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OPINION REVIEW

Trial eligibility in advanced hepatocellular carcinoma: Does it support clinical practice in underrepresented subgroups?

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Abstract

Although hepatocellular carcinoma is considered a highly lethal malignancy, recent therapeutic advances have been achieved during the last 10 years. This scenario resulted in an unprecedented improvement in survival for patients with advanced hepatocellular carcinoma, almost reaching 20-26 mo of overall survival after first-second line sequential treatment. The advent of the combination of atezolizumab with bevacizumab showed, for the first time, superiority over sorafenib with improvement in overall survival. However, first and second-line trials were correctly based on the premise that a strict selection of patients enhances the power to capture the positive effect of treatment by excluding competing risks for mortality such as liver failure, decompensated cirrhosis or other underlying medical conditions. As a result, the inclusion criteria used in clinical trials do not support the use of novel therapies in several real-world scenarios involving underrepresented subgroups, such as patients with unpreserved liver function, other comorbid conditions, a history of solid-organ transplantation, autoimmune disorders and those with a high risk of bleeding. The present text aims at discussing treatment strategies in these subgroups.

Key Words: Eligibility; Systemic therapies; End-stage; Hepatocellular carcinoma

Core Tip: The strict criteria used in clinical trials in advanced hepatocellular carcinoma have led to a scarcity of available data in a considerable proportion of patients in the real-world practice. The daily challenge of treating these underrepresented subgroups

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can be overcome by future clinical trials addressing special situations, collaborative studies and real-world data.

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INTRODUCTION

Although hepatocellular carcinoma (HCC) is considered a highly lethal malignancy, recent therapeutic advances have been achieved during the last 10 years. These achievements were unthinkable 20 years before. Historically, patients with HCC at advanced stages or refractory to locoregional therapies (such as surgery, ablation or intra-arterial treatments) were associated with a dismal prognosis[1]. This scenario has fortunately changed.

In 2008, the first positive phase III trial (SHARP trial) using a systemic agent for HCC was published, showing that sorafenib improved overall survival over placebo in a selected population[2]. This result was observed in the Asia-Pacific trial, repeating similar observations yet in another population[3]. Sorafenib has succeeded due to its activity against different tumor pathways, particularly angiogenesis and proliferation signaling activation even in the absence of significant tumor shrinkage. It showed a favorable safety profile, particularly in patients with a well-preserved liver function (Child-Pugh A), a performance status of 2 or less and no other organ failure.

Following sorafenib, several drugs with similar or different targets were tested with disappointing results in phase III trials[4]. On the other hand, lenvatinib, shown to be non-inferior to sorafenib in the phase III REFLECT trial in patients without main portal trunk tumor invasion or without more than 50% of liver involvement[5], became an alternative in the first-line setting. Other agents such as regorafenib[6], cabozantinib[7] and ramucirumab[8] were incorporated as second-line options after sorafenib failure. This scenario resulted in an unprecedented improvement in survival for patients with advanced HCC, almost reaching 20-26 mo of overall survival after first-second line sequential treatment[9,10].

The advent of immune checkpoint inhibitors (ICI) with impressive results in solid tumors underpinned trials in advanced HCC. ICIs were rapidly incorporated after encouraging results with nivolumab and pembrolizumab in phase II trials with HCC patients, with durable objective response rates in 15%-20% of the patients[11,12]. In 2020, the combination of atezolizumab (a programmed death ligand 1 inhibitor) with bevacizumab [an anti-vascular endothelial growth factor-vascular endothelial growth factor (VEGF)-antibody] showed for the first time superiority over sorafenib in the phase III IMBRAVE150 trial[13]. This result was followed by approval of this combination as the standard first-line treatment for advanced HCC in different countries.

However, first and second-line trials were correctly based on the premise that a strict selection of patients enhances the power to capture the positive effect of treatment by excluding competing risks of mortality such as liver failure, decompensated cirrhosis or other underlying medical conditions (Figure 1). As a result, the inclusion criteria used in clinical trials do not support novel therapies in several real-world scenarios involving underrepresented subgroups. Moreover, due to the mechanism of action of ICIs and the risk of immune-related adverse events, the IMBRAVE trial did not enroll specific subgroups, such as patients with a history of solid-organ transplantation, auto-immune disorders, and a high risk of bleeding. The present text aims at discussing treatment strategies in these subgroups.

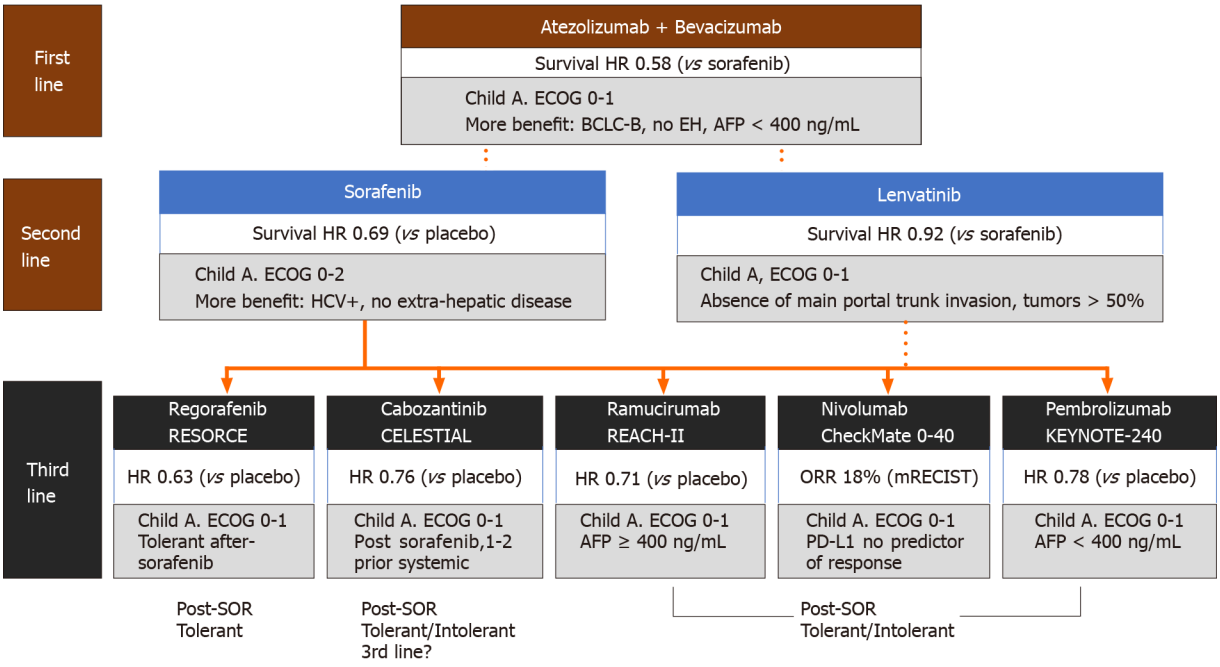

Figure 1 Systemic therapy for advanced hepatocellular carcinoma. Note: First and second-line options may be presented as first-line options in parallel. Exclusion criteria in the REFLECT trial shown for lenvatinib[5]. AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; ECOG: Eastern Cooperative Oncology Group; EH: Extrahepatic; HCV: Hepatitis C virus; HR: Hazard ratio; ORR: Overall response rate; PD-L1: Programmed death ligand 1; SOR: Sorafenib.

CHALLENGES IN REAL-WORLD SCENARIOS

Etiology of underlying liver disease: does it really matter for decision making?

In Western countries (mainly Europe and the United States), the leading risk factor for HCC is chronic hepatitis C virus (HCV) infection. In contrast, hepatitis B virus (HBV) chronic infection is predominant in China, Asia and sub-Saharan Africa, where a higher burden of HCC is found compared to the rest of the world[14]. In Latin America, HCV represents the most prevalent risk factor for HCC[15,16], but other etiologies, such as nonalcoholic fatty liver disease, are steadily increasing[17,18].

This geographic heterogeneity directly impacts the recruitment of patients. Trials that restrict enrollment to a specific region are likely to be enriched with the predominant local etiology. Clinical trials that recruit globally tend to have HBV as the most frequent etiology when the Asian population predominates. A noticeable transition in risk factors has been observed in Western countries, with growing evidence that metabolic-associated fatty liver disease is an increasing cause of HCC, often associated with other comorbidities such as obesity, hypertension and diabetes [19].

This geographical eligibility is exemplified by the pivotal trials exploring sorafenib. In the SHARP trial, only 18.4% of the enrolled population had HBV-related HCC[2], while 73% of the patients enrolled in the Asia-Pacific trial had HBV-related HCC[3]. Although both trials showed a benefit in overall survival irrespective from underlying liver disease, a combined analysis of these two trials demonstrated a significant benefit in patients with HCV[20].

Nonviral etiologies represent only 30% to 45% of the included population in recent immunotherapy trials (Table 1). Whether immunotherapy is equally effective across all etiologies is still uncertain[13], shown through subgroup analysis in the IMBRAVE150 study suggesting that atezolizumab plus bevacizumab may have a lower benefit over sorafenib in nonviral etiologies [hazard ratio (HR): 0.91, 95% confidence interval (CI): 0.51-1.60], when compared to HBV (HR: 0.51; 95%CI: 0.32-0.81) or HCV-associated HCC (HR: 0.43; 95%CI: 0.22-0.87). This was also shown in the first-line trial comparing nivolumab *vs* sorafenib (Checkmate-459 trial, NCT02576509), in which nivolumab did not reach superiority over sorafenib in the overall population. Stratified subgroup analysis showed an HR of 0.91 (95%CI: 0.72-1.16) in nonviral etiologies when compared to HBV (HR: 0.79; 95%CI: 0.59-1.07) or HCV-associated HCC (HR: 0.72; 95%CI: 0.51-1.02). More recently, it has been shown that chronic inflammation in nonalcoholic fatty liver leads to liver injury and promotes liver cancer, impairing tumor surveillance. A meta-analysis of three randomized-control trials (IMbrave150,

Table 1 Studies reporting the effect and safety of first-line therapies in advanced hepatocellular carcinoma

	IMbrave: Phase III, open-label		REFLECT: Phase III, open-label		CheckMate-459: Phase III, open-label	
	Atezo + Bev	Sorafenib	Lenvatinib	Sorafenib	Nivolumab	Sorafenib
n	336	165	478	476	371	372
Age, median	64	66	63	62	65	65
≥ 65 yr, %	48	55	44	41	NR	NR
Male, %	82	83	85	84	85	85
Asia, %	56	58	70	68	NR	NR
ECOG 1, %	38	38	36	37	27	30
AFP ≥ 200 ng/mL, %	43	45	46	39	39	43
HBV, %	49	46	53	48	31	31
HCV, %	21	22	19	26	23	23
Nonviral, %	30	32	28	26	45	45
MVI trunk			Excluded		Excluded	
MVI, %	38	43	23	19	NR	NR
EH, %	63	56	61	62	NR	NR

AFP: Alpha-fetoprotein; Atezo + Bev: Atezolizumab + bevacizumab; ECOG: Eastern Cooperative Oncology Group; EH: Extrahepatic tumor disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; MVI: Macrovascular invasion or neoplastic thrombosis of the main portal trunk; NR: Not reported.

Checkmate-459 and KEYNOTE-240), showed that treatment with ICIs in these patients is associated with reduced survival compared to other etiologies[21].

Although not conclusive, subgroup analysis might offer partial information and generate a hypothesis for future trials. In regard with etiology, there is some molecular background that supports the existence of different molecular activated pathways according to molecular and transcriptomic-based features, which may lead to distinct activation of antitumor immunity or even to ICI resistance[22].

Elderly patients: discrepancy between trials and real-world scenario?

Approximately 70% of patients with cancer are aged 65 or older. The number of patients with cancer in this age group is projected to increase over the next decades significantly[23]. The aging process has been associated with changes in antineoplastic agents' pharmacokinetics due to a number of age-related changes, including modifications in renal and liver function, leading to altered drug absorption, metabolism and distribution.

The mean age of patients with HCC included in clinical trials is around 65-years-old. However, a substantial proportion of HCC patients are older. In HCC and other malignancies, elderly patients are underrepresented in clinical trials. In HCC, exceptionally, the concomitance of advanced age with chronic liver disease raises concerns about toxicity and clinical benefit.

A pooled analysis of both sorafenib pivotal trials (SHARP and Asia-Pacific) did not demonstrate prognostic differences between patients < or ≥ 75 years[20], suggesting that well-selected individuals could derive benefit from systemic treatment irrespective of age.

A concern that elderlies may present a poor tolerance to systemic treatment primes a trend for early discontinuation of sorafenib in field-practice studies[24]. However, in this cohort study (SOFIA Italian study), patients with half dosing sorafenib were associated with improved overall survival and discontinuation with worse outcomes. Consequently, early reduction avoiding definitive treatment discontinuation should be mandatory[24]. On the other hand, other studies did not show significant differences in overall survival and class-specific adverse events with lenvatinib in older patients [25]. A subanalysis of the IMBRAVE150 trial evaluating patients aged < or ≥ 65 years showed a similar toxicity profile, patient-reported outcomes and survival outcomes [26].

Most available data come from retrospective studies and subanalysis of prospective trials, mainly with sorafenib. Most of these data support that age alone should not

restrict treatment in advanced HCC, but a multidisciplinary approach and frailty metrics, apart from Eastern Cooperative Oncology Group grades, can be helpful in managing this group.

The limits of liver function: Is it unquestionable?

Cirrhosis and its complications (ascites among others) are the most significant competing risk for mortality in patients with HCC. In fact, prior evaluation of liver function, liver decompensation (prior history of ascites and its complications) or clinically significant portal hypertension are mandatory before systemic therapy initiation or selection (*e.g.*, presence of gastric or esophageal varices, other abdominal collaterals, enlarged spleen more than 120 mm, low platelet count < 150000 mm^3, among others). Due to this fact, clinical trials specifically selected those populations in which HCC determines the risk of mortality so that the antitumor treatment effect is more likely to be captured without distortion by cirrhosis-imposed threats. The majority of trials strictly included patients with preserved liver function, Child-Pugh A, or without liver decompensation events. It results in the lack of robust data showing how to manage patients with advanced HCC and impaired liver function. On the other hand, liver decompensation during systemic therapy leads to a significant impact on overall survival and an exclusion of sequencing systemic options[27,28].

The GIDEON study[29], the most extensive real-world data including patients treated with sorafenib, demonstrated that the median survival of patients with unpreserved liver function or Child-Pugh B and C was 5.2 mo and 2.6 mo, respectively. On the one hand, this result shows almost futility and discourages systemic agent use in patients with very poor liver function (Child-Pugh C) due to lack of treatment benefit. On the other hand, Child-Pugh B patient data suggests that well-selected patients can be considered for treatment, although more robust data is lacking. It seems that the presence of clinically significant ascites is a mandatory exclusion criterion. For example, in patients treated with sorafenib, those with a Child-Pugh B7 score without ascites presented similar outcomes than Child-Pugh A6[30]. Another retrospective study showed poor survival in patients with Child-Pugh B treated with lenvatinib[31].

Some authors recommend against grading ascites due to its subjective assessment, showing that the albumin-bilirubin score may be an alternative tool to evaluate prognosis in candidates for systemic treatment[32]. However, events of liver decompensation, such as ascites, jaundice or encephalopathy, have been associated with a significant worse prognosis and should always be part of eligibility criteria in trials and in the real-world setting.

Safety and efficacy in patients with liver dysfunction should not be extrapolated to all tyrosine kinase inhibitors (TKIs). The GIDEON cohort study showed an increasing incidence rate of serious adverse events from Child Pugh A to B or C, with a rising rate of sorafenib discontinuation[29]. Moreover, almost 20% of the patients may experience clinical deterioration due to liver impairment with the treatment with TKIs, particularly during the first 4 wk of therapy[6,24,33]. Nevertheless, in the second-line setting, patients allocated to cabozantinib in the CELESTIAL trial who presented worsening in liver function by week 8 had a manageable safety profile and maintained treatment benefit compared to the total cohort[7].

Whether these data could be extrapolated to ICIs is a matter of debate. Nivolumab was tested in a prospective Child-Pugh B cohort (75% of Child-Pugh B7)[11]. The median overall survival was 7.6 mo, with a disease control rate of 55.1%. Although safety profile may be more favorable, there is a paucity of data on other immune-oncology drugs in the setting of liver dysfunction.

The safety of combined therapies, including ICIs and VEGF targeted pathways (TKIs or anti-VEGF), in patients with unpreserved liver function is a matter to be clarified in prospective real-world data. Tyrosine kinase inhibitors and immunotherapy seems to be feasible in patients with a mild liver alteration. A close follow-up and multidisciplinary management are paramount to secure safety and better outcomes.

Recurrent HCC after liver transplant: An orphan situation in clinical trials

Liver transplantation has been an exclusion criterion in all clinical trials enrolling patients with advanced HCC, TKIs or ICIs. Safety concerns and overall survival in immunosuppressed patients has been one of the main explanations of this exclusion criteria.

However, the use of TKIs has been reported in retrospective cohort studies with acceptable results. Sorafenib was shown to be safe and effective, with a median overall survival of 20.1 mo[34]. The toxicity profile and the risk for liver graft deterioration

have been reported to be similar and lower than patients with no history of transplantation, respectively[35]. Favorable outcomes were observed in a multicenter retrospective study exploring the sorafenib-regorafenib sequencing therapy in the post-transplant setting. The median survival was 12.9 mo (95%CI: 6.7-19.1) since regorafenib initiation and 38.4 mo (95%CI: 18.5-58.4) since sorafenib discontinuation [36]. Other studies have already reported outcomes with lenvatinib in the post-transplant setting.

The risk of allograft rejection with ICI therapy precludes these patients from being treated with ICIs, either monotherapy or in combination with TKIs or anti-VEGF[37]. Therefore, sequencing TKIs is the optimal approach for patients with tumor recurrence after liver transplantation not amenable to local treatment.

Risk of bleeding events associated with systemic treatments

Patients with HCC and coexisting cirrhosis have an increased risk of bleeding events due to portal hypertension. However, the risk of spontaneous bleeding in other organs is rare, and these patients are paradoxically at a higher risk of thrombotic events[38, 39]. The risk of bleeding goes in parallel with the presence and severity of portal hypertension. In patients without prior endoscopy, at least during the last 6 mo, the risk of variceal hemorrhage should be assessed before systemic therapy, particularly with bevacizumab. Primary or secondary prophylaxis of variceal hemorrhage should be implemented according to International Consensus guidelines (*e.g.*, BAVENO VI), either with beta-blockers or endoscopic variceal banding or both for secondary prophylaxis. In some patients without any surrogate marker of clinically significant portal hypertension (*e.g.*, presence of enlarged spleen more than 120 mm, low platelet count < 150000 mm^3 or other abdominal collaterals), upper endoscopy may be replaced by transient elastography as a first or additional approach to rule-out gastroesophageal varices.

Bleeding can occur either due to variceal cause or spontaneous tumor rupture, both dramatic events associated with dismal outcome in patients with advanced HCC. In fact, HCC leads to an increase in portal hypertension, and consequently the risk of bleeding should be reassessed in these patients. Drugs with antiangiogenic activity, TKIs or anti-VEGF are associated with an increased risk of bleeding that usually does not require significant interventions. In pivotal trials, sorafenib was associated with a low risk of severe bleeding events (7% any grade, 1% grade 3) as well as ramucirumab (1% of grade 3-4)[2,8].

