways that are closely associated with inflammatory responses and gastric mucosal protective mechanisms. qRT-PCR showed that altered expression of the selected genes, such as *CYP2b2*, *UGT2b1*, *A2m*, and *MASP1*, was consistent with the microarray results.

Research conclusions

SL-4 exerted protective effects against PL-induced gastroduodenal ulcers *via* reducing inflammatory cytokines and elevating the expression of gastric acid inhibitory factors. Downregulation of the *CYP2b2* and *UGT2b1* genes in retinol metabolism and upregulation of the *A2m* and *MASP1* genes in the complement and coagulation cascade pathways may have been involved in the protection against gastroduodenal ulcers.

Research perspectives

SL-4, a Mongolian folk medicine, is a promising gastroprotective agent with potential clinical use as a treatment of gastric ulcers.

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ORIGINAL ARTICLE

Basic Study

Depletion of MRPL35 inhibits gastric carcinoma cell proliferation by regulating downstream signaling proteins

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Abstract

BACKGROUND

Gastric carcinoma (GC) is a digestive system disease with high morbidity and mortality. However, early clinical detection is difficult, and the therapeutic effect for advanced disease is not satisfactory. Thus, finding new tumor markers and therapeutic targets conducive to the treatment of GC is imperative. MRPL35 is a member of the large subunit family of mitochondrial ribosomal protein. MRPL35 shows the characteristic of oncogene in colorectal cancer and esophageal cancer, which promotes the exploration of the correlation between MRPL35 and GC. We proposed that the expression of MRPL35 might be critical in GC.

AIM

To study the effect of *MRPL35* knockdown on GC cell proliferation.

METHODS

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The expression of *MRPL35* in GC was evaluated based on data from the public tumor database UALCAN (www.ualcan.path.uab.edu). The effect of the expression of *MRPL35* on the prognosis was evaluated with KMplot (www.kmplot.com). The expression of MRPL35 was assessed on the tissue microarray by immunohistochemistry and the level of *MRPL35* mRNA in 25 pairs of clinical GC tissues and matched adjacent tissues was detected by quantitative reverse transcription-polymerase chain reaction. Celigo cell count assay, colony formation assay, and flow cytometry were used to assess the role of MRPL35 in GC cell proliferation and apoptosis *in vitro*. Additionally, tumor formation experiment in BALB/c nude mice was utilized to determine the effect of *MRPL35* on GC cell proliferation. After knockdown of *MRPL35*, related proteins were identified by isobaric tags for relative and absolute quantification analysis, and the expression of related proteins was detected by Western blot.

RESULTS

The expression of MRPL35 was up-regulated in GC ($P = 1.77 \times 10^{-4}$). The Kaplan-Meier plots of the overall survival indicated that high expression of MRPL35 was associated with a poor survival in GC. Compared with adjacent tissues, the expression of MRPL35 in GC tissues was increased, which was related to age ($P = 0.03$), lymph node metastasis ($P = 0.007$), and pathological tumor-node-metastasis stage ($P = 0.024$). Knockdown of *MRPL35* inhibited GC cell proliferation and colony formation and induced apoptosis. Animal experiment results showed that knockdown of *MRPL35* inhibited tumor formation in BALB/c nude mice. Western blotting analysis showed that after knockdown of *MRPL35*, the expression of PICK1 and BCL-XL proteins decreased, and that of AGR2 protein increased.

CONCLUSION

Collectively, our findings demonstrate that knockdown of *MRPL35* inhibits GC cell proliferation through related proteins including PICK1, BCL-XL, and AGR2.

Key Words: Gastric carcinoma; MRPL35; Apoptosis; Proliferation; Tissue microarray; Isobaric tags for relative and absolute quantification

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Core Tip: MRPL35 is a member of the large subunit family of mitochondrial ribosomal protein. Our results showed that compared with adjacent tissues, the expression of MRPL35 in gastric carcinoma (GC) tissues was increased significantly, which was related to age, lymph node metastasis, and pathological tumor-node-metastasis stage of GC patients. Knockdown of MRPL35 inhibited GC cell proliferation, clone formation, and tumor formation in BALB/c nude mice, induced apoptosis, reduced the expression of PICK1 and BCL-XL proteins, and increased that of AGR2 protein. These data indicate that MRPL35 might be an oncogene and be used as a new therapeutic target for GC.

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INTRODUCTION

Gastric carcinoma (GC) is a common digestive system disease, the fourth most common cancer, and the second leading cause of cancer-related mortality worldwide. About 1033701 new cases of GC and 782685 GC-related deaths occurred worldwide in 2018, accounting for 8.2% of all cancer deaths in this year[1,2]. In China, the incidence of GC has always been high, and the mortality rate of the disease has also increased

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gradually, which is threatening human life and health.

At present, the treatment for GC mainly includes surgery, chemotherapy, radiotherapy, and drug treatment[3]. Most patients with early and advanced GC can be treated by surgery. However, the metastasis of GC is a major problem faced by surgery. The surgical treatment cannot cope with the distant metastasis of GC. Chemotherapy and radiotherapy reduce the recurrence and metastasis of GC, treat residual lesions, protect surrounding normal tissues and important organs, and improve survival[4]. During chemotherapy and radiotherapy, patients have to bear a specific degree of discomfort and pain, resulting in lower patient compliance, which in turn, affects the treatment effect. Therefore, finding effective drug treatment target is a major issue in the treatment of GC.

MRPL35, a 39S large subunit, encoded by a nuclear gene, is a member of the large subunit family of mitochondrial ribosomal protein. It is a specific component of mitochondrial ribosomes and plays a key role in the assembly of cytochrome C oxidase, involved in the translation of mitochondrial proteins[5,6]. The *MRPL35* gene is localized on chromosome 2p11.2, and the protein contains 188 amino acids and has a molecular weight of about 25 ku. Survival analysis revealed that high expression of MRPL35 indicated a prolonged survival in patients with glioblastoma[7]. The expression of MRPL35 in colorectal cancer tissues is higher than that of the matched adjacent tissues[8]. Furthermore, down-regulation of MRPL35 leads to increased production of reactive oxygen species and DNA damage and can inhibit cell proliferation, block the G2/M phase cell cycle, reduce the mitochondrial membrane potential, and induce apoptosis and autophagy *in vitro*. After the transfection of esophageal cancer TE-1 cells with lentivirus, compared with shCtrl (negative control virus) group, the expression of MRPL35 was reduced in the shMRPL35 (lentiviral particles of MRPL35) group, and *MRPL35* silencing suppresses the proliferation of esophageal cancer TE-1 cells and promotes apoptosis[9]. Other studies found that MRPL35 was negatively correlated with the survival time of patients after surgery[10]. *MRPL35* knockdown in colorectal cancer HCT116 cell and SW480 cell inhibited cell proliferation, promoted apoptosis, and blocked the G2/M phase of the cell cycle.

The MRP family regulates cells by participating in the transcriptional translation function of ribosomal proteins, which is closely related to tumor genesis and development[11]. Previous studies have confirmed that *MRPS18-2* is an oncogene[12]; *MRPL44* is up-regulated in the tissues of cervical lymph node metastasis of thyroid cancer[13]; *MRPS22*, which is found in large cell lung cancer, is highly expressed in squamous cell carcinoma and adenocarcinoma, and is low in other types of cancer[14]; *MRPL41* is down-regulated in colorectal cancer, pancreatic cancer, and other cancers[15]; *MRPS23*, down-regulated in breast cancer, can activate P21 WAF1/CIP1 and P53, inhibit cell proliferation, and promote apoptosis[16]; and MRPL35 is associated with the occurrence and development of glioblastoma, esophageal cancer, and colorectal cancer. However, the expression and functional role of MRPL35 in GC remain largely unknown. Therefore, we analyzed the correlation between MRPL35 and GC by tissue microarray, cell function experiments, nude mouse tumor formation experiments, and proteomic analysis. The expression of MRPL35 might be critical in GC according to the experimental results, and it could be a potential therapeutic target for GC.

MATERIALS AND METHODS

Patients

GC tissues and matched adjacent tissues were obtained from Department of Hepatobiliary Surgery, General Hospital of Ningxia Medical University. Following surgical removal, the tissue samples were immediately stored in liquid nitrogen before total RNA extraction. All tissues were histopathologically identified as GC or adjacent tissues. None of the patients had received preoperative adjuvant therapy, and all patients provided informed consent in accordance with the protocol approved by the Ethics Committee of Ningxia Medical University.

Immunohistochemistry staining

Immunohistochemistry (IHC) staining was performed on the tissue microarray (TMA)[17,18]. The method of staining is immunohistochemical streptavidin-peroxidase (SP) method. The section contained 64 pairs of GC tissues and matched adjacent tissues. The section was deparaffinized in xylene and washed in 700 mL/L ethanol. The antigen was recovered using citrate buffer (pH 6.0) under high pressure for 10

min. Endogenous peroxidase was blocked with 30 mL/L H_2O_2 (MERCK, Germany) in methanol for 10 min. Then, the section was incubated overnight with primary antibody (mouse polyclonal anti-MRPL35 antibody, HPA026631; Sigma-Aldrich, St. Louis, MO, United States) at 4 °C and secondary antibody successively, and developed with diaminobenzidine (DAB) reagent. The section was washed in TBST three times for 5 min, rinsed with tap water for 5 min, and then counterstained with hematoxylin. A negative control was run by omission of primary antibody (phosphate buffered saline instead of primary antibody) on another slide that was for testing the proper antibody concentration. Subsequently, the TMA was analyzed independently by two pathologists without knowing the patients' clinical information. The areas of the brown-yellow particles are the signs of positively stained tumor cells which represent the MRPL35 protein in the tissues. The staining intensity was scored from 0-3 as negative (0), weak (1), medium (2), or strong (3). The degree of staining was scored by the area of positively stained tumor cells relative to the entire tumor area: 0 (0%), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). The overall protein expression score (the overall score range was 0-12) was calculated by multiplying the intensity and the positive score.

Cell culture and transfection

Human GC cell lines (AGS, NCI-N87, MKN-45, and HGC-27) were purchased from the Cell Bank of the Chinese Academy of Sciences. The cell lines were cultured in a cell incubator at 37 °C and 50 mL/L CO_2 in RPMI-1640 medium (Gibco, New York, United States) containing 100 mL/L fetal bovine serum (FBS, Gibco) and penicillin and streptomycin (Invitrogen, Carlsbad, United States). shMRPL35 and shCtrl were designed and produced by GeneChem (Shanghai, China). Lentiviral transfection and the specific steps were performed according to the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction

Total RNA of the cultured cells was extracted using TRIzol reagent (PuFei, Shanghai, China). PrimeScript RT Master Mix Perfect Real-Time (TaKaRa, Shiga, Japan) was used to synthesize first-strand cDNA from total RNA. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using SYBR Master Mixture (TaKaRa) according to the manufacturer's instructions. The primers used are as follows: *MRPL35*: Forward, 5′-TTGGCATCTTCAACCTACCGC-3′ and reverse, 5′-GGAGGAAACAACTGGTGTCTGA-3′; *GAPDH*: Forward, 5′-TGACTTCAACAG CGACACCCA-3′ and reverse, 5′-CACCCTGTTGCTGTAGCCAAA-3′. Data are presented as the mean ± SD for duplicate runs. The relative quantification of *MRPL35* expression was evaluated using the $2^{-\Delta\Delta CT}$ method. All experiments were repeated three times.

Celigo cell count assay

The infected cells were seeded in a six-well plate at a density of 2000 cells/well and cultured at 37 °C and 50 mL/L CO_2. On the following day, the Celigo assay was performed daily for 5 consecutive days according to the manufacturer's instructions. All experiments were repeated three times.

Colony formation assay

The infected cells were inoculated in a six-well plate at a density of 1000 cells/well. The medium was changed every 3 d during the interval. After 2 wk, the colonies were fixed with 4% paraformaldehyde for 40 min and stained with 0.1% crystal violet (Sigma-Aldrich) for 4 min. The visible colonies were counted manually. Triplicate wells were assessed for each treatment group. All experiments were repeated three times.

Flow cytometry analysis of apoptosis

Apoptotic and necrotic cells were evaluated with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (KeyGEN, Jiangsu, China). All the samples were analyzed in triplicate by flow cytometry (BD Bioscience, Bedford, MA, United States). All experiments were repeated three times.

Tumor formation experiment in BABL/c nude mice

Four-week-old male BABL/c nude mice of specific pathogen-free (SPF) grade were purchased from the Animal Experiment Center of Ningxia Medical University. All animals were housed in polypropylene cages and fed standard laboratory chow and water *ad libitum*, and maintained at a temperature of 22 ± 1 °C, with 50% ± 5% relative

humidity and 12:12 h light-dark cycles. First, MKN-45 cells were infected with shMRPL35 and shCtrl (n = 10 per group). A microsyringe was used to extract 200 μL of the infected and uninfected MKN-45 cell suspension at a concentration of 1.25 × 10^7 /mL, and injected into the skin of nude mice. The tumor volume was measured daily using the formula $V = W^2 \times L \times 0.5$ (V, volume; W, width; L, length). The animal protocols (IACUC-NYLAC-2019-083) were approved by the Institutional Animal Care and Use Committee of Ningxia Medical University.

Isobaric tags for relative and absolute quantification analysis

SDT lysis method was used for GC cell sample preparation, and the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) was used for protein quantification. After protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie brilliant blue staining, and filter aided sample preparation enzymolysis, the resulting peptides were quantified using NanoDrop (Thermo Scientific ND2000, Shanghai, China), and the sample peptides were labeled according to the instructions of the isobaric tags for relative and absolute quantification (iTRAQ) labeling kit (AB SCIEX, Framingham, MA, United States). The labeled peptides of each group were mixed and graded using Agilent 1260 infinity II HPLC system, and Easy nLC chromatographic system (Thermo Scientific) analysis and mass spectrometry were performed. The high-resolution mass spectrometer Q Exactive Plus (Thermo Scientific) and Proteome Discoverer 2.1 (Thermo Scientific) software were used for iTRAQ analysis, and the database search was performed on the MASCOT2.5 (Matrix Science, Boston, MA, United States) server. The Protein Database used was Uniprot_HomoSapiens_159615_20170811.fasta (http:// www.uniprot.org). A homology search was first performed for the identified sequences with a localized sequence comparison tool NCBI BLAST+ against the NCBI database, and top 10 aligned sequences were selected according to the E value from the lowest to the highest for subsequent analysis. Then, Blast2GO Command Line was utilized to perform GO (Gene Ontology) annotation on the identified proteins. The enrichment analysis of GO annotation or KEGG pathway annotation was performed on the identified proteins through Fisher's exact test. The expression of identified proteins was analyzed with Cluster 3.0 software, and hierarchical clustering heat map was generated with Java Trewview software.

Western blot analysis

Total protein was extracted using the Whole Cell Lysis Assay (KeyGEN, Jiangsu, China), and the concentration was determined by BCA Protein Quantitation Assay (KeyGEN, Jiangsu, China). The protein was separated by SDS-PAGE (60-100 mL/L gel) with a sample amount of 50 μg and transferred to polyvinylidene difluoride (PVDF) membranes that were probed with specific primary antibodies overnight at 4 °C. Then, the membranes were incubated with secondary antibodies for 1 h. The immune-reactive bands were visualized using ECL Reagent (Affinity Biosciences, Jiangsu, China) and images were analyzed by Bio-Rad Laboratories (Inc., Hercules, United States). All the primary antibodies and secondary antibodies were from Abcam (MA, United States).

Statistical analysis

The statistical methods of this study were reviewed by Sun J from Department of Occupational and Environmental Health and Department of Medical Statistics, Institute of Public Health and Management, Ningxia Medical University. All analyses were performed using GraphPad Prism 7.0. All the data are expressed as the mean ± SE. Statistical significance was analyzed using one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

The expression of MRPL35 is up-regulated in GC tissues

The expression of MRPL35 between GC tissues and adjacent tissues was compared through the public tumor database UALCAN (www.ualcan.path.uab.edu). The results showed that the expression of MRPL35 was increased in GC tissues in comparison with matched adjacent tissues (P = 1.77 × 10^{-4}; 10-4; Figure 1A). In different pathological grades, the expression of MRPL35 in moderate and well grades was higher than that in matched adjacent tissues (P = 8.25 × 10^{-5}; P = 4.19 × 10^{-5}; 10-5;

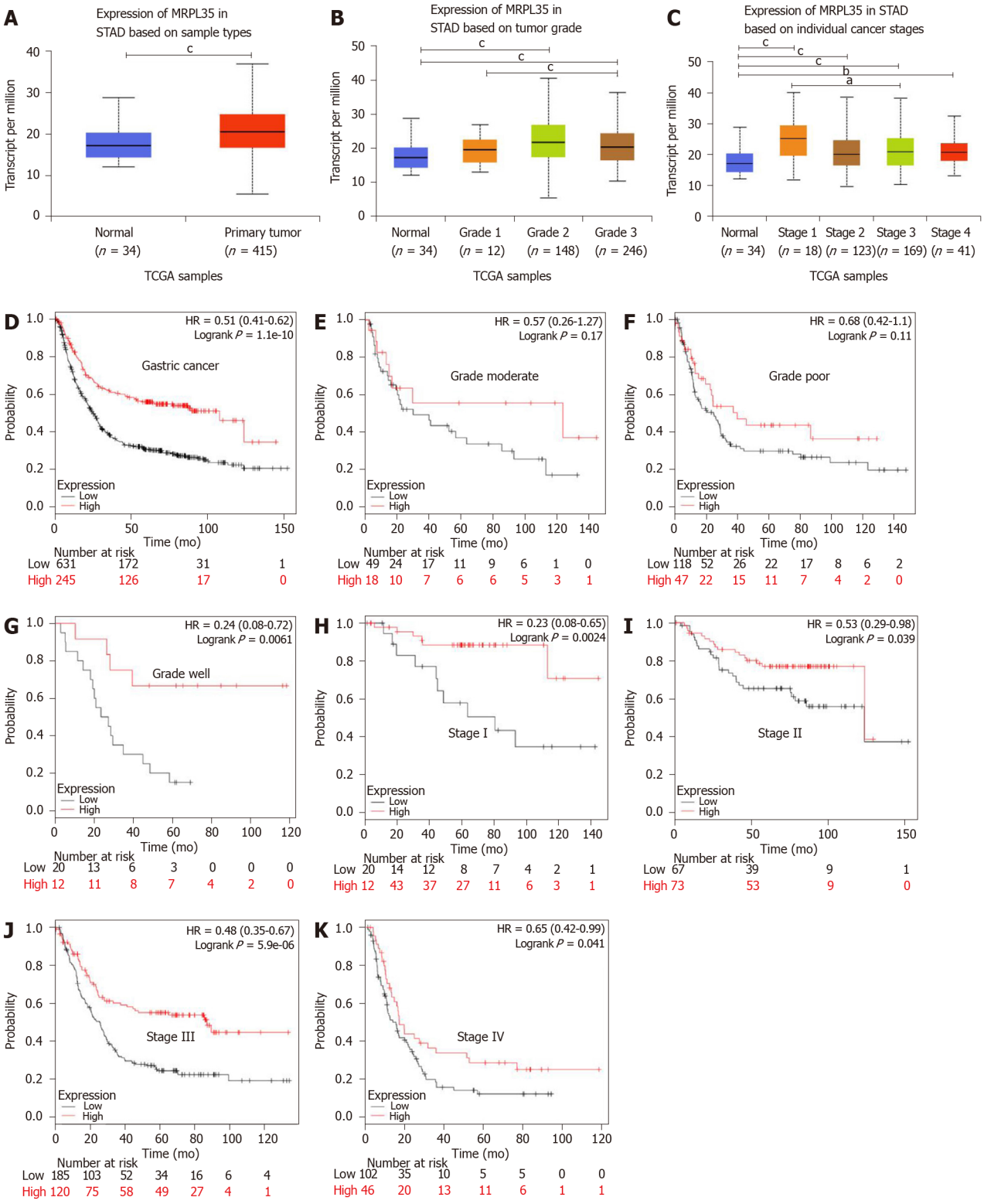


Figure 1 MRPL35 is highly up-regulated in gastric carcinoma. A-C: The expression of MRPL35 in stomach adenocarcinoma based on sample type (A), tumor grade (B), and individual cancer stage (C). The results were based on data from the public database UALCAN (www.ualcan.path.uab.edu); D: Kaplan-Meier overall survival chart. The results were based on data from the public database KMplot (www.kmplot.com); E-G: Overall survival of patients with high and low expression of MRPL35 in different pathological grades of gastric carcinoma (GC) (E, moderate; F, poor; G, well); H-K: Overall survival of patients with high and low expression of MRPL35 in different pathological stages of GC (H, stage I; I, stage II; J, stage III; K, stage IV). The results were based on data from the public database KMplot (www.kmplot.com). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. STAD: Stomach adenocarcinoma; HR: Hazard ratio.

Figure 1B). In different pathological stages, the expression of MRPL35 in stages I, II, III, and IV was higher than that in matched adjacent tissues ($P = 0.0012$, $P < 6.42 \times 10^{-4}$, $P = 4.36 \times 10^{-4}$, and $P = 0.0021$, respectively; Figure 1C). Next, the effect of MRPL35 expression on the prognosis was evaluated with KMplot (www.kmplot.com). The

Kaplan-Meier plots of the overall survival indicated that high expression of MRPL35 was associated with a poor survival in GC (Figure 1D). When stratified according to different histological types and disease stages, patients with high MRPL35 expression had a poor overall survival in well grade and in stage I, stage II, stage III, and stage IV (Figure 1G-K), but not in poor and moderate grades (Figure 1E and F).

Furthermore, the expression of MRPL35 in clinical samples was detected by IHC method in 64 pairs of GC tissues and matched adjacent tissues. The immunostaining of MRPL35 protein was mainly located in the cell (Figure 2A-C). The negative control pictures are shown in the supplementary materials (Supplementary Figure 1). According to the staining site, the MRPL35 protein expression score was calculated by multiplying the intensity and the positive score (total score range 0-12). The expression of MRPL35 protein in GC tissues was higher than that in matched adjacent tissues ($P < 0.05$) (Figure 2D and E). These data indicated that up-regulation of MRPL35 might play an important role in the development of GC. In addition, the expression of *MRPL35* mRNA in 25 pairs of clinical GC tissues and matched adjacent tissues was detected by qRT-PCR, and the expression of *MRPL35* mRNA was up-regulated in GC tissues ($P = 0.009$; Figure 2F and G).

The correlation between the high expression of MRPL35 and clinicopathological factors in patients with GC proved the importance of high expression of MRPL35 in GC patients (Table 1). According to the average expression (staining score), 68 patients were divided into two groups; for the expression of MRPL35, < 6 was defined as low expression and ≥ 6 defined as high expression. The result of statistical analysis showed that high expression of MRPL35 was correlated with age ($P = 0.03$), lymph node metastasis ($P = 0.007$), and pathological tumor-node-metastasis stage (p-TNM stage) ($P = 0.024$). On the other hand, the correlation between the expression of MRPL35 and other clinicopathological features was not significant.

Expression of MRPL35 in GC cells

Next, qRT-PCR analysis was performed to determine the expression of *MRPL35* in four human GC cell lines. The result showed that the expression of *MRPL35* was elevated in AGS and HGC-27 cell lines, but was lower in MKN-45 and NCI-N87 cell lines (Figure 3A). Thus, lentiviral vectors were used to knock down *MRPL35* in both cell lines. At 72 h post-transfection, bright light and fluorescence observation under a microscope showed that the cell transfection rate was > 80% (Figure 3B and C). qRT-PCR data revealed that the expression of *MRPL35* mRNA (PSC32114 and PSC32115) in AGS and HGC-27 cells was inhibited after *shRNA* lentivirus transfection ($P < 0.05$), and the knockdown efficiency reached 73.7% and 76.5% in AGS cells, and 67% and 77.5% in HGC-27 cells (Figure 3D and E). Subsequently, the expression of MRPL35 protein after *MRPL35* knockdown in AGS cells was assessed by Western blot; in contrast with the shCtrl group, the expression of MRPL35 (PSC32114 and PSC32115) was reduced in AGS cells ($P < 0.05$) (Figure 3F).

Effect of MRPL35 knockdown on GC cell proliferation, colony formation, and apoptosis

In contrast with the shCtrl group, the shMRPL35 (PSC32114 and PSC32115) group had inhibited proliferation of the two cell lines as monitored by Celigo cell count assay ($P < 0.01$), indicating that knockdown of *MRPL35* had an inhibitory effect on cell proliferation (Figure 4A and B). In addition, knockdown of *MRPL35* greatly reduced the colony-forming ability of AGS and HGC-27 cells (Figure 4C and D). Finally, the apoptosis was analyzed using flow cytometry. The numbers of apoptosis of AGS cells and HGC-27 cells in the shMRPL35 (PSC32114 and PSC32115) group increased compared with the shCtrl group ($P < 0.01$) (Figure 4E and F). These result indicated that MRPL35 might be essential for the proliferation of GC cells.

Knockdown of MRPL35 inhibits tumor formation in BABL/c nude mice

The tumor volume of the shMRPL35 group was smaller than that of the shCtrl group (Figure 5A and B). Within 3 wk of tumor formation, the tumor growth curve was drawn. Compared with the shCtrl and the NC groups, the tumor growth rate of the shMRPL35 group was lower (Figure 5C) ($P < 0.01$).

Proteomic and bioinformatic analysis identified proteins

Among 5993 proteins, 100 differentially expressed proteins (DEPs) were identified (Figure 6A). In comparison with the shCtrl group, 49 DEPs were up-regulated and 51 were down-regulated in the shMRPL35 group. The DEPs were screened based on the multiple of expression difference > 1.2 times and P value (t-test) < 0.05. The

Table 1 Correlation of expression of MRPL35 with various clinicopathological features in 68 gastric carcinoma patients

Pathological feature	*n*	Expression of MRPL35		
		High	Low	*P* value
	68	28	40	
Gender				
Male	48	19	29	0.685
Female	20	9	11	
Age (yr)				
< 65	33	18	15	0.03[a]
≥ 65	35	10	25	
p-TNM stage				
I/II	33	9	24	0.024[a]
III/IV	35	19	16	
Size of tumour				
≤ 5 cm	29	9	20	0.1
> 5 cm	39	19	20	
Pathological T grade				
T1/T2	11	3	8	0.313
T3/T4	57	25	32	
Lymph node metastasis (pN)				
N0/N1	30	7	23	0.007[b]
N2/N3	38	21	17	

[a]$P < 0.05$.
[b]$P < 0.01$. p-TNM stage: Pathological tumor-node-metastasis stage.

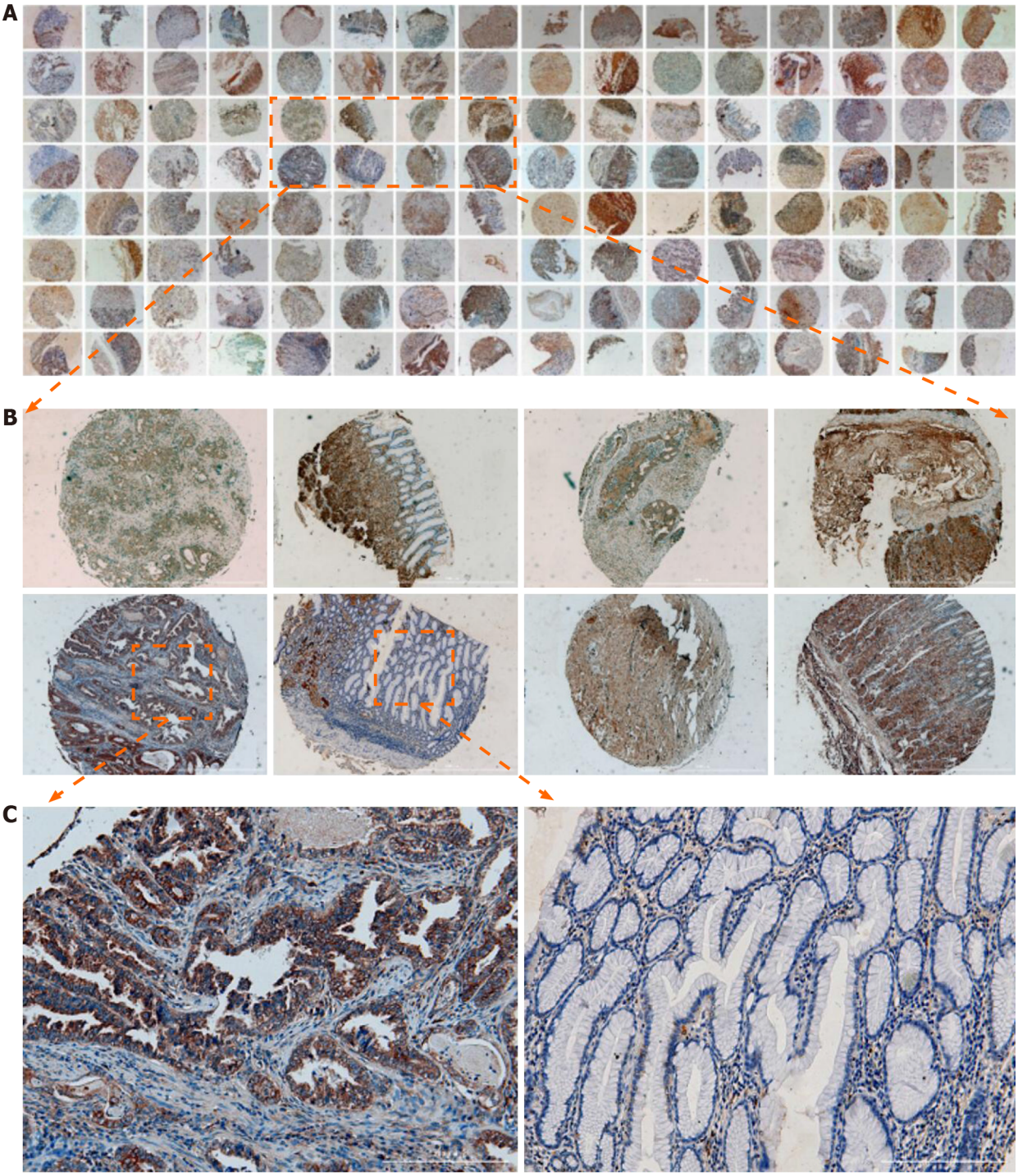
enrichment analysis was performed through GO functional annotation, describing the differential proteins from a large variety of biological processes, including cellular process, single-organism process, biological regulation, regulation of the biological process, and metabolic process (Figure 6B and C). The significance level of protein enrichment in each pathway was obtained by analyzing DEPs based on the KEGG database pathway annotation analysis (Figure 6D). The result of cluster analysis showed that the expression of all DEPs was different in the groups of shMRPL35 and shCtrl (Figure 6E).

Knockdown of MRPL35 alters the expression of PICK1, BCL-XL, and AGR2 proteins in GC cells

PHLDA1, MAPK8, PICK1, BCL-XL, AGR2, and ZFP36L1 proteins were selected for Western blot verification, and these proteins ranked the top 30 in biological process, molecular function, and cellular component. The expression of PHLDA1, MAPK8, PICK1, BCL-XL, AGR2, and ZFP36L1 proteins in AGS cell was detected by Western blot, and these proteins were contained in DEPs. Compared with the shCtrl group, the expression of PICK1 and BCL-XL proteins was decreased, and that of AGR2 was increased in the shMRPL35 group ($P < 0.01$) (Figure 7A and B). Based on the above result, knockdown of *MRPL35* may inhibit the proliferation of GC cells through PICK1, BCL-XL, and AGR2 proteins.

DISCUSSION

A major finding of this study is that knockdown of *MRPL35* could inhibit the proliferation of human GC cells and promote apoptosis through nude mouse tumor


A
B
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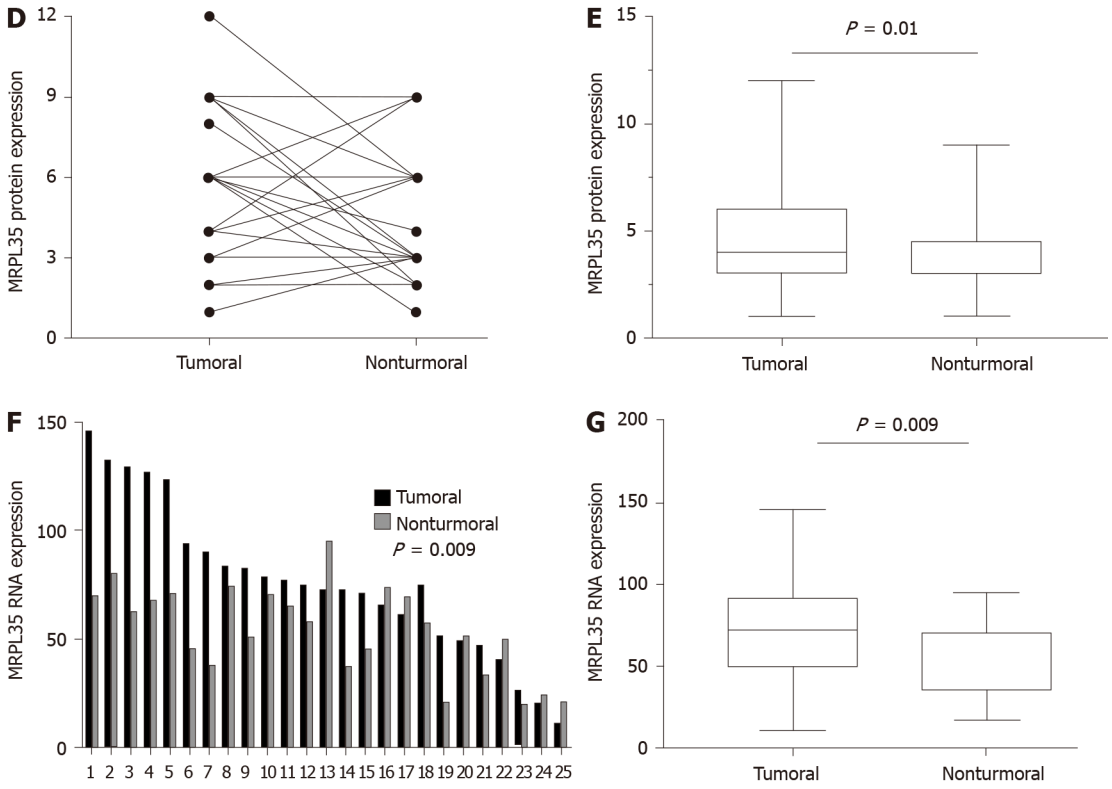


Figure 2 The expression of MRPL35 is up-regulated in gastric carcinoma tissues. A-C: Immunohistochemistry was performed on 64 pairs of gastric carcinoma (GC) tissues and matched adjacent tissues. Magnification: 4 × (A), 40 × (B), 200 × (C); D and E: The relative expression of MRPL35 in GC tissues and matched adjacent tissues; F and G: The relative expression of *MRPL35* mRNA in GC tissues and matched adjacent tissues (n = 25).

formation experiment *in vivo*, together with cell function experiment *in vitro*. In addition, proteomic analysis found that related downstream proteins changed after knockdown of *MRPL35*. And then, the six proteins were assessed by Western blot, which showed that the expression of PICK1 and BCL-XL proteins decreased, while that of AGR2 protein increased. In addition, the expression of MRPL35 was up-regulated in GC tissues, and high expression of MRPL35 was associated with a low survival rate in GC. Sixty-four pairs of clinical samples of GC tissues and matched adjacent tissues were examined for the expression of MRPL35 by IHC. The expression of MRPL35 in GC tissues was increased compared with matched adjacent tissues, and high expression of MRPL35 was correlated with age, lymph node metastasis, and p-TNM stage. Moreover, the results of other studies on MRPL35 in other cancers were similar to those of the current study, suggested that MRPL35 has the potential to serve as a new therapeutic target for GC. It is necessary to explain the number of tissues on the TMA. Originally, there were 75 pairs of GC tissues and matched adjacent tissues on the section, but a few of them were worn out during the IHC process. Finally, the tissues that could present on the slide and be used for analysis included 68 GC tissues and 64 matched adjacent tissues, that is to say, there were 64 GC tissues and 64 matched adjacent tissues, as well as the extra four GC tissues of which the matched adjacent tissues were worn out. We performed clinicopathological feature analysis on the remaining 68 GC tissues, and then obtained the data in Table 1. However, we carried out the IHC analysis on the remaining 64 pairs of GC tissues and matched adjacent tissues, because it should make a comparative analysis between GC tissues and the matched adjacent tissues. So only 64 pairs of tissues are shown in Figure 2A, for there were no matched adjacent tissues to be compared with the extra four GC tissues. The figures of the extra four GC tissues are shown in the supplementary materials (Supplementary Figure 2).

From the previous studies, we noticed that the expression of MRPL35 was decreased and cell proliferation inhibited, while apoptosis was promoted after knockdown of *MRPL35* in esophageal cancer TE-1 cells[9]. This phenomenon was similar to the results of the current study. The expression of MRPL35 in cancer tissues was higher than that in matched adjacent tissues in colorectal cancer, and *MRPL35* knockdown *in vitro* can inhibit cell proliferation and promote apoptosis[8]. The expression of MRPL35 was decreased in the nude mouse xenograft model of colorectal

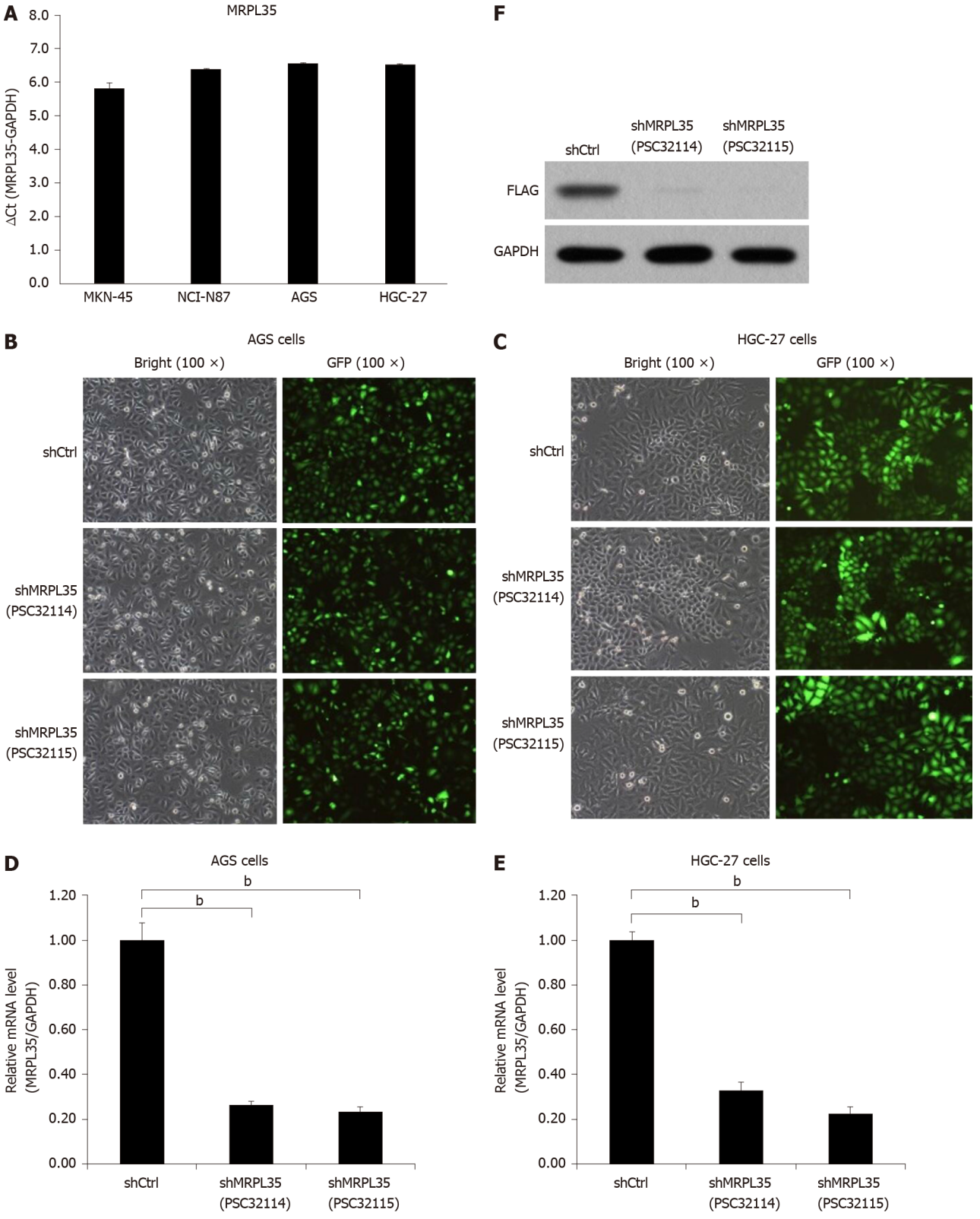
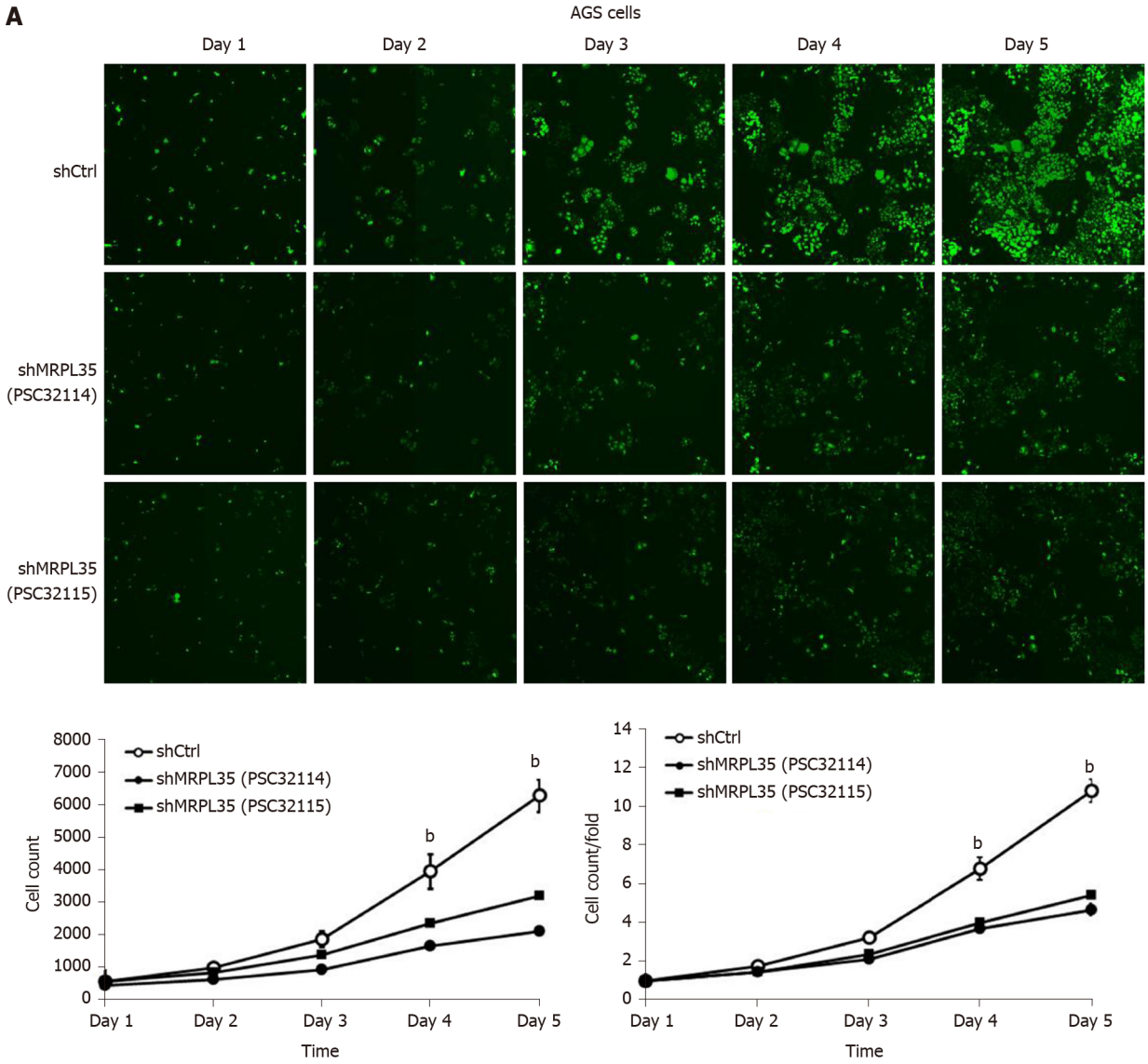
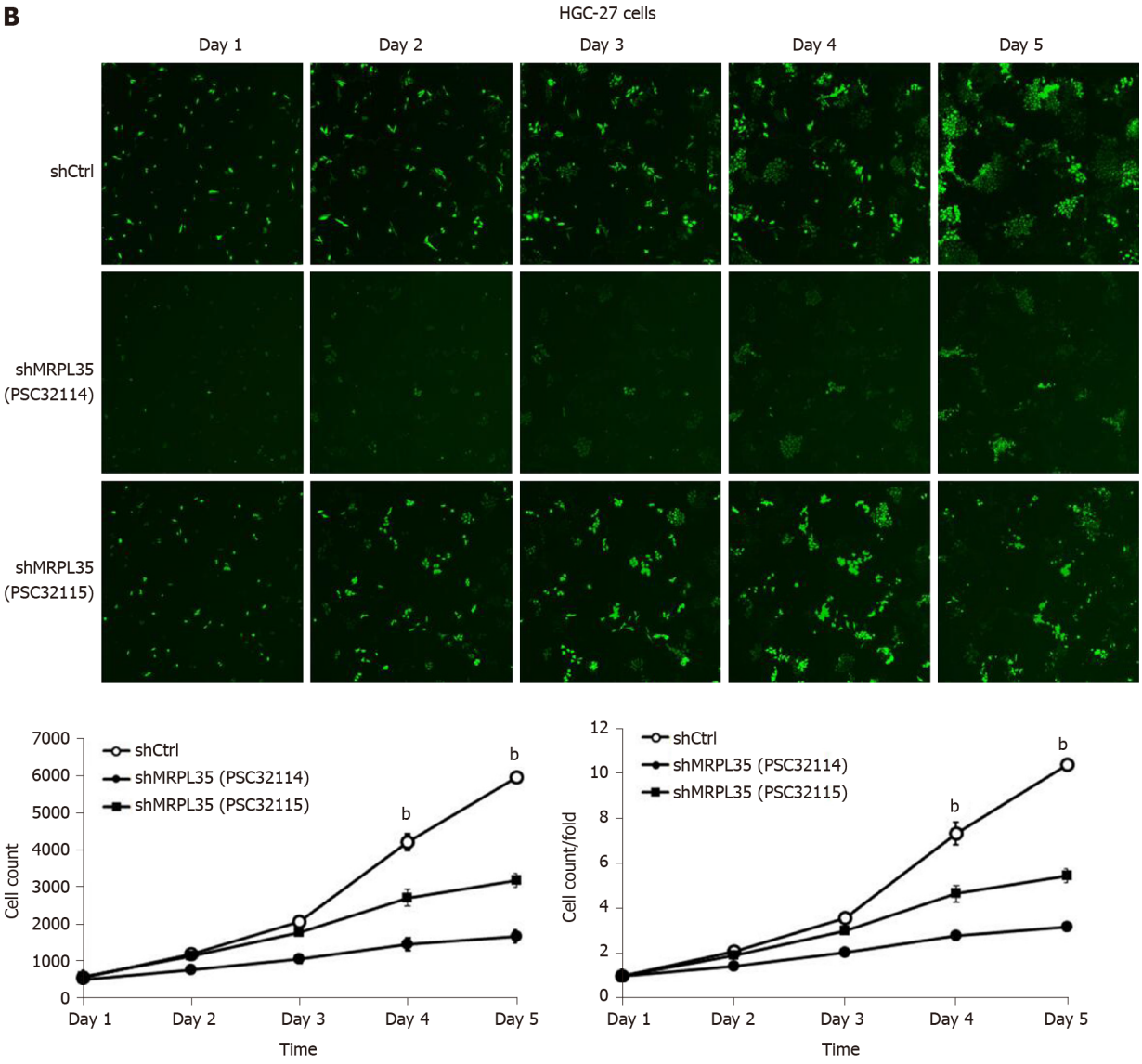
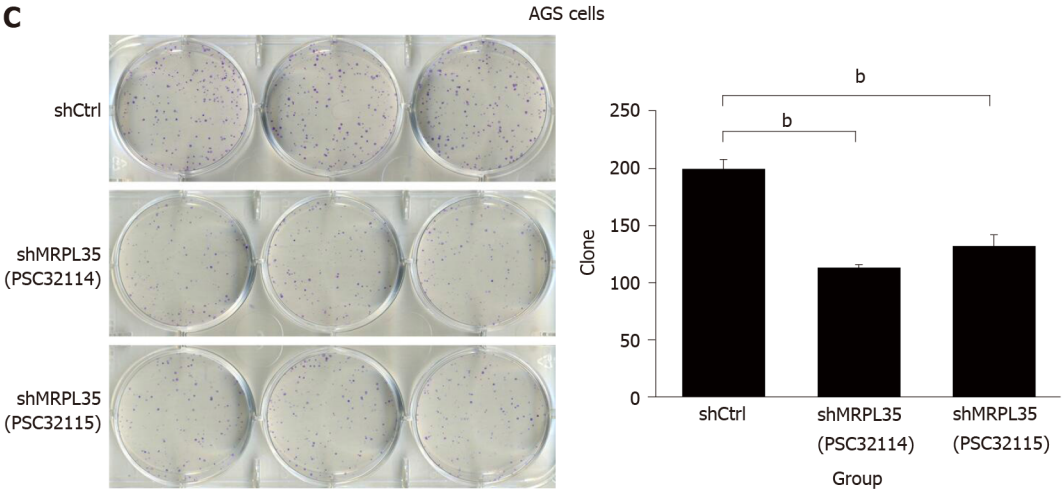
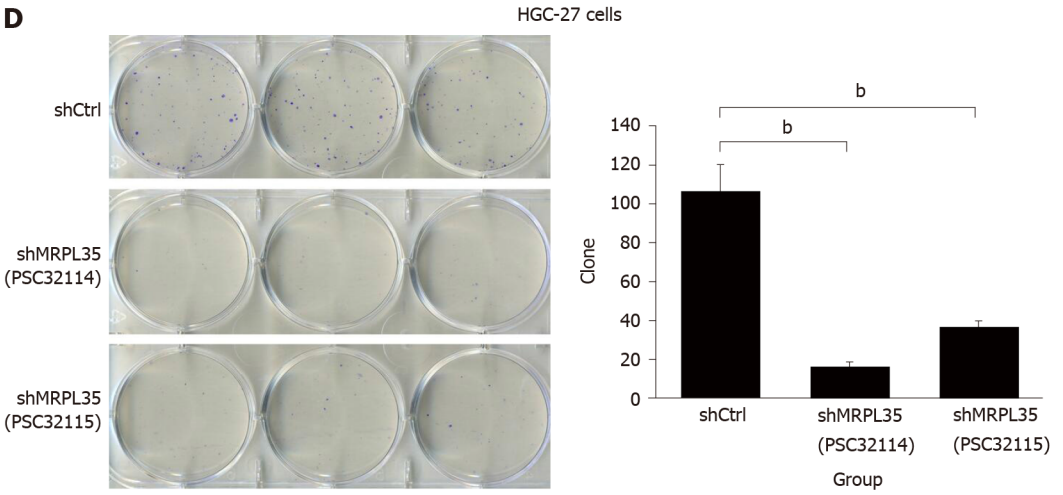