On the contrary, the IMBRAVE150 trial did not include patients with untreated or incompletely treated esophageal or gastric varices (according to local clinical practice, either beta-blockers or endoscopic procedures)[13]. This concern was based on the risk of tumor-associated hemorrhage with bevacizumab (3%-5%), with reported fatal bleeding cases in earlier trials[40]. Despite the exclusion of high-risk patients and a well-balanced risk of bleeding (26% of each group had varices), there was a 25.2% rate of any grade bleeding events in the atezolizumab-bevacizumab arm, and fatal bleeding events occurred in 6 patients in the IMBRAVE150 trial (1.8%). Specifically, variceal bleeding occurred more frequently in the atezolizumab plus bevacizumab arm *vs* sorafenib (7% *vs* 4.5%)[13].

The risk of bleeding should be extensively assessed in systemic treatment candidates, and a careful follow-up should be carried out in the real-world setting. Particular attention is required for those patients considered for atezolizumab and bevacizumab, patients using anticoagulants and those with a recent history or higher risk of variceal bleeding (*e.g.*, esophageal or gastric varices with red spots).

Common comorbidities and other conditions excluded in HCC trials

The classes of agents used for treating advanced HCC have particular prescribing concerns due to their mechanism of action. TKIs with antiangiogenic properties may increase the risk of cardiovascular disease and ischemic events. Consequently, patients with risk for cardiovascular events, such as diabetes or prior cardiovascular complications, are underrepresented in clinical trials, although they were not entirely excluded from enrollment. The challenge in such situation relies on the proper management of risk factors. ICIs, on the other hand, carried a low risk of cardiovascular events.

Drug interaction is a crucial topic, particularly with antiretroviral therapy for HIV. Patients with HIV are not included in clinical trials, but a real-world study showed that sorafenib does not impact viral load and CD4-T cell count[41]. Data with immunotherapy for HIV-positive patients lack as they were excluded from pivotal trials with ICIs.

Patients under supportive renal care or hemodialysis have been excluded from clinical trials, and more recent real-world data has been reported with sorafenib treatment[42]. Finally, ICIs may exacerbate autoimmune disorders. Some of these disorders are associated with an increased risk of HCC, such as autoimmune hepatitis.

Exacerbation of immune disorders and immune-related adverse events may occur in up to 75% of the cases. In this regard, ICIs should be used with caution in this population[43]. Many of these events can be managed without discontinuing therapy, but further data are required. Also, there is a deep concern with extrapolating the management of these adverse events in patients with cirrhosis. Most clinical guideline recommendations are based on non-cirrhotic patients[43]. Although immune-related events should be promptly recognized and adequately treated, the use of high steroid doses should be cautiously implemented in cirrhosis[44]. It is already known that the use of steroids may accelerate or result in liver decompensation (*e.g.*, ascites development, among other events).

Sequencing therapies beyond clinical trials

In the second-line setting, all effective options were explored after sorafenib, either intolerance or tumor progression. There is no comparative study that evaluated how second-line drugs perform after lenvatinib or atezolizumab plus bevacizumab. Regorafenib was superior to placebo in sorafenib-tolerant patients[6], ramucirumab was effective in patients with high alpha-fetoprotein (AFP) levels[8], and cabozantinib showed better survival in second or third-lines over placebo[7]. In addition, the combination of nivolumab-ipilimumab (a dual ICI combination) was granted approval after sorafenib based on an encouraging phase II trial[45].

Although more recent retrospective studies have compared nivolumab *vs* regorafenib efficacy, all second-line competitors have not been compared face-to-face in clinical trials[46]. Head-to-head comparisons between all these options are unlikely to be addressed in future trials, so sequencing strategies will be an unmet knowledge requiring real-world data outside clinical trials. Some assumptions are reasonable to be considered when choosing the best strategy (Figure 2).

The selection based on the safety profile is crucial. For example, risk of bleeding, cardiovascular events or immune-related adverse events may impact negatively if not correctly assessed. Survival is the primary objective, but patients with tumor-related symptoms may also benefit from therapies with a higher response rate, such as lenvatinib or atezolizumab plus bevacizumab. Special subgroups not included in trials may be more safely managed based on real-world data showing favorable results. For example, this is the case of sorafenib-regorafenib therapy in transplanted patients.

Alternating treatments with different mechanisms of action instead of using sequences of drugs directed to the same target is a reasonable strategy, although not evidenced-based in clinical trials, particularly for third or even fourth-line therapies. For example, after progression on immunotherapy-based therapy, a TKI is more likely to be effective and vice versa. This issue will be a major discussion when novel therapies are incorporated following the results of ongoing clinical trials.

There is still an unmet need in HCC. The use of biomarkers for treatment selection, except high AFP levels for ramucirumab therapy, is lacking. Even the expression of programmed death ligand 1 in tumor tissue has not been associated with a predictive response. While the neutrophil-lymphocyte ratio has already been associated with better response with sorafenib[20] and lenvatinib[47], other biomarkers in other settings have been extensively explored without clinical implication[48,49].

CONCLUSION

The strict criteria used in clinical trials in advanced HCC have led to a scarcity of available data in a considerable proportion of patients in real-world practice. The daily challenge of treating these underrepresented subgroups can be overcome by future clinical trials addressing special situations, collaborative studies and real-world data [50]. A critical view of study design is essential to avoid excessive extrapolation and not limit efforts to provide better care to some subgroups that are not widely included in clinical research.

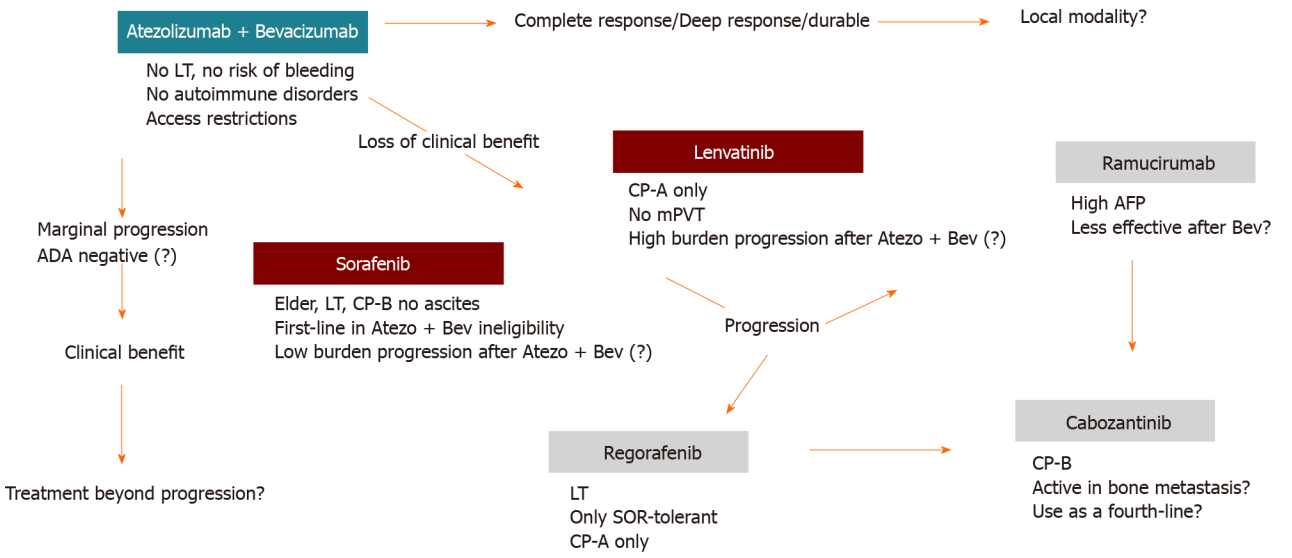

Figure 2 Sequencing systemic therapies in real-world setting. Note: These recommendations should be individualized for each patient. ADA: Anti-drug antibodies; AFP: Alpha-fetoprotein; Atezo + Bev: Atezolizumab + bevacizumab; CP: Child-Pugh; LT: Liver transplantation; mPVT: Main portal vein thrombosis; SOR: Sorafenib.

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REVIEW

Chronic intestinal failure and short bowel syndrome in Crohn's disease

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Abstract

Chronic intestinal failure (CIF) is a rare but feared complication of Crohn's disease. Depending on the remaining length of the small intestine, the affected intestinal segment, and the residual bowel function, CIF can result in a wide spectrum of symptoms, from single micronutrient malabsorption to complete intestinal failure. Management of CIF has improved significantly in recent years. Advances in home-based parenteral nutrition, in particular, have translated into increased survival and improved quality of life. Nevertheless, 60% of patients are permanently reliant on parenteral nutrition. Encouraging results with new drugs such as teduglutide have added a new dimension to CIF therapy. The outcomes of patients with CIF could be greatly improved by more effective prevention, understanding, and treatment. In complex cases, the care of patients with CIF requires a multidisciplinary approach involving not only physicians but also dietitians and nurses to provide optimal intestinal rehabilitation, nutritional support, and an improved quality of life. Here, we summarize current literature

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on CIF and short bowel syndrome, encompassing epidemiology, pathophysiology, and advances in surgical and medical management, and elucidate advances in the understanding and therapy of CIF-related complications such as catheter-related bloodstream infections and intestinal failure-associated liver disease.

Key Words: Chronic intestinal failure; Short bowel syndrome; Crohn's disease; Inflammatory bowel disease; Parenteral nutrition; Intestinal failure-associated liver disease

Core Tip: Chronic intestinal failure (CIF) is a rare but feared severe complication of Crohn's disease, with 60% of patients permanently dependent on parenteral nutrition. This review aims to summarize the knowledge available in the current literature describing recent advances in the management and treatment of adult patients with CIF, with emphasis on patients with Crohn's disease. Moreover, it aims to further understanding of modern approaches to CIF complications such as catheter-related bloodstream infections and intestinal failure-associated liver disease.

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INTRODUCTION

Chronic intestinal failure (CIF) is a rare but feared severe complication of Crohn's disease (CD). Sixty percent of patients with CD and CIF are permanently dependent on parenteral nutrition (PN). According to the recommendations of the European Society for Clinical Nutrition and Metabolism (ESPEN)[1], CIF is defined as "a reduction of gut function below the minimum necessary for the absorption of macronutrients and/or water and electrolytes, such that intravenous supplementation is required to maintain health and/or growth". ESPEN suggests classifying CIF on the basis of anatomical, functional, pathophysiological, or clinical characteristics (Figure 1) and has defined five major pathophysiological conditions causing CIF: Short bowel, intestinal fistula, intestinal dysmotility, mechanical obstruction, and extensive mucosal disease[1]. In CD, the most common cause of CIF is short bowel syndrome (SBS), in which the small bowel length, by definition, is less than 200 cm[1]. Concerning nutritional-medicinal management, patients with CIF are most frequently subdivided according to the type and extent of bowel resection and additional surgical procedures (*e.g.*, stomata).

CLASSIFICATION

According to the recommendations of ESPEN, the classification of CIF can be based on pathophysiological, anatomical, functional, or clinical criteria (Figure 1).

From a pathophysiological point of view, a classification into five types (of which SBS is one) has been proposed based on the presence of various gastrointestinal and/or systemic diseases (Table 1)[2].

Anatomically, according to the type and extent of bowel resection and additional surgical procedures (stomata), three different categories of short bowel can be distinguished as prognostic criteria for future disease progression (Figure 2)[1,3]: Type I: Terminal jejunostomy ("very SBS" if remnant bowel length < 50 cm); Type II: Jejunoascendostomy, jejunotransversostomy, or jejunodescendostomy (rarely with colostoma); Type III: Jejunoileotransversostomy (very rarely with colostoma).

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Table 1 Pathophysiological classification of intestinal failure (adapted from Pironi *et al*[2], 2015)

Type	Primary cause	Underlying disease
Short bowel type	Quantitative/qualitative loss of resorptive surface	Post-operative, in patients with mesenteric infarction, Crohn's disease, radiation enteritis, familial polyposis, abdominal traumata, necrotizing enterocolitis[1], bariatric obesity surgery (biliopancreatic diversion with or without duodenal switch), gastroileal anastomosis, extensive tumor resection.
		Congenital, in patients with gastroschisis[1], intestinal atresia[1], intestinal malformation, omphalocele
Fistula type	Bypass of resorptive surface due to jejunocolic fistula	Inflammatory bowel disease (Crohn's disease[2], diverticulitis, radiation enteritis)
		Post-operative[3] in patients with neoplasia (colorectal carcinoma, ovarian carcinoma, Crohn's disease)
		Iatrogenic (post-op, percutaneous drainage)
		Traumata
		Foreign bodies
Dysmotility type	Restricted (insufficient) nutrition intake due to postprandial exacerbation of symptoms, to the point of non-mechanic ileus in severe cases	Ogilvie syndrome (acute non-mechanic obstruction of the colon)
		Chronic intestinal pseudo-obstruction: Primary/idiopathic (neuropathic/myopathic); Secondary (collagenous vascular disease, *e.g.*, PSS, LE, Ehlers-Danlos syndrome; neurological disorders such as Morbus Parkinson, intestinal hypoganglionosis; endocrinopathies)
Obstruction type	Reduced nutrition intake; Increased secretion of liquid and electrolytes in obstructive segments of the intestine; Loss of liquids and nutrients due to recurrent vomiting and/or "overflow sensors"	"Frozen abdomen" in patients with peritoneal carcinomatosis, extensive intestinal adhesion, recurrent peritonitis.
		Neoplastic stenoses and/or strictures
		Incarceration/strangulation of the intestine (hernia)
		Volvulus
Mucosa type	Extensive loss or damage of mucosa results in insufficient resorption of nutrients and pronounced enteral loss	Microvillus inclusion disease[1]
		Tufting enteropathy (intestinal epithelial dysplasia)
		Tricho-hepato-enteric syndrome
		Autoimmune enteropathy
		Intestinal lymphangiectasia
		Protein-losing enteropathy (Morbus Waldman)
		Radiation enteritis
		Chemotherapy-induced/associated enteritis

[1]Causes 70% of pediatric cases[4].
[2]75%-85% of enterocutaneous fistulae (EF).
[3]15%-25% of EF[3].
LE: Lupus erythematosus; PSS: Progressive systemic sclerosis.

The functional classification of intestinal failure, first described by Shaffer[4], is based on its time frame, metabolic course, and long-term progression: Type I: Acute and short-term. Usually a self-limiting condition, often observed in the perioperative setting and/or in association with critical illness, where patients require PN for a few days or weeks; Type II: Prolonged acute condition, often arises in patients with metabolically unstable conditions and requires complex multidisciplinary treatment and intravenous supplementation over a period of weeks to months; Type III: Chronic condition found in metabolically stable patients who need to be intravenously substituted for months or years. May be (partially) reversible or irreversible.

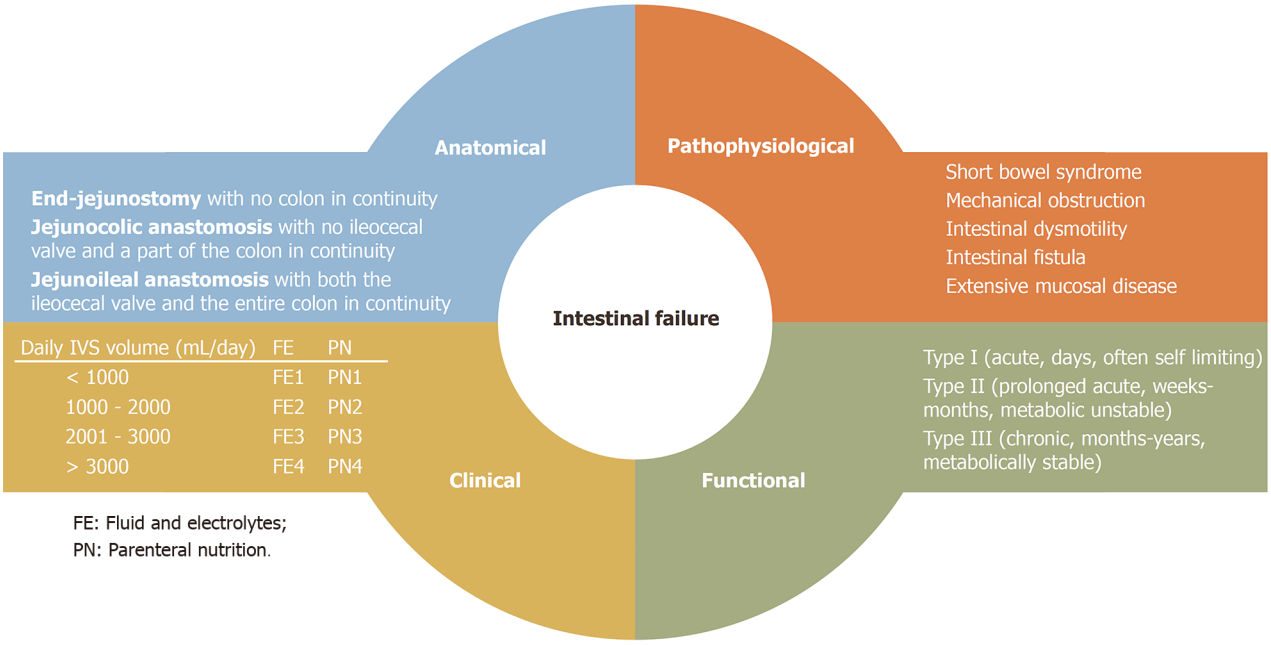


Figure 1 Four domains of chronic intestinal failure. Disease severity is defined by the content of the supplementation — fluid and electrolytes (FE) only, or admixture containing energy (parenteral nutrition (PN)). Each group is subdivided into four categories of mean daily intravenous supplementation (IVS), with volume calculated as average infusion volume per day × number of infusions per week/7.

The clinical classification is based on the patient's energy and fluid requirements for PN in the context of clinical management. It does not aim at risk stratification and is defined according to 16 categories (Figure 1).

EPIDEMIOLOGY

While intestinal failure can occur due to a number of different underlying conditions, CD has been found to be the most common reason for intestinal failure (Figure 3)[5]. However, incidence of CIF as a complication of CD can only be indirectly evaluated based on data from home PN registries. Recent reports from national intestinal failure units in the United Kingdom show that CD is the underlying disease in 30%–32% of patients with long-term intestinal failure[6,7]. Longitudinal data from Japan indicate an incidence of CIF in CD of 8.5%-18.2% during the first 20 years after initial presentation[8,9].

Mechanisms of CIF in CD are poorly understood. Among recognized pre-operative risk factors are systemic steroid treatment[10-13], recent weight loss[11,14], intraabdominal abscess[15-17], and smoking[14,18-20]. Whether recent administration of antitumor necrosis factor medication is associated with post-operative morbidity in CD is currently under investigation.

The peri-/post-operative events that most frequently lead to CIF in CD have been purported to be multiple resections, enterocutaneous fistulation, and malabsorption with multiple resections, leading to cumulative loss of the small bowel. However, Soop *et al*[21], in a longitudinal cohort study of 121 patients referred to a national intestinal failure unit from 2000 to 2018 who were diagnosed with CD and subsequently treated with PN for at least 12 mo, identified septic complications following abdominal surgery to be the most frequent event leading directly to CIF.

ANATOMICAL-PHYSIOLOGICAL ADAPTATION

Three phases of intestinal adaptation can be distinguished. Although it is not possible to define specific biological or functional demarcation points between the hypersecretion, adaptation, and stable (chronically adapted) phases, their differentiation can be a useful aid in therapy planning.

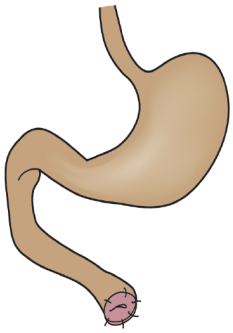
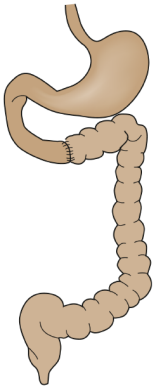
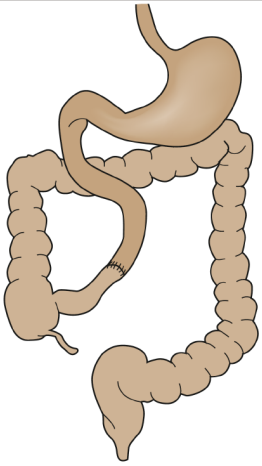
	Type I: Terminal jejunostomy	Type II: Jejunocolic anastomosis	Type III: Jejunoileotransversostomy
Surgical procedure	Complete resection of ileum and colon; Jejunum preserved, represents the end of the intestine	Resection of most of the Ileum, colon preserved	Jejunal resection, at least 10 cm of terminal ileum and colon retained
			
Presence of ileocaecal valve	No	No	Yes
Preservation of colon	No	Partial	Complete
Clinical features	Immediate postsurgical dehydration with risk of electrolyte imbalances; Jejunal output increases after food and fluid intake	Diarrhoea/steatorrhoea; Small intestinal bacterial overgrowth Malabsorption/weight loss Vitamin B_{12} deficiency Oxalate-induced nephrolithiasis	Malnutrition rare
Risk for permanent dependence on parenteral nutrition	Very high	Mostly as supplemental PN (in patients who partially satisfy energy requirements *via* oral/enteral feeding)	Usually no need for parenteral support

Figure 2 Main characteristics of different types of short bowel syndromes according to the anatomical criteria (adapted from Massironi *et al*[3], 2020).

Phase I: Acute (hypersecretory) phase

The hypersecretory phase occurs during the immediate post-operative period and may last for 2 or 3 mo, and in some cases even longer. This phase is characterized by poor absorption of almost all nutrients, including water, electrolytes, proteins, carbohydrates, fats, vitamins, and trace elements. Depending on the extent of resection, daily fluid loss of up to 5 L may occur, while some patients with jejunostomy may lose as much as 6-8 L[22].

Gastric hyperacidity (GH) — increased stomach acidity due to a transient increase in gastric acid secretion (over a period of weeks to months) — affects over half of patients with small bowel resection of 30% or more. GH has been suggested to be caused by the temporary discontinuation of an indirectly or directly acting intestinal inhibitor of gastric secretion (*e.g.*, vasoactive intestinal peptide or gastric inhibitory polypeptide). Alternatively, it may arise due to the diminished breakdown or increased production of a stimulant. Serum levels of gastrin, for example, have been found to be increased after surgery.

Alongside diminished contact time, which leads to inadequate mixing with the chyme, GH-induced inactivation of pancreas ferments is considered vitally important. Following resection of the proximal small bowel, serum levels of cholecystokinin and secretin have been found to be substantially diminished. This generates, in effect, a disturbed positive feedback that leads to a reduction in pancreas stimulation. On the other hand, a reduction in enterokinase activity, observed mainly following duod-

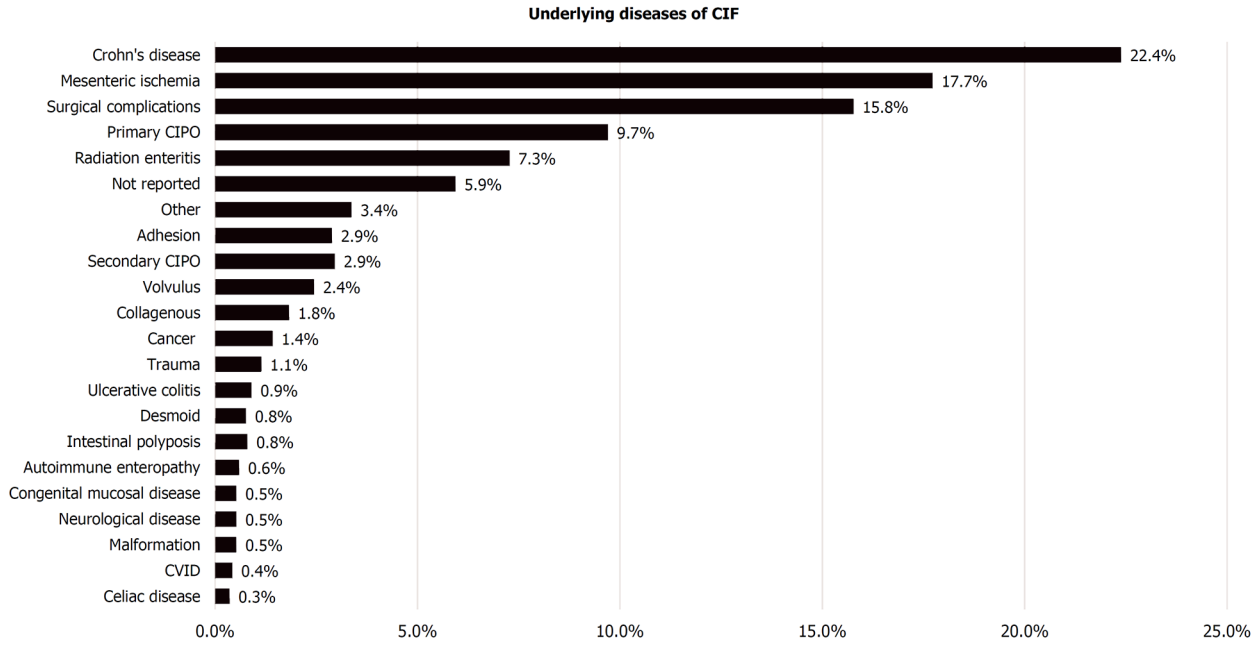


Figure 3 Underlying diseases of chronic intestinal failure. CIF: Chronic intestinal failure; CIPO: Chronic intestinal pseudo-obstruction; CVID: Common variable immunodeficiency.

enum resection, is of no significance.

In patients whose terminal ileum and proximal colon have been surgically removed (type I), a lack of L-cells leads to reduced synthesis of enterohormones [peptide tyrosine-tyrosine, glucagon-like peptide (GLP)-1, GLP-2], effecting an acceleration of gastrointestinal transit duration[23].

Phase II: Adaptation of the residual intestine

Following intestinal resection, structural and functional adaptation processes in the remnant bowel begin even during the early post-operative hypersecretion phase. Distal resection tends to trigger a greater adaptive response than more proximal resection; during this phase, fluid losses should fall to less than 2.5 L. Several years can pass before a stage of maximal adaptation is reached. Generally, 90%-95% of adaptation potential of the remnant bowel is realized within 2 years after resection surgery. In the stabilization phase, improvement of bowel efficiency due to enhanced adaptation leads to a reduction in diarrhea and steatorrhea. Intestinal adaptation is induced by enterotrophic hormones and growth factors, such as epidermal growth factor, growth hormone, insulin-like growth factor-1 (ILG-1) and glucagon-like peptide-2 (GLP-2), an amino acid peptide created by specific post-translational proteolytic cleavage of proglucagon. In addition, intraluminal nutrient supply and the resultant secretion of biliary and pancreatic enzymes play a vital role (for review, see[23]).

Phase III: CIF

Around 50% of patients with prolonged acute intestinal failure go on to develop CIF [24]. Patients with CIF are metabolically stable but need intravenous nutritional support for months or years (reversible CIF) or even lifelong (irreversible CIF). Home-based PN (HPN) is the mainstay of treatment. During this phase, symptoms of SBS are usually addressed with medication. Overall, the probability of weaning off HPN has been reported to be about 50% in adults and up to 73% in children. Complete commutation off HPN in patients with SBS is unlikely[24]. PN can be necessary either to substitute total caloric-nutritional needs when patients are fasting and totally dependent on HPN (total PN) or as supplemental intake in patients who partially satisfy energy requirements *via* oral/enteral feeding (supplemental PN).

DIAGNOSIS AND WORK-UP

Diagnostics include on the one hand the correct diagnosis of SBS/CIF, *i.e.*, determination of the post-operative bowel anatomy (intraoperative bowel length, radiological assessment of bowel length) and the resorptive capacity of the remnant bowel, and on the other hand, the determination of specific nutrient deficiencies and their symptoms and, if applicable, any complications of enteral nutrition (EN)/PN.

As a first step, diarrhea is quantified and further characterized by stool frequency, stool weight, and 24-h steatorrhea/creatorrhea[25,26]. The resorptive surface area can be calculated using the D-xylose absorption test and/or serum citrulline[27,28]. However, data published on the validity of serum citrulline determination as a biomarker for bowel length and a prognostic parameter for successful intestinal adaptation are the subject of controversy. Assessment of early onset nutrient deficiencies requires analysis of iron (ferritin), folic acid, calcium, phosphate, and copper in serum in addition to urine concentrations of zinc and magnesium. In addition, if steatorrhea is present, serum concentrations of the fat-soluble vitamins (A, D, and E) can be utilized for dose-finding and/or correction (cave: Hypervitaminosis). Symptoms of late onset deficiencies, which often remain unnoticed for years before clinical manifestations become apparent, include megaloblastic anemia as a consequence of vitamin B_{12} deficiency (the liver's stores are sufficient for 3-5 years). In this context, and also as an early marker of folic acid or vitamin B_6 deficiency, homocysteine levels serve as a simple and efficient screening parameter (Table 2)[29].

MANAGEMENT OF CIF

Management of CIF focuses on optimizing nutritional status and fluid balance and achieving and/or maintaining adequate weight, while simultaneously minimizing risks associated with long-term complications[30]. The choice of therapy is contingent upon the underlying disease, concomitant disorders, and complications of therapy as well as the localization and extent of resection. Both strategies aim to compensate the diminished resorptive capacity of the remnant bowel, striving in the long-term for oral autonomy. For optimal management, ESPEN recommends that patients with CIF are supported by an expert multidisciplinary team (MDT) addressing all relevant domains of care, such as underlying disease therapy, catheter care, psychological well-being, and PN monitoring[1]. Thus, the ideal MDT should include physicians, surgeons, nurses specializing in stoma and catheter care, dieticians, pharmacists, and psychologists[31].

OPTIMIZING NUTRITIONAL STATUS

There is no specific diet for patients with CIF. Individual remnant intestinal anatomy is a crucial consideration when planning nutritional management and should be clarified prior to commencement of PN. As mentioned above, it is recommended that patients with SBS-IF are divided into three groups based on the absence or presence of the terminal ileum and the colon, since these are the main factors influencing the type and amount of nutrient supplementation required[32].

Specific caloric requirements will vary on an individual basis; however, observational studies have found that patients with SBS consume between 35 and 58 kcal/kg/d in order to meet their nutritional needs[33].

Depending on the adaptation stage, nutritional therapy measures can take the form of overlapping or combined therapy with oral and (long-term) PN/EN, with adjunctive medication as necessary. Patients with jejunostoma (type I) and a residual bowel length of less than 100 cm, as well as those with less than 50 cm continual remnant colon (Table 3), are almost certain to require PN on a permanent basis. If, however, a greater length of remnant bowel is present, it is usually possible to gradually discontinue parenteral feeding or at least progressively convert to partial PN or intravenous fluid supplementation[24,32,34].

There are no controlled data describing the protein requirements of adults with CIF. Prior studies show that protein absorption depends on the length of the small bowel remnant and may increase over time as the bowel adapts, ranging from 61% to 80% [33]. In patients with IBD, supplementation must additionally compensate protein loss *via* the bowel[35]. Current ESPEN guidelines recommend protein substitution of 0.8-2.0 g/kg body weight (BW) per day according to individual needs, representing 20%-

Table 2 Recommended laboratory monitoring for patients receiving parenteral nutrition (adapted from Lappas *et al*[29], 2018)

Parameter	Frequency	
	Initiation of therapy (acute care)	Long-term therapy
Capillary glucose	Every 6 h until advanced to goal and as needed to maintain 140-180 mg/dL	Not routine, as needed basis to coordinate with PN infusion cycle
Basic metabolic panel Phosphorus, magnesium	Daily, until advanced to goal and stable; then 1-2 times/wk	Weekly, then decrease frequency as stable
CBC (with differential)	Baseline; then 1-2 times/wk	Monthly, then decrease frequency as stable
Liver function: ALT, AST, ALP, total bilirubin	Baseline; then weekly	Monthly, then decrease frequency as stable
Serum triglycerides	Baseline if at risk; then as needed	Not routine, as needed
Iron studies, 25-OH vitamin D	Not routine (see Table 4)	Baseline, then every 3-6 mo
Zinc, copper, selenium, manganese	Not routine (see Table 4)	Baseline, then every 6 mo

25-OH vitamin D: 25-hydroxyvitamin D; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; CBC: Complete blood count; PN Parenteral nutrition.

Table 3 Nutrition therapy (macronutrients) according to presence or absence of colon (modified from Pironi *et al*[46], 2016)

Nutrient	Colon present (partial/complete)	Colon removed or disabled
Carbo-hydrates	Slow increase of the proportion of complex carbohydrates up to approx. 40%-50% of total calorie intake, no low molecular weight sugars	Slowly increase the proportion of complex carbohydrates up to approx. 40%-50% of total calorie intake, no low molecular weight sugars, modified FODMAP diet
Protein	Up to 20%-30% of total energy intake	Protein: up to 20%-30% of total energy intake
Fat	Up to 20%-30% of total energy intake	Fat: up to 40% of total energy intake
	Ensure adequate essential fatty acids (high EFA content)	Ensure adequate essential fatty acids (high EFA content)
	Consider MCTs	Limit MCTs
Fiber	Dietary fiber supplements: up to 5-10 g/d	Dietary fiber supplements: up to 5-10 g/d
Oxalate	Diet low in oxalic acid and fats; Replacement of up to 50%-75% of dietary fat with MCTs	No restriction of oxalic acid necessary
Fluids	Even if liquids are well tolerated, iso- and hypotonic drinks are preferable	Oral rehydration solutions frequently required
Lactose	Low lactose diet (10-12 g)	Unrestricted lactose intake

EFA: Essential fatty acids; FODMAP: Fermentable oligo-, di-, monosaccharides and polyols diet; MCT: Medium-chain triglyceride.

30% of daily calorie requirements[1]. The supply of proteins (enteral) and/or amino acids (parenteral) should be chosen to offset undermet needs and ensure the prompt correction of existing deficits (catabolism). The sparse data available on the utility of peptide-based EN are contradictory. However, a considerable increase in protein supply can be achieved through continual nasogastric application of standard polymer feeds, either alone or supplementary to oral (*ad libidum*) nutrition, above and beyond the immediate post-operative phase. Peptide-based enteral feeding is possibly only of additional value in SBS type I[32].

Fat restriction is not necessary in patients who lack a colon. In patients with a preserved colon, it is important to limit fat intake to 20%-25% of total calories in order to reduce the risk of nephrolithiasis from calcium oxalate stones (Table 3). In these patients, a low-fat diet has been shown to increase absorption of calcium, magnesium, and zinc[36] and to improve utilization of medium chain triglycerides for nutrient absorption without increasing stool volume and diminishing calcium and magnesium absorption[37,38]. Fat assimilation can be improved through the addition of pancreas enzyme formulations in powder or granulate form. Controlled studies are not available for CIF[32].

MANAGEMENT OF FLUID AND ELECTROLYTE BALANCE

The primary challenge in patients with jejunostomy is the management of major losses of fluid and electrolytes, particularly zinc, copper, and magnesium (Table 4)[39,40]. It is important for these patients to avoid drinking hypotonic solutions or tap water, as this can lead to worsening electrolyte disturbances by increasing stool sodium content. Conversely, hypertonic solutions such as soda and fruit juice should also be avoided as these solutions are hyperosmolar and can draw water into the gastrointestinal tract and worsen dehydration[33]. Ideally, fluids should be given in small portions between mealtimes, initially in the form of oral rehydration salts (ORS) that offer a sodium-glucose ratio optimized to enhance resorption[41]. In patients with a colon, ORS solutions based on rice starch have been shown to be particularly effective[42]. Sport electrolyte solutions, on the other hand, are not only costly but generally unsuitable, since they contain relatively large amounts of sugar and/or sweeteners in relation to their sodium content. Common sweeteners such as sorbitol or aspartame are known to cause osmotic diarrhea. In addition, fruits, fruit juice, and sweets/candies may increase the frequency of diarrhea. Glutamine-containing ORS solutions are not to be recommended, since glutamine diminishes the intake of sodium and fluids[43].In the adaptation phase (see above), filling foods such as potatoes, rice, oat flakes, or bananas can help solidify the stool. Fluid binding and swelling formulations with pectin may also be beneficial[44].

Ostomy patients should be made aware that every intake of food can be expected to result in stool emptying *via* the ostomy outlet. These patients have a daily fluid requirement of approximately 3 L. Adequate fluid intake can be assessed on the basis of urine volume, with a urine volume of at least 1 L/d considered ideal (urine volume monitoring). Daily intake of 6-9 g salt (*e.g.*, salted meat or vegetable broth) and 25-30 g dietary fiber are also recommended.

Cave: Ileo-/jejunostomy patients (type I) are extremely susceptible to thirst and increased fluid loss due to sweating, diarrhea, *etc.* The resulting rise in oral fluid intake can lead to increased stool volume. Thus, these patients are at increased risk of rapid dehydration, to the point of prerenal kidney failure.

MINERAL SUBSTITUTION

The electrolyte disturbance most frequently seen in SBS is sodium deficiency. As a rough guide, it can be assumed that about 100 mmol/L of sodium are lost with each liter of jejunostomy fluid. Sodium deficiency is better detected by measuring urinary sodium, which decreases before hyponatremia occurs: Urinary sodium concentration below 10 mmol/L is a diagnostic criterion for sodium depletion. Overall, the aims of treatment are to maintain normal hydration and a daily urine volume of at least 800 mL, with a urinary sodium concentration greater than 20 mmol/L[1,40,45,46].

A urine sodium to potassium ratio of ≤ 1 indicates secondary hyperaldosteronism caused by volume depletion as a result of inadequate fluid substitution[47,48]. This marker therefore requires close monitoring and therapy should seek to maintain a sodium to potassium ratio > 1[49].

While hypokalemia may occur in patients with SBS, net intestinal loss occurs only in the case of end-jejunostomy with less than 50 cm remnant jejunum. The effluent from a jejunostoma with a longer remnant jejunum or an ileostoma contains approximately 15 mmol/L potassium. Low serum potassium is most commonly due to urinary losses of potassium associated with secondary hyperaldosteronism elicited by dehydration and sodium depletion. Hypokalemia may also be caused by magnesium depletion, which disrupts many of the potassium transport mechanisms and increases renal potassium excretion; in this case, hypokalemia is resistant to potassium substitution but responds to magnesium replacement[1,50,51].

Due to potassium loss *via* the stool or stoma output, daily potassium requirements of patients with CIF are increased compared to healthy individuals, for whom the recommended intake is 1-1.5 mmol/kg BW per day. Potassium loss in patients with small bowel ostomy amounts to approximately 10-30 mmol/L (Table 4)[49].

Daily calcium requirements are estimated at 0.15-0.25 mmol/kg BW (300-400 mg). Calcium intake should be aimed at maintaining normal levels of parathyroid hormone, with calcium excretion in 24 h urine providing a useful additional marker[49]. Extreme depletion of magnesium stores can disrupt parathyroid hormone excretion, leading to false low measurements[52].