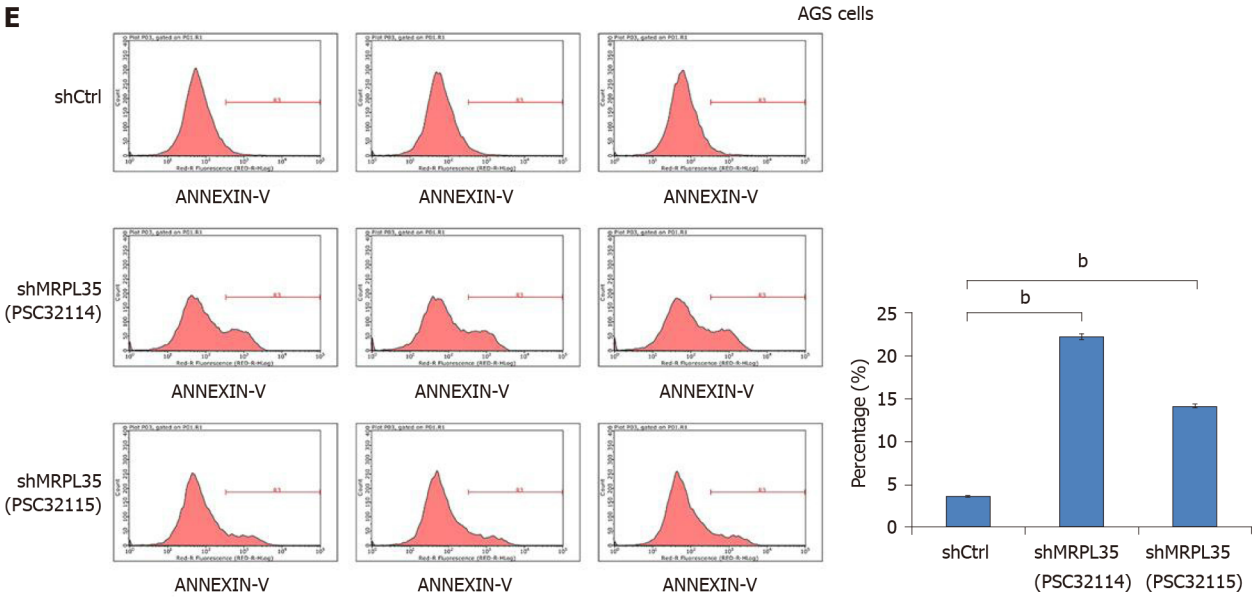
Figure 3 The expression of MRPL35 in gastric carcinoma cells. A: Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) detection of MRPL35 in gastric carcinoma cell lines (AGS, NCI-N87, MKN-45 and HGC-27); B and C: shCtrl (negative control virus) and shMRPL35 (lentiviral particles of MRPL35) (PSC32114 or PSC32115) infected AGS cells (B) and HGC-27 cells (C) at 72 h with bright and fluorescent micrographs (100 × magnification); D and E: qRT-PCR was used to analyze the expression of MRPL35 in AGS cells (D) and HGC-27 cells (E) infected with shCtrl and shMRPL35; F: Western blot was used to detect the expression of MRPL35 in AGS cells transfected with shCtrl and shMRPL35, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. $n = 3$. $^{b}P < 0.01$. shCtrl: Negative control virus; shMRPL35: Lentiviral particles of MRPL35; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GFP: Green fluorescent protein.


A
AGS cells
Day 1
Day 2
Day 3
Day 4
Day 5
shCtrl
shMRPL35 (PSC32114)
shMRPL35 (PSC32115)
Cell count
Time
shCtrl
shMRPL35 (PSC32114)
shMRPL35 (PSC32115)
b
Cell count/fold
Time


B
HGC-27 cells
Day 1
Day 2
Day 3
Day 4
Day 5
shCtrl
shMRPL35 (PSC32114)
shMRPL35 (PSC32115)
Cell count
Time
Cell count/fold
b


C
AGS cells
shCtrl
shMRPL35 (PSC32114)
shMRPL35 (PSC32115)
Clone
250
200
150
100
50
0
b
b
shCtrl
shMRPL35 (PSC32114)
shMRPL35 (PSC32115)
Group


D
HGC-27 cells
shCtrl
shMRPL35 (PSC32114)
shMRPL35 (PSC32115)
Clone
140
120
100
80
60
40
20
0
b
b
shCtrl
shMRPL35 (PSC32114)
shMRPL35 (PSC32115)
Group


E
AGS cells
shCtrl
shMRPL35 (PSC32114)
shMRPL35 (PSC32115)
ANNEXIN-V
Percentage (%)
25
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shMRPL35 (PSC32115)

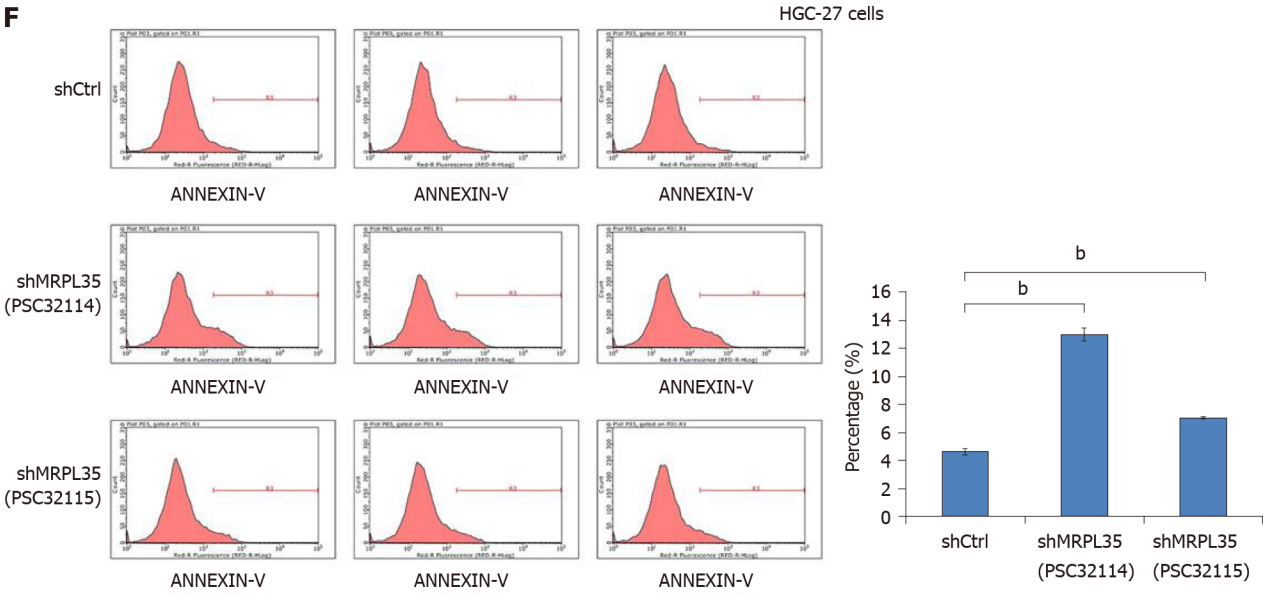


Figure 4 Knockdown of *MRPL35* inhibits gastric carcinoma cell proliferation and monoclonal formation and promotes gastric carcinoma cell apoptosis. A and B: The Celigo cell count assay was used to analyze the proliferation of AGS cells (A) and HGC-27 cells (B) transfected with shCtrl (negative control virus) and shMRPL35 (lentiviral particles of MRPL35); C and D: Colony formation assay of shCtrl and shMRPL35 infected AGS cells (C) and HGC-27 cells (D); E and F: Flow cytometry analysis of apoptosis of shCtrl and shMRPL35 infected AGS cells (E) and HGC-27 cells (F). $n = 3$. $^{b}P < 0.01$. shCtrl: Negative control virus; shMRPL35: Lentiviral particles of MRPL35.

cancer[19]. Indeed, the tumor growth rate in the experimental group was slow, and the tumor was smaller than that in the control group, which was the same as our experimental results. The gastrointestinal tract contains the mouth, esophagus, stomach, small intestine, and large intestine. The cells contained in these tissues were developed from the endoderm of the early embryo. Perhaps because the esophagus, stomach, and large intestine all come from the endoderm, the inhibitory effects of MRPL35 in GC, esophageal cancer, and colorectal cancer are similar.

Furthermore, the PICK1 protein contains a PDZ domain, which binds to a variety of membrane proteins and organizes the subcellular localization of adaptor protein[20]. It may also participate in receptor aggregation by acting on the level of receptor internalization. Decreased expression of MRPL35 regulates the expression of MRPL27, reducing the expression of PICK1. The protein encoded by *BCL-XL* belongs to the BCL-2 protein family[21]. It can be used as an anti- or pro-apoptotic regulator involved in various cell activities and regulation of the opening of the mitochondrial outer membrane channel (VDAC). MRPL35 regulates the expression of BCL-XL by regulating the family member BAX. AGR2 encodes a member of the disulfide isomerase family of endoplasmic reticulum protein and plays a role in cell migration, cell differentiation, and growth, and is associated with cancer progression[22]. Consequently, the expression of MRPL35 and MRPL2 is altered, which in turn, affects that of AGR2. These results fully indicated that *MRPL35* might be an oncogene, and the correlation between MRPL35 and GC is worthy of in-depth exploration.

In short, the current study suggested that MRPL35 has a causal correlation with GC, and related downstream proteins of MRPL35 have been identified. Nevertheless, the current study has some deficiencies. At present, only a few studies have investigated MRPL35, which is not sufficient to clarify its role as a therapeutic target for GC. On the premise that there are no relevant studies on MRPL35 and GC, follow-up research would be divided into three stages. In the first stage, the correlation between *PICK1*, *BCL-XL*, and *AGR2* genotype and GC phenotype would be explored. In the second stage, the correlation between MRPL35 and its downstream proteins PICK1, BCL-XL, and AGR2 would be examined. In the third stage, the mechanism underlying the interaction between MRPL35 and PICK1, BCL-XL, and AGR2 would be assessed, and two modes of action were considered: Direct and through tool protein. Also, covalent and non-covalent interactions occurred between the proteins. Anyway, our study aimed to provide a scientific basis for the treatment of GC and find effective drug target for the early prevention of GC.

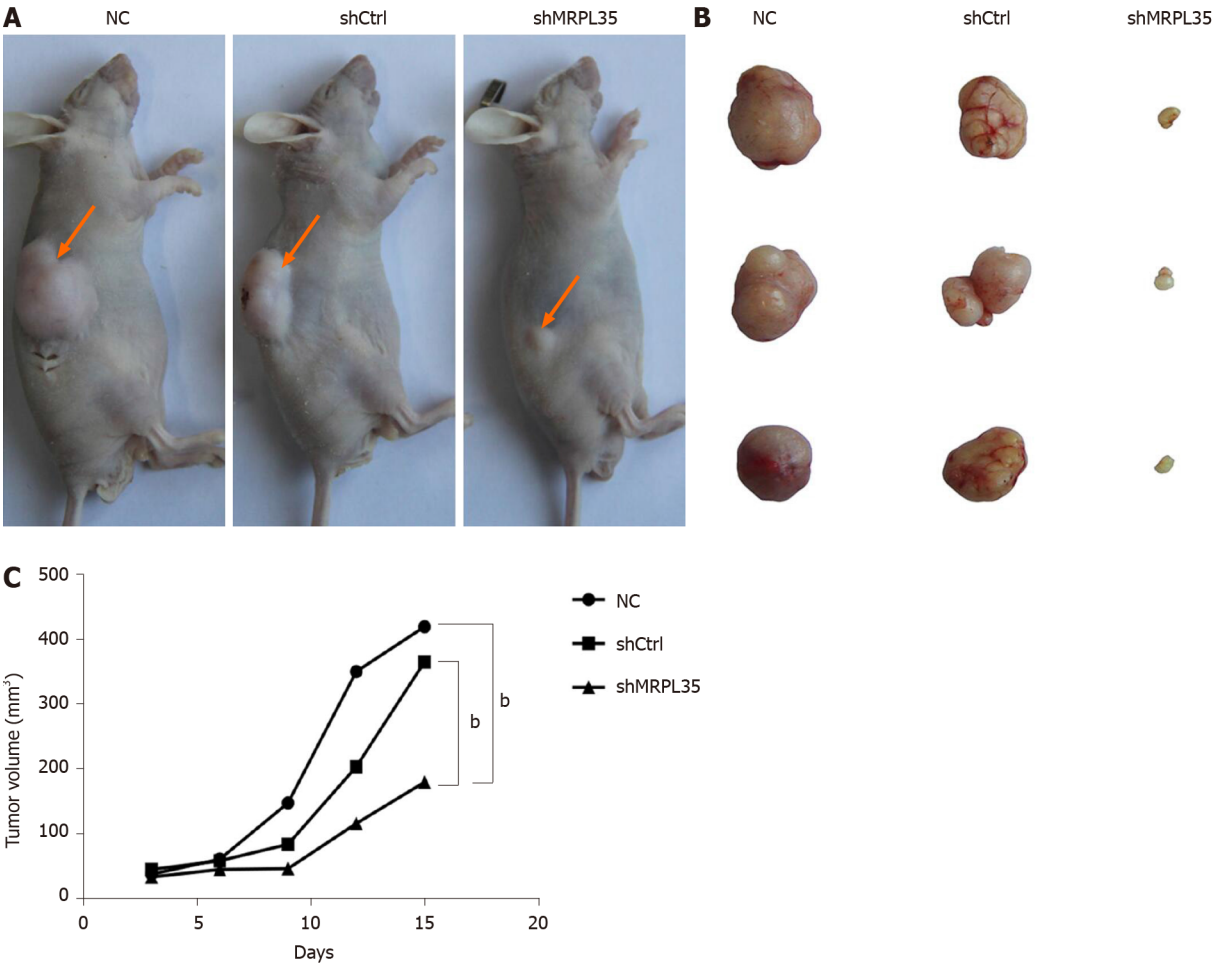


Figure 5 Knockdown of *MRPL35* inhibits tumor formation in nude mice. A: Nude mouse gastric carcinoma xenograft model; B: Subcutaneously transplanted tumor in nude mice; C: Tumor growth curve of transplanted tumor model. n = 30. $^{b}P < 0.01$. NC: Negative control; shCtrl: Negative control virus; shMRPL35: Lentiviral particles of MRPL35.

CONCLUSION

MRPL35 is related to esophageal cancer and colorectal cancer, and has not been reported in GC. Our experimental data combined with the data in the public tumor database show that MRPL35 is of extraordinary importance in GC tissues and cells, which has also been verified in nude mice. The expression of MRPL35 in GC tissues is higher than that of the matched adjacent tissues, and knockdown of *MRPL35* can inhibit the proliferation of GC cells and induce apoptosis. This suggests that MRPL35 is a potential target for the treatment of GC, and it is necessary to further study the effect of MRPL35 on GC.

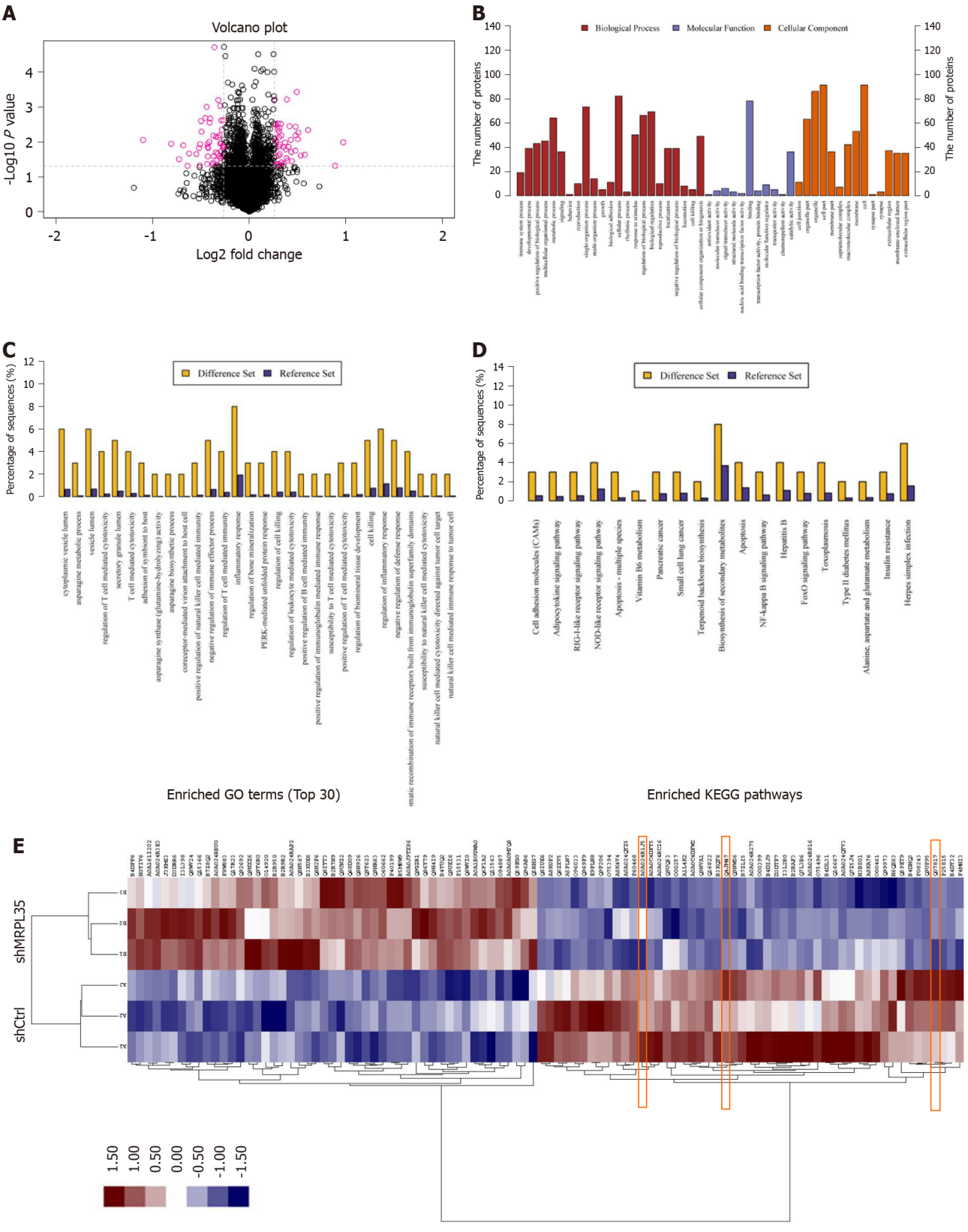


Figure 6 Proteomic and bioinformatic analysis of identified proteins. A: A total of 5993 proteins were identified by isobaric tags for relative and absolute quantification. Red circle indicates 100 differentially expressed proteins (DEPs); B: The result of Gene Ontology annotation. The identified proteins were categorized into three types: Biological process, molecular function, and cellular component; C: Enriched Gene Ontology annotation statistics of DEPs; D: Enriched KEGG pathway statistics of DEPs; E: Protein cluster analysis of DEPs. The red box part is AGR2, PICK1, and BCL-XL. shCtrl: Negative control virus; shMRPL35: Lentiviral particles of MRPL35.