Table 4 Symptoms, prophylactic supplementation, and therapy of frequent (critical) nutrient deficiencies in chronic intestinal failure

Nutrient	Symptoms of deficiency	Normal values (blood/serum)	Additional lab tests	Prophylaxis	Therapy in case of deficiency
Calcium	Neuromuscular hyperarousal, cardiovascular symptoms, osteopathy	2.1-2.7 mmol/L	↑Alkaline phosphatase; ↑intact PTH; ↓Bone mineral density	Calcium citrate, oral 1200–2000 mg/d	Bisphosphonate if T-Score < 2.5
Magnesium	Neuromuscular hyperarousal, osteopathy (PTH effect ↓)	0.75-1.15 mmol/L	↓Magnesium in urine	Magnesium citrate, oral 300 mg/d	10 – 15 mmol magnesium, *e.g.*, in 1000 mL NaCl 0.9 %
Vitamin A	Night blindness, wound healing disorders	1.05-2.80 µmol/L	↓Plasma retinol; ↓ Retinol binding protein	10000-50000 U/d, if liver function normal	No corneal changes: 10000–25000 IU/d oral for 1–2 wk; Corneal changes: 50000–100000 IU i.m. followed by 50000 IU/d i.m. for 2 wk
Vitamin D	Osteopathy, wound healing disorders, immune system disorders	< 20 µg/L: deficiency; 20-30 g/L: insufficiency; > 30 µg/L: sufficient supplies	↑Alkaline phosphatase; ↑intact PTH; ↓Bone mineral density	Oral vitamin D (400–800 U/d) [ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3)] or 100000 U/3–6 mo orally	50000–150000 IU oral 3-5 times a week; If required: calcitriol [1,25(OH)2D] oral
Vitamin K	Hemorrhagic diathesis	INR < 1.2	PIVKA	10 mg/wk	N/A
Vitamin B_1	Polyneuritis ("dry form"), edema, tachycardia, Cardiac insufficiency ("wet form"); Wernicke encephalopathy, ataxia ("central form")	10.64 µg/L	↓Thiamine pyrophosphate; ↓ Erythrocyte transketolase activity	If vomiting, aggressive oral thiamine supplementation with 100 mg/d for 7–14 d	Treatment of Wernicke Encephalopathy: 500 mg i.v. 3 ×/d for 2-3 d; ≥ 250 mg/d i.v. for 5 d; 30 mg oral 2 ×/d
Vitamin B_{12}	Megaloblastic anemia, glossitis, skin and mucous membrane pallor, paresthesia, polyneuropathy, funicular myelosis	156-675 pmol/L	↓Holo-TC; ↓MMA; ↑ Homocysteine	Oral: 1000 µg/wk or 250–350 µg/d, i.m./s.c.: 1000 µg/mo or 3000 µg every 6 mo	1000 or 2000 µg/d oral or 1000 µg/wk i.m.
Zinc	Wound healing disorders, hair loss, taste disturbances, predisposition to infection	11-23 mol/L	↓Zinc in urine	In presence of fistula, diarrhea or stomata: 12 mg; Otherwise: 3-4 mg	30-45 mg (as zinc histidine), 220-440 mg (as zinc sulphate). For each 8–15 mg of elementary zinc, 1 mg copper should be substituted
Iron	Anemia, hair loss, cognitive disorders, predisposition to infection	CRP < 5 µg/L: > 30 µg/L; CRP ≥ 5 µg/L: ≥ 100 g/L	↓Transferrin saturation; ↑Soluble transferrin receptor; ↑ Zinc protoporphyrin	Oral max: 100–150 mg iron	Parenteral, depending on iron status: Aim: normalization of Hb plus transferrin saturation 35%-50% (calculated according to Ganzoni)
Copper	Neutropenia, iron deficiency anemia, central venous development disorders	11-22 µmol/L	↓Copper/zinc superoxide dismutase	Copper gluconate, oxide or sulphate equivalent to 2 mg elementary copper; 1 mg copper for each 8-15 mg zinc	Copper sulphate equivalent to 2.4 mg elementary copper in 100 ml 0.9% NaCl i.v. administered one hour/d for 5 d. Subsequently, oral substitution as required

CRP: C-reactive protein; Holo-TC: Holo-transcobalamin; INR: International normalized ratio; MMA: Methylmalonic acid; PTH: Parathyroid hormone.

Magnesium requirements, estimated at 0.1-0.2 mmol/kg BW (200-300 mg) per day for healthy individuals, can be more than doubled as a consequence of magnesium losses *via* stool or stoma (Table 4). Magnesium supply should be sufficient to maintain normal serum magnesium levels (0.75-1.15 mmol/L). When interpreting serum magnesium values, it is important to consider that magnesium deficiency may be present even at serum levels of up to 0.85 mmol/L[53]. Magnesium secretion of < 9.7 mg/24 h in urine is regarded as a more sensitive parameter for magnesium deficiency than serum magnesium: 24 h urine levels should therefore be determined when serum magnesium is 0.75-0.85 mmol/L[54]. Since magnesium is not well absorbed, oral magnesium substitution is often inadequate. Nocturnal infusions once or twice a week are recommended, each containing 10-15 mmol magnesium, *e.g.*, in 1000 mL NaCl 0.9%[55]. For oral substitution of calcium or magnesium, Ca^{2+} or Mg^{2+} citrate are preferable due to their superior bioavailability[56].

Daily phosphate requirements of healthy persons are estimated at 0.3-0.5 mmol/kg BW. Phosphate supply should be calculated to maintain normal serum phosphate levels while taking account of parathyroid hormone levels (there are no data from controlled trials). When initiating parenteral and/or enteral feeding in undernourished patients, the possibility of nutrition-associated hypophosphatemia must be borne in mind (refeeding syndrome)[57].

MICRONUTRIENTS

The most common micronutrient deficiencies are of vitamin D, zinc, iron, and vitamin B_{12}. These deficiencies can be observed even in patients receiving full ("total") PN, especially during weaning from PN to EN[58].

Vitamin B_{12} malabsorption occurs frequently after terminal ileum resection, even after short resection of only 50 cm. Resection of the ileocecal valve reduces intestinal transit duration and increases the risk of bacterial miscolonization of the small bowel, increasing the likelihood of additional bile acid deconjugation and depletion of the vitamin B_{12} intrinsic factor complex. The resulting pernicious anemia manifests as megaloblastic anemia, thrombocytopenia, and Hunter (atrophic) glossitis, along with neurological disturbances consistent with funicular myelosis (except in folic acid deficiency). To prevent vitamin B_{12} deficiency, 1000 IE should be given prophylactically every 2-3 mo. In case of manifest deficiency, treatment should commence with daily administration of B_{12} at a dose of 1000 IE intramuscular/subcutaneous for 5 d, followed by monthly applications of 1000 IE (Table 4).

Deficiencies of fat-soluble vitamins arise as a direct result of disrupted fat resorption. If untreated, these deficiencies can lead to night blindness (vitamin A), coagulation disturbances (vitamin K), and, in the long term, bone metabolism disorders and even osteoporosis (vitamin D)[59] (Table 4).

Trace element deficiencies may also occur, especially of iron, zinc, and copper[58,60,61]. Patients with intestinal failure are prone to iron deficiency as result of malabsorption, gastrointestinal blood loss, and multiple surgical procedures. Accordingly, iron deficiency is the most common micronutrient deficiency during and after transition from TPN to EN, with reported incidences of 60%-80% for iron deficiency and 30%-37% for iron deficiency anemia[62,63]. A study from the Mayo Clinic (Rochester, MI, United States), including 185 patients, showed that iron deficiency anemia developed much more rapidly in patients with fistula and bowel obstruction than in those with SBS and dysmotility[63].

Despite the high prevalence of iron deficiency, iron is not routinely added to PN formulations because of the risk of anaphylaxis and concerns about incompatibilities. Although data describing the compatibility of iron supplementation with parenteral formulations are conflicting[64], iron dextran has been found to be compatible with lipid-free solutions at an amino acid concentration > 2%. A safer approach would prescribe the intermittent infusion of therapeutic iron doses[65]. Dosage requirements for intravenous iron replacement should be calculated according to Evstatiev *et al*[66].

In patients with diarrhea, large quantities of zinc are lost, with losses of 12 mg zinc per liter stoma output not unusual (Table 4). This considerably exceeds not only normal total zinc requirements but also the content of standard oral mineral supplements[67,68]. In the case of manifest zinc deficiency, 30-45 mg zinc per day can be taken orally (approximately 1 h before breakfast) as zinc histidine or zinc gluconate [69]. However, response to oral supplementation is frequently inadequate, in which case parenteral substitution is indicated[68,70].

PHARMACOLOGICAL TREATMENTS

Analogous to the modified resorption of micro- and macronutrients in CIF, and depending on the underlying disease and the extent and localization of bowel resection, alterations in the bioavailability of any and every kind of drug therapy are to be anticipated. While the scale of these changes is subject to wide interindividual variation, larger drug doses may be required than are typically recommended[30,50]. Data on specific drug classes are scarce, and those that have been published are either case reports or very small cohort studies[71,72]. Therefore, sublingual, transdermal, or transnasal drug application should be chosen whenever suitable options are available [41]. Pharmacological treatments for CIF are primarily based on antisecretory and antimotility treatments intended to minimize gastrointestinal fluid losses. Based on the

identification of GLP-2 as a tissue-specific intestinal growth factor in the late 1990s, hormonal treatment promoting intestinal hyperadaptation has been proposed, aimed at maximizing absorption in the remnant bowel, decreasing intestinal losses, and reducing the need for PN[73,74]. Table 5 shows current drug therapy options for the treatment of CIF.

Antisecretory treatment

For the majority of these medications, only scant scientific evidence is available, or none at all. For example, despite the complete absence of data from controlled studies to endorse the application of proton pump inhibitors in the hypersecretion phase, they are widely recommended as a means of reducing fluid volume and improving the effectiveness of pancreas ferments. Their use in the case of high output stomata is at least supported by two small monocentric studies, whereby intravenous is evidentially superior to oral application[75,76]. Longer-term or permanent intake of proton pump inhibitors is, however, associated with increased risks for osteoporosis and vitamin B_{12} deficiency[41].

Typical second-line agents used to combat gastric hypersecretion include histamine type 2 receptor (H_2) antagonists (*e.g.*, famotidine, ranitidine, cimetidine) and α_2-adrenergic receptor agonists (*e.g.*, clonidine)[75,77]. As with antimotility agents, acid suppressors should be initiated at a low dose and titrated upward to yield maximal efficacy with minimal adverse events. The optimal duration of post-operative antacid therapy is unknown, but it should be discontinued in the event of worsening diarrhea [30].

Data from two smaller trials in patients with CD and ileostoma demonstrated that topically acting steroids may improve the absorptive capacity of the intestinal mucosa for water, independently of their anti-inflammatory effects[78,79].

A number of randomized controlled trials (RCTs) have been published examining the efficacy of crofelemer, a dual inhibitor of cyclic adenosine monophosphate- and calcium-mediated chloride secretion, in chronic acquired immunodeficiency syndrome-associated diarrhea (for reviews, see[80,81]).

Antimotility treatment

The use of antimotility drugs to control diarrhea in CIF has been validated mainly by small studies in patients with ileostomy[59,77]. Recommendations for their use are additionally based on extensive practical experience of their application in diarrhea of infectious and non-infectious etiology[49].

Accelerated intestinal motility is typically treated with opioids or the opioid receptor agonists loperamide and diphenoxylate-atropine as the first-line choices. Unlike diphenoxylate, which crosses the blood-brain barrier, loperamide, a peripherally restricted μ-opioid receptor agonist, does not engender undesirable central nervous system effects such as sedation, euphoria, or addiction[30,82,83]. Because loperamide enters the enterohepatic circulation, higher doses (up to 16 tablets/d) may be needed in patients whose ileum has been shortened or removed. Other antimotility agents include codeine[82,84], morphine, and opium tincture[30]. However, these opioids are not restricted to the peripheral nervous system and can thus generate central nervous system effects[30]. When used together, loperamide and codeine may have a synergistic effect[82].

Besides alleviating gastric hypersecretion, clonidine, a centrally acting α_2 adrenergic and imidazoline receptor agonist (which can also be administered transdermally), has been shown to diminish stomach and colon motility and inhibit intestinal chloride secretion. As with H_2-antagonists, evidence supporting clonidine use in patients with CIF is based only on small case series[85-88]. Dosage of the selected antimotility agent-administered 30 min before meals and at bedtime-should be escalated in a stepwise manner at intervals of 3-5 d until benefit is observed, adverse events occur, or the recommended maximum dosage is reached[30]. Whereas the development of tolerance to analgesic properties of opioids or opioid receptor agonists is well recognized, tolerance to the antidiarrheal effect is rare and the effective dose may remain constant for months to years[30].

Hormonal treatment

In patients with SBS-type CIF, intestinal adaptation can be additionally stimulated by the application of enterotrophic hormones and growth factors, such as growth hormone with or without glutamine or the glucagon-like peptides GLP-1 and GLP-2 [89-93]. Treatment with somatostatin analogues has also been studied.

Table 5 Symptomatic treatment options in short bowel syndrome

Symptom	Drug/therapy	Dose (per day)
Gastral hypersecretion	Proton pump inhibitors	20-40 mg i.v. (p.o.)
	Clonidine	2 × 75-150 μg s.c./p.o.
	Octreotide (sandostatin)[1]	3-4 × 50-100 μg s.c.
Hypermotility	Loperamide	4-6 mg p.o. (max daily dose 16 mg)
	Diphenoxylate	4 × 2.5-7.5 mg (max daily dose 20-25 mg)
	Codeine	30 mg p.o.
	Opium tincture	4 × 0.3-1 mL (10-60 mg) p.o.
Secretory diarrhea	Octreotide (sandostatin)[1]	2-3 × 50-100 μg s.c.
	Budesonide (*e.g.*, entocort)	3 × 3 mg p.o.
	Clonidine	2 × 75-150 μg s.c.
Fat malabsorption	Pancrelipase (*e.g.*, Creon)	40000 IU with main meals (15000 IU with snacks)
Lactose malabsorption	Lactase formulations (L-products)	Depending on severity
Reduced fluid resorption	Locust/carob bean gum flour added to drinks (yoghurt)	Approx. ½-1 tablespoon per glass/pot
	Kaopectate (kaolin/pectin)	4 × 1 tablespoon

[1]Not in the adaptive phase.

Since the pilot trial of Jeppesen *et al*[94] in 2003, a number of trials of teduglutide, a long-acting GLP-2 analogue, have shown an overall 20%-40% reduction in PN dependence, with some patients able to discontinue PN entirely. On the evidence of two 24-wk RCTs, teduglutide is recommended to minimize the number of infusion days in patients with stable infusion-dependent CIF. Improvements in stool consistency and general condition of the patients were demonstrated in both of the aforementioned trials[95,96].

Most recently, first results from a European interdisciplinary center-based retrospective data analysis on teduglutide treatment for SBS in clinical practice have been published. The results show that, even when applied in routine medical care to patients with anatomically and clinically heterogeneous SBS-CIF, teduglutide induced functional and structural changes, allowing a gradual reduction of parenteral support. The data suggest that treatment with teduglutide results in an improved intestinal function and a compensatory effect on nutritional status[97].

It must, however, be kept in mind that all existing GLP-2 trials were conducted in patients at a late phase of intestinal adaptation, months or years after surgery. If therapy were initiated immediately after resection, adaptation would probably be induced more quickly (accelerated) and potentially to a greater degree (supraphysiologic). Conversely, and importantly, because GLP-2 is trophic to the intestinal mucosa, it may carry a risk of promoting or inducing the growth of localized polyps, or more ominously, malignancies. A recent systematic review indicated that treatment with teduglutide for up to 30 mo in individuals without known pre-existing cancer did not confer an increased risk of intestinal neoplasia[98]. However, based on animal data showing that GLP-2 may promote the growth of existing neoplasia, endoscopic examination of the remnant colon is mandatory before beginning teduglutide therapy [74].

In 2013, an open-label, placebo-controlled study showed that GLP-1 decreased diarrhea and fecal excretion in SBS patients. More recently, liraglutide, a GLP-1 analogue, was administered daily subcutaneously in patients with end-jejunostomy over 8 wk in an open-label pilot trial[72]. Liraglutide reduced ostomy wet weight output (by 474 ± 563 g/d), increased intestinal wet weight output (by 464 ± 557 g/d), and improved intestinal energy absorption (by 902 ± 882 kJ/d), with statistical significance in all three instances. Combination therapy with GLP-1 and GLP-2 has been shown to be more effective than GLP-2 alone[99].

Over the past two decades, several clinical studies evaluating the effects of growth hormone, alone or in combination with a high-carbohydrate, low-fat diet and/or glutamine, on intestinal adaptation and absorption in pediatric and adult populations,

have demonstrated conflicting findings[89,100-107]. The positive effects of high-dose growth hormone treatment, mainly on intestinal water absorption, have been described in patients with SBS with a colon in continuity[74]. Although high-dose growth hormone therapy is already in use, no recommendation for routine application of growth hormone is given in the latest guidelines of either the German Nutrition Society[49] or ESPEN[108].

Octreotide, a long-acting analogue of the peptide hormone somatostatin, has been demonstrated to improve diarrhea in patients with CIF by inhibiting gastrin and other gastrointestinal hormones, by inactivating adenylate cyclase, thereby inhibiting intestinal ion secretion and prolonging intestinal transit time[109-111]. Longer-term application of octreotide is associated with an increased risk of gallstones, a recognized complication of SBS, and impairment of intestinal adaptation[30,112].

COMPLICATIONS AND SPECIAL SITUATIONS

Nephrolithiasis and enteric hyperoxaluria

Enteric hyperoxaluria (EH) was first described as a complication in the early 1970s, upon recognition of the association of small bowel resection and subsequent hyperoxaluria and nephrolithiasis: When significant lengths of the ileum are resected (> 60 cm), bypassed, or dysfunctional due to inflammation (*e.g.*, in CD), both fatty acids and bile acids are delivered to the colon at an increased rate, subsequently complexing calcium and thereby reducing free calcium and increasing free oxalate in the colon.

The management of EH focuses on lowering diet oxalate intake and reducing colonic oxalate absorption. However, many patients find restricting oxalate intake to a therapeutic target level of approximately 50 mg/d (< 10 mg oxalate per mealtime) through avoidance of oxalate-rich foods (*e.g.*, rhubarb, spinach, sorrel, cocoa, chocolate, and cola)[58] difficult on an ongoing basis. Since steatorrhea leads to the complexation of calcium by free fatty acids in the colon, and thus to an increase in free oxalate concentration, reducing fat intake is a useful additional dietary intervention. As an alternative, medium chain fatty acids can be used as a substitute for long chain fatty acids[22,113]. As the mainstay of EH therapy, calcium (1000-1200 mg/d) should be orally substituted. If gastric suppression is required, calcium citrate supplements should be favored over the more commonly-used calcium carbonate salts, since the latter are insoluble at a neutral pH[113].

Choleretic diarrhea/bile acid loss syndrome

Colestyramine has been recommended on the basis of clinical consensus and practical experience to treat choleretic diarrhea associated with compensated bile acid malabsorption (*i.e.*, without significant steatorrhea)[49]. While few controlled studies of colestyramine have been conducted, a recent trial comparing the drug to hydroxypropyl cellulose (which is not a placebo) found colestyramine to have a significantly greater effect. A systematic review demonstrated a dose-response relationship between severity of bile acid diarrhea and treatment response, ranging from 96% in patients with severe to 70% in patients with mild bile acid diarrhea[114].

Newer bile acid sequestrants such as colestipol and colesevelam, which are much more selective bile acid binders than colestyramine[115], are reportedly associated with better compliance and fewer side effects[116,117]. The efficacy of colesevelam has now been confirmed in a first RCT[118]. Neither colesevelam nor colestyramine is suitable for the treatment of uncompensated bile acid loss syndrome, since the associated steatorrhea is exacerbated by both compounds (Table 6).

A promising therapeutic approach in the treatment of (decompensated) bile acid loss syndrome is the administration of cholylsarcosine, a conjugated bile acid that has no secretagogue effects. Smaller studies showed a significant increase in calcium and fat absorption in patients treated daily with 4-6 g cholylsarcosine[119-121].

A recent proof-of-concept study assessed the usefulness of obeticholic acid, a potent farnesoid x receptor agonist, in patients with bile acid diarrhea, and found statistically significant increases in fasting serum FGF19, both in patients with idiopathic bile acid diarrhea and in those with secondary disease and ileal resection of less than 45 cm [122].

Gallstones

Gallstones have been reported in 31%-45% of patients with SBS (with or without a colon). Reported risk factors are prolonged periods of starvation, large fluctuations in BW, medications such as opiates, and lipid emulsions (in TPN). In addition, diseases

Table 6 Major complications of short bowel syndrome: risk factors, prevention and treatment (adapted from Pironi *et al*[46], 2016)

	Risk factors	Prevention and/or treatment
Bacterial overgrowth/ miscolonization	Ileocecal valve resection; Reduced intestinal motility (Ogilvie syndrome; chronic intestinal pseudo-obstruction)	Metronidazole (500 mg, 2 times per day), vancomycin (125 mg, 4 times per day), neomycin (500 mg, 3 times per day), clindamycin (300 mg, 3 times per day) tetracycline (500 mg, 3 times per day), rifaximin (550 mg, 2 times per day)
Renal failure	Dehydration; CRBSI; Nephrocalcinosis; Kidney stones	Optimize fluid and sodium balance; Optimize CVC care; Prevent urinary calcium oxalate formation
Calcium oxalate, kidney stones	SBS with colon in continuity and fat malabsorption (enteric hyperoxaluria); Pyridoxine or thiamine deficiency; Excess of ascorbic acid; Dehydration; Low urinary citrate; Low urinary magnesium	Reduce or avoid excess lipid in the diet; Reduce food with high oxalate content; Oral calcium at mealtime (1 g); Oral cholestyramine; Optimize fluid balance; Optimize acid-base balance; Optimize magnesium status; Limit ascorbic acid supplementation
BAMS		
—Compensated	Extent of resection < 100 cm; Fecal bile acid excretion increased; Adequate hepatic compensation of bile acid loss; ≥ reduction of bile acid pool; no or minimal steatorrhea	Colestyramine/Colesevelam
—Decompensated	Extent of resection > 100 cm, fecal bile acid excretion increased; Inadequate hepatic compensation of bile acid loss; ≥ reduction of bile acid pool ≥ steatorrhea	Fat-modified/-reduced diet; Cholylsarcosine/ox gall[1]
Gallstones	Prolonged oral fasting; Interrupted bile acid entero-hepatic circulation; Prolonged treatment with anticholinergic and narcotic drugs	Limit periods of oral fasting; Limit narcotic or anticholinergic treatment; Use oral and/or enteral feeding as much as possible
IFALD-cholestasis	SBS with < 50 cm of residual small bowel; SBS without colon; CRBSI episodes; Chronic intraabdominal inflammation and/or small bowel bacterial overgrowth; Interrupted enterohepatic circulation of bile acid; Oral fasting; PN-overfeeding; i.v. soya-based lipid emulsion ≥ 1 g/kg/d	Avoid oral fasting; Optimize CVC care; Treat intraabdominal inflammation foci; Rehabilitative surgical procedures; Optimize i.v. feeding; i.v. soya-based lipid emulsion < 1 g/kg/d and/or i.v. fish oil lipid emulsion
D-lactic acid acidosis	SBS with a colon in continuity; Carbohydrate and soluble fiber-based diet; Ingestion of rapidly fermentable simple sugars; Feeding D-lactate containing food; High blood and urinary oxalate; Thiamine deficiency; Antibiotic and/or probiotic courses; Dehydration; Decreased renal function; Decreased liver function	Low carbohydrate and simple sugar diet; Antibiotics active against D-lactate-producing bacteria orally, such as metronidazole (500 mg, 2 times per day), vancomycin (125 mg, 4 times per day), neomycin (500 mg, 3 times per day), clindamycin (300 mg, 3 times per day), tetracycline (500 mg, 3 times per day), rifaximin (550 mg, 2 times a day); Thiamine supplementation; Reduction of oxalate absorption; Optimize fluid balance

[1]If colon has been removed or disabled. BAMS: Bile acid malabsorption syndrome; CRBSI: Catheter-related bloodstream infection; CVC: Central venous catheter; i.v.: Intravenous; PN: Parenteral nutrition; PPI: Proton pump inhibitor; PTH: Parathyroid hormone; SBS: Short bowel syndrome.

affecting the ileum (*e.g.*, CD) and absence of ileum and/or ileocecal valve following surgical resection alter enterohepatic circulation and cause a loss of bile salts (Table 7), thus leading to cholesterol supersaturation and sludge formation[123-125].

Small intestinal bacterial overgrowth

Especially after ileocecal valve resection, patients with CIF are at risk of small intestinal bacterial overgrowth (SIBO), *i.e.*, the miscolonization of the upper small bowel by colon-derived bacteria. By promoting bile acid deconjugation, SIBO not only exacerbates steatorrhea, thereby disrupting the absorption of fat and fat-soluble vitamins[126,127], but also hinders intestinal adaptation[127,128]. SIBO is also perceived as a predisposing factor for intestinal failure-associated liver disease (IFALD), since hepatotoxins (*e.g.*, lithocholic acid) produced by anaerobic bacteria in the small bowel may lead to hepatic injury. While diagnosis can be made using H^2 or C^{13} breath tests, their sensitivity is not very high[127]. Some authors therefore suggest a "therapeutic trial" approach on the basis of clinical suspicion, even in non-responders[62,129,130]. Adjunctive intermitting-alternating antibiotic therapy should be administered for a period of 7-10 d (Table 6)[63].

D-lactic acidosis

D-lactic acidosis is a rare, but often overlooked, condition observed only in patients with a preserved colon. It is caused by an increased intake of refined carbohydrates that, having been broken down by bacteria into short-chain fatty acids, lactate when they pass into the colon. The associated decrease in colonic pH promotes the growth of gram-positive, acid-resistant, D-lactate producing anaerobes (*e.g.*, *Bifidobacterium*,

Table 7 Pathophysiological characteristics of short bowel syndrome with and without colon in continuity (adapted from Pironi *et al*[46], 2016)

Characteristic	End-jejunostomy	Jejunocolic or jejunoileal anastomosis
Structural and functional adaptation, to increase nutrient absorption	No evidence thereof at any time after surgery	Possible up to 2 yr after surgery
Gastric hypersecretion (up to 6 mo after resection)	Present	Present
Gastric emptying and small bowel transit	Accelerated gastric emptying for liquids Accelerated small bowel transit	Slowed
GI hormone secretion (PYY, GLP-1, GLP-2)	Decreased/absent	Increased
Energy absorption from microbiota SCFA, production in the colon	Absent	Increased up to 1000 kcal (4.2 MJ) per day
Water and sodium absorption in the remnant small bowel	Possible "net secretion" when jejunum length < 100 cm (more fluid and sodium lost than ingested)	Colon adaptation can increase the absorption of water up to 6 liters and sodium up to 800 mmol per day
Vitamin B12 and bile salt absorption	Absent	Partially conserved or absent
Magnesium absorption	Decreased	Decreased
Remnant small bowel cut-off length for HPN weaning	> 115 cm	Jejunocolic anastomosis > 60 cm Jejunoileal anastomosis with ICV and entire colon > 35 cm

GI: Gastrointestinal; GLP: Glucagon-like peptide; HPN: Home parenteral nutrition; IVC: Ileocecal valve; PYY: Peptide tyrosine-tyrosine; SCFA: Short chain fatty acid.

Lactobacillus, Eubacteriaceae). This results in insufficient metabolism of human D-lactate, and D-lactic acidosis occurs, with associated neurological symptoms such as vision disturbances, confusion, and gait insecurity. Such symptoms are often mistakenly assumed to be signs of alcohol abuse. Diagnosis is made by determining D-lactate levels in the blood[131,132]. The pathogenesis of neurological symptoms associated with D-lactate acidosis remains unclear. One hypothesis is that D-lactate itself is toxic to the brain or alters neuro-transmitter production. Other potentially neurotoxic substances or false neurotransmitters produced in variable quantities during periods of D-lactate elevation may also be involved[46] (Table 6). Due to the similarity of symptoms of D-lactic acidosis to those of Wernicke encephalopathy, prophylactic administration of thiamine has been recommended[131,132].

Diseases of the bone metabolism

Diseases of the bone metabolism, such as osteoporosis and osteomalacia, may occur as long-term complications of TPN and have been reported to occur in 40%-100% of patients with CIF[133-135]. The main causal factors include not only malabsorption of calcium, magnesium[136], and vitamin D but also the presence of underlying inflammatory disease (*e.g.*, CD[137]), combined, as a rule, with many years of steroid intake [135]. Patients on long-term HPN are recommended to undergo bone density testing every 2 years, with frequent assessment of calcium and phosphorus balance, as well as vitamin D levels. Vitamin D substitution should be orientated at 25-hydroxy levels > 30 ng/mL[138].

MANAGEMENT OF THERAPY-RELATED COMPLICATIONS

Therapy-related complications most commonly arise from long-term HPN. While catheter-associated complications are frequent, a variety of organic and metabolic disorders may also occur (for detailed review, see[139]).

Catheter-associated complications

Direct catheter-associated complications in patients receiving long-term HPN can be characterized as either catheter occlusion or thrombotic or infectious complications.

Catheter occlusion can be partial or complete. Factors that influence its occurrence include duration of catheter placement, catheter size and material, type and meticulousness of care of the central venous access route, and the composition of applied infusion solutions. Signs and symptoms of occlusion include resistance when flushing, sluggish flow, inability to infuse fluids, frequent occlusion alarm on the infusion pump, infiltration, extravasation, swelling or leaking at the insertion site upon infusion or during flushing, inability to withdraw blood, and sluggish blood return upon aspiration of blood[140-143].

Possible causes of catheter occlusion include: (1) mechanical problems; (2) non-thrombotic obstruction (precipitate of formulations such as medications or PN constituents within the catheter lumen); and (3) thrombotic obstruction (clot, thrombus, or fibrin deposition).

Any obvious mechanical obstruction should be ruled out before checking for non-thrombotic and thrombotic occlusion[144]. Mechanical occlusions arise from internal or external problems with the catheter, such as kinking in the catheter or tubing, catheter migration or malpositioning, clogging of the cap/needleless connector or filter, or excessive tightness of the retaining suture[145]. Depending on its underlying cause(s), mechanical occlusion can be remedied by removing the venous catheters, avoiding twisting/kinking when dressing, or, if occlusion or malpositioning of the needle is suspected, replacing the non-coring needle.

Catheter-associated infections (CAI) — in particular, catheter-related bloodstream infections (CRBSIs) — remain the "Achilles' heel" of HPN treatment. Infections are classified as follows: Local infections: Catheter exit site infections, port recess infections, and tunnel infections in long-term subcutaneous tunnel catheters (Hickmann catheters); CRBSI: Defined as an infection in which the same organism can be isolated in cultures from the catheter and from peripheral blood, and clinical symptoms of sepsis are present, in the absence of an alternative focus of infection. CRBSI occurs in association with 5%-10% of all central venous catheters, equivalent to 0.3-30 cases/1000 catheter d, and accounts for approximately 70% of all home PN-related hospital admissions[146-148].

More than 50% of all CAIs are caused by gram-positive pathogens, 33% by coagulase-negative staphylococci, and approximately 20%-22% by pathogenic fungi. About a quarter are mixed infections[149]. The chances of saving an infected (tunneled) catheter vary from 50% to 85%, depending on the pathogen responsible[49].

Different catheter-locking solutions have been studied for their effectiveness in preventing CRBSIs, including antiseptic agents, antibiotics, and anticoagulants. Due to the supposed need for an anticoagulant, heparin has most commonly been used as a catheter-locking solution. Its use, however, is no longer recommended, as it has been shown to potentially increase the risk of CRBSI by promoting the formation of an intraluminal biofilm[1,150,151].

Based on its ability to prevent microbial adhesion to the inner catheter surface, and to destroy microbial cell membranes and toxins, use of the broad-spectrum antiseptic agent taurolidine as a catheter-locking solution was described in 1998. Subsequent studies and a meta-analysis confirmed that, compared with heparin, taurolidine decreases CRBSI incidence in patients with catheters. Based on these data and the most recently published RCT by Wouters *et al*[152], and in view of its favorable safety and cost profile, taurolidine solution (*e.g.*, 0.2%) can be applied as a secondary prophylactic locking solution as soon as infection occurs and may also be used for primary CRBSI prophylaxis. However, effective training of the patient (and/or their family) and nursing staff is recognized as the single most effective primary prophylaxis[1,153].

IFALD

Based on the multifactorial nature of IFALD, a recent international position paper suggested defining it as "liver injury as a result of one or more factors relating to intestinal failure including, but not limited to, PN and occurring in the absence of another primary parenchymal liver pathology"[154,155]. Based on retrospective data in adult patients with CIF, Sasdelli *et al*[156] recently reported an IFALD prevalence of 13%-40% for cholestasis, 27%-90% for steatosis, 2%-5% for fibrosis, and 8%-75% for unclassified IFALD, depending on the criteria adopted.

The pathogenesis of IFALD is not yet fully understood and likely multifactorial, with both PN and patient-dependent associated factors playing a role (Table 8)[123,157]. Recommended measures for the prevention and therapy of IFALD are[158,159]: (1) maintenance of suitable proportions of nutrients, avoiding in particular high-calorie and high-glucose feeds, and insulin application, with 20% up to maximum 50% of total energy intake as lipids (approximately 1g fat/kg BW); (2) cyclical infusion feeding periods of 12-16 h, maintaining an overnight fasting period of 8-12 h; (3)

Table 8 Intestinal failure-associated liver disease risk factors in adult patients with home-based parenteral nutrition (adapted from Van Gossum *et al*[157], 2019)

PN-related	Patient-related
Energy overfeeding	Lack of oral feeding
Glucose overload > 7 mg/kg/min	Short bowel syndrome (small bowel remnant < 50 cm)
Lipid emulsion overload	Inflammation/infection
Soya-based lipid emulsion > 1 g/kg/d	Sepsis (*e.g.*, central venous catheter related)
Continuous infusion (24/24 h)	Small intestinal bacterial overgrowth
Contaminants (phytosterols)	Gut inflammation
Antioxidant deficiency	Viral infection (*e.g.*, hepatitis B, C)
Nutrient deficiency (choline, carnitine, methionine, taurine, essential fatty acid deficiency, *etc.*)	Autoimmune
	Hepatotoxic medications

PN: Parenteral nutrition.

enteral feeding, even if the quantities are insufficient to cover requirements, *e.g.*, as a supplement; (4) in patients with cholestasis and/or "sludge" production, administration of ursodesoxycholic acid (10-15 mg/kg BW, *e.g.*, as juice) or cholecystokinin (cave: possibility of anaphylactoid reaction), with additional dose reduction of fat-soluble vitamins, copper, and manganese; and (5) administration of "essential" substrates such as carnitine, taurine, or choline in infants and children. On the basis of affirmative results from a number of randomized studies, fish oil-based lipid solutions are recommended for IFALD therapy in children. Furthermore, an initial study showed a positive effect of fish oil on hepatopathy in adults with CIF[158] (for review, see[160]). Medium-chain triglyceride- and/or olive oil-based lipid solutions are not recommended either for primary or secondary prophylaxis of IFALD[158]. The same applies to probiotics.

SURGICAL PROCEDURES

Reconstructive surgery

A range of operative techniques have been developed over the past years to allow patients with CIF at least the possibility of enteral feeding. These techniques have two common goals: To increase gastrointestinal transit duration and to increase the resorptive surface of the bowel. In principle, three surgical procedures may be applied (for review, see[161-163]): (1) in cases where bowel length is critical, longitudinal lengthening and tailoring, first described by Bianchi in 1980, accomplishes intestinal tapering without loss of surface; (2) in the serial transverse enteroplasty procedure described by Kim *et al*[164] in 2003, the intestinal lumen is narrowed by firing a series of staples perpendicularly to the long axis of the bowel in a zig-zag pattern; (3) if the remaining small bowel is too short (*i.e.*, < 70-80 cm) to achieve weaning from TPN, a reversed antiperistaltic intestinal segment (10-15 cm in adults) can be placed to slow intestinal transit and thereby enhance nutrient and fluid absorption.

Intestinal transplantation

Intestinal transplantation, alone or in combination with other organs (visceral transplantation), is reserved for patients with life-threatening complications of PN defined by significant liver injury with elevated hepatic enzymes, multiple line infections, a single episode of life-threatening catheter-related sepsis, thrombosis of two of the central veins, or frequent episodes of dehydration (for review, see[165]).

CONCLUSION

While considerable advances have been made in recent years with regard to the

understanding and management of CIF and its various related complications, it remains a challenging disorder. Home-based PN has brought distinct benefits, particularly in terms of higher survival rates and improved patient quality of life. Nevertheless, more than half of patients with CIF require PN on a permanent basis. While the traditional mainstays of pharmacological management are antimotility and antisecretory drugs, new hormonally acting drugs that stimulate intestinal adaptation, such as teduglutide, have had encouraging results, adding a new dimension to CIF therapy. More work is needed to improve understanding of CIF mechanisms, and to advance preventive and therapeutic approaches. Optimization of patient outcomes in complex CIF requires a dedicated multidisciplinary team of physicians, dietitians, and nurses, whose foci of care include maximizing intestinal rehabilitation, providing nutritional support, and improving quality of life.

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REVIEW

Non-cirrhotic hepatocellular carcinoma in chronic viral hepatitis: Current insights and advancements

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Abstract

Primary liver cancers carry significant morbidity and mortality. Hepatocellular carcinoma (HCC) develops within the hepatic parenchyma and is the most common malignancy originating from the liver. Although 80% of HCCs develop within background cirrhosis, 20% may arise in a non-cirrhotic milieu and are referred to non-cirrhotic-HCC (NCHCC). NCHCC is often diagnosed late due to lack of surveillance. In addition, the rising prevalence of non-alcoholic fatty liver disease and diabetes mellitus have increased the risk of developing HCC on non-cirrhotic patients. Viral infections such as chronic Hepatitis B and less often chronic hepatitis C with advance fibrosis are associated with NCHCC. NCHCC individuals may have Hepatitis B core antibodies and occult HBV infection, signifying the role of Hepatitis B infection in NCHCC. Given the effectiveness of current antiviral therapies, surgical techniques and locoregional treatment options, nowadays such patients have more options and potential for cure. However, these lesions need early identification with diagnostic models and multiple surveillance strategies to improve overall outcomes. Better understanding of the NCHCC risk factors, tumorigenesis, diagnostic tools and treatment options are critical to improving prognosis and overall outcomes on these patients. In this review, we aim to discuss NCHCC epidemiology, risk

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factors, and pathogenesis, and elaborate on NCHCC diagnosis and treatment strategies.