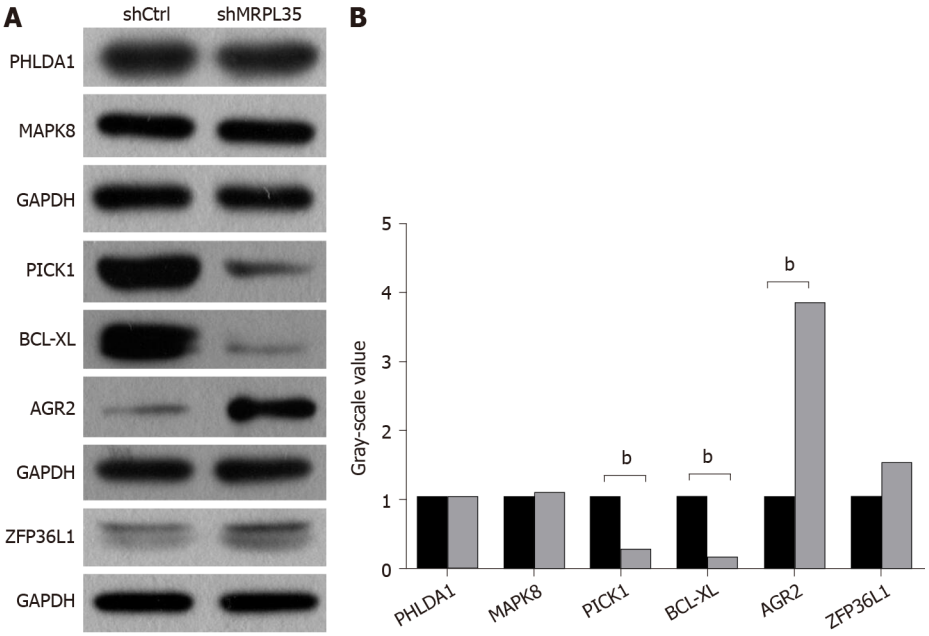


Figure 7 Expression of related proteins in AGS cells after knockdown of *MRPL35*. A and B: The expression of PHLDA1, MAPK8, PICK1, BCL-XL, AGR2, and ZFP36L1 proteins was detected by Western blot. GAPDH was used as an internal control. A: Western blot images; B: Gray-scale value. $n = 3$. $^{b}P < 0.01$. shCtrl: Negative control virus; shMRPL35: Lentiviral particles of MRPL35.

ARTICLE HIGHLIGHTS

Research background

Gastric carcinoma (GC) is one of the most common cancers, and the existing treatment methods cannot meet the treatment needs for GC. At the same time, MRPL35, a member of the large subunit family of mitochondrial ribosomal protein, shows the characteristics of oncogene in certain cancers.

Research motivation

The existing treatment methods for GC mainly include drugs, chemotherapy, and surgery, all of which have certain defects. Finding new therapeutic targets will be of great benefit to patients with GC.

Research objectives

The present study aimed to explore the correlation between MRPL35 and GC, and the effect of knockdown of *MRPL35* on GC cells.

Research methods

The expression of MRPL35 in GC and the effect of MRPL35 on the prognosis of GC were evaluated based on data from public databases. Immunohistochemistry staining and pathological factors analysis were performed on 64 pairs of GC tissues and matched adjacent tissues. The effect of MRPL35 on the proliferation and apoptosis of GC cells was determined by Celigo cell count assay, flow cytometry, and tumor formation experiment in BABL/c nude mice. The related proteins that changed after knockdown of *MRPL35* was identified by proteomic analysis and tested by Western blot.

Research results

The expression of MRPL35 was up-regulated in GC and high expression of MRPL35 was associated with a poor survival in GC. The expression of MRPL35 in GC tissues was increased in comparison with matched adjacent tissues, which was related to age, lymph node metastasis, and pathological tumor-node-metastasis stage. Knockdown of *MRPL35* inhibited GC cell proliferation and colony formation and induced apoptosis. After knockdown of *MRPL35*, the expression of PICK1 and BCL-XL protein decreased, and that of AGR2 protein increased.

Research conclusions

MRPL35 is up-regulated in GC, and knockdown of *MRPL35* could inhibit the proliferation of GC cells and induce apoptosis.

Research perspectives

MRPL35 can be used for targeted therapy of GC, and can also be used as a new biomarker for GC.

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ORIGINAL ARTICLE

Basic Study

Mitochondrial pathway of the lysine demethylase 5C inhibitor CPI-455 in the Eca-109 esophageal squamous cell carcinoma cell line

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Author contributions: Xue XJ analyzed the data, drafted the article, and contributed to study design; Li FR contributed to data gathering; Yu J contributed to study design, editing, and revising the paper; all authors read and approved the final manuscript.

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Abstract

BACKGROUND

Esophageal cancer is a malignant tumor of the digestive tract that is difficult to diagnose early. CPI-455 has been reported to inhibit various cancers, but its role in esophageal squamous cell carcinoma (ESCC) is unknown.

AIM

To investigate the effects and mechanism of the lysine demethylase 5C inhibitor, CPI-455, on ESCC cells.

METHODS

A methyl tetrazolium assay was used to detect the inhibitory effect of CPI-455 on the proliferation of Eca-109 cells. Apoptosis, reactive oxygen species (ROS), and mitochondrial membrane potential were assessed by flow cytometry. Laser confocal scanning and transmission electron microscopy were used to observe changes in Eca-109 cell morphology. The protein expression of P53, Bax, lysine-specific demethylase 5C (KDM5C), cleaved Caspase-9, and cleaved Caspase-3 were assayed by western blotting.

RESULTS

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Grade D (Fair): 0
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Compared with the control group, CPI-455 significantly inhibited Eca-109 cell proliferation. Gemcitabine inhibited Eca-109 cell proliferation in a concentration- and time-dependent manner. CPI-455 caused extensive alteration of the mitochondria, which appeared to have become atrophied. The cell membrane was weakly stained and the cytoplasmic structures were indistinct and disorganized, with serious cavitation when viewed by transmission electron microscopy. The flow cytometry and western blot results showed that, compared with the control group, the mitochondrial membrane potential was decreased and depolarized in Eca-109 cells treated with CPI-455. CPI-455 significantly upregulated the ROS content, P53, Bax, Caspase-9, and Caspase-3 protein expression in Eca-109 cells, whereas KDM5C expression was downregulated.

CONCLUSION
CPI-455 inhibited Eca-109 cell proliferation *via* mitochondrial apoptosis by regulating the expression of related genes.

Key Words: Lysine-specific demethylase 5C; CPI-455; Esophageal squamous cell carcinoma; Caspase; P53

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Core Tip: This study confirmed that the lysine demethylase 5C inhibitor CPI-455 inhibited the proliferation of Eca-109 esophageal squamous cell carcinoma cells. CPI-455 played a role in esophageal squamous cell carcinoma cells proliferation and invasion that may have been associated in part with p53, Bax, Caspase-9, and Caspase-3 expression. CPI-455 induced Eca-109 cell apoptosis *via* a mitochondria-mediated intrinsic apoptotic signaling pathway by regulating the expression of related genes.

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INTRODUCTION

Esophageal cancer is a common digestive tract cancer, of which esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma represent the main histological types[1,2]. The incidence of esophageal cancer is relatively high, ranking seventh worldwide. ESCC accounts for more than 90% of esophageal cancers. As it is difficult to diagnose ESCC early due to a lack of specific clinical symptoms and insufficient preventive measures, it is often diagnosed at an advanced stage and has a poor prognosis. In addition, the 5-yr postoperative survival rate is less than 50%[3-5].The lysine-specific demethylase 5C (KDM5C) inhibitor, CPI-455, regulates the apoptosis and proliferation of tumor cells by regulating the methylation state of lysine in KDM5C[6]. CPI-455 activity been reported in cervical[7], gastric[8], and prostate cancer[9], and in other diseases[10,11]; but not in the treatment of esophageal cancer. In this study, human ESCC Eca-109 cells were used as a research model to preliminarily study the effect and mechanism of CPI-455 against ESCC and to explore novel approaches for the treatment and prevention of ESCC.

MATERIALS AND METHODS

Cell lines

Eca-109 human esophageal cancer cells and HPT-1A normal human esophageal epithelial cells were provided by the Cell Resource Center of the Shanghai Life Sciences Institute, Chinese Academy of Sciences (Shanghai, China). Other materials

included CPI-455 (MSDS, 1628208-23-0; Gibco, Ltd., United States), DMEM and RPMI 1640 cell culture media and fetal bovine serum (Gibco, Ltd). Anti-human P53 (cat no. sc-6243), Bax, KDM5C (cat no. sc-81623), Caspase-9 (cat no. 9746), Caspase-3 (cat no. 9502), GAPDH (glyceraldehyde-3-phosphate dehydrogenase, cat no. sc-47778) polyclonal antibody (1:200; Abcam, United Kingdom) were used for western blotting. Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kits (cat no. F7250) were from Sigma-Aldrich (United States). polyvinylidene difluoride (PVDF) membranes were from Biyuntian Biotech (China) and bicinchoninic acid (BCA) protein quantitative detection kits were from Thermo Fisher (United States). An Epics Ultra flow cytometer (Beckman Coulter, United States), JEM-100sx transmission electron microscope (Jeol Ltd., Japan), and a TCS SP2 laser confocal microscope (Leica, Germany) were used.

Cell culture

Eca-109 cells were cultured in DMEM and HET-1A cells were cultured in RPIM-1640 medium. Both were supplemented with 10% pre-inactivated FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. Cells were adherently cultured in a 37 °C constant temperature incubator with a volume fraction of 5% CO_2 and 95% humidity; 0.25% trypsin-EDTA was used for cell digestion and passage. Cell growth in the culture flask was observed every 1-2 d, and the cell culture medium was replaced for passage culture.

Methyl tetrazolium assay

For assay of growth and the logarithmic phase, cells were collected by trypsin digestion and centrifugation and 90 mL of a single cell suspension (3×10^3/mL) were inoculated into 96-well culture plates and cultured at 37 °C in an atmosphere containing 5% CO_2. overnight to ensure adherence. When the cells grew to the appropriate density, the supernatant was discarded and 100 mL of the CPI-455 KDM5C inhibitor was added to each well, giving a final concentration of 15 mmol/L. The plates were cultured in an atmosphere containing 5% CO_2 at 37 °C for 0, 24, 48, 72, and 96 h (5 wells/time point). Following this, the cells were collected and centrifuged at 150 *g* for 10 min. The supernatant was discarded and the precipitate was washed once with phosphate buffered saline (PBS). A total of 200 μL of serum-free RPMI-1640 medium was added, followed by addition of 10 μL of methyl tetrazolium (MTT; 5 mg/mL). Following culture for a further 4 h, the supernatant was removed and 150 μL DMSO was added to each well to dissolve the purple crystals. The optical density (OD) was read at 490 nm after the crystals had completely dissolved. The survival rate was calculated as S% = (OD of the experimental group/OD of the control group) × 100.

Laser confocal scanning microscope assay of the ectropion of membrane phosphatidylserine

The fluorescence of cell smears and suspension cultures after polylysine treatment was observed by confocal microscopy. Red (488/590 + 42 nm) and green (488/530 + 30 nm) dual channel detection and differential interference contrast imaging were performed. A total of 100 fields of vision were screened and 30 typical fields of vision were selected for inclusion in the preliminary statistical analysis. Annexin V-FITC (fluorescein isothiocyanate), PI double staining was performed and the fluorescence of Eca-109 cell membranes and necrotic nuclei was observed and analyzed as described above. DAPI (4', 6-diamidine-2-phenylindole, dihydrochloride) was used to stain the nuclei in cell smears and glycerol patches and analyzed by fluorescence microscopy, as described above.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay of nucleation and DNA fragmentation of Eca-109 cells

Eca-109 cells (5×10^6/mL) were seeded in 24-well plates and cultured for 48 h. Cell smears were prepared from 50 μL of cell suspension on polylysine-treated slides that were fixed with 4% paraformaldehyde at room temperature for 30-60 min and washed twice with PBS. After treatment with terminal deoxynucleotidyl transferase and termination, the preparations were washed four times with PBS and incubated with FITC-labeled antibody for 30 min. Nuclei were stained with PI (1.0 g/mL) and the preparations were sealed with glycerin-mounted coverslips and marked. The cells were observed with a confocal laser scanning microscope, with FITC and PI dual channel fluorescence at an excitation wavelength of 490 nm and an emitted wavelength of 520 nm).

Flow cytometry assay of reactive oxygen species (ROS)

Eca-109 cells were cultured in a six-well plates (3×10^3 cells/well), treated with 15 μmol/L CPI-455 for 48 h, and collected. The cells were washed three times in PBS, centrifuged for 5 min, and the supernatant was discarded. The cells were resuspended in serum-free medium, dichloro-dihydro-fluorescein diacetate (DCFH-DA) was added to a final concentration of 10 mmol/L, and the plates were incubated at 37 °C for 40 min. The cells were centrifuged for 10 min, and the pellet was collected, washed, and resuspended in precooled PBS. The cells were centrifuged to obtain a pellet and were stained for fluorescence detection. Each assay was performed in triplicate.

Flow cytometry assay of mitochondrial membrane potential

Cells were harvested (2×10^6/mL) at scheduled times after treatment with 15 μmol/L CPI-455 for 48 h. The Mitocapture dye and antibody incubation solutions were freshly prepared. The cells were washed and centrifuged and the mitochondria were stained. The cells were immediately transferred to collecting tubes for flow cytometry.

Western blotting assay of P53, Bax, KDM5C, cleaved Caspase-9, and cleaved Caspase-3

Eca-109 cells were cultured for 48 h, collected, and centrifuged at 150 *g* for 5 min (r = 6 cm). Total protein was extracted using a protein extraction kit. The supernatant was collected, and the protein concentration was determined using a BCA protein kit. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. After blocking in 5% skim milk at room temperature for 2 h, anti-human mouse P53, Bax, KDM5C, Caspase-9, Caspase-3, and GAPDH monoclonal antibodies (1:1000; Santa Cruz Biotechnology, Inc., United States) were added and the membranes were incubated overnight at 4 °C and then with horseradish peroxidase (HRP)-labeled mouse secondary antibody (1:3,000; Origene Technologies Inc., China). The films were developed, fixed, scanned and analyzed using Image J software to calculate the gray values. Protein expression of the target protein was reported relative to GAPDH protein expression.

Statistical analysis

GraphPad Prism 6.0 software was used to statistical analyze the data. The data were reported as means ± SD. All assays were repeated independently three times. Between-group differences were compared with independent samples *t*-tests. One-way analysis of variance was used to compare data with normal distributions. Least significant difference (LSD)-*t*-tests were used for pairwise comparisons of multiple groups. The significance level was = 0.05.

RESULTS

CPI-455 inhibits Eca-109 cell proliferation

The MTT results demonstrated that CPI-455 significantly inhibited the proliferation of Eca-109 cells in a time- and a concentration-dependent manner. The CPI-455 LD_{50} was 15 mol/L CPI-455 at 48 h. Therefore, the 48 h concentration of 15 mol/L CPI-455 was selected for the follow-up procedures (Figure 1).

CPI-455 induces Eca-109 cell apoptosis

The laser confocal scanning microscopy results showed that prior to CPI-455 treatment, the Eca-109 cells had developed a complete mitochondrial structure. Mitocapture revealed a bright red color and "pipe-like network" that surrounded the nuclear lobule and was distributed throughout the entire cell. Following treatment with CPI-455 (15 μmol/L) for 48 h, both groups showed the presence of dye in the cytoplasm, with red mitochondrial staining and green cytoplasmic staining. Some cells contained visible "point and flake" red-stained mitochondria scattered in the cytoplasm, not present in clusters surrounding the nucleus, and gradually merging to form a "structureless aggregation". Substantial changes to the mitochondrial structure were observed, which appeared to indicate mitochondrial atrophy. Following CPI-455 treatment, the Eca-109 cell membranes were weakly stained and the internal cytoplasmic structures indistinct and disorganized, with severe cavitation (Figure 2A and B).

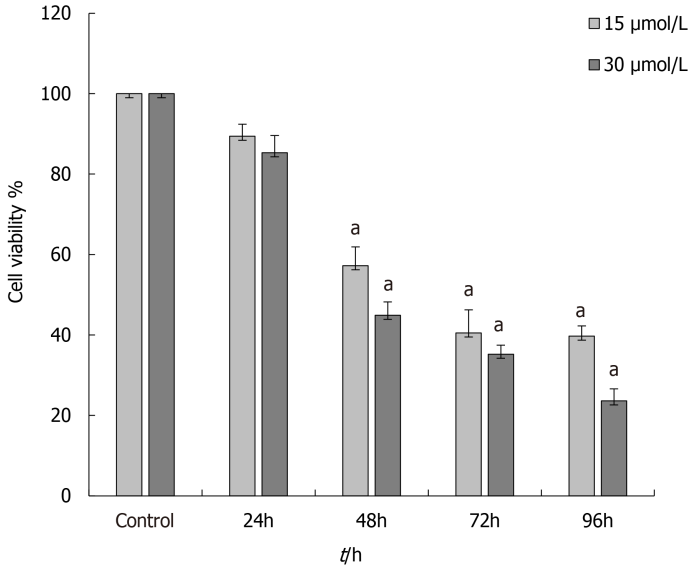


Figure 1 Eca-109 cell viability following treatment with CPI-455 at different times and concentrations. Data are expressed as means ± SE (n = 3). [a]P < 0.05 *vs* control.

TUNEL and laser confocal scanning microscopy showed green fluorescence generated by the combination of FITC and the staining of 3'-OH terminal fragments produced by DNA fragmentation in the nucleus of apoptotic cells. Red fluorescence was emitted by PI-stained nuclei, which revealed the location of cells. Double-positive cells were seen in cells treated with CPI-455. Most Eca-109 cells in the CPI-455 group were double-positive for FITC and PI fluorescence. Most Eca-109 cells in the control group were negative for FITC and positive for PI, indicating that TUNEL assay of the CPI-455-stimulated group was positive. In terms of cell morphology, compared with the control group, most Eca-109 cells in the control group had a lower nuclear staining density, an unclear nuclear boundary, and larger nuclei. However, most ECA-109 cells in gemcitabine group had dense chromatin and small nuclei (Figure 2C and D).

CPI-455 upregulates ROS content, P53, Bax, Caspase-9, and Caspase-3 expression and downregulates KDM5C expression and mitochondrial membrane potential in Eca-109 cells

Flow cytometry was used to detect the effect of CPI-455 on the level of ROS in Eca-109 cells. With an extension of the induction time, the level of intracellular ROS in the Eca-109 cells was increased significantly, with statistically significant differences observed at 24, 48, and 72 h (P < 0.01, Figure 3). Compared with the control group, the mitochondrial membrane potential was depolarized and decreased (P < 0.01, Figure 4). The western blot results found increased expression of p53, Bax, Caspase-9, and Caspase-3 at 24, 48, and 72 h following CPI-455 treatment (Figure 5). However, KDM5C protein expression in the treated cells was significantly decreased compared with the controls (P < 0.01, Figure 6).

DISCUSSION

Esophageal cancer is a digestive tract malignant tumor that is difficult to diagnose early. The most common treatments are surgery, radiotherapy, chemotherapy, and biologically targeted therapy[12]. The prognosis remains poor. The occurrence and development of esophageal cancer is complex and involves multiple gene changes, commonly resulting from histone methylation and epigenetic regulation of tumor-related genes.

CPI-455 is a KDM5C inhibitor that modulates the apoptosis and proliferation of tumor cells by regulating the lysine methylation status KDM5C. Recent reports have confirmed that the anti-tumor effect of CPI-455 in ovarian, breast, stomach, lung, and liver cancer is achieved by inducing the apoptosis and autophagy of tumor cells *via* modulating KDM5C gene transcription and expression[13,14]. In this study, the anti-Eca-109 effect of CPI-455 was studied and the associated mechanism was explored.

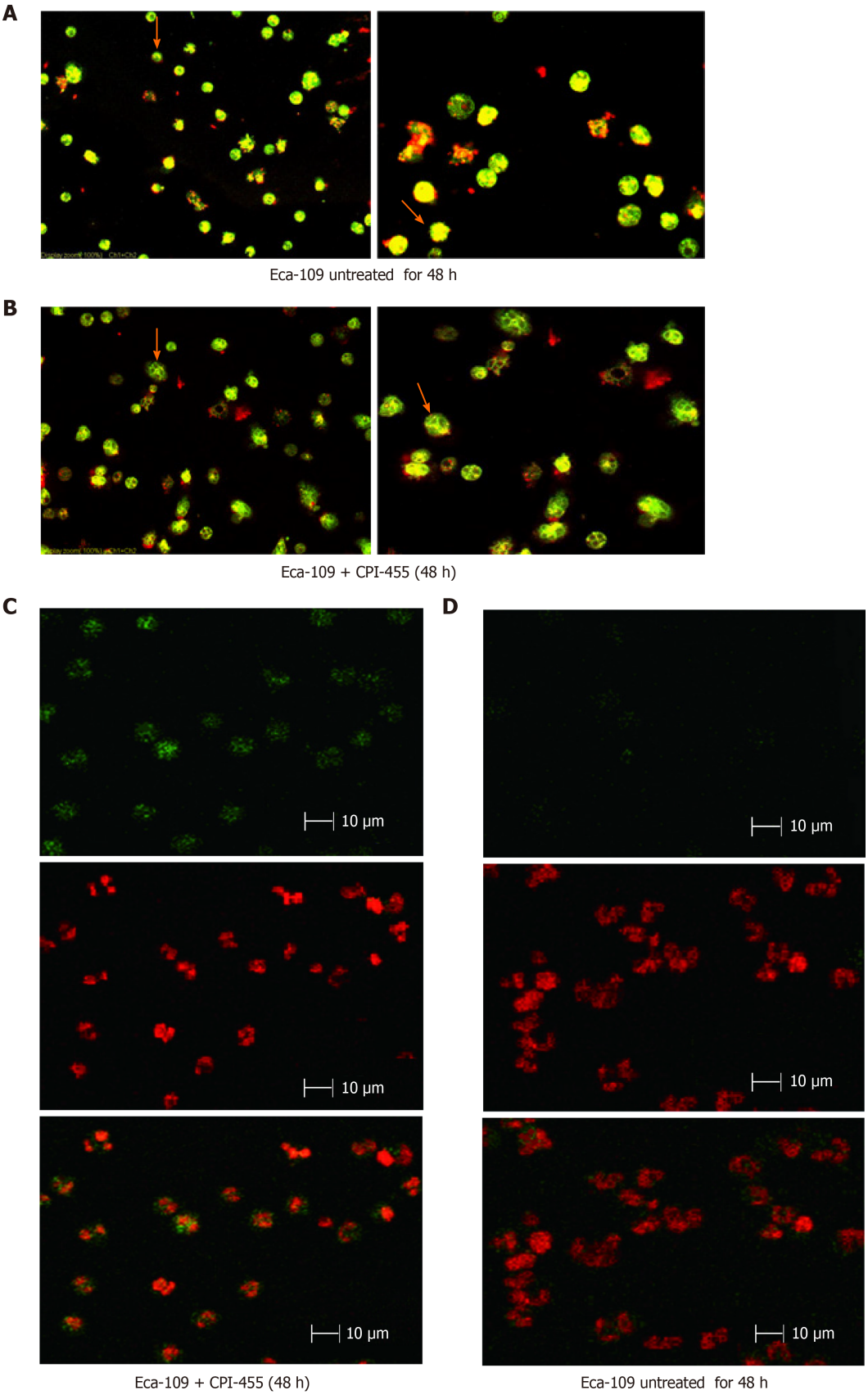


Figure 2 Changes in Eca109 cells after 48 h culture with and without CPI-455 treatment. Left panels show the morphology of the mitochondria of Eca109 cells. A: Arrows indicate mitochondria with complete and clear structure. B: Arrows indicate mitochondria with a fuzzy structure and weak staining of cells. Right panels show TUNEL assay results (bar = 10 μm) and laser confocal scanning microscopy × 600 of nuclear staining and DNA fragmentation of Eca109 cells C: without, and D: with CPI-455 treatment. Data are reported as means ± SE (n = 3).

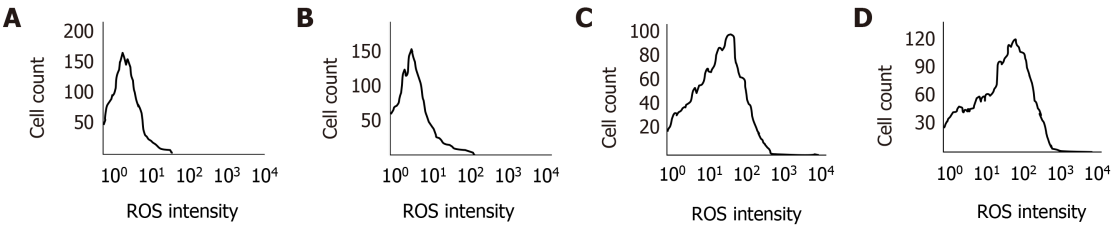


Figure 3 Eca-109 induced by CPI-455 is dependent on the generation of reactive oxygen species. A: Eca109 untreated (0 h); B: Eca-109 induced by CPI-455 for 24 h; C: Eca-109 induced by CPI-455 for 48 h; D: Eca-109 induced by CPI-455 for 72 h. Data are reported as means ± SE (n = 3). ROS: Reactive oxygen species.