Key Words: Cirrhosis; Hepatic fibrosis; Non-alcoholic liver disease; Primary liver cancer; Hepatocellular carcinoma; Hepatoma; Liver cancer; Hepatitis B virus; Hepatitis C virus; Liver resection; Liver transplantation

Core Tip: Non-cirrhotic hepatocellular carcinoma (HCC) accounts for 20% of reported HCCs. Such tumors are typically diagnosed late, compromising the outcome. The discovery of direct antivirals, loco-regional treatments and systemic novel immune-chemotherapies, along with advancements of complex hepatobiliary surgery, and the genesis of transplant oncology have revolutionized the management of these aggressive primary liver tumors. Coordinated care at tertiary high-volume HCC, preferably liver transplant centers, remains critical. It is time the stakeholders pursued a consensus approach in developing universal HCC surveillance and treatment strategies on non-cirrhotic patients at risk, such as patients with non-alcoholic steatohepatitis and/or patients with advanced fibrosis.

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INTRODUCTION

Primary liver cancer originates from the liver parenchyma, the bile ducts or both. Worldwide, as per 2018 statistics, primary liver cancer is the second most lethal cancer (only next to pancreatic cancer), fourth leading cause of cancer-related mortality and the sixth most frequently diagnosed with an incidence of 841000 cases per year[1]. Hepatocellular carcinoma (HCC) is the most common primary malignant tumor (90%) originating from the liver[2]. HCC commonly develops within a background of chronic liver disease, characterized by progressive hepatic fibrosis, loss of architecture and formation of regenerative nodules (cirrhosis). This is referred to as cirrhotic-HCC and is present in the majority of the cases (80%). However, 20% of HCC cases develop on a non-cirrhotic background, and therefore referred to as non-cirrhotic-HCC (NCHCC)[3]. Fibrolamellar HCC, angiosarcoma, lymphoma, embryonic sarcoma are other non-cirrhotic liver malignancies, but are rare in occurrence. Due to the lack of surveillance strategies, NCHCC is often diagnosed late, leading to poor prognosis[4,5].

NCHCC risk factors include alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), hepatitis B [hepatitis B virus, (HBV)], hepatitis C [hepatitis C virus (HCV)], hepatitis D virus, tobacco abuse, diabetes mellitus, genetic factors and environmental toxins. Among these, NAFLD and viral hepatitis (HBV and HCV) have been the most common. Given the obesity pandemic, NAFLD burden on the health systems has been expanding. Chronic HBV can be associated with high viral loads, hepatitis D coinfection, prolonged replication phase, concomitant tobacco use and alcohol intake, which can predispose to NCHCC pathogenesis. Similarly, chronic HCV can be associated with carcinogenesis due to multiple gene products development during viremia, altered cell cycle resulting in NCHCC. Further, this risk can continue even after eradication of HCV. It is critical to understand the underlying mechanisms associated with chronic viral hepatitis leading to NCHCC. A comprehensive strategy is needed for surveillance, diagnosis, and management of these tumors[6].

In this review, we discuss NCHCC epidemiology, risk factors, and pathogenesis, and elaborate on NCHCC diagnosis and treatment strategies.

METHODS

An online search was performed using databases PubMed/Medline, EMBASE, Cochrane, Web of Science and CINAHL from January 1, 2000 to January 1, 2021 to identify published reports on HCC in chronic viral hepatitis without cirrhosis. We used following search terms- "carcinoma, hepatocellular" or "cancer, hepatocellular" and "viral hepatitis" and "HBV" and "HCV" excluding "liver cirrhosis." This resulted in 705 published reports. With use of filters (human species and English language), 648 published reports were obtained. After removing articles not relevant/duplicates/non-English language including a manual search, 677 published articles were reviewed.

EPIDEMIOLOGY

HCC is the most common primary liver cancer and a leading cause of cancer-related deaths[5]. HCC-related deaths have been increasing globally. In the United States, in 2001-2006, average HCC incidence increased from 2.7 (2001) per 100,000 to 3.2 per 100,000 (2006)[7]. During this time, HCC was considered ninth leading cause of cancer death. From 2011-2014, mortality from HCC increased by 2.7%[8]. Geographically, HCC is 72% in Asia[9]. World's highest incidence is noted in Mongolia with an incidence of 93.7 per 100,000[1]. Racial and ethnic variations are present in HCC with Asian/Pacific Islands, Blacks, Native Americans having a higher prevalence compared to whites[7,10].

Up to 20% of HCC can grow in a non-cirrhotic liver[11]. While HCC shows a unimodal distribution with peak at the seventh decade of life, NCHCC shows bimodal age distribution, with peaks at second and seventh decade of life[11,12]. This could be related to HBV infection at birth and during adult life[13]. Fibrolamellar HCC is commonly seen in younger adults (second and third decades of life). Male to female ratio is 3:2 in HCC *vs* 1.3-2:1 for NCHCC[14]. Fibrolamellar HCC does not show any sex predilection. Contrary to HCC which is seen frequently in Asians, prevalence of fibrolamellar HCC is higher in Europe and North America[15]. Shim *et al*[16] reported that in Korea, prevalence of HBV infection among NCHCC can range up to 77%. NCHCC individuals, even without active HBV infection, can have antibodies against HBV core (indicative of prior HBV infection) and occult HBV infection [HBV DNA in the liver/blood without hepatitis B surface antigen (HBsAg)], signifying the role of HBV infection in NCHCC[16]. Despite the disappearance of HBV antigen (with treatment or spontaneous regression), some patients continue to be at risk of developing HCC[17].

NON-VIRAL RISK FACTORS FOR NCHCC

Obesity, overweight, and diabetes mellitus are considered NCHCC risk factors. Fatty liver disease can lead to inflammation and hepatocellular carcinogenesis (Table 1)[18]. Tumor suppressor gene dysregulation plays an important role in steatosis development, hepatocyte injury, and NCHCC tumorigenesis[4,19]. Increased tumor necrosis factor-alfa, interleukin-6, leptin, resistin and decreased adiponectin contribute to carcinogenesis in non-cirrhotic livers[20]. Recently, Sydor *et al*[21] reported an association with gut microbiota and primary conjugated bile acid composition in CHCC and NCHCC carcinogenesis among NASH patients[21]. Microbiota-associated changes in bile acid homeostasis and farnesoid X receptor signaling *via* fibroblast growth factor-19 might contribute to the tumorigenesis in these patients[21]. Given the promising results obtained from direct-acting antiviral (DAA) agents in the treatment of HCV, metabolic syndrome and NAFLD will likely predominate and may become the leading risk factor for the pathogenesis of HCC and NCHCC.

Other risk factors include toxin exposure (alcohol, aflatoxin B1, industrial agents, genotoxins, anabolic steroids, iron excess)[22-25], genetic conditions, such as Wilson's disease, glycogen storage disease, Alpha-1 antitrypsin deficiency, hereditary hemochromatosis, acute hepatic porphyria's, hypercitrullinemia, Budd-Chiari syndrome, nodular regenerative hyperplasia[26-30], and germline mutations (telomerase reverse transcriptase gene mutation is the most commonly described mutation in NCHCC)[31]. Excess alcohol intake may play a role in NCHCC carcinogenesis[32]. Studies reported that prevalence of alcohol abuse among patients with NCHCC range up to 12%-21%[4,11,33,34]. If alcohol leads to significant inflammation

Table 1 Key differences between non cirrhotic hepatocellular carcinoma and hepatocellular carcinoma

	HCC	NCHCC
Epidemiology	Eighty percent of HCC develops with a cirrhotic background. A unimodal age distribution (peak in 7th decade) noted. Male:female ratio - 3:2	Twenty percent of tumors develop in non-cirrhotic liver. A bimodal age distribution (peak in 2nd and 7th decade) noted. Male:female ratio-2:1
Risk factors	Development of cirrhosis from any etiology can progress to HCC. Hepatotropic viruses, environmental and life-style factors (alcohol, tobacco), metabolic conditions (nonalcoholic fatty liver disease, diabetes mellitus, obesity) play a predominant role	NCHCC develops without a background of underlying cirrhosis. Viral (HBV, HCV infection) and non-viral risk factors (obesity, diabetes mellitus, toxin exposure, germline mutations and genetic disorders) noted
Clinical features	Symptoms could be related to underlying cirrhosis (from portal hypertension) or HCC (early satiety, upper abdominal pain) itself. Paraneoplastic signs such as hypercalcemia, hypoglycemia have been reported	Generalized fatigue, abdominal pain and weight loss are common symptoms. Can present at late stage with large tumor burden, extrahepatic metastasis
Diagnosis	High quality cross-sectional imaging (CT/MRI) are used with typical arterial phase hyper-enhancement and portal venous washout. LI-RADS classification is used in classification of radiological findings in HCC	Although CT and MRI are increasingly utilized for diagnosis, liver biopsy are utilized in patients when cross-sectional imaging is equivocal. LI-RADS classification cannot be utilized for NCHCC and instead tumor characteristics (size, imaging features) are utilized for staging
Treatment	Given the underlying cirrhosis, liver transplant candidacy need to be evaluated for HCC patients. Resectability of the lesion, amount of liver reserve, vascular invasion, performance status determines the treatment outcomes	Antiviral treatment recommended when etiology of NCHCC is HBV/HCV. Surgery remains the main treatment modality. Systemic and local therapy options are increasingly being utilized for NCHCC

Key differences in epidemiology, risk factors, clinical presentations, diagnosis and treatment for non-cirrhotic hepatocellular carcinoma and hepatocellular carcinoma. A multidisciplinary team evaluation is frequently utilized for diagnosis and treatment. CT: Computed tomography; MRI: Magnetic resonance; HCC: Hepatocellular carcinoma; NCHCC: Non cirrhotic hepatocellular carcinoma; LI-RADS: Liver Reporting and Data System; HBV: Hepatitis B; HCV: Hepatitis C.

with or without fibrosis in these NCHCC is unclear. Further, a synergism with other risk factors, such as viral hepatitis and metabolic syndrome could likely play a role in hepatic carcinogenesis.

VIRAL RISK FACTORS FOR NCHCC

Hepatotropic viral infections such as HBV and less often HCV are major contributors to NCHCC. Hepatitis D can cause coinfection with HBV and lead to high risk of HCC compared to HBV alone [odds ratio (OD) 1.28, 95% confidence interval (CI) 1.05-1.57] [35]. Few studies report hepatitis E may affect chronic HBV infection as they already have compromised liver function[36,37]. If similar risk exists for NCHCC is unclear. Additionally, NCHCC can exhibit more genomic variants, suggestive of a separate tumor biology in these patients[38,39]. Further, HBV-human immunodeficiency virus (HIV) coinfection has been reported to cause higher risk of HCC (OD 7.1, 95%CI: 2.8-17.9)[40].

HBV AND NON-CIRRHOTIC HCC CARCINOGENESIS

HBV virus is a DNA virus (Figure 1), a member of the *Hepadnaviridae* family. It is composed of viral core [nucleocapsid, HBV core antigen (HBcAg) and DNA polymerase] and surface [formed by surface antigen (HBsAg)]. The genes coding for HBcAg code for HBV e antigen (HBeAg). Worldwide, there are 292 million people living with chronic HBV, with 2.2 million reported in the United States[41,42]. HBV (+/- HDV) associated HCC is more common in low and middle socio-economic population. Chronic HBV coinfection can been seen with HDV and/ or HCV.

Ten genotype variants (A to J) are known for HBV of which genotype B and C are highly relevant to HCC development[43]. In general, patients with genotype B have lower risk of progression towards cirrhosis (due to less active disease and earlier HBeAg seroconversion)[44], compared to genotype C. Few studies from Japan demonstrated that genotype B is more common in younger NCHCC[45]. Some studies suggest that individuals infected with either B or C HBV genotype with high T1762/A1764 basal core promotor mutation have a higher risk of HCC development, especially among non-cirrhotic younger adults (< 50 years)[46,47]. Notably, most of

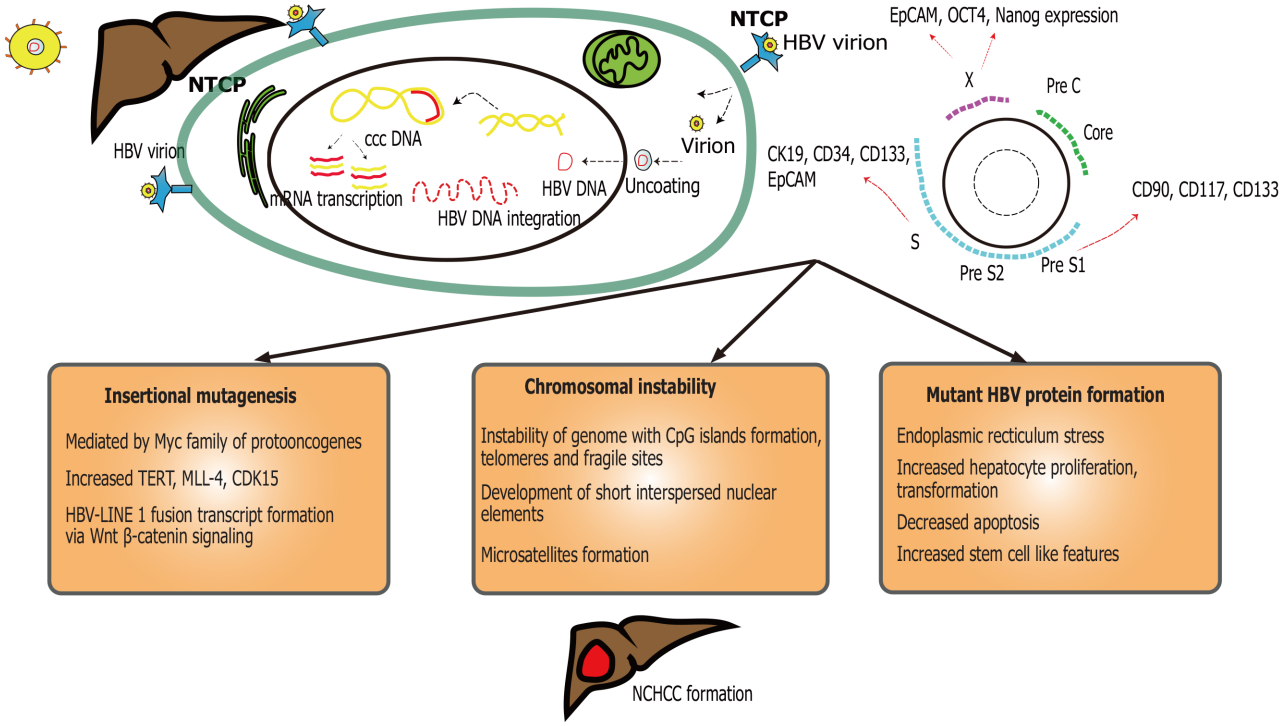

Figure 1 Mechanisms of oncogenesis in hepatitis B virus induced non-cirrhotic hepatocellular carcinoma. Entry of hepatitis B virus (HBV) virion into hepatocytes results incorporation of DNA into the host genome resulting in covalently closed circular DNA. Integration of HBV DNA leads to expression of multiple stem cell markers. These markers differ based on the region of the HBV genome (preS1, S, core, X). Three mechanisms of oncogenesis with insertional mutagenesis, chromosomal instability and mutant protein formation shown. HBV: Hepatitis B virus; NTCP: Na+-taurocholate co-transporting polypeptide; cccDNA: Covalently closed circular DNA; EpCAM: Epithelial cell adhesion molecule; OCT4: Octamer-binding transcription factor 4; Nanog: Homeobox protein; CK19: Cytokeratin 19; CD: Cluster of differentiation; TERT: Telomerase reverse transcriptase; MLL4: Myeloid lymphoid leukemia 4; CKD15: Cyclin dependent kinase 15; NCHCC: Noncirrhotic hepatocellular carcinoma.

these studies were of Asian origin, known to have higher HBV prevalence compared to the West. Mutations are frequently noticed during HBV replication, due to its lack of proofreading activity during reverse transcription. Girones *et al*[48] reported that hepadnavirus mutation rate is 100 times higher than other DNA viruses[48]. Thirty percent of these HCC patients have no underlying cirrhosis (NCHCC)[49]. Pooled HCC surveillance adherence is much lower in non-cirrhotics (23%) compared to their cirrhotic counterparts, despite recommendations[50]. This can lead to tumors, which are advanced at the time of diagnosis, limiting treatment options and outcomes.

Transforming hepatocytes without significant fibrosis or inflammation remains a hallmark of HBV-induced NCHCC[51]. The underlying mechanism remains unclear, even though related to cellular transformation with epigenetic alteration, telomere shortening and instability of the genome system, insertional proto-oncogenes/tumor suppressor mutagenesis, and expression of mutant HBV proteins following integration [52,53]. A variety of factors can predispose HBV positive individuals to develop HCC through either cirrhosis or non-cirrhotic pathways. HBV genome, due to its ability to integrate into the host, can cause genomic instability, disrupting normal regulatory mechanisms. Further, and most interestingly, these mechanisms continue to exist even after seroconversion, due to HBV genome integration. This has been related to HBV highly stable minichromosome and/ or covalently closed circular DNA (HBV cccDNA) which resides inside the hepatocyte nucleus[54]. Tu *et al*[53] compared HBV integrated sites between tumor and matched non-tumor tissues and found that HCC tumors have higher integration event frequency in coding/promoter regions. In the non-cirrhotic setting, recurrent enhancer II/core HBV promoter integration near telomerase reverse transcriptase, myeloid/lymphoid or mixed leukemia 4 genes cause upregulation of oncogenes during early and late tumorigenesis[53,55]. This is critical for HBV non-cirrhotic carcinogenesis, even after HBV treatment or spontaneous resolution. High viral loads of HBV are independently associated with NCHCC, indicative of genetic transformation of the hepatocytes induced by the HBV[47].

Many cellular processes modulate the HBV cccDNA such as histone acetylation, epigenetic modification and activation of signal transduction[56]. These processes could be a target for pharmaceutical agents, however remaining challenges are

cccDNA longevity and stability in the host and viremia recurrence once the antiviral therapy is stopped[57]. The host immune response is unable to eliminate infected cells, resulting instead in immune-mediated damage. This can lead to repeat bouts of hepatitis and inflammation-necrosis-proliferation cycles, resulting in production of reaction oxygen species, genetic mutations and carcinogenesis[58].

NCHCC individuals are more likely African American (OR 6.8, 95%CI: 2.1-22.4), Asian (OR 11.6, 95%CI 2.63-50.8) or have a family history of HCC (OR 32.9, 95%CI: 3.76-288)[51]. In addition, some of the NCHCC from chronic HBV can have multiple risk factors, such as HCV coinfection, alcohol abuse and cryptogenic etiologies[16]. It has been widely contested that viral infection alone might not be sufficient for carcinogenesis; an interplay of host factors, environment and time are needed for full blown cancer development. This probably explains the variation in HCC prevalence among different population groups, and the protracted timeline for HBV-related tumorigenesis, which may span at least one to two decades of life.

HCV AND NON-CIRROTIC HCC CARCINOGENESIS

HCV is a part of *Flaviviridae* family. It is an single-stranded enveloped RNA virus which primarily infects hepatocytes (Figure 2) and targets liver-specific cellular host factors[59]. Precise HCV NCHCC prevalence is unclear. Few reports suggested a possible incidence of up to 10.6%[60]. This risk decreases to 4.2% after achieved sustained viral response (SVR)[61]. Few risk factors, such as male sex, advance age (> 60 years), F3 fibrosis, steatosis, elevated ALT at the end of treatment and history of alcohol abuse contribute to this risk. Furthermore, HCV genotype 3 with steatosis may correlate with HCC[62].

Similar to HBV, successful HCV treatment would not completely eliminate HCC risk, even though risk would be much lower compared to HBV[63]. Attributed risk factors are HCV-genotype 1b[64], viral co-infection infections (HBV, HIV), alcohol or tobacco abuse, and metabolic syndrome. HCV core protein can alter the telomerase activity and immortalize the hepatocytes (along with loss of tumor suppressor function)[65]. Upon hepatocyte entry, the viral RNA undergoes replication and translation in the rough endoplasmic reticulum[66]. This translated product is cleaved by host and viral proteases to form different proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B). Among these, NS3, NS4B, NS5A can induce carcinogenesis coupled with host cellular proteins[65]. Release of reactive oxygen species remains a critical part for HCV-induced HCC. Chronic HCV infection leads to milder liver inflammation, facilitating release of pro-inflammatory cytokines and growth factors, and the generation of inflammation-necrosis-proliferation cycles[67,68]. This is precipitated by increased reactive oxygen species production, mediated by calcium release and high expression of HCV core protein, and cytochrome p450 2E1 oxidase [69]. Decades of this cycles lead to fibrosis, cirrhosis and, eventually HCC.

Due to the ongoing risk of carcinogenesis despite achieving SVR, active evaluation for non-viral risk factors (co-infection, alcohol, tobacco, fatty liver, and metabolic syndrome) should be performed. Recommendations on the surveillance of non-cirrhotic patients with stage 2 or 3 fibrosis and history of treatment-naïve or SVR HCV infection have been unclear. The American Association for the Study of Liver Diseases do not recommend active surveillance. The European Association of the Study of Liver recommended that surveillance would be useful[70].

VIRAL CO-INFECTION AND HCC

Co-infection of hepatotropic viruses can occur among each other and rarely with other virus infection such as the HIV. Due to identical modes of transmission (contaminated needles, blood and sexual routes), co-infection with viral hepatitis and HIV have been observed in certain populations (sub-Saharan Africa)[71]. Although the rates of infection differ based on region and risk-based groups, 5%-20% of HIV patients could be co-infected with HBV. Further, the rates of morbidity and mortality are significantly higher among HIV and HBV co-infection even after adequate viral suppression[72]. HCV and HIV co-infection, although lower (1%-7%) compared to HBV, can lead to evolution of fibrosis (especially with low CD4 counts) and early onset cirrhosis[73]. Chronic viral hepatitis D, being a defective virus, depends on HBV for propagation and occurs in patients concomitantly infected with HDV and HBV. HDV could infect up to 5%-15% of all HBV carriers[74]. This co-infection can cause severe form of

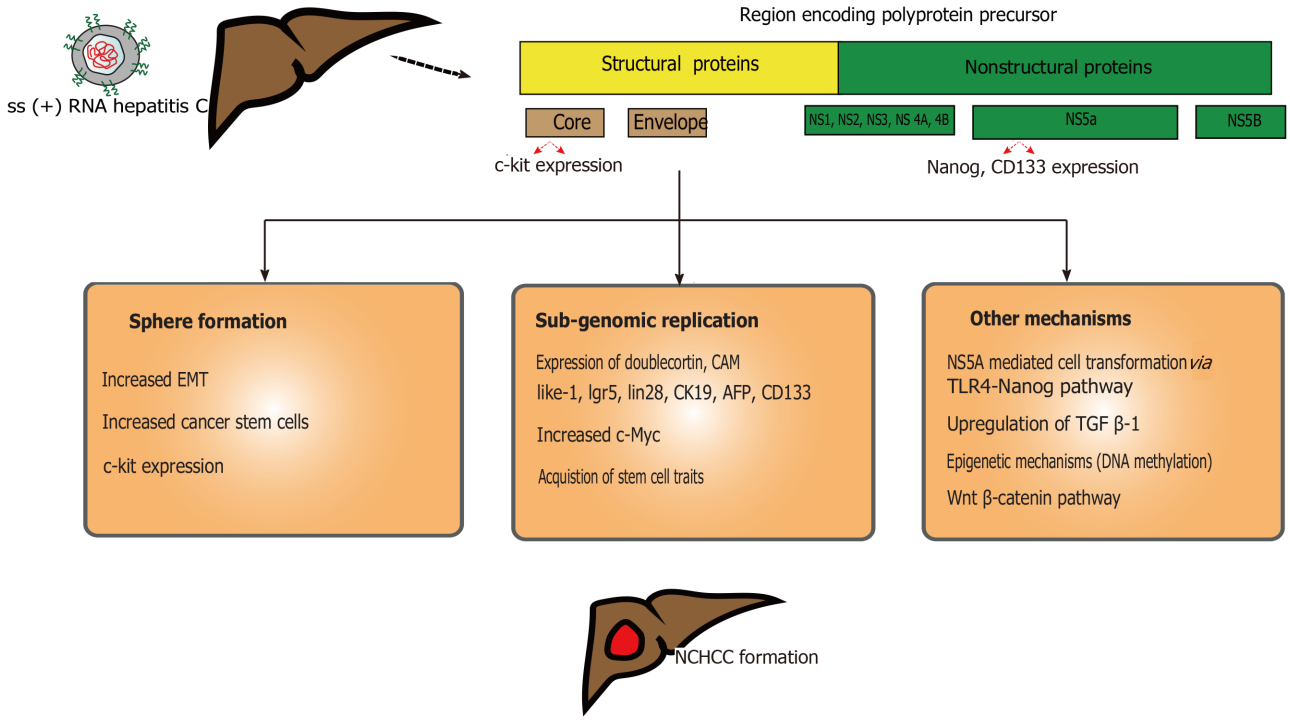

Figure 2 Mechanisms of oncogenesis in hepatitis C virus induced non-cirrhotic hepatocellular carcinoma. Entry of single stranded hepatitis C virus (HBV) RNA into hepatocytes leads to expression of multiple oncogenic proteins. The core section and NS5A sections of the HCV genome produces c-Kit and Nanog-CD133 proteins respectively. HCV infected hepatocytes can lead to sphere formation, sub-genomic replication formation and multiple other mechanism are noted in the schema. Ss: Single stranded; NS: Non-structural; c-Kit: Proto-oncogene, Cam Kinase-like-1; Lgr5: Leucine rich G-protein receptor; CD: Cluster of differentiation; Lin28: RNA binding protein; CK19: Cytokeratin 19; AFP: Alfa fetoprotein; TLR4: Toll-like receptor 4; TGFβ-1: Transforming growth factor-1; NCHCC: Noncirrhotic hepatocellular carcinoma.

chronic viral hepatitis, with 2-fold risk of mortality and 3-fold risk of cirrhosis[75]. The risk of HCC increased 1.2 fold with this co-infection and even higher (7.1%) with HIV-infection. HDV-associated HCC can be due to oncogenesis induced by impact of viral replication/epigenetic events and abnormal cell methylation processes[76]. Despite these findings, future studies are needed to evaluate the carcinogenesis induced by co-infection and long-term effects in the patients.

SYMPTOMS AND SIGNS

NCHCC usually presents at late stage, with a large tumor size, extrahepatic metastases, and heavy tumor burden. Symptoms may include malaise, fatigue, weight loss, abdominal pain, gastrointestinal bleeding and distension, even though often patients are asymptomatic[77-79].

DIAGNOSIS

Imaging

Diagnosis of HCC is mostly performed with non-invasive techniques and rarely histological diagnosis is needed (Table 2). Imaging has replaced histology and conventional angiography[80]. Most of the radiological features of CHCC and NCHCC are similar, except, of course, for the background of cirrhosis among the CHCC patients. Multiple imaging modalities are available, including ultrasound (US), contrast enhanced US, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography scan[81], CT-based radiomics nomogram [82,83], and angiography-assisted CT hepatic angiography[84,85]. Classical CT/MRI findings are arterial phase hyper-enhancement (due to hepatic arterial supply of the tumor), followed by portal venous washout.

Table 2 Imaging characteristics of cirrhotic and non-cirrhotic hepatocellular carcinoma

	CHCC	NCHCC
Imaging modality	Background of advance fibrosis (cirrhosis)	No background of advance fibrosis (cirrhosis)
CT	Homogenous with irregular but well defined margin	Initially hypoattenuating mass which can be come heterogenous (areas of necrosis/hemorrhage within the tumor) when tumor attains bigger size
	Multiple masses	Large solitary mass (/dominant mass) with satellite nodules
	Extrahepatic extension less common	Extrahepatic extension (with direct adjacent organ) is more often seen
	Vascular invasion (encasement) more common (85%)	Metastasis frequently seen, vascular invasion less common (15%)
		Lymphadenopathy seen in 20% of cases.
MR	T1: Variable but mostly hypointense. T2: Hyperintense/isointense compared to surrounding liver	Unenhanced T1 image - Hypointense lesion (presence of hemorrhage/fat can increase the signal). T2 - Hyperintense (low grade/well differentiated can be iso/hypointense)
	DWI-high ADC when lesion is well differentiated	DWI - Used for small lesions. Shows low ADC

CHCC: Cirrhotic hepatocellular carcinoma; NCHCC: Non-cirrhotic hepatocellular carcinoma; CT: Computed tomography; MRI: Magnetic resonance; DWI: Diffuse weighted imaging; ADC: Apparent diffusion coefficient.

Few studies evaluated clinico-radiological characteristics in NCHCC and found that they differ compared to CHCC[86]. Time of contrast washout correlates with histopathological grade in HCC[86]. US is rarely used for diagnosis of the NCHCC due to its non-specific findings, especially for tumors which are less than 2 cm in size, obesity and background of cirrhosis[87]. In rare conditions, such as undifferentiated embryonal sarcoma, ultrasound shows solid component of the tumor (and can exclude purely cystic lesions which are benign)[88]. The sensitivity of US can be significantly limited (up to 21%) due to abdominal fat and obesity; CT/MR is frequently used in these situations[89].

Computed tomography

CT is one of the most commonly used modality of imaging for diagnosis of HCC and can be critical in identifying salient features of HCC. Patients with NCHCC can present with large tumor size lesions, solitary mass (with or without satellite lesions)[90]. They can have well-defined margins with mosaic pattern of enhancement, complete capsule and delayed washout on the CT[86]. This in comparison to CHCC which showed smaller lesions, ill-defined margins, heterogeneous enhancement, no capsule and portal venous phase washout (Table 2). Extrahepatic lesions are more commonly noted in NCHCC (20.5%) compared to CHCC (6.5%). Calcifications are rare in HCC, but in fibrolamellar subtype, they can be found up to 70% and can be associated with central scar.

Magnetic resonance

MRI imaging is superior and often diagnostic. Although several imaging protocols and contrast media are available, hepatocyte specific contrast media are beneficial for accurate diagnosis in NCHCC. MRI Imaging characteristics of NCHCC can differ from classical CHCC in few important aspects (Table 2). Some of independent predictors that were utilized in prior studies include T1-hypointensity, T2-hypo/hyperintensity, lack of central tumor-enhancement, and satellite lesions. Use of these features improved the sensitivity and specificity of identifying lesions as high as 91% and 98% respectively[91]. Although superior in nature, MRI features can differ. T1 images are mostly hypointense. Degree of tumor differentiation, iron or glycogen, lipid, copper could make the lesion appear hyperintense. Further lipid could be distributed diffusely, which can manifest as signal drop outs on the MRI[92]. The mosaic pattern often seen in the NCHCC may be better seen on MRI compared to CT, due to better soft tissue enhancement. HCCs are mildly hyperintense or isointense compared to the surrounding liver on T2- weighted images.

Liver biopsy

Histological diagnosis for CHCC is rarely needed due to availability of high quality imaging such as CT and MRI. However, NCHCC often requires biopsy confirmation as the standard LIRADS classification can only be applied in the presence of cirrhosis.

If lesions are smaller (< 1 cm) especially in NCHCC with atypical features, it is reasonable to repeat imaging in 6 months or obtain histological diagnosis with a liver biopsy based upon the pretest probably and HCC risk factors. Multiple routes of liver biopsy can tried; percutaneous or endoscopic ultrasound guided[93]. Risk of bleeding, pain, tumor seeding of the needle track should be weighed against obtaining adequate tissue for histological diagnosis[94]. Multidisciplinary team HCC diagnosis and management has now become the standard of care. Multidisciplinary approach can improve the overall care in these patients[95].

TREATMENT

Treatment options for NCHCC depend on the etiology, tumor size, extent, vascular invasion, performance status of the individual, and their transplant candidacy (Table 3). Surgery remains the mainstay, if the lesion is resectable. With the introduction of antiviral agents for HCV and HBV, there has been significant improvement on HCC occurrence. Advanced surgical techniques have improved survivability in these patients. In recent years, the implementation of locoregional and novel systemic therapies have been added to the armamentarium of the treatment options in these patients.

Antiviral therapy

All patients with NCHCC should be evaluated for underlying cause. If HBV or HCV infection is detected, the patient should be offered the option of antiviral treatment.

HBV

Entecavir and tenofovir are used as first-line therapies for CHB infection. Multiple risk prediction models have been developed for the study of HCC prevalence in HBV patients[96]. Some of these models include individual prediction model, Chinese University-HCC, Guide with Age, Gender, HBV DNA, Core Promoter Mutation and Cirrhosis-HCC, Risk Estimation for Hepatocellular Carcinoma in Chronic HBV and Nomogram-HCC[97]. Arends *et al*[98] noted that HCC incidence is lower in patients who have received antiviral therapy, even though it did not eliminate the risk: The reported 5-year cumulative incidence rate of NCHCC was 2.1%. Although both therapeutic agents reduce the risk of HCC, few studies compared their effectiveness. A systematic review reported that tenofovir was associated with lower risk of HCC development [adjusted hazard ratio (HR) 0.81; 95%CI: 0.62-0.85], however the beneficial effect did not reach statistical significance for non-cirrhotic patients (adjusted HR 0.83; 95%CI: 0.51-1.35)[99].

HCV

DAA agents have revolutionized HCV care. This in turn, has decreased the risk of HCC on both cirrhotics and non-cirrhotics. Tanaka *et al*[100] analyzed 5814 patients (5646 SVR and, 168 non-SVR) from Asia and noted that HCC incidence was higher in the non-SVR group (5.26 *vs* 1.94, $P < 0.001$). Among the SVR group, in non-cirrhotic SVR patients, baseline alfa fetoprotein of ≥ 10 ng/mL was significant (adjusted HR: 4.26, $P = 0.005$)[100]. SVR in HCV patients depended on the type of treatment regimen. Few studies showed that these regimens might achieve SVR (especially in genotype-2) at higher rate in non-cirrhotics compared to cirrhotics (98.2% *vs* 89.4%)[101]

Surgery

Surgical resection remains the mainstay of NCHCC treatment. Survival of these patients is excellent if the lesions are at an early stage[5]. Unfortunately, NCHCC lesions are usually large due to their late diagnosis[102]. Long-term overall survival (OS) after hepatic resection for NCHCC showed an OS rate of 62%-100% (1 year), 46%-78% (3 year) and 30%-64% (5 year)[103,104]. Multiple synchronous lesions, large tumor size, non-clear resection margin, poor tumor staging and lymphatic invasion were indicators of poor prognosis[104]. Subject to the presence of adequate future liver remnant, these patients may undergo repeat resections. Advanced surgical resection modalities, such as non-anatomical (parenchymal sparing liver resections), ex-situ resections along with the application of future liver remnant growth techniques, such as portal venous embolization, two-stage hepatectomies, associating liver partition with portal vein ligation, hepatic venous deprivation with/without locoregional treatments, have allowed for extended repeat resections on these patients, extending survival.

Table 3 Treatment options for non-cirrhotic hepatocellular carcinoma

Treatment	Comments
Antiviral therapy	If HBV or HCV are identified as potential causes of NCHCC, aggressive treatment should be pursued. Entecavir, tenofovir have been used for HBV and DAA agents are used for HCV infection
Surgery	Mainstay for the treatment of NCHCC. BCLC staging cannot be used for NCHCC patients. Tumor size, elevated bilirubin level, low platelet count, vascular invasion can predict prognosis in NCHCC individuals
Locoregional therapy	Limited data available in NCHCC patients. Isolated cases and case series showed improved prognosis with these treatment options
Systemic therapy	Multikinase inhibitors (sorafenib, regorafenib), immunotherapy (nivolumab), chemotherapeutic agents (epirubicin, cisplatin, 5-fluororuacil, capecitabine, docetaxel, GEMOX) have been used in NCHCC with various success

Potential treatment options for non-cirrhotic hepatocellular carcinoma. Antiviral therapy is indicated if hepatitis C virus or hepatitis C virus is identified as a potential cause. While surgery remains the mainstay of the treatment, locoregional and systemic therapy options have been tried. HCC: Hepatocellular carcinoma; DAA: Direct acting antiviral; NCHCC: Non cirrhotic hepatocellular carcinoma; BCLC: Barcelona-Clinic Liver Cancer; HBV: Hepatitis B virus; HCV: Hepatitis C virus; GEMOX: Gemcitabine and oxaliplatin regimen.

Tumor size (> 5 cm), elevated bilirubin levels (> 5.6 mg/L), low platelet count, portal vein thrombus development can predict vascular invasion and prognosis in the patients[105,106]. Few studies in the past debated if tumor size along predicts survival in these patients. Pommergaard *et al*[39] studied 22787 HCC patients from European Liver Transplant Registry transplanted between 1990 and 2016 and noted that HCC in non-cirrhotic livers had similar overall mortality (adjusted HR 1.11, 95%CI: 0.99-1.25), but higher HCC-specific mortality (adjusted HR 1.62, 95%CI: 1.31-2); perhaps due to a more aggressive biology of the tumors in the non-cirrhotic livers. NCHCC patients were younger, had lower MELD scores and higher risk of microvascular invasion and received more locoregional treatment. Irrespective of the background liver quality, vascular invasion remains critical, underlining the need for early diagnosis and management strategies[107].

In the absence of extrahepatic disease or vascular invasion, liver transplantation (LT) has been the gold standard of HCC treatment among cirrhotics based on the Milan criteria, with the number and size of lesions the being the main determinants [108]. If outside Milan criteria, the patients' lesions may be down-staged under specific guidelines (University of California San Francisco criteria), and then be reassessed for transplantability. On patients beyond the established criteria, novel surgical approaches can be pursued, such as living donor transplantation, split transplantation or the use of marginal grafts, such as donation after circulatory death allografts[109]. On non-cirrhotics, macrovascular invasion and extrahepatic spread are the only recommended exclusion criteria for LT[39]. As per Mazzaferro *et al*[108] if transplanted within Milan criteria, the four-year actuarial survival is 76%, and the recurrence free survival 83%. The recurrence risk increases if the pre-transplant lesions is higher: 78% recurrence is noted with lesions higher than 8 cm compared to 40% with 4-8 cm lesions[110,111]. Higher NCHCC recurrence may reflect more advanced tumors and multicentric carcinogenesis[39,112].

Locoregional therapies

Locoregional therapies such as radiofrequency ablation or transarterial embolization modalities, have been highly successful in the management of HCC on cirrhotics. However, little has been reported in the non-cirrhotic setting. Wagle *et al*[79] retrospectively noted ten NCHCC patients with outcomes with LRT and surgery. One patient underwent trans-arterial embolization and three underwent sequential TACE-portal vein embolization. No mortality was noted in these patients and risk-free survival in all these ten patients was 100% at year 1, 62% at year 3 and OS was 100% at year 1, 72% at year 3 and 72% at year 5. These findings indicate the developing role for LRTs for NCHCC and overall improved prognosis.