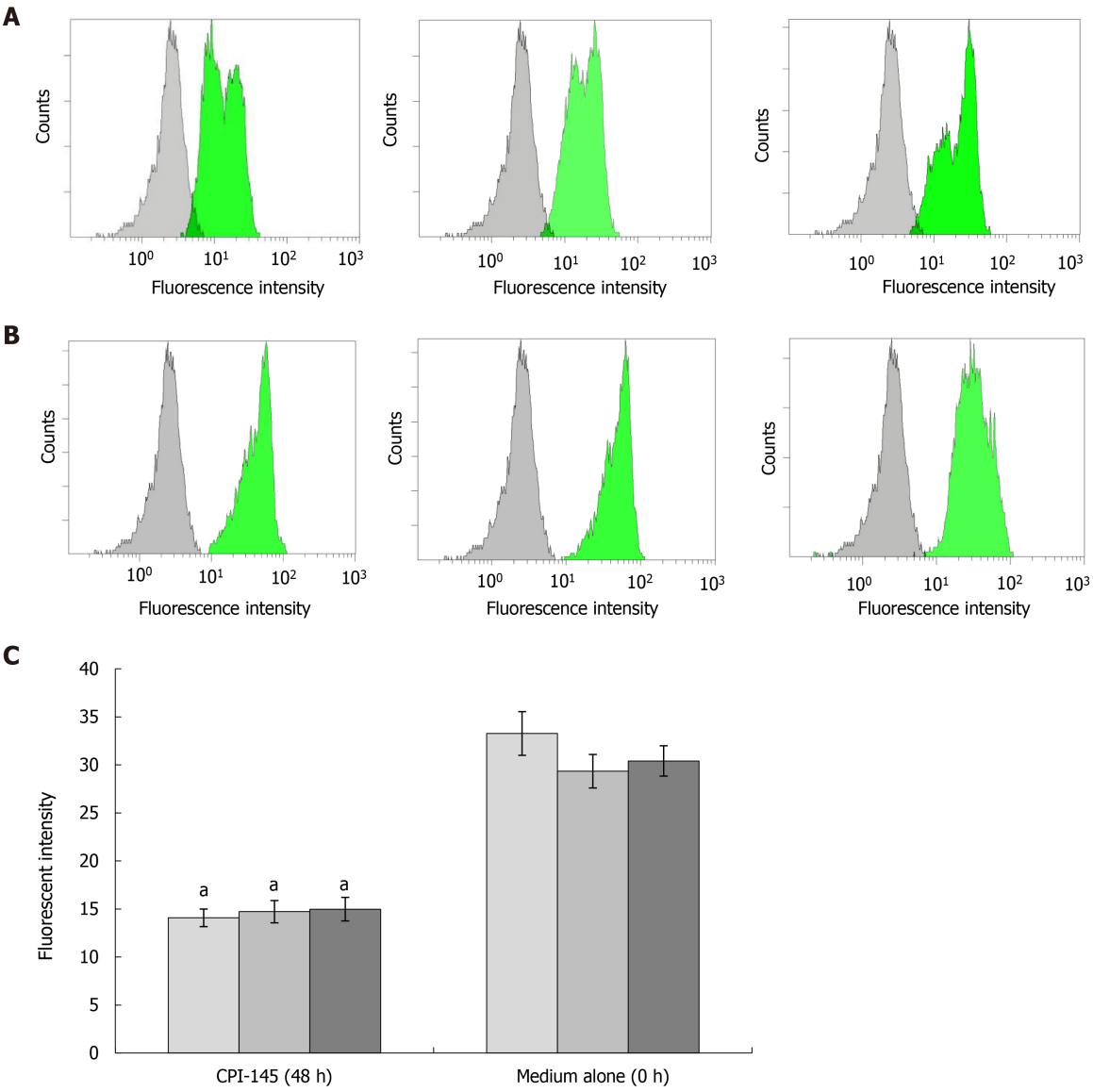


Figure 4 Mitochondrial membrane potential assayed by flow cytometry. A: Eca109 untreated (0 h); B: Eca109 treated with CPI-455 for 48 h; C: Gray scale results of membrane potential energy of mitochondria (Blue: Results of the first assay; Red: Results of the second assay; Green: Results of the third assay). Data are reported as means ± SE (n = 3). $^{a}P < 0.05$ *vs* control.

The study results found that CPI-455 inhibited the proliferation of esophageal cancer cells in a time-and concentration-dependent manner. However, at its effective concentration, CPI-455 was less toxic to normal esophageal cells than to Eca-109 cells. The inhibitory effect of CPI-455 on Eca-109 cell proliferation was confirmed by the assay of cell-membrane permeability by laser confocal scanning microscopy. This finding indicates that CPI-455 has favorable effects by inhibiting Eca-109 cell

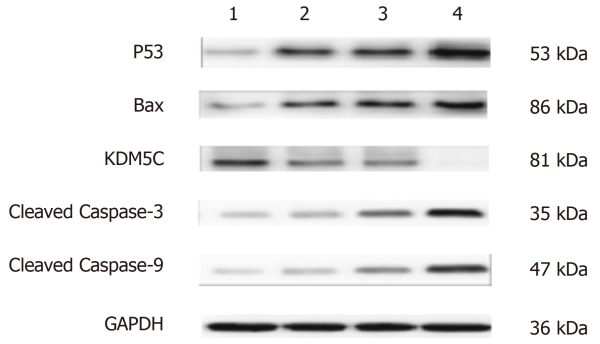


Figure 5 Western blot assays of p53, Bax, lysine-specific demethylase 5C, cleaved Caspase 3, and cleaved Caspase 9 protein expression in Eca-109 cells. Lane 1: Eca109 untreated (0 h); Lane 2: Eca109 induced by CPI-455 for 24 h; Lane 3: Eca109 induced by CPI-455 for 48 h; Lane 4: Eca109 induced by CPI-455 for 72h. KDM5C: Lysine-specific demethylase 5C; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

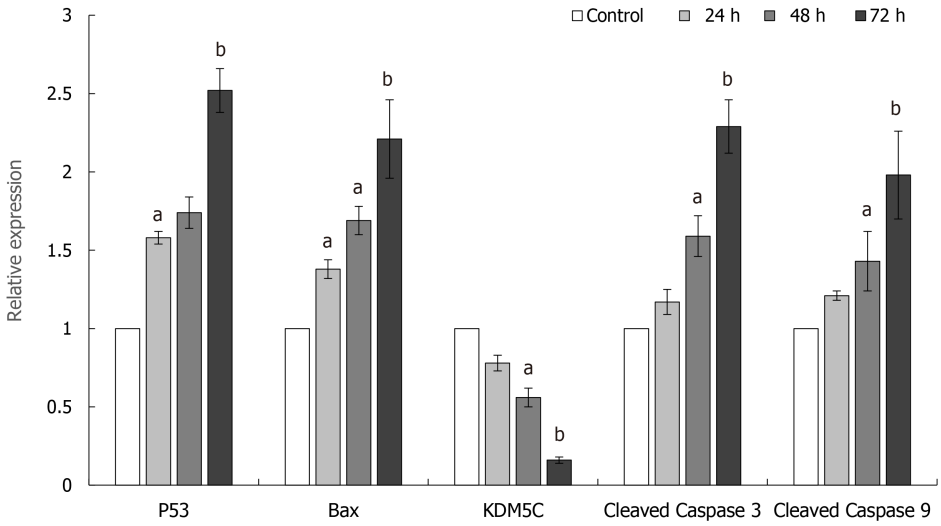


Figure 6 Densitometry analysis of the immunoblotting data of P53, Bax, lysine-specific demethylase 5C, cleaved Caspase 3, and cleaved Caspase 9 proteins in Eca-109 cells. Data are reported as means ± SE (n = 3). $^{a}P < 0.05$ *vs* control; $^{b}P < 0.01$ *vs* control.

proliferation and growth, as well as the selective killing on ESCC cells (Figures 1 and 2).

Flow cytometry was used to determine the ROS content of Eca-109 cells, which is considered to be the by-product of oxygen consumption and cell metabolism. Previous studies have found that various chemotherapy drugs can induce an increase in the ROS content in tumor cells, and that at a certain level, ROS can induce the apoptosis of tumor cells[15-18]. Decrease of the mitochondrial membrane potential is the initial manifestation of the hypoxia apoptosis cascade[19]. In this study, the level of intracellular ROS significantly increased in treated Eca-109 cells (Figure 3), and depolarization of mitochondrial membrane was increased compared with controls (Figure 4). CPI-455 significantly induced the apoptosis of Eca-109 cells.

Previous studies have confirmed that the mechanism by which tumor cell apoptosis is induced varies widely depending on the various pathways that are involved. These includes mitochondrial pathways, which play an important role in the maintenance of stability, and those involving activation of multiple upstream and downstream genes (*e.g.*, p53 and Bax). Activation of Caspase-2, 3, and 9 on the mitochondrial membrane is linked to the activation of mitochondrial apoptotic pathways[22-24].

Therefore, the inhibitory effect of the KDM5C inhibitor, CPI-455, on Eca-109 cell proliferation may be related to the downregulation of KDM5C expression, activation of Caspase-3 and Caspase-9, change in mitochondrial membrane permeability, promotion of ROS production, depolarization of the mitochondrial membrane, release of the proapoptotic proteins, Bax and p53, in the mitochondria into the cytoplasm, and induction of tumor cell apoptosis (Figures 5 and 6). The above results indicate that CPI-455 induced apoptosis through an ROS-dependent mitochondrial signaling

pathway[25].

Our study had some limitations, the mechanism involved in inducing apoptosis of tumor cells is extensive, and the mitochondrial apoptosis pathway is only one of them that plays an important role in maintaining cell stability. The mechanism has not been fully elucidated and needs further study. The results of this study provide a theoretical basis for the application of CPI-455 for the treatment of ESCC.

CONCLUSION

CPI-455 inhibited ECA-109 cell proliferation *via* the mitochondrial apoptosis pathway by regulating the expression of related genes.

ARTICLE HIGHLIGHTS

Research background

Esophageal cancer is a malignant tumor of the digestive tract that is difficult to diagnose early. Although CPI-455 is reported to inhibit various cancers, the role of CPI-455 in esophageal squamous cell carcinoma (ESCC) is unknown.

Research motivation

KDM5C, a lysine-specific demethylase 5C, inhibited ESCC proliferation and invasion, which may be partly associated with the expression of p53, Bax, Caspase-9, and Caspase-3. Mitochondria-mediated intrinsic apoptotic signaling plays an important role in the process of Eca-109 cell apoptosis induced by CPI-455.

Research objectives

The study objective was to investigate the effect and mechanism of the KDM5C inhibitor, CPI-455, on ESCC cells.

Research methods

The changes in proliferation, apoptosis, ROS content, and mitochondrial membrane potential of Eca-109 cells induced by CPI-455 were observed. The expression of P53, Bax, Caspase-9, Caspase-3, and KDM5C in Eca-109 cells was assayed.

Research results

CPI-455 inhibited Eca-109 cell proliferation. P53, Bax, Caspase-9, and Caspase-3 were upregulated in Eca-109 cells, and KDM5C was significantly downregulated, by CPI-455. CPI-455 increased the level of intracellular ROS in Eca-109 cells, and decreased the mitochondrial membrane potential compared with the control group.

Research conclusions

CPI-455 inhibited Eca-109 cell proliferation *via* the mitochondrial apoptosis pathway by regulating the expression of related genes.

Research perspectives

CPI-455 might be a potential candidate for the development of novel chemotherapeutic agents to treat ESCC.

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ORIGINAL ARTICLE

Observational Study

Health-related quality of life after curative resection for gastric adenocarcinoma

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Abstract

BACKGROUND

With improved survival in gastric cancer patients, health-related quality of life has become an important clinical endpoint alongside primary oncological outcomes.

AIM

To investigate health-related quality of life after various surgical procedures for gastric cancer treatment.

METHODS

The validated Slovenian version of the European Organization for Research and Treatment of Cancer Quality of Life Core Questionnaire (QLQ-C30) and its gastric cancer-specific module (QLQ STO-22) was sent for self-completion to patients that underwent curative resection for gastric adenocarcinoma between January 2014 and December 2018 at our centre. In total, 116 patients responded. Scores were compared between patients after subtotal distal *vs* total gastrectomy and patients after subtotal distal gastrectomy with Billroth II *vs* Roux-en-Y reconstruction.

RESULTS

Interestingly, the extent of resection did not influence daily functioning; however, more dysphagia and eating restrictions were reported in patients after total gastrectomy when compared to patients after subtotal distal gastrectomy. Moreover, patients with Billroth II reconstruction after subtotal distal resection experienced worse physical and role functioning and reported more pain, fatigue and reflux compared to Roux-en-Y reconstruction.

CONCLUSION

Based on our results, Roux-en-Y reconstruction after subtotal distal gastrectomy

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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should be preferred over Billroth II reconstruction. The data obtained from this study will help surgeons when preoperatively informing their patients about expected functional outcomes after gastrectomy and enable them to ensure proper supportive care of their patients in the postoperative period.

Key Words: Gastric cancer; Quality of life; Roux-en-Y; Billroth II; Gastrectomy

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Core Tip: Quality of life assessment is an important tool to guide and evaluate treatment interventions, especially after a major surgery like gastrectomy. We conducted a cross-sectional survey among patients with gastric adenocarcinoma treated at our centre to provide insight into overall wellbeing after curative resection. The information provided will guide surgeons in selecting an optimal treatment approach and informing patients about expected treatment outcomes.

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INTRODUCTION

Gastrectomy is a major operation that alters the physiological functions of the digestive tract. Consequently, patients that undergo this treatment commonly experience a broad range of metabolic disorders, including malnutrition, weight loss and several postgastrectomy symptoms that negatively impact patients' wellbeing[1]. With modern gastrectomy techniques and other treatment modalities, survival in gastric cancer patients has improved and health-related quality of life (HRQoL) has become an important aspect when evaluating treatment outcomes[2].

Various surgical procedures have been described for achieving oncological radicality in gastric cancer. In previous comparative studies, subtotal distal gastrectomy was associated with shorter operative duration, reduced postoperative complications and better recovery when compared to total gastrectomy[3-6]. On the other hand, total gastrectomy could still be performed safely with low morbidity while reducing the risk of inadequate safety margins or remnant carcinoma[7,8]. Following subtotal distal gastrectomy, there is no consensus regarding the best type of reconstruction[9,10]. Billroth II reconstruction is often performed due to its simplicity, but associated reflux gastritis and esophagitis have been the limiting concerns. Roux-en-Y reconstruction is a recommended alternative, but it can result in delayed gastric emptying, nausea, vomiting and abdominal pain[11]. Selection of the procedure is usually made based on tumour location, preoperative staging, the patient's general physical status and the surgeon's preference. In addition, quality of life assessment has become an increasingly important index for evaluating and selecting treatment interventions. Patient-reported measures regarding their physical and emotional state after treatment as opposed to objectively defined short-term perioperative outcomes should be taken into consideration to achieve optimal care.

We conducted a cross-sectional survey among patients with gastric adenocarcinoma treated at a tertiary referral centre to provide insight into overall wellbeing after curative resection. The European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Core Questionnaire (QLQ-C30) and its gastric cancer-specific module (QLQ STO-22) were used to assess HRQoL[12]. Its various aspects were compared among different surgical procedures.

MATERIALS AND METHODS

Patients

Patients with gastric cancer that underwent curative resection at the Ljubljana University Medical Centre between January 2014 and December 2018 were recruited for the study. Patients were eligible for inclusion if they were older than 18, had undergone curative subtotal distal or total gastrectomy for histologically confirmed adenocarcinoma, and had not undergone previous gastric surgery. We excluded patients that were deemed unable to answer the questionnaires, provided questionnaires with missing items that prevented final scoring, suffered from other gastrointestinal diseases (*e.g.*, chronic inflammatory bowel disease, exocrine pancreatic insufficiency or cholestasis) or other malignant diseases, had undergone emergency or palliative surgery, or had experienced disease recurrence. The study was approved by the National Medical Ethics Committee of Republic of Slovenia (No. 0120-315/2019/3; July 9, 2019). Patients that met the inclusion criteria and provided written informed consent were included for further analysis.

Data collection

Data on the patients included demographics, comorbidities, American Society of Anesthesiologists score, tumour stage, type of resection, type of reconstruction, postoperative complications, hospital stay, histopathological characteristics of the tumour, cancer recurrence and possible (neo)adjuvant therapy and were collected from electronic patient records.

Surgery

All patients underwent open gastrectomy, either subtotal distal or total, depending on the tumour location and preoperatively determined tumor-node-metastasis stage. Gastric adenocarcinoma was confirmed upon histopathological examination of the specimen.

After total gastrectomy, reconstruction was performed with Roux-en-Y esophagojejunostomy in all cases. After subtotal distal gastrectomy, either a Billroth II or Roux-en-Y anastomosis was constructed. Braun enteroenterostomy was routinely performed in all cases of Billroth II gastrojejunostomy. Billroth I reconstruction was not performed in our patient cohort due to the surgeon's preference and lack of early-stage gastric cancer.

HRQoL assessment

To assess postoperative HRQoL of our patient cohort, the validated Slovenian versions of the EORTC QLQ-C30 (version 0.3) and QLQ STO-22 were used[12].

The EORTC QLQ-C30 questionnaire consists of 30 questions divided into five functional scales (physical, role, cognitive, emotional and social), three symptom scales (fatigue, pain, and nausea and vomiting), the global health status/quality of life scale and six single items to report other complaints (dyspnoea, loss of appetite, insomnia, constipation, diarrhoea and financial difficulties).

The EORTC QLQ-STO22 is a gastric cancer–specific module to assess HRQoL of patients with adenocarcinoma of the stomach. It comprises 22 questions divided into five multi-item scales (dysphagia, chest and abdominal pain, reflux, eating restrictions and anxieties) and four single items (dry mouth, taste problems, body image and hair loss).

A study description with an informed consent form plus the EORTC QLQ-C30 and EORCT QLQ-STO-22 questionnaires were sent to the patients for self-completion.

All completed questionnaires were scored and linearly transformed to a 0-100 scale according to the EORTC QLQ-C30 Scoring Manual[13]. On the functional scale higher scores represent better functioning, whereas on the symptom scale higher scores indicate higher symptom burden. Missing values were processed as follows: if at least half of the items from the scale were answered, the missing items were assumed to have values equal to the average of the completed items on the scale.

HRQoL was compared between patients after subtotal distal *vs* total gastrectomy and between patients after subtotal distal gastrectomy with Billroth II *vs* Roux-en-Y reconstruction.

Statistical analysis

Means with standard deviation as well as medians with interquartile ranges of EORTC QLQ-C30 and EORTC QLQ-STO-22 scores were obtained. The Shapiro–Wilk test was used to test the normal distribution of the data.

The time that elapsed from surgery to completion of the questionnaires was compared among different surgical procedures using the Kruskal-Wallis test and Mann-Whitney *U* test.

Scores of EORTC QLQ-C30 and EORTC QLQ-STO-22 of patients after subtotal distal *vs* total gastrectomy and Billroth II *vs* Roux-en-Y reconstruction were compared with the Mann-Whitney *U* test.

To assess the correlation between the type of operation and general health status adjusted for some demographic and clinical characteristics of patients, multiple linear regression was used. The assumptions of absence of multicollinearity (assessed by variance inflation factor), normal distribution of the residuals and homoscedasticity were met.

A double-sided P value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 27.0 (IBM Corporation, Armonk, NY, United States).

RESULTS

Patients

Invitations for study participation were sent to 234 patients. A total of 116 (49.6%) patients that provided informed consent with completed questionnaires were further analyzed. There were 63 men and 53 women 44 to 88 years old. Ten questionnaires had missing items. In six questionnaires, only one item was missing. In four questionnaires, there were two missing items, which were not part of the same scale.

The time that elapsed from surgery to completion of the questionnaires is shown in Table 1 and was not statistically different among different surgical procedures (P = 0.161).

Respondents and non-respondents did not differ significantly according to sex, type of gastric resection, type of reconstructive procedure and postoperative complications. Respondents were younger (P = 0.016) and had a significantly less advanced disease stage (P = 0.001). The baseline characteristics of patients eligible for inclusion are presented in Table 2.

EORTC QLQ-C30 questionnaire evaluation

Patients after total *vs* subtotal distal gastrectomy: No statistically significant differences were observed in HRQoL of patients after total gastrectomy when compared to patients after subtotal distal gastrectomy. The details are shown in Table 3.

Patients after subtotal distal gastrectomy with Billroth II *vs* Roux-en-Y reconstruction: HRQoL of patients after subtotal distal gastrectomy with Billroth II reconstruction was significantly lower on the physical (P = 0.038) and role functioning (P = 0.034) scale when compared to patients with Roux-en-Y reconstruction. Patients with Billroth II reconstruction also reported more pain (P = 0.01) and fatigue (P = 0.028). No differences were observed between the two groups in global health status/quality of life scores (P = 0.635). The details are summarized in Table 4.

Reported scores on different functional scales and the global health status/quality of life scale among various surgical procedures are presented in Figure 1. Patients after subtotal distal gastrectomy with Roux-en-Y reconstruction scored highest on cognitive, role functioning and physical scales when compared to patients after total gastrectomy or subtotal distal gastrectomy with Billroth II reconstruction.

Quality of life and type of surgery: The type of surgery was significantly associated with the stage of the disease (P = 0.002) (Table 5). Patients with stage III gastric adenocarcinoma underwent either total gastrectomy (43.9%) or subtotal distal gastrectomy with Billroth II reconstruction (41.3%) rather than Roux-en-Y reconstruction (22.2%).

When adjusted for demographic data, disease stage and postoperative complications in a multiple linear regression model, no statistically significant differences in the global health status/quality of life scale were observed among different surgical procedures. Global health status/quality of life scores were significantly negatively associated with disease stage (β = -0.21, P = 0.029; Table 6).

Patients with Roux-en-Y reconstruction had significantly higher scores on the emotional (β = 0.24, P = 0.041), role functioning (β = 0.24, P = 0.034) and physical scale (β = 0.23, P = 0.048) when compared to patients after Billroth II reconstruction, even

Table 1 The time elapsed from surgery to completion of the questionnaires among different surgical procedures

	Median (IQR), yr
Total gastrectomy	3 (2-4)
Subtotal distal gastrectomy	3 (2-4)
Billroth II reconstruction	3 (1-4)
Roux-en-Y reconstruction	2.5 (1-4)

The difference among total gastrectomy, subtotal distal gastrectomy with Billroth II reconstruction and Roux-en-Y reconstruction was not statistically significant (P = 0.161, Kruskal-Wallis test), nor was the difference among total and subtotal distal gastrectomy (P = 0.056, Mann-Whitney U test). IQR: Interquartile range.

Table 2 Baseline characteristics of patients eligible for inclusion (n = 234)

	Respondents (n = 116)	Non-respondents (n = 118)	P value
Gender, n (%)			0.192
Male	63 (54.3)	74 (62.7)	
Female	53 (45.7)	44 (37.3)	
Age at surgery (yr, median, IQR)	66 (58-74)	72 (63-78)	0.016
Performed procedure, n (%)			0.299
Total gastrectomy	43 (37.1)	55 (46.6)	
Distal-Billroth II	26 (22.4)	20 (16.9)	
Distal-Roux-Y	47 (40.5)	43 (36.4)	
Postoperative complications, n (%)			
Yes	28 (24.1)	38 (32.2)	0.170
TNM stage, n (%)			0.001
0	3 (2.6)	4 (3.4)	
I	52 (44.8)	25 (21.2)	
II	32 (27.6)	36 (30.5)	
III	29 (25.0)	53 (44.9)	

IQR: Interquartile range; TNM: Tumor-node-metastasis.

when adjusted for other variables in a regression model (Table 7).

EORTC QLQ STO-22 questionnaire evaluation

Patients after total *vs* subtotal distal gastrectomy: Patients after total gastrectomy reported more dysphagia (P = 0.020) and eating restrictions (P = 0.017) when compared to patients after subtotal distal gastrectomy. No differences were found in other scales of the EORTC QLQ STO-22 questionnaire (Table 8).

Patients after subtotal distal gastrectomy with Billroth II *vs* Roux-en-Y reconstruction: Patients after subtotal distal gastrectomy with Billroth II reconstruction reported more problems with reflux when compared to patients with Roux-en-Y reconstruction. No differences were found in other scales of the EORTC QLQ STO-22 questionnaire (Table 9).

DISCUSSION

The results of this cross-sectional survey show that the type of gastric resection influences different aspects of HRQoL. Patients after total *vs* subtotal gastrectomy had similar functional scores, but the former experienced more dysphagia and eating

Table 3 European Organization for Research and Treatment of Cancer Quality of Life Core Questionnaire (QLQ-C30) questionnaire scores by the type of resection (Mann-Whitney *U* test)

	Subtotal gastrectomy (*n* = 73)		Total gastrectomy (*n* = 43)		*P* value
	mean (SD)	Median (IQR)	mean (SD)	Median (IQR)	
Functional scales					
Physical functioning	79.9 (21.4)	87 (67-100)	80.3 (19.5)	87 (73-100)	0.954
Role functioning	80.2 (26.7)	100 (67-100)	77.9 (26.1)	83 (67-100)	0.509
Cognitive functioning	81.9 (21.6)	83 (67-100)	82.9 (19.7)	83 (67-100)	0.842
Emotional functioning	82.1 (20.5)	92 (75-100)	76.1 (21.5)	79 (58-96)	0.116
Social functioning	81.5 (22.5)	83 (67-100)	73.7 (26)	83 (50-100)	0.115
Symptoms					
Dyspnoea	10.5 (22.1)	0 (0-0)	10.8 (21.5)	0 (0-0)	0.869
Insomnia	28.7 (29.1)	33 (0-33)	27.8 (28.1)	33 (0-33)	0.917
Appetite loss	10 (19.7)	0 (0-0)	12.3 (20.6)	0 (0-33)	0.489
Nausea/vomiting	10.7 (17.4)	0 (0-17)	4.7 (9.8)	0 (0-0)	0.079
Constipation	10.5 (22.1)	0 (0-0)	6.9 (13.6)	0 (0-0)	0.665
Diarrhoea	13.6 (22.7)	0 (0-33)	19.3 (24.4)	0 (0-33)	0.141
Fatigue	30.5 (23.3)	22 (17-33)	34.2 (23.4)	33 (22-50)	0.263
Pain	18.3 (22.4)	17 (0-33)	17.9 (21.6)	17 (0-33)	0.906
Financial problems	16.4 (26.7)	0 (0-33)	27.1 (32.8)	0 (0-67)	0.069
Global health status	66.6 (22.9)	67 (50-83)	67.4 (19.9)	67 (58-83)	0.846

IQR: Interquartile range; SD: Standard deviation.

restrictions. At the same time, patients after subtotal gastrectomy with Billroth II (compared to Roux-en-Y) reconstruction reported worse physical and role functioning scores and complained of symptoms such as pain, fatigue and reflux. However, these differences appear to be clinically less relevant because similar global health scores were reported by patients after different surgical procedures. The information provided should guide the surgeon on the optimal treatment approach after considering oncological feasibility of the technique. Moreover, it should be used to inform patients about expected functional sequelae.

Previous studies evaluated longitudinal changes of HRQoL after gastrectomy for gastric cancer and used preoperative scores as a reference[14-21]. However, these scores are highly influenced by circumstances surrounding the diagnosis as well as symptoms associated with the disease itself, such as nausea and vomiting, dysphagia, postprandial fullness, loss of appetite, fatigue due to anaemia and so on, resulting in worse HRQoL[16,17]. In their multicentre study, Brenkman *et al*[22] compared EORTC QLQ-C30 scores of patients after gastrectomy to a Dutch reference population and concluded that global HRQoL is more or less comparable between the two cohorts despite patients' worse scores on several functional and symptom scales. Similarly, Lee *et al*[23,24] found no significant difference in global HRQoL between patients more than 5 years after surgery for gastric cancer and healthy volunteers awaiting a routine screening exam.

A limited number of studies focused on HRQoL after various surgical procedures[5,11,15,16,18,20]. Hence, we opted to conduct a cross-sectional analysis to compare HRQoL after various types of gastric resection and to evaluate the actual life quality deviation caused by surgical treatment. Based on our results, the type of reconstruction appeared to have a greater effect on HRQoL than the extent of gastric resection, which is somehow unexpected. Our data show that proximal gastric preservation has marginal advantages for improving patients' quality of life by reducing dysphagia and eating restrictions postoperatively whereas no differences in daily functioning were found. In line with our finding, subtotal distal gastrectomy was generally better tolerated in several previous studies, especially due to a higher

Table 4 European Organization for Research and Treatment of Cancer Quality of Life Core Questionnaire (QLQ-C30) questionnaire scores by the type of reconstruction (Mann-Whitney *U* test)

	Billroth II (*n* = 26)		Roux-Y (*n* = 47)		*P* value
	mean (SD)	Median (IQR)	mean (SD)	Median (IQR)	
Functional scales					
Physical functioning	73.1 (23)	73.5 (53-93)	83.7 (19.6)	92 (73-100)	0.038
Role functioning	70.5 (31.4)	67 (50-100)	85.5 (22.4)	100 (67-100)	0.034
Cognitive functioning	76.9 (26.3)	83 (67-100)	84.7 (18.3)	83 (83-100)	0.301
Emotional functioning	74.9 (27.7)	83 (58-100)	86 (14)	92 (75-100)	0.220
Social functioning	79.5 (25.9)	83 (67-100)	82.6 (20.5)	83 (67-100)	0.792
Symptoms					
Dyspnoea	16.7 (30.3)	0 (0-33)	7 (15.4)	0 (0-0)	0.285
Insomnia	37.1 (33.2)	33 (0-67)	24 (25.8)	33 (0-33)	0.102
Appetite loss	12.8 (25.1)	0 (0-33)	8.4 (16.2)	0 (0-0)	0.651
Nausea/vomiting	15.4 (22.1)	0 (0-33)	8.1 (13.8)	0 (0-17)	0.194
Constipation	19.2 (31.5)	0 (0-33)	5.6 (12.5)	0 (0-0)	0.054
Diarrhoea	14.1 (31.6)	0 (0-0)	13.3 (16.4)	0 (0-33)	0.200
Fatigue	41 (28.6)	33 (22-67)	24.6 (17.5)	22 (17-33)	0.028
Pain	28.9 (28.1)	17 (0-50)	12.4 (16.1)	0 (0-17)	0.010
Financial problems	19.2 (28.6)	0 (0-33)	14.8 (25.8)	0 (0-33)	0.510
Global health status	68.2 (24.7)	67 (50-83)	65.7 (22)	67 (50-83)	0.635

IQR: Inter-quartile range; SD: Standard deviation.

Table 5 Surgery type among different stages of the disease

	Total gastrectomy, *n* (%)	Subtotal-Billroth II, *n* (%)	Subtotal-Roux-Y, *n* (%)	*P* value
Stage				0.002
0	4 (4.1)	3 (6.5)	0 (0)	
I	25 (25.5)	14 (30.4)	38 (42.2)	
II	26 (26.5)	10 (21.7)	32 (35.6)	
III	43 (43.9)	19 (41.3)	20 (22.2)	

symptom burden reported with total gastrectomy such as nausea and vomiting, dysphagia, eating restrictions and reflux[16,21]. In subtotal gastrectomy, gastric physiology is at least partly preserved, possibly leading to superior HRQoL. However, several studies found no difference in global HRQoL between the two groups[5,15,18,25]. A possible explanation for this finding is the time interval from the surgical procedure. HRQoL changes over time are well documented. In longitudinal analyses, significantly worse scores on almost all HRQoL scales were observed 1 mo to 6 mo postoperatively compared to the preoperative scores[14-17,20]. Several functional scales recovered to the baseline by 1 year after surgery, however, symptoms such as nausea and vomiting, reflux and eating restrictions persisted even 5 years after surgery[19,23,24]. In a long-term analysis by Lee *et al*[25], HRQoL inferiority of patients after total gastrectomy when compared to subtotal distal gastrectomy generally disappeared beyond 5 years postoperatively, remaining inferior only in eating restrictions. In our study, the median time interval from the surgery to the completion of the questionnaires was 3 years, possibly diminishing some differences between the two groups.

Regarding the type of digestive tract reconstruction after subtotal distal gastrectomy, the choice of the technique is often driven by the surgeon's preferences,

Table 6 Association between surgery type and global health status scores adjusted for demographic and clinical characteristics

	β (*P* value)
Male	-0.11 (0.229)
Age (yr)	-0.05 (0.625)
Total *vs* Billroth II	-0.001 (0.990)
Roux-Y *vs* Billroth II	-0.08 (0.492)
Postoperative complications-yes	-0.15 (0.116)
Stage II/III *vs* 0/I	-0.21 (0.029)

β: Standardized regression coefficient.

Table 7 Association between surgery type and scores on functional scales adjusted for demographic and clinical characteristics

	Emotional		Social		Role		Physical		Cognitive	
	β	*P* value	*β*	*P* value	*β*	*P* value	*β*	*P* value	*β*	*P* value
Male	0.01	0.901	0.10	0.254	0.09	0.334	0.16	0.089	0.04	0.666
Age (yr)	0.09	0.343	0.05	0.557	0.08	0.379	-0.09	0.343	0.18	0.054
Total *vs* Billroth II	0.04	0.729	-0.11	0.343	0.15	0.199	0.15	0.201	0.16	0.194
Roux-Y *vs* Billroth II	0.24	0.041	0.04	0.763	0.24	0.034	0.23	0.048	0.17	0.154
Postoperative complications yes	-0.03	0.730	-0.13	0.150	-0.15	0.101	-0.10	0.292	0.01	0.915
Saage II/III *vs* 0/I	-0.17	0.062	-0.20	0.031	-0.27	0.003	-0.16	0.079	-0.09	0.324

β: Standardized regression coefficient.

Table 8 European Organization for Research and Treatment of Cancer Quality of Life Core Questionnaire gastric cancer-specific module (QLQ STO-22) questionnaire scores by the type of resection (Mann-Whitney *U* test)

	Subtotal gastrectomy (*n* = 60)		Total gastrectomy (*n* = 51)		*P* value
	mean (SD)	Median (IQR)	mean (SD)	Median (IQR)	
Dysphagia	11.1 (15.8)	11 (0-11)	17.2 (17.1)	11 (0-33)	0.020
Pain	19.7 (17.9)	17 (8-33)	23.3 (20.9)	17 (8-33)	0.477
Reflux	16 (20.9)	11 (0-22)	22.7 (23.6)	11 (0-44)	0.089
Eating restrictions	14.7 (17.4)	8 (0-17)	21.6 (18.2)	17 (8-33)	0.017
Anxiety	33.1 (23.4)	33 (17-44)	31.4 (25.8)	22 (11-44)	0.591
Dry mouth	24.1 (28.5)	33 (0-33)	24.7 (28.3)	33 (0-33)	0.871
Taste	6.4 (18.1)	0 (0-0)	11.6 (21.7)	0 (0-33)	0.109
Body image	18.7 (29.9)	0 (0-33)	20.1 (27.4)	0 (0-33)	0.567
Hair loss	16.4 (27.9)	0 (0-33)	8.7 (20.9)	0 (0-0)	0.124

IQR: Interquartile range; SD: Standard deviation.

and no clear recommendations exist in the current literature[9-11]. Several studies suggested Roux-en-Y reconstruction to be superior to Billroth II reconstruction in terms of preventing bile reflux and remnant gastritis, thus allowing better quality of life[10,26-28]. However, in a proportion of patients, Roux-en-Y may be associated with a Roux stasis syndrome causing delayed gastric emptying with postprandial pain, nausea and vomiting[29,30]. Our results are partly in line with previous studies reporting reduced HRQoL following Billroth II *vs* Roux-en-Y reconstruction[10,26-28]. Patients after

Table 9 European Organization for Research and Treatment of Cancer Quality of Life Core Questionnaire gastric cancer-specific module (QLQ STO-22) questionnaire scores by the type of reconstruction (Mann-Whitney *U* test)

	Billroth II (*n* = 18)		Roux-Y (*n* = 42)		*P* value
	mean (SD)	Median (IQR)	mean (SD)	Median (IQR)	
Dysphagia	15.8 (22.3)	5.5 (0-22)	8.5 (10.2)	11 (0-11)	0.526
Pain	24.3 (23.9)	17 (8-33)	17.2 (13.3)	17 (8-25)	0.471
Reflux	28.5 (26.3)	22 (0-44)	9.1 (13)	0 (0-11)	0.001
Eating restrictions	22.3 (22.8)	17 (0-42)	10.4 (11.7)	8 (0-17)	0.069
Anxiety	36.6 (26.9)	33 (22-55)	31.2 (21.3)	33 (11-44)	0.541
Dry mouth	23 (26.3)	33 (0-33)	24.7 (29.9)	0 (0-33)	0.965
Taste	11.5 (26.6)	0 (0-0)	3.5 (10.3)	0 (0-0)	0.247
Body image	26.8 (36.5)	0 (0-33)	14.1 (24.8)	0 (0-33)	0.127
Hair loss	14.1 (27)	0 (0-33)	17.7 (28.6)	0 (0-33)	0.541

IQR: Inter-quartile range; SD: Standard deviation.

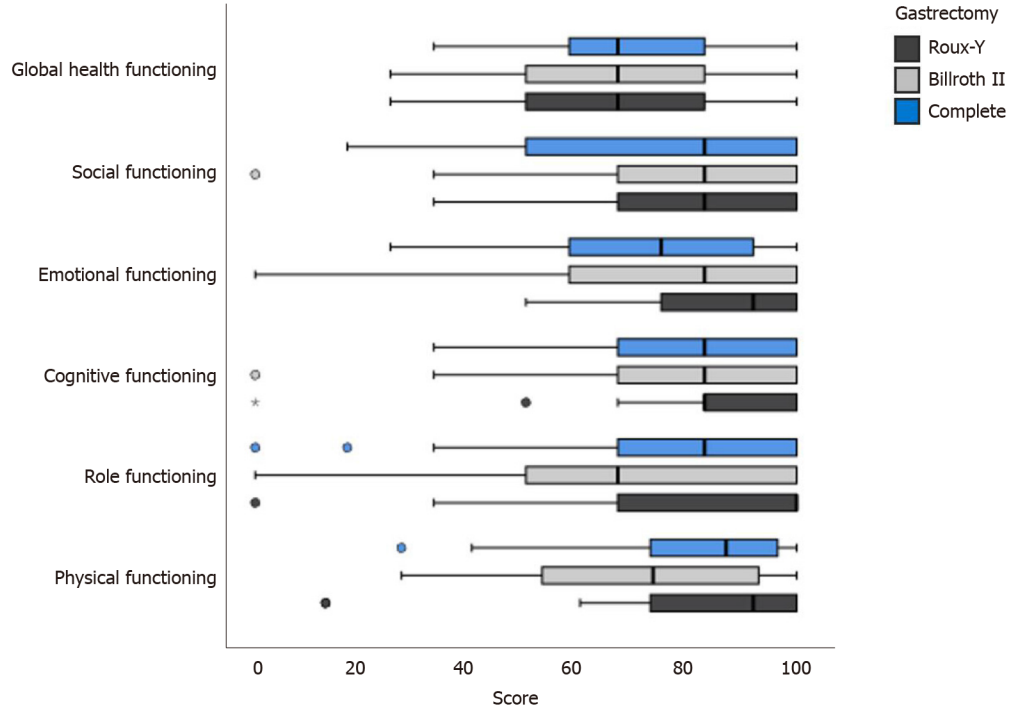


Figure 1 Median scores with interquartile ranges of functional scales and global health status/quality of life scale based on the type of surgery.

Billroth II reconstruction scored lower on some of the functional scales and reported more pain, fatigue and reflux symptoms. This occurred despite routine construction of Braun anastomosis, which supposedly diverts bile from the remnant stomach, relieving reflux symptoms, dumping syndrome or other disturbances in food intake[31]. Although patients after Billroth II reconstruction were more likely to have a higher disease stage than those after Roux-en-Y reconstruction, statistically significant superiority of the Roux-en-Y procedure on the emotional, role and physical functioning scale remained after adjustment for demographic data, disease stage and postoperative complications.

Study limitations

This study has some limitations. First, the cross-sectional nature does not allow

longitudinal assessment of HRQoL. Nonetheless, we believe this kind of design allows us to gain important insight into the overall HRQoL of patients following gastrectomy, which represents the basis for clinical decision making. Second, the number of participating patients is relatively low (116), representing 49.6% of patients that were eligible for study inclusion. Third, mail surveys lack data related to the actual health status of the patient. We did not see the patients to obtain their health status objectively; therefore, their subjective measures could not be compared to their actual physical findings. Nonetheless, HRQoL is a multimodal construct of physical, psychological and social wellbeing in relation to disease treatment. Therefore, objective and subjective measures are not necessarily related. Even if a patient is objectively well, he or she may at the same time be subjectively unwell, which should be addressed separately from objective measures.

CONCLUSION

Our study shows that patients after gastrectomy for gastric cancer experience several functional and symptom complaints affecting quality of life. Based on our results, with regard to HRQoL, subtotal distal gastrectomy with Roux-en-Y reconstruction should be preferred over subtotal distal gastrectomy with Billroth II reconstruction. Patients should be informed preoperatively about expected functional sequelae after surgery and should be regularly monitored postoperatively to ensure proper symptomatic and supportive care.

ARTICLE HIGHLIGHTS

Research background

Gastrectomy is a major operation that alters the physiological functions of the digestive tract. Consequently, these patients experience malnutrition, weight loss and several postgastrectomy symptoms that negatively impact patients' wellbeing.

Research motivation

With improved survival in gastric cancer patients, health-related quality of life has become an important clinical endpoint alongside oncological outcomes.

Research objectives

The aim of this study was to investigate health-related quality of life after various surgical procedures for gastric cancer treatment.

Research methods

Patients that underwent curative resection for gastric adenocarcinoma at a tertiary centre between January 2014 and December 2018 were recruited for inclusion in this cross-sectional survey. The validated Slovenian version of the European Organization for Research and Treatment of Cancer Quality of Life Core Questionnaire (QLQ-C30) and its gastric cancer specific module (QLQ STO-22) were sent to all eligible patients for self-completion. The scores of both questionnaires were compared between patients after subtotal distal *vs* total gastrectomy and patients after subtotal distal gastrectomy with Billroth II *vs* Roux-en-Y reconstruction using the Mann-Whitney *U* test. The association between the type of operation and general health status adjusted for some demographic and clinical characteristics was assessed with multiple linear regression.

Research results

Out of 234 patients that were eligible for study inclusion, 116 (49.6%) patients completed the questionnaires. No statistically significant differences were observed in scores on global or functional scales among patients after total or subtotal distal gastrectomy. However, patients after total *vs* subtotal gastrectomy did experience more dysphagia (P = 0.020) and eating restrictions (P = 0.017). Patients after subtotal distal gastrectomy with Billroth II reconstruction reported significantly worse scores on the physical (P = 0.038) and role functioning (P = 0.034) scales and had more problems with pain (P = 0.010), fatigue (P = 0.028) and reflux (P = 0.001). When adjusted for demographic data, disease stage and postoperative complications, no

differences were observed in reported global health status/quality of life scores among different surgical procedures. However, Roux-en-Y was superior over Billroth II reconstruction in emotional (β = 0.24, P = 0.041), role (β = 0.24, P = 0.034) and physical (β = 0.23, P = 0.048) functioning when adjusted for other variables in a regression model.

Research conclusions

Patients after gastrectomy for gastric cancer experience several functional and symptom complaints. Based on our results, subtotal distal gastrectomy with Roux-en-Y reconstruction should be preferred over subtotal distal gastrectomy with Billroth II reconstruction.

Research perspectives

The data obtained from this study will help surgeons when preoperatively informing their patients about expected functional outcomes after gastrectomy and enable them to ensure proper supportive care for their patients in the postoperative period.

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SYSTEMATIC REVIEWS

Surrogate markers of mucosal healing in inflammatory bowel disease: A systematic review

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Abstract

BACKGROUND

Mucosal healing (MH) has emerged as a key therapeutic target in inflammatory bowel disease (IBD), and achievement of this goal is documented by endoscopy with biopsy. However, colonoscopy is burdensome and invasive, and substitution with an accurate noninvasive biomarker is desirable.

AIM

To summarize published data regarding the performance of noninvasive biomarkers in assessing MH in IBD patients.

METHODS

We conducted a systematic review of studies that reported the performance of biomarkers in diagnosing MH in patients with IBD. The main outcome measure was to review the diagnostic accuracy of serum and fecal markers that showed promising utility in assessing MH.

RESULTS

We screened 1301 articles, retrieved 46 manuscripts and included 23 articles for full-text analysis. The majority of the included manuscripts referred to fecal markers (12/23), followed by circulatory markers (8/23); only 3/23 of the included manuscripts investigated combined markers (serum and/or fecal markers). Fecal calprotectin (FC) was the most investigated fecal marker for

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assessing MH. In ulcerative colitis, for cutoff levels ranging between 58 mcg/g and 490 mcg/g, the sensitivity was 89.7%-100% and the specificity was 62%-93.3%. For Crohn's disease, the cutoff levels of FC ranged from 71 mcg/g to 918 mcg/g (sensitivity 50%-95.9% and specificity 52.3%-100%). The best performance for a serum marker was observed for the endoscopic healing index, which showed a comparable accuracy to the measurement of FC and a higher accuracy than the measurement of serum C-reactive protein.

CONCLUSION
Several promising biomarkers of MH are emerging but cannot yet substitute for endoscopy with biopsy due to issues with reproducibility and standardization.

Key Words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Biomarkers; Serum; Fecal; Mucosal healing

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Core Tip: Identification of performant biomarkers that can substitute for repeated and cumbersome invasive procedures is a priority. Although therapeutic success in both Crohn's disease and ulcerative colitis remains to be clearly defined, mucosal healing has been one of the main therapeutic targets stipulated by recent recommendations. In inflammatory bowel disease mucosal healing, several serum- and fecal-based markers have shown promising results; however, a multimarker may improve the diagnostic accuracy of any single independent biomarker.

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INTRODUCTION

Endoscopic inspection plays a pivotal role in the diagnosis and monitoring of inflammatory bowel disease (IBD). According to current guidelines and recommendations, each individual patient is likely to undergo several endoscopic evaluations during the course of their disease, sometimes at relatively short intervals. Endoscopy is crucial to establish a diagnosis and to evaluate disease extent and severity. Endoscopic reassessment is recommended on a case-by-case basis in patients not responding to treatment or with frequent relapse and remains the gold standard for confirming the response to treatment[1].

Although therapeutic success in both Crohn's disease (CD) and ulcerative colitis (UC) remains to be clearly defined, mucosal healing (MH) has been one of the main therapeutic targets stipulated by recent recommendations[2]. MH is associated with lower risks of relapse, hospitalization and surgery[3,4]. While there is currently no clear consensus regarding the definition of MH despite the many proposed scoring systems for endoscopic activity in IBD, most experts agree that a Mayo endoscopic subscore of 0 or 1 or a SES-CD (simple endoscopic score for CD) less than 3 constitutes an acceptable measure of MH, both of which have been widely used in recent clinical trials of UC and CD, respectively[5,6].

Invasive procedures, such as colonoscopy, have a great impact on patients. Patients with IBD report significantly more embarrassment and burden from the bowel preparation phase and more pain during the colonoscopy[7]. Although the risk of complications related to colonoscopy in IBD patients is very low (< 1%), it is higher than that in other groups of patients[8,9] and involves significant costs[10]. Therefore, identifying reliable surrogate noninvasive markers for disease activity is desirable to reduce patient burden and costs.

In recent years, a wealth of publications regarding various noninvasive biomarkers in IBD have reported conflicting results on various clinical endpoints. The aim of this

systematic review was to evaluate published data regarding the diagnostic accuracy of biomarkers in assessing MH in IBD patients.

MATERIALS AND METHODS

Rationale

We conducted a systematic review of studies that reported on the accuracy of noninvasive biomarkers in diagnosing MH in patients with colonic IBD with the aim of identifying and assessing the diagnostic accuracy of these biomarkers. Neither imaging markers nor urine-based markers were included in this analysis. Studies investigating the role of C-reactive protein (CRP) in detecting MH were also not included, as a comprehensive analysis regarding this subject was recently published[11]. In this review, 30 eligible publications regarding CRP were included. CRP positively correlated with the endoscopic activity of the disease, yielding correlation coefficients from 0.29 to 0.63 for UC and from 0.31 to 0.71 for CD. At the time of this publication, no new data have emerged; thus, reanalysis of published data is unnecessary at this time.

Literature search

A structured search was conducted on December 12, 2020 of the PubMed (MEDLINE) database. Our search terms included the following medical subject headings (Mesh) and text words: For IBD: inflammatory bowel disease, ulcerative colitis, Crohn's disease; For MH: mucosal healing, endoscopic remission, colonoscopy, sigmoidoscopy, remission induction; For biomarkers: biomarker, predict*, clinical, laborator*, serol*, serum, fecal*, feces, blood, cytokine*. The entire search algorithm was the following: ((((((((((((marker*[tw])) OR (biomarker*[tw])) OR ("Biomarkers"[Mesh])) OR (predict*[tw])) OR (clinical[tw])) OR (laborator*[tw])) OR (serol* [tw])) OR ("Serum"[Mesh])) OR (fecal* [tw])) OR ("Feces"[Mesh])) OR (blood [tw])) OR ("Blood"[Mesh])) OR (cytokine* [tw])) OR ("Cytokines"[Mesh])) AND ("Colonoscopy"[Mesh Terms] OR "Sigmoidoscopy"[Mesh Terms] OR "Remission Induction"[Mesh Terms] OR ("mucosal"[Text])) OR "endoscopic remission"[Text Word])) AND ((((((inflammatory bowel disease [tw]) OR (ulcerative colitis [tw])) OR (Crohn's disease [tw])) OR ("Inflammatory Bowel Diseases"[Mesh])) OR ("Colitis, Ulcerative"[Mesh])) OR ("Crohn's Disease"[Mesh])).

Inclusion criteria

All manuscripts published from January 2010, with the full text version available online, either *via* open access or pay per view, were included in the screening process. To avoid potential bias related to the use of older generation endoscopes and to account for the evolving definition of "mucosal healing", we restricted our review to manuscripts published in the last 10 years.

Exclusion criteria

Manuscripts concerning pediatric patients, veterinary studies, noncolonic CD, unspecified IBD, narrative reviews, case reports and research that did not include an endoscopic evaluation to confirm MH and a clear definition of MH were not included.

Data extraction

These criteria were applied for the title screening process, followed by a full-text read of the filtered results. For all articles that met the criteria, using an a priori designed extraction form, two independent reviewers collected data regarding the type of study that was conducted, the investigated marker and its source (serum, feces) and the marker accuracy in predicting MH and the definition of MH considered for each study. The reference sections of the included studies were analyzed to retrieve relevant studies not identified by the original search.

Articles included in the final analysis were evaluated for quality and risk of bias using the Cochrane Risk of Bias Tool[12] and the Methodological Index for Non-Randomized Studies criteria[13] (Supplementary Tables 1 and 2).

Our review was registered on PROSPERO (December 19, 2020) and is currently awaiting publication on the registry site. Reporting of the review was conducted using the PRISMA guidelines. The statistical methods of this study were reviewed by a biomedical statistician at Colentina Clinical Hospital.

RESULTS

In total, 1301 articles were retrieved with the search strategy, and 46 manuscripts were included for full-text analysis (Figure 1)[14]. After excluding studies that did not provide a clear definition of MH and studies that did not report at least a measure of performance for the investigated markers, 23 articles were included for complete data extraction and final analysis. Another reason for exclusion was reference to unclear characteristics of the study populations (ileal or colonic CD); MH was not one of the stated endpoints.

The selected manuscripts were further categorized by the source of investigated markers (fecal, serum). Studies investigating the performance of combined markers were discussed separately. Of 23 studies, 14 referred to prospective cohorts, with only 2 having a multicentric design. We also included 5 retrospective cohort analyses, 2 cross-sectional cohort studies, one case-control pilot study and a post hoc analysis of a clinical trial. The number of patients included in each study ranged from 20 to 311. The majority of included manuscripts referred to fecal markers (12/23), followed by circulatory markers (7/23), and only 4/23 investigated combined markers (serum and/or fecal markers). Most studies were performed in patients with UC (13/23): 7/12 studies investigated fecal markers, 4/7 studies investigated circulatory markers, and only one of the 3 included studies investigated combined markers. For CD patients, only 2/12 studies investigated fecal markers, and 2 studies referred to combined markers. Seven studies included both UC and CD patients (3/12 studies dedicated to fecal-based markers and 4/7 studies investigating circulatory markers).

For the definition of MH in CU patients, a Mayo endoscopic score (MES) of 0/0-1, UCEIS (UC endoscopy index of severity) of 0 or a Matts score of 1-2 (MH) Matts = 1/MES = 0 (complete MH) was applied. For CD patients, the MH definition was variable among studies: a SES-CD < 2/≤ 2/≤ 3/3-7, CDEIS (CD activity index) ≤ 4, or absence of mucosal lesions was used.

Role of fecal biomarkers in detecting MH

Of the 23 studies included in this analysis, 12 investigated the association between fecal marker levels and MH.

Fecal calprotectin: Calprotectin is an antimicrobial protein mainly secreted by neutrophils that remains stable for several days at room temperature[15] and has been widely investigated in IBD patients for disease activity detection. In 9/12 studies, the authors investigated fecal calprotectin (FC) performance in assessing MH. Two studies compared FC and fecal immunochemical test (FIT) performance, and one investigated immune fecal occult blood test (iFOBT). Most studies were conducted in cohorts of UC patients (6/9) that involved a low number of participants (minimum 44, maximum 128). There was a large variation in the selected optimal cutoff values, and several commercial assays were used (ELISA, QPOCT).

In UC, the reported cutoff levels of FC for MH detection ranged between 58 mcg/g (sensitivity 89.7% and specificity 93.3%) and 490 mcg/g (sensitivity 100% and specificity 62%). For CD, the cutoff levels of FC ranged from 71 mcg/g (sensitivity 95.9% and specificity 52.3%) to 918 mcg/g (sensitivity 50% and specificity 100%) (Table 1).

FIT: Another fecal marker widely investigated for the assessment of IBD patients is FIT, which quantifies the globin (protein) component of hemoglobin found in stool. Quantitative FIT estimates the degree of colonic inflammation by measuring the concentration of hemoglobin in feces using an antibody that targets human hemoglobin[16]. When compared to FC, the specificity for MH in UC was similar, but FIT appeared to be more sensitive than FC for predicting MH (95% *vs* 77% for MES = 0 in a study by Hiraoka *et al*[17], 98.0% *vs* 78.4% for MES = 0, 94.9%, *vs* 74.6% for UCEIS 0-1 in a study by Ryu *et al*[18]). One of the main advantages of using FIT over FC refers to cutoff values of FIT, which are relatively stable and almost equivalent to the cutoff values used in colorectal cancer screening[19].

iFOBT: In addition to FIT, another fecal marker is the iFOBT, which measures the heme (nonprotein) component of hemoglobin from blood in the stool. This method has many limitations that cause false-positive results. Food and medication, as well as blood from dietary sources or upper GI bleeding, can result in a positive test[20]. When compared to FC in a retrospective analysis in UC patients, iFOBT showed higher sensitivity and specificity for assessing MH (80.6% and 100% *vs* 74.1% and 84.2%, respectively)[21].

Table 1 Synthetic presentation of data on fecal marker performance in detecting mucosal healing

Ref.	Study type	Investigated marker	MH definition	CU/CD	Number of patients	AUC	95%CI	Sn (%)	Sp (%)	Cutoff level
E Penna *et al*[22]	Prospective cohort	FC	SES-CD ≤ 2	CD	65 + 21 individuals in the control group	0.77	0.65-0.88	96	78	155 mcg/g
Hiraoka *et al*[17]	Prospective cohort	FC	MES = 0	UC	84	0.67	0.56 - 0.78	77	67	180 mcg/g
Hiraoka *et al*[17]	Prospective cohort	FIT	MES = 0	UC	84	0.62	0.50- 0.74	95	62	NA
Lee *et al*[23]	Prospective cohort	FC-ELISA; FC-QPOCT	MES = 0; SES-CD < 4	UC + CD	93	0.88	NANA	81.8(ELISA); 85.7 (QPOCT)	100 ELISA; 100 QPOCT	201 mcg/g ELISA; 150.5 mcg/g QPOCT
Urushikubo *et al*[24]	Cross-sectional, observational	FC	MES = 0; REI = 0; UCEIS = 0	UC	50	0.823; 0.780; 0.777	0.707-0.939; 0.658-0.903; 0.645-0.909	100; 100; 88	62; 70; 71	490 mcg/g; 288 mcg/g; 288 mcg/g
Ryu *et al*[18]	Retrospective cohort	FIT	MES = 0; UCEIS = 0-1	UC	128	NA	NA	98.0; 94.9	37.4; 38.3	100 ng/mL
Ryu *et al*[18]	Retrospective cohort	FC	MES = 0; UCEIS = 0-1	UC	128	NA	NA	78.4; 74.6	74.4; 76.5	170 mcg/g
Lin *et al*[25]	Multicentric prospective cohort	FC	UCEIS < 3; CDEIS < 6	UC + CD	88	0.87; 0.74	NA	88; 50	75; 100	191 mcg/g; 918 mcg/g
Vázquez Morón *et al*[26]	Prospective cohort	FC	SES-CD ≤ 2	CD	71	NA	NA	95.9	52.3	71 mcg/g
Ryu *et al*[27]	Retrospective cohort	FIT	MES = 0-1	UC	63	0.81	0.59-0.94	73.33	81.82	< 7 ng/mL
Hassan *et al*[28]	Prospective cohort	FC	MES = 0-1	UC	44	0.949	0.838-0.992	89.7	93.3	58 mcg/g
Kostas *et al*[29]	Retrospective cohort	FC	MES = 0-1; absence of mucosal lesions for CD	UC + CD	149	0.956	NA	91.9	87.2	174 mcg/g
Yen *et al*[21]	Retrospective cohort	FC	MES = 0-1	UC	50	0.812	NA	74.19	84.21	156 mcg/g
Yen *et al*[21]	Retrospective cohort	iFOBT	MES = 0-1	UC	50	0.906	NA	80.65	100	≤ 43 ng/mL
Kristensen *et al*[30]	Prospective cohort	FC	MES = 0-1	UC	20	NA	NA	82.4	100	250 mcg/g

UC: Ulcerative colitis; CD: Crohn's disease; FC: Fecal calprotectin; FIT: Fecal immunoassay test; MES: Mayo endoscopic subscore; UCEIS: Ulcerative colitis endoscopy index of severity; REI: Rachmilewitz endoscopic index; M2-PK: M2 pyruvate kinase, iFOBT: Immune fecal occult blood test; NA: Not available; QPOCT: Quantitative point-of-care test; AUC: Area under the curve; CI: Confidence interval; Sn: Sensitivity; Sp: Specificity.

A systematic presentation of the data regarding fecal marker performance discussed in this review is listed in Table 1.

Role of circulatory biomarkers in detecting MH

We analyzed 7 studies discussing the role of various serum biomarkers in detecting MH. Four of 7 studies were conducted in UC cohorts; in 3 of the 7 studies, both UC and CD patients were included. We identified 3 studies that investigated the utility of trefoil factor 3 (TFF3) as a marker for MH in patients with UC, but only one study met the inclusion criteria. TTF3 is a mucin-associated peptide that is widely expressed in a tissue-specific manner in the gastrointestinal tract and is predominantly secreted by goblet cells of the small and large intestine[31]. In a study by Nakov *et al*[32], the TFF3

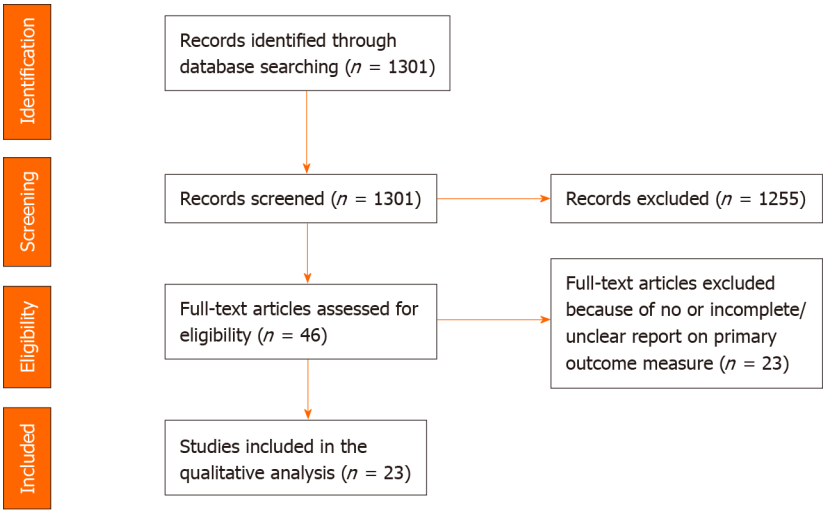


Figure 1 Flow chart of the manuscripts included in the final analysis according to the PRISMA reporting guidelines.

cutoff level of 6.74 ng/mL indicated complete MH (MES = 0; UCEIS = 0), with a sensitivity and specificity of 87.9% and 86.9% in patients with UC, respectively. An interesting aspect is that the area under the receiver operating characteristics (ROC) curve (AUC) of TFF3 + CRP showed higher accuracy than the AUC of TFF3 alone (Z = 2.210, P = 0.027) for predicting complete MH. However, when investigated in CD patients treated with tumor necrosis factor (TNF)-α antagonists, TTF3 was not a convenient or reliable surrogate marker of MH[33].

Budzynska *et al*[34] investigated serum neutrophil gelatinase-associated lipocalin (NGAL), a low-molecular-weight protein released from activated neutrophils and the intestinal epithelium, whose mRNA expression is increased in inflamed intestinal tissue. NGAL performed well in UC patients and was able to distinguish endoscopically active from inactive UC, with an AUC-ROC of 0.758 (sensitivity 96% and specificity 54%). However, NGAL levels showed no significant relationship with either the clinical or endoscopic activity of CD.

In a study by Planell *et al*[35], transcriptional changes in whole-blood samples from UC patients were investigated. A significant correlation with the degree of endoscopic activity was observed in several genes, including *haptoglobin* (*HP*), *CD177*, *GPR84*, and *S100A12*. Within the subset of genes whose expression best correlates with endoscopic disease severity, *HP* was the best gene to predict mucosal lesions. The logistic regression model for endoscopic activity prediction (endoscopic Mayo score: 0 *vs* ≥ 1) demonstrated a significant effect of *HP* (P < 0.001), with an odds ratio of 1.9 [95% confidence interval (CI): 1.35-2.78] and an AUC = 0.75 (95%CI: 0.64-0.85). Defining a cutoff of -2.9 DCt, the sensitivity and specificity were 63.5% and 80.0%, respectively.

Nicotinamide phosphoribosyltransferase (Nampt) is another serum marker showing good potential in assessing MH. Nampt is an intracellular enzyme that is primarily associated with the induction and persistence of inflammatory responses. In a study by Neubauer *et al*[36], serum Nampt in UC positively correlated with its clinical and endoscopic activity as well as with proinflammatory cytokines. Serum Nampt ≤ 1.54 ng/mL was a good indicator of MH (sensitivity 76%, specificity 75%, AUC 0.768).

One recent study proposed serum amyloid A (SAA) as a surrogate marker for MH. Both SAA and CRP are mainly secreted from the liver, but SAA is reported to be more effective than CRP in diseases other than IBD[37]. The diagnostic accuracy of SAA for MH was superior to that of CRP. SAA levels < 5.8 could discriminate mucosal inflammation from MH, with a sensitivity of 72.2%, specificity of 85%, and accuracy of 79.9%. In contrast, CRP levels < 0.060 could distinguish mucosal inflammation from MH, with a sensitivity of 62%, specificity of 75%, and accuracy of 70.4%[38].

Dierckx *et al*[39] investigated glycoprotein acetylation (GlycA) in the serum or plasma of patients with IBD. GlycA is a novel nuclear magnetic resonance biomarker that summarizes the signals originating from glycan groups of certain acute-phase glycoproteins. In this study, GlycA concentration in both CD and UC patients achieving MH dropped back to HC levels (P = 0.90, P = 0.910) and showed potential to identify MH even in patients without elevated CRP. This should therefore be tested in large prospective cohorts.

Serum levels of leucine-rich alpha-2 glycoprotein, investigated in a prospective setting, were significantly higher in endoscopically active patients with UC having extensive and left-sided colitis than in those with MH, although marker performance measures were not clearly reported[40].

A systematic presentation of the data regarding serum marker performance discussed in this review is listed in Table 2.

Role of combined biomarkers in detecting MH

Because of the suboptimal performance of individual noninvasive markers at detecting MH, further combining fecal and serum biomarkers in panels has been attempted to increase accuracy in several studies.

A multicentric international study conducted by D'Haens *et al*[41] aimed to develop and validate a multimarker, serologic, algorithm-based diagnostic test that could reflect the severity of endoscopic inflammation in CD patients. The endoscopic healing index (EHI) model includes serum concentrations of 13 biomarkers: angiopoietin 1 (ANG1) and 2 (ANG2); carcinoembryonic antigen-related cell adhesion molecule 1; CRP; SAA1; interleukin (IL) 7; transforming growth factor α; vascular cell adhesion molecule 1; extracellular matrix metalloproteinase inducer; and matrix metalloproteinases 1 (MMP1), 2 (MMP2), 3 (MMP3), and 9 (MMP9). EHI was constructed as a scale of 0-100 arbitrary units of EHI activity, with a higher score indicating more severe disease activity. The EHI identified patients with resolution of endoscopic disease activity, with good overall accuracy, although with variation between the 2 cohorts assessed. The EHI AUC-ROC values were comparable to the measurement of FC (0.950 *vs* 0.923 $P = 0.147$ in validation cohort 1 and 0.803 *vs* 0.854, $P = 0.298$ in validation cohort 2) and higher than that of serum CRP.

Other serum markers that have shown promising results are anti-inflammatory serum cytokine profiles. Gubatan *et al*[42] reported that the serum cytokine ratio IL-4 + IL-10/IL-17A + TNF-α has the ability to identify UC patients with baseline histologic MH (AUC of 0.641, $P = 0.044$). A serum cytokine ratio IL-4 + IL-10/IL-17A + TNF-α threshold of 0.1522 had the greatest sensitivity (52.6%, 95%CI: 35.8-69.0) and specificity (75.0%, 95%CI: 56.6-88.5), with a positive likelihood ratio of 2.015.

Other authors have tried to evaluate the combined role of serum, fecal and clinical scores in detecting MH. A post hoc analysis from the CALM study[43] reported the performance of the combination of FC, CRP and clinical disease activity index (CDAI) in detecting MH. Using the cutoffs FC < 250 μg/g, CRP < 5 mg/L, and CDAI < 150, it had a sensitivity/specificity of 72%/63% and positive/negative predictive values of 86%/42% for CDEIS < 4 and no deep ulcers 48 wk after randomization. Data regarding the performance of combined biomarkers are listed in Table 3.

Cytokines, such as IL-6 and soluble IL-2 receptor (sIL-2R), have been shown to modulate the intestinal immune system. Mavropoulou *et al*[44] analyzed serum IL-6 and sIL-2R levels in a cohort of IBD patients. They evaluated the correlation between these laboratory markers and clinical and endoscopic disease activity status in IBD patients. In this study, serum levels of sIL-2R ($P < 0.001$) and CRP ($P = 0.003$) as well as FC values ($P < 0.001$) were associated with endoscopic remission in UC patients by univariate analysis. For CD patients, the threshold value for IL-6 to discriminate patients with endoscopic remission or active disease was 5.5 pg/mL, with an AUC-ROC of 0.80 (95%CI: 0.71-0.89).

DISCUSSION

Over the past decade, the standard of care and management of IBD have greatly improved as a result of newly approved drugs as well as a wide array of invasive and noninvasive diagnostic tools. Novel "treat-to-target" strategies have recently been shown to impact disease progression and improve outcomes for IBD patients. MH has been one of the main targets suggested as part of current therapeutic goals[42]. Compared to previous strategies that focused on symptom control, recent studies have shown that achieving MH is associated with lower risks of relapse, hospitalization and surgery[3,4]. However, because MH is objectivated by intrusive colonoscopy, it is becoming increasingly apparent that surrogate noninvasive markers are needed for close and efficient disease monitoring and control. A reliable surrogate marker for MH would have a significant clinical impact, by reducing the number of endoscopic evaluations required during the course of disease.

Based on our findings, other than FC, which has a clear established role in current real-life clinical practice, none of the biomarkers was accurate enough to replace

Table 2 Synthetic presentation of data on serum marker performance in detecting mucosal healing

Ref.	Study type	Investigated marker	MH definition	CU/CD	Number of patients	AUC	95%CI	Sn (%)	Sp (%)	Cutoff level
Shinzaki *et al*[40]	Prospective cohort	LRG	Matts = 1-2 (MH); Matts = 1/MES = 0 (complete MH)	CU	129	0.849; 0.759	NA	NA	NA	NA
Dierckx *et al*[39]	Case control pilot study	GlycA	MES = 0-1; SES-CD ≤ 2	CU + CD	58 + 10 healthy controls	NA	NA	NA	NA	NA
Neubauer *et al*[36]	Prospective cohort	S-Nampt	MES = 0-1; NA for CD	CU + CD	240 + 40 non-IBD controls	0.768	0.67-0.85	76	75	≤ 1.54 ng/mL
Planell *et al*[35]	Cross-sectional cohort + case control	HP	MES = 0	UC	126 + 20 healthy controls + 16	0.75	0.64-0.85	63.5	80	-2.9 DCt
Nakov *et al*[32]	Prospective cohort	TTF3	MES = 0; UCEIS = 0	UC		0.927	0.877-0.976	87.9	86.9	6.74
Budzynska *et al*[34]	Prospective cohort	NGAL	MES = 0-1; SES-CD = 3-7	UC + CD	120	0.79;0.608	0.65-0.93; NA	96; 48	50; 83	43.6 ng/mL; 72.5 ng/mL
Wakai *et al*[38]	Retrospective cohort	Serum amyloid A	MES = 0-1	UC	108	0.807	0.748-0.867	72.2	85	< 5.8

UC: Ulcerative colitis; CD: Crohn's disease; LRG: Leucine-rich alpha-2 glycoprotein; GlycA: Glycoprotein acetylation; Nampt: Nicotinamide phosphoribosyltransferase; HP: Haptoglobin; TFF3: Trefoil factor 3; NGAL: Serum neutrophil gelatinase-associated lipocalin; NA: Not available; SES-CD: Simple endoscopic score for Crohn's disease; AUC: Area under the curve; CI: Confidence interval; Sn: Sensitivity; Sp: Specificity.

endoscopy. The most accurate serum marker was a multimarker based on 13 serum proteins, called the EHI, which showed comparable accuracy to the measurement of FC and higher accuracy than the measurement of serum CRP. We observed that most studies focused on assessing individual markers among a small number of patients in a wide variety of study designs and heterogeneous groups of patients.

However, endoscopic activity scores and MH definitions are not homogenous, which could represent an impediment in establishing the performance of various investigated markers. Additionally, there are several commercial assays for FC and other marker measurements available, making the establishment of cutoff values an insurmountable obstacle. Few studies compared the investigated markers to FC, which has been widely investigated and is commonly used in clinical practice.

It is mandatory to assess MH at different key time points during the disease course, especially to gauge the response to therapy or to address newly developed symptoms. FC has emerged as one of the few noninvasive tools commonly used in IBD clinical practice, and there is now considerable evidence confirming the good accuracy of FC in detecting MH[2].

Even though some factors may influence its concentration (*e.g.*, the amount of mucus and blood in stool[45]), FC is one of the most sensitive noninvasive tools in differentiating IBD from functional disorders[46]. For IBD patients, different cutoff levels have been proposed for diagnostic purposes to differentiate between endoscopic active and nonactive disease, histologic healing[47] or prediction of relapse[2], but a consensus has not yet been established. At present, an FC level over 250 mcg/g is considered to indicate active colonic inflammation[48], while levels below 100 mcg/g may indicate endoscopic remission[49].

One major limitation of FC use refers to the so-called "gray zone" level between 100 mcg/g and 250 mcg/g, which is difficult to interpret. Bodelier *et al*[50] investigated the occurrence of indefinite FC levels in a real-life IBD cohort and studied the additional value of a combination of biochemical markers and clinical activity indices. In the studied cohort, 24% of CD and 15% of UC patients had FC in this "gray zone". Finally, if given the option, patients usually prefer blood over fecal tests[51], and this further limits the widespread use of FC in clinical practice.

Some of the circulatory markers investigated in the studies included in this review showed promising results. However, none of the investigated blood- or fecal-based markers showed great accuracy for MH prediction and therefore cannot replace endoscopy at this point.

Table 3 Synthetic presentation of data on combined marker performance in detecting mucosal healing

Ref.	Study type	Investigated marker	MH definition	CU/CD	Number of patients	AUC	95%CI	Sn (%)	Sp (%)	Cutoff level
Reinisch *et al*[43]	Post hoc analysis of clinical trial	FC, CRP and CDAI	CDEIS < 4	CD	244	0.68,	0.62-0.74	74	64	FC < 250 mcg/g, CRP < 5 mg/L, and CDAI < 150
D'Haens *et al*[41]	Prospective-specimen collection, retrospective–blinded-evaluation	EHI	SES-CD of ≤ 2 and ≤ 1 in each segment	CD	311 in 2 cohorts (116 + 195)	0.962; 0.693	0.942-0.982; 0.619-0.767	97.1; 83.2 (for cutoff of 20)	100; 87.8 (cutoff of 50)	≤ 20
Gubatan *et al*[42]	Prospective cohort	IL-4 + IL-10/IL-17A + TNF-α	Geboes Histologic Grade of < 3	CU	70	0.641		52.6	75	0.1522
Mavropoulou *et al*[44]	Prospective cohort	sIL-2R (CU); IL-6 (CD); FC (UC); FC (CD)	MES = 0-1; SES-CD ≤ 3	UC + CD	299 (84 + 145)	0.80; 0.80; 0.92; 0.84	0.68-0.91; 0.71-0.89; 0.86-0.99; 0.76-0.92	63; 69; 91; 70	85; 80; 89; 100	< 646 IU/mL; < 5.5 pg/mL; 340 mg/kg; < 180 mg/kg

EHI: Endoscopic healing index measuring 13 proteins in blood [angiopoietin 1 (ANG1) and 2 (ANG2), carcinoembryonic antigen-related cell adhesion molecule 1, C-reactive protein, serum amyloid A1, interleukin 7, transforming growth factor α, vascular cell adhesion molecule 1, extracellular matrix metalloproteinase inducer, and matrix metalloproteinases 1 (MMP1), 2 (MMP2), 3 (MMP3), and 9 (MMP9)]; UC: Ulcerative colitis; CD: Crohn's disease; AUC: Area under the curve; CI: Confidence interval; Sn: Sensitivity; Sp: Specificity; SES-CD: Simple endoscopic score for Crohn's disease; CRP: C-reactive protein; CDAI: Crohn's disease activity index; sIL-2R: Soluble interleukin 2 receptor; MES: Mayo endoscopic subscore; FC: Fecal calprotectin.

An ideal marker or multimarker should be noninvasive, sensitive, disease-specific, easy to perform, cost-effective[52] and ideally patient-friendly; such a marker remains to be identified by future research.

An important observation concerns newly established therapeutic targets in IBD management, which could soon be included as a standard of care achievement of histologic healing. Several studies have already assessed the performance of biomarkers in detecting this endpoint, such as the study conducted by Gubatan *et al*[42], which explored the accuracy of serum cytokines in assessing histologic healing, defined as a Geboes Histologic Grade of < 3. There is evidence that FC levels correlate more closely with histological evaluation than macroscopic findings, suggesting that this biological marker is more sensitive than endoscopy[53].

Widely used serum markers, such as CRP, white blood cell count, and erythrocyte sedimentation rate, combined with clinical activity scores are currently used in IBD monitoring. This approach lacks the diagnostic accuracy needed for decision making in patients with UC, and colonoscopy is still required for proper evaluation of disease activity.

For future research, a combined marker approach benchmarked against a well-investigated marker, such as FC, could be a promising alternative to endoscopic examination.

There are certain limitations to our analysis. We decided not to include data on CRP or imaging markers (magnetic resonance imaging, ultrasound) in detecting MH. A previous comprehensive analysis regarding the role of CRP in this setting was recently published by Krzystek-Korpacka *et al*[11]. In this review of 30 studies, there was a large variation in the selected optimal cutoff values, ranging from 0.4 mg/L to 28 mg/L. CRP performance as an MH marker displayed better sensitivity than specificity for CD, with respective median values of 79.5% *vs* 61%, and better specificity than sensitivity for UC, with respective median values of 82% *vs* 66%[1]. CRP is a widely used marker in clinical practice for IBD monitoring, but it still fails to provide sufficient accuracy to replace endoscopy as an independent marker for MH. Expanding the search to imaging modalities would have diluted the information provided and was beyond the scope of our paper. Both magnetic resonance imaging and ultrasound are indispensable tools in IBD management, but operator-dependent

and costly, limiting their widespread use and accessibility. Our search was we relied exclusively on PubMed (MEDLINE) database for identification of potentially eligible studies.

CONCLUSION

Various biomarkers for MH are currently under investigation and show promising results. However, at present, no biomarker is accurate enough to replace endoscopy. Therefore, it is our strong belief that future high-quality studies should further focus on establishing panels of biomarkers that would have higher predictive values than biomarkers alone.

ARTICLE HIGHLIGHTS

Research background

Mucosal healing (MH) has been one of the main therapeutic targets stipulated by recent recommendations. MH is associated with lower risks of relapse, hospitalization and surgery. In recent years, a wealth of publications regarding various noninvasive biomarkers for MH have reported conflicting results.

Research motivation

MH is objectivated by intrusive colonoscopy, and it is becoming increasingly apparent that surrogate noninvasive markers are needed for close and efficient disease monitoring and control. A reliable surrogate marker for MH would have a significant clinical impact, by reducing the number of endoscopic evaluations required during the course of disease.

Research objectives

We aimed to summarize published data regarding the performance of noninvasive biomarkers in assessing MH in inflammatory bowel disease patients.

Research methods

We conducted a systematic review of studies that reported the performance of biomarkers in diagnosing MH in patients with inflammatory bowel disease. The main outcome measure was to review the diagnostic accuracy of serum and fecal markers that showed promising utility in assessing MH.

Research results

We screened 1301 articles, retrieved 46 manuscripts and included 23 articles for full-text analysis. Fecal calprotectin (FC) was the most investigated fecal marker for assessing MH. The best performance for a serum marker was observed for the endoscopic healing index, which showed a comparable accuracy to the measurement of FC and a higher accuracy than the measurement of serum C-reactive protein.

Research conclusions

Several promising biomarkers of MH are emerging but cannot yet substitute for endoscopy with biopsy due to issues with reproducibility and standardization.

Research perspectives

For future research, a combined marker approach benchmarked against a well-investigated marker, such as FC, could be a promising alternative to endoscopic examination.

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CASE REPORT

Managing esophagocutaneous fistula after secondary gastric pull-up: A case report

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Abstract

BACKGROUND

Gastric pull-up (GPU) procedures may be complicated by leaks, fistulas, or stenoses. These complications are usually managed by endoscopy, but in extreme cases multidisciplinary management including reoperation may be necessary. Here, we report a combined endoscopic and surgical approach to manage a failed secondary GPU procedure.

CASE SUMMARY

A 70-year-old male with treatment-refractory cervical esophagocutaneous fistula with stenotic remnant esophagus after secondary GPU was transferred to our tertiary hospital. Local and systemic infection originating from the infected fistula was resolved by endoscopy. Hence, elective esophageal reconstruction with free-jejunal interposition was performed with no subsequent adverse events.

CONCLUSION

A multidisciplinary approach involving interventional endoscopists and surgeons successfully managed severe complications arising from a cervical esophagocutaneous fistula after GPU. Endoscopic treatment may have lowered the perioperative risk to promote primary wound healing after free-jejunal graft interposition.

Checklist (2016), and the manuscript was prepared and revised according to the CARE Checklist (2016).

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Core Tip: Gastric pull-up (GPU) reconstruction after esophagectomy may be complicated by leaks, fistulas, and stenoses. In such cases, endoscopic interventions, including endoscopic vacuum therapy, stenting, or dilatation, are the corrective treatments of choice, but surgery is preferred when esophageal reconstruction becomes necessary. Here, we report a case of esophageal reconstruction after a secondary GPU procedure by combining endoscopic and surgical techniques to perform subtotal esophageal resection and reconstruction using a free-jejunal graft interposition.

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INTRODUCTION

Gastric pull-up (GPU) procedures may be complicated by leaks, fistulas, and stenoses. In such cases, restoration of the esophagus and adequate recovery of alimentation becomes challenging[1]. Endoscopic interventions, including endoscopic vacuum therapy (EVT), stenting, or dilatation, are the corrective treatments of choice[2], but surgery is preferred when esophageal reconstruction becomes necessary[3]. Here, we report a case of esophageal reconstruction after a secondary GPU procedure by combining endoscopic and surgical techniques.

CASE PRESENTATION

Chief complaints

A 70-year-old male patient underwent emergency esophageal diversion after injury to the cervical esophagus during spinal surgery.

History of present illness

Six months later, retrosternal GPU procedure was performed but postoperative anastomotic leakage occurred. The leakage was successfully managed by endoscopy and the patient was discharged with a fully covered esophageal stent in place. Three weeks later, local cervical infection and sepsis developed. At this time, our tertiary university hospital was contacted for patient transfer.

History of past illness

Notable previous illnesses were arterial hypertonus, non-active smoking status and an ischemic stroke with incomplete senso-motoric hemiparesis. Furthermore, the patient had a thyroidectomy in the past and open prostatectomy due to prostate cancer.

Personal and family history

The patient had no family history that was related to the present illness.

Physical examination

On admission, the patient showed clear signs of malnutrition and systemic inflammation with jugular and cervical phlegmon.

Laboratory examinations

Initial laboratory data revealed a hemoglobin level of 6.7 g/dL, white blood cell count of 6400 cells/μL, and platelet count of 210 × 10^3/μL. The creatinine level was 0.76 mg/dL, unremarkable liver and cholestasis parameters, and albumin level was 2.8 g/dL.

Imaging examinations

A chest computed tomography (CT) scan and endoscopy revealed a dislodged esophageal stent, with the proximal flare abutting an extraesophageal air and fluid-filled cavity (Figure 1). During stent removal, a partially epithelialized esophageal perforation 2 cm distal to the pharynx with an infected cavity (Figure 1B and C) as well as a 5 cm-long stenosis of the remaining esophagus were detected.

FINAL DIAGNOSIS

Diagnosis of cervical esophagocutaneous fistula and anastomotic stenosis after GPU with signs of chronic malnutrition and systemic inflammation.

TREATMENT

The patient received calculated antibiotic treatment and was transferred to the intensive care unit. An immediate endoscopy was carried out, and the dislocated stent (partially covered metal stent, length 80 mm, diameter 18 mm) was removed. Endoscopy showed an old, partially epithelialized and wide esophageal perforation 2 cm distal of the pharynx with an infected cavity behind it. Distal of the fistula, a long-segment (5 cm) stenosis of the esophagus ranging up to the esophago-gastrostomy was confirmed. Notably, the perforation and the fistula cavity were likely to be a secondary injury due to the dislocated cervical stent tulip (diameter 24 mm). After pus evacuation and debridement of the fistula, a fully covered self-expanding metal stent (length 100 mm, diameter 20 mm) was inserted in order to cover the fistula and to treat the stenosis. Due to an expected long-term course, a percutaneous jejunal feeding catheter was surgically inserted to ensure sufficient enteral nutrition. The first re-endoscopy revealed a slippage of the stent so that the cranial fistula ostium was not sufficiently covered. The stent position was successfully corrected but dislocated again during follow-up.

Thus, endoscopic management was switched to EVT. An EsoSponge system (B. Braun Melsungen, Germany) was placed into the esophagocutaneous fistula ostium (cavity). The jugular and cervical phlegmon resolved during EVT within two weeks and repeated endoscopic balloon dilatation was performed to resolve the esophageal stenosis. However, it became obvious that endoscopic management could not resolve the broad and rigid fistula with the concomitant partially obliterated esophagus. The issue was discussed interdisciplinarily and the therapeutic aim of endoscopy was redefined: Profound consolidation of the cervical infection and cleaning of the fistula cavity until salvage surgery (Figure 1D).

The cervical phlegmon resolved within two weeks, allowing subtotal esophageal resection and reconstruction using a free-jejunal graft interposition according to Ott *et al*[4]. Pre-operative work up included CT angiography to establish the feasibility of vascular reconstruction. Partial sternotomy was necessary to provide access to the esophagus and the stomach (Figure 2A). After resection of the cervical esophagus and fistula, the exploration confirmed that the GPU could not be mobilized up to the pharynx. Thus, laparotomy was performed and a suitable jejunal segment was harvested. The left carotid artery and the left jugular vein were used as recipient vessels. The graft was implanted in an isoperistaltic position. The cervical anastomosis was performed in an end-to-end fashion and the upper mediastinal gastro-jejunostomy in a side-to-side fashion (Figure 2B). A part of the upper sternum was resected to avoid any pressure on the graft and gastrojejunostomy. The soft tissue defect was covered with a sternocleidomastoid muscle flap. Abdominal reconstruction was achieved by an end-to-end jejunojejunostomy.

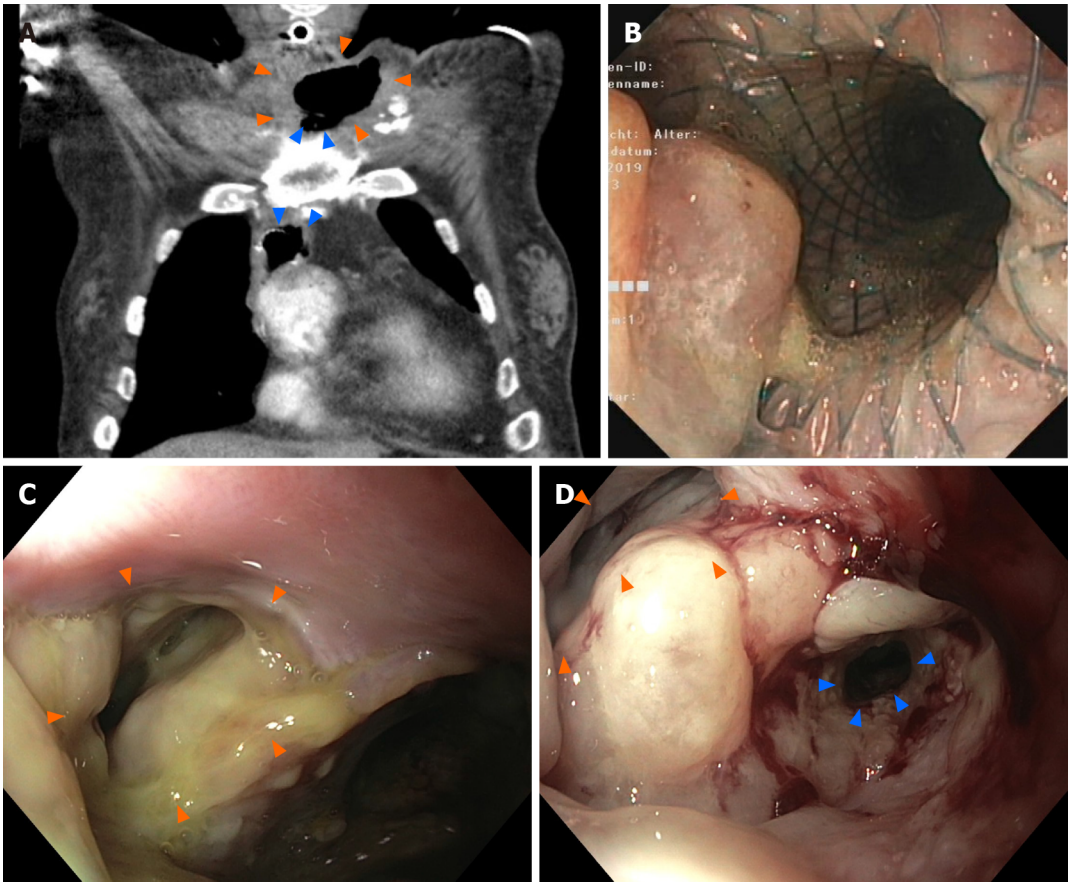


Figure 1 Preoperative findings. A: Contrast-enhanced computed tomography with oral and intravenous contrast application showing the jugular abscess (indicated by orange arrowheads) and the fully covered self-expandable metallic stent with its proximal end in the fistula (indicated by blue arrowheads); B: Endoscopy showing fully covered self-expandable metallic stent placed in the distal stenotic esophagus with the esophagocutaneous fistula directly orally of the stent. The fistula orifice is partially covered by the stent flare; C: Endoscopy after stent extraction with the large prestenotic fistula orifice (indicated by orange arrowheads); fistula filled with pus; D: Endoscopy after endoscopic vacuum therapy with cleaned fistula (indicated by orange arrowhead). The fistula is entirely epithelized. The distal esophagus segment remains stenotic (indicated by blue arrowheads).

OUTCOME AND FOLLOW-UP

The postoperative course was uneventful. Oral alimentation was reestablished accompanied with daily speech therapy. Anastomotic healing was confirmed radiologically and endoscopically and the patient was transferred to a rehabilitation clinic in good clinical condition.

DISCUSSION

Esophagectomy with diversion and later reconstruction of the upper gastrointestinal (GI) tract is reserved for complex esophageal disease[5]. Despite numerous advantages over alternative reconstruction techniques, anastomotic complications following GPU procedures occur in up to 12% of cases[6].

Managing anastomotic leakage or stricture of GPU can be extremely challenging because of local tissue damage from previous surgery or chronic infection[1,7]. Endoscopic management of leaks, fistulas and stenosis in the upper GI tract has therefore not only become a valuable tool, but the standard approach for successful management even in complex situations[8,9]. Here, it is extremely important to carefully consider and constantly evaluate the local tissue and wound conditions. In our case, the constant pressure caused by the stent itself may have further deteriorated the esophageal wall and, thus, may have caused perforation and fistula with abscess formation since no external drainage had been established.

The surgical options available for the management of a complicated GPU procedure are limited and usually contain a high risk of morbidity and mortality[5]. Esophageal reconstruction with a free jejunal graft was introduced by Seidenberg and colleagues

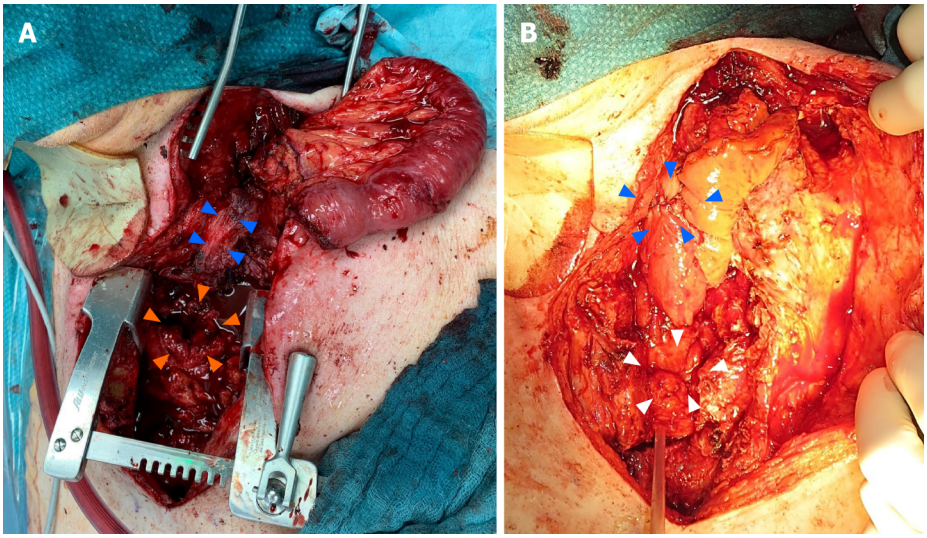


Figure 2 Findings during surgery. A: Free jejunal segment after reperfusion before intestinal anastomosis; orange arrowheads indicating the orifice of the gastric pull-up; blue arrowheads indicating the stenotic distal esophagus; B: Free jejunal segment after intestinal anastomosis; blue arrowheads at the mesentery of the free jejunal graft indicating the proximal esophago-jejunostomy; white arrowheads indicating distal jejuno-gastrostomy.

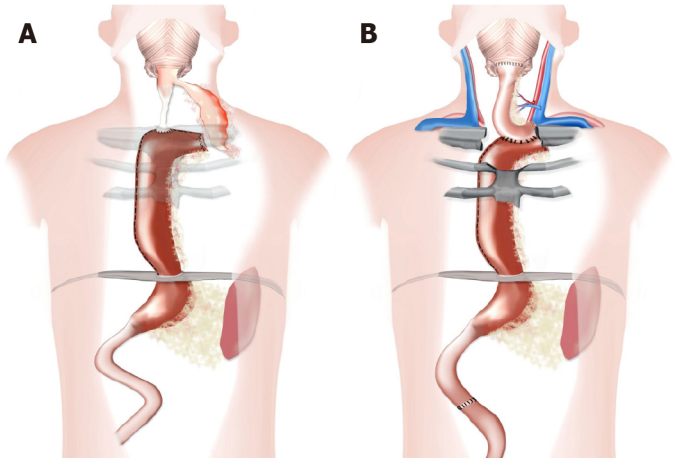


Figure 3 Anatomic schemes. A: Anatomy on admission; B: Reconstruction after surgery.

in 1959 and later modified by Siewert and colleagues in 2008 for treating cancer of the cervical esophagus[4,10]. The use of a salvage option after failed GPU has also been described[12]. Recent data regarding follow-up results in esophageal reconstruction strongly suggest the superiority of intestinal flaps over other reconstructions such as skin flaps.

High failure rates for secondary attempts after failed esophageal reconstruction have been described[1], with a complication rate in excess of 70%[11]. However, free jejunal grafts have considerable advantages. They are usually easy to harvest, provide low donor-site morbidity, have good size approximation to the cervical esophagus, tolerate reflux well, and have a low rate of peptic ulceration incidence[12]. Disadvantages include the need for transient ischemia that requires advanced microsurgical skills for swift revascularization and a challenging surgical site environment because of previous surgery and infection.

CONCLUSION

A multidisciplinary approach involving interventional endoscopists and surgeons was necessary to enable successful salvage management. Despite prior complications, operations and chronic infection, restoration and reconstruction of the esophagus was ultimately achieved (Figure 3). Thus, patients with chronic postoperative fistulas or

stenoses of the esophagus should be referred to specialized centers in a timely manner.

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