Systemic treatment

Systemic therapy with multikinase inhibitors (Sorafenib, regorafenib) and programmed cell death protein 1 inhibitor (nivolumab) have been tried for HCC and extended their use in NCHCC[113]. Sorafenib is effective in advanced HCC and can prolong survival in these patients. However, owing to its adverse events (nausea,

excessive fatigue, diarrhea and skin reactions), its widespread use is limited[114]. Use of chemotherapeutic agents such as epirubicin (E), cisplatin (C), 5-fluororuacil (F), capecitabine, docetaxel, GEMOX haven tried in NCHCC and fibrolamellar HCC. Variable responses have noted with these regiments. For instance, epirubicin, cisplatin, capecitabine showed 52% disease control and epirubicin, cisplatin and 5-flurouracil. GEMOX showed complete response in fibrolamellar HCC. Combination therapy such as use of Sorafenib and mammalian target of rapamycin inhibitors[115] has been attempted with one year survival of 82% and five year survival of 33%[116]. New immunotherapy protocols such as atezolizumab-bevacizumab combination has not been studied in NCHCC, however future trials can be expected with these agents in NCHCC[117]. Although these modalities seem promising, further studies are needed to evaluate their role in survivability in NCHCC patients.

FUTURE RESEARCH DIRECTION

Improved cross-sectional imaging characteristics are expected to identify NCHCC at an earlier stage and provide increasing treatment options in the future. Survival and recurrence rates in NCHCC have been improving and expected to reach HCC patients with efficacy of antiviral treatment options, living donor liver transplantation, parenchymal sparing liver resection and two stage liver resections. Use of artificial intelligence, deep learning models (convolutional neural network) are being utilized for identification of NCHCC[118]. Messenger RNA (mRNA) is a family of RNA molecules which are involved in coding proteins and convey genetic information. On the contrary, microRNAs (miRNAs) are non-coding molecules (22 nucleotide length) that regulate gene expression especially in post-transcriptional state[119]. Dysregulated miRNA can lead to DNA damage with alter gene expression playing a role in NCHCC tumor pathogenesis. Use of micro RNA and messenger RNA (miRNA-mRNA) networks with bioinformatic analysis and experimental validation are being development for therapeutics for NHCCC[119]. Use of miRNA as a potential serum biomarker for diagnosis, prognostication, survival after liver resection and systemic therapy have been studied[120,121]. Despite these advances, further research on molecular mechanism of mRNA and miRNA regulation in NCHCC, and validation of genes involved in NCHCC are urgently needed.

CONCLUSION

Following the introduction of direct antiviral treatments and the increasing prevalence of NAFLD, NCHCC has been on the rise. Even though less common than the hepatocellular carcinoma encountered on cirrhotics, NCHCC still accounts for 20% of reported hepatomas. Such tumors are typically diagnosed late, thus compromising the outcome. The discovery and generalization of use of direct antivirals, loco-regional treatments and systemic novel immune-chemotherapies, the advancements of complex hepatobiliary surgery and the genesis of transplant oncology have added to the treatment armamentarium of these aggressive primary liver tumors. Multidisciplinary approach and coordinated care at tertiary high-volume HCC, preferably liver transplant centers, remain critical. It is time the stakeholders pursued a consensus approach in developing universal HCC surveillance and treatment strategies on non-cirrhotic patients at risk, such as NAFLD and/or patients with advanced fibrosis.

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REVIEW

Natural killer cells in pancreatic cancer stroma

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Abstract

Pancreatic cancer remains one of medicine's largest areas of unmet need. With five-year survival rates of < 8%, little improvement has been made in the last 50 years. Typically presenting with advance stage disease, treatment options are limited. To date, surgery remains the only potentially curative option, however, with such late disease presentation, the majority of patients are unresectable. Thus, new therapeutic options and a greater understanding of the complex stromal interactions within the tumour microenvironment are sorely needed to revise the dismal outlook for pancreatic cancer patients. Natural killer (NK) cells are crucial effector units in cancer immunosurveillance. Often used as a prognostic biomarker in a range of malignancies, NK cells have received much attention as an attractive target for immunotherapies, both as cell therapy and as a pharmaceutical target. Despite this interest, the role of NK cells in pancreatic cancer remains poorly defined. Nevertheless, increasing evidence of the importance of NK cells in this dismal prognosis disease is beginning to come to light. Here, we review the role of NK cells in pancreatic cancer, examine the complex interactions of these crucial effector units within pancreatic cancer stroma and shed light on the increasingly attractive use of NK cells as therapy.

Key Words: Pancreatic cancer; Natural killer cells; Tumour microenvironment; Pancreatic cancer stroma; Stromal cells

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Core Tip: Increasing evidence has found natural killer (NK) cells to be crucial players in the prognosis and progression of cancer. Whilst pancreatic cancer remains one of medicine's largest areas of unmet need, NK cells may prove to be an exciting new therapeutic option for pancreatic cancer patients. Here we provide an overview of the complex interactions between NK cells and pancreatic cancer stroma, suggest a role for NK cells as prognostic biomarkers and highlight exciting new NK cell-based treatment options which may transform the therapeutic landscape of pancreatic cancer.

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INTRODUCTION

Pancreatic cancer

Pancreatic cancer is a malignancy with a dismal prognosis. Set to become the second leading cause of cancer-related death worldwide by 2030, little progress has been made in the treatment of pancreatic cancer over the past five decades[1,2]. Surgery remains the only potentially curative option, however, with the majority of patients typically presenting with advance stage disease, most cases are unresectable. Further to this, with approximately 80% of surgery patients relapsing, frequently within two years, pancreatic cancer has solidified itself as one of medicine's most urgent areas of unmet need[3].

Pancreatic ductal adenocarcinoma tumour microenvironment

Characterised by its strong desmoplastic reaction, the pancreatic ductal adenocarcinoma (PDAC) tumour microenvironment (TME) plays a crucial role in disease progression[4,5]. Primary tumour sites display extensive fibrosis characterised by overexpression of extracellular matrix proteins (such as laminin, collagen and fibronectin) and activation of fibroblastic cells. Multiple cell types, both cancer and stromal, are present in the pancreatic TME, including; pancreatic stellate cells (PSCs), myeloid-derived suppressor cells (MDSCs), tumour associated macrophages (TAMs) and regulatory T-cells (Tregs), amongst many other cell types[6,7]. The dense fibrosis associated with PDAC (largely orchestrated by PSCs) results in tumour hypoxia, a feature characteristic of PDAC, which is exacerbated by the secretion of anti-angiogenic factors (such as endostatin and angiostatin) by both pancreatic cancer and stellate cells[6,8]. Development of a hypoxic TME has been linked to disease aggressiveness and progression, as well as chemotherapy resistance. Importantly, in addition to developing resistance to chemotherapeutics, the pancreatic TME is highly immunosuppressive, limiting the efficacy of the immune-mediated cancer surveillance [6]. MDSCs release reactive oxygen species (ROS) and reactive nitrogen species which have been shown to inhibit T cell proliferation and migration into the TME. In addition, release of immunosuppressive cytokines including interleukin (IL)-10 and transforming growth factor beta (TGF-β) sustains the development of Tregs which further modulate the TME[9]. Tregs secrete immunosuppressive cytokines such as IL-10 and TGF-β which recruit additional immunosuppressive cells to the TME and stimulate the transition of $CD4^+$ T cells to $FoxP3^+$ regulatory cells, facilitating immune evasion[6,10]. Through the release of IL-10 and TGF-β, tumour associated macrophages are also able to induce T-cell anergy leading to the development of an immune-privileged microenvironment[11]. Finally, pancreatic cancer cells can downregulate Fas, resulting in resistance to $CD8^+$ T cell-induced Fas/FasL apoptosis[6,12]. Key components of the PDAC TME are shown in Figure 1.

NATURAL KILLER CELLS

NK cells are large granular lymphocytes which are key components of the innate immune system, and are poorly understood compared to other lymphocytes (T and B

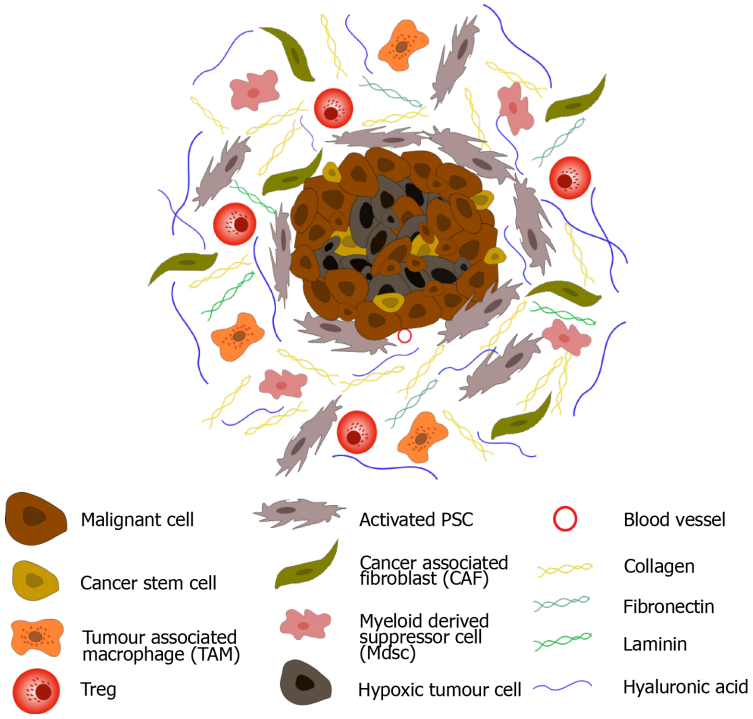

Figure 1 Pancreatic ductal adenocarcinoma tumour microenvironment. Upon activation, pancreatic stellate cells secrete an abundance of extracellular matrix proteins including collagen, fibronectin, laminin, and hyaluronic acid, leading to dense desmoplasia. In addition, fibroblastic cells (CAFs) become active, and immune suppressive cells (myeloid-derived suppressor cells, regulatory T-cells and tumour associated macrophages) are sequestered to the tumour microenvironment (TME). Secretion of anti-angiogenic factors, in addition to dense desmoplasia, results in the development of a hypoxic TME. Cancer stem cells are also observed in pancreatic ductal adenocarcinoma.

cells) belonging to the adaptive immune system. Acting as the first line of defence against viral infected and malignant cells[13,14], NK cells are classified as $CD56^{+}CD3^{-}$ cells. This classification can be further sub-divided into two main effector populations: immunomodulatory $CD56^{bright}CD16^{-}$ cells which regulate their function through cytokine release [specifically interferon (IFN)-γ], and cytotoxic $CD56^{dim}CD16^{+}$ effector cells[14]. Activation of NK cells relies on the balance of signals received from inhibitory and activating cell surface receptors (Figure 2)[13]. Inhibitory receptors, comprised of killer cell immunoglobulin-like receptors (KIRs) and C-type lectin-like receptors, including natural killer group 2 member A (NKG2A), specifically recognisemajor histocompatibility complex (MHC) class 1 molecules. These ligands are highly expressed on non-transformed 'self' cells, and consequently prevent harmful activation of NK cells against host cells. Conversely, malignant cells often downregulate the expression of surface MHC-1 molecules to evade detection by T cells. This 'missing self' signal prevents the inhibition of the NK cells, resulting in cytotoxic efficacy[13,14]. NK cells can also be negatively regulated by checkpoint proteins such as programme death 1 (PD-1), which binds to its' ligands programme death ligand 1 and 2 (PDL-1, PDL-2)[13]. Activating receptors include the type 1 transmembrane natural cytotoxicity receptors (NCRs) NKp46 (which is exclusive to NK cells), NKp30 and NKp44 (which are also expressed on T cells), the C-type lectin-like receptors NKG2C and NKG2D, the activating KIRs and the DNAX accessory molecule 1 (DNAM1)[13-15]. Ligands for the activating KIR receptors include the HLA-C2 and HLA-A ligands, however, the interaction between ligand and receptor is less well understood for activating KIRs than for their inhibitory counterparts[16]. NKG2D receptors recognise stress-induced proteins on transformed and virally infected cells. These include the MHC class 1 related genes MICA and MICB and UL-16 binding proteins[16]. Whilst recognising nectin adhesion molecule and the poliovirus receptor, DNAM1 also interacts with the β2 integrin leukocyte function-associated antigen 1 (LFA-1) which is involved in the formation of the immunological synapse[15]. It is important to note that in addition to independent activation, specific combinations of activating receptors are synergistic, increasing the overall signal received by the effector cell and consequently, increasing its cytotoxic response[17].

$CD16^{+}$ NK cells, in addition to direct receptor-ligand binding, express a propensity to carry out antibody-dependent cellular cytotoxicity (ADCC)[18]. CD16 (FcγRIII) is a transmembrane receptor which can bind the Fc region of IgG1 and IgG3 antibodies,

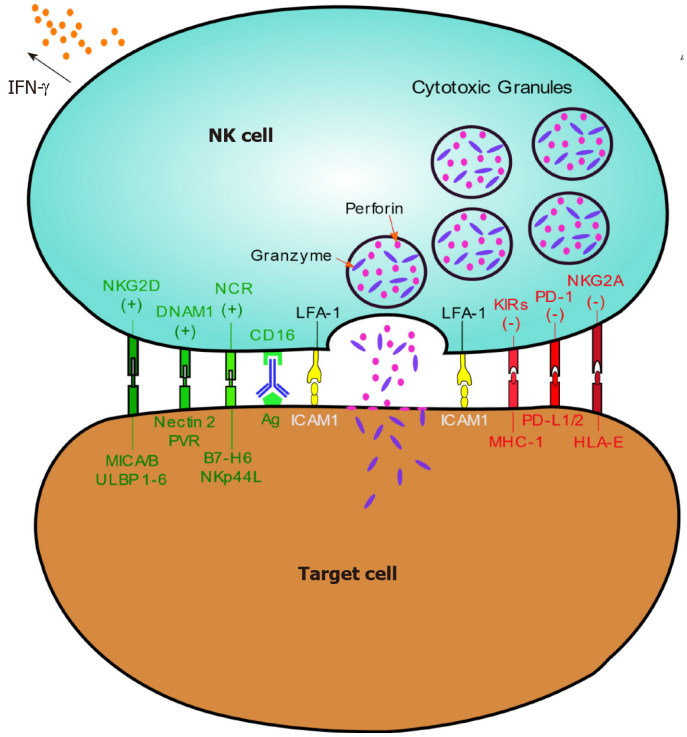

Figure 2 Activation and cytotoxicity of natural killer cells. Natural killer (NK) cells recognise a multitude of ligands on both healthy and transformed cells. Inhibitory receptors (red) recognise 'self-antigens' on healthy tissue preventing activation. However, these molecules are lost on aberrant cells as a result of viral transformation or malignancy ('missing-self') leading to NK cell activation. Alternatively, NK cells may become active through engagement of activating receptors (green) *via* stress ligands expressed on transformed cells. Binding of leukocyte function-associated antigen 1 to ICAM1 stabilises the immunological synapse between NK and target cells and ensures effective cytotoxicity. Upon activation NK cells release cytotoxic granules which contain perforin and granzymes to initiate target cell death *via* necrotic or apoptotic pathways. NK cells can also execute antibody dependent cellular cytotoxicity through Fc engagement of the CD16 receptor. Finally, NK cells secrete cytokines, such as interferon-γ, facilitating crosstalk between the adaptive and innate immune system, resulting in dendritic and T cell recruitment.

enabling NK cells to kill immunoglobulin labelled cells[16], a crucial concept for monoclonal antibody-based therapies[13].

NK Cytotoxicity

Following initial interactions between activating/inhibitory receptors and target cells, the balance of signals received by the NK cell determines its activation status. If activated, NK cells must form an immunological synapse with the target cell. Formation of this synapse enables stable adhesion, polarisation of cytotoxic granules and subsequent lysis of the target cell. This is achieved through binding of the β2 integrin, LFA-1[17]. Consisting of two chains, α_L and β_2, LFA-1 is a heterodimer whose reactivity to its ligands (ICAM family members) can be modified *via* conformational changes. Specifically, a bent conformation exhibits a low affinity for its ligands, intermediate affinity can be achieved through a closed/extended conformation, whilst an open/extended conformation results in high-affinity binding (Figure 3)[17,19,20].

In contrast to T cells, NK cells do not require inside-out signals (such as chemokines and T cell receptor activation) to stimulate LFA-1 binding and can signal autonomously[15,21]. Following integrin activation, a signalling network is employed to facilitate the convergence of cytotoxic granules to the microtubule organising centre (MTOC) which is subsequently polarised towards the target cell, allowing degranulation (Figure 4)[21-26]. Actin remodelling is also carried out at the immunological synapse in response to NK cell activation, facilitating docking of the cytolytic granules [27]. Integrin-linked kinase, paxillin, Pyk2 and RhoGEF7 signalling cascades are employed for the polarisation of MTOC, with Cdc42, CLIP-170, Par6 and APC components of this signalling cascade being key for granule polarisation[21,28]. During this process, the mitochondria and Golgi complex have also been shown to polarise towards the immunological synapse[29,30]. Following polarisation of the cytotoxic granules, the pore-forming protein, perforin, and the serine proteases, granzymes, are exocytosed leading to target cell death *via* perforin induced necrosis, or granzyme stimulated caspase-dependent apoptosis[28,31,32]. Non-granule dependent cytotoxic mechanisms include the binding of death ligands such as TNF-related

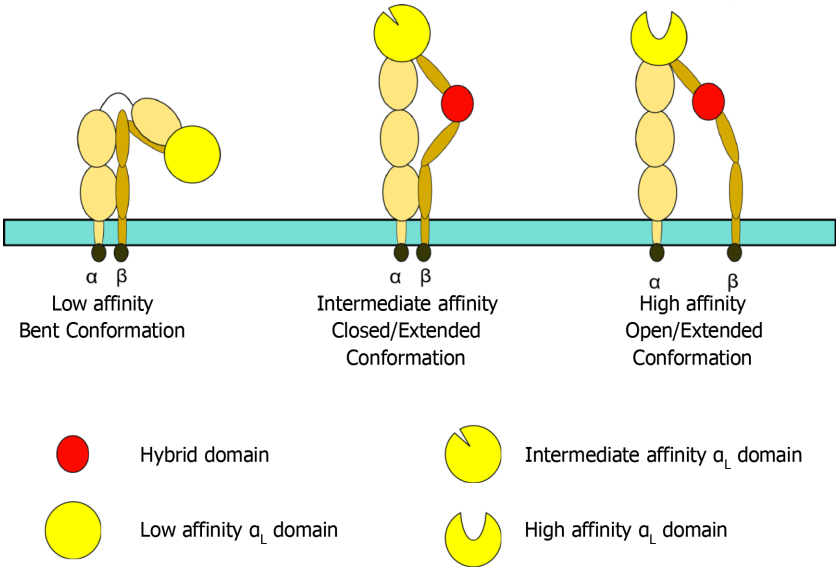

Figure 3 Leukocyte function-associated antigen 1 conformation. Leukocyte function-associated antigen 1 (LFA-1) affinity can be altered by its conformation. When bent, LFA-1 exhibits low affinity for its ligand, ICAM1. Intermediate affinity is achieved through a closed/extended conformation, whilst an open/extended conformation results in high affinity binding to ICAM1 and generation of an effective, stable immunological synapse.

apoptosis-inducing ligand and FasL to their receptor on target cells result in extrinsic apoptosis *via* caspase-8 activation[33] and release of IFN-γ[13,34,35].

NK cells in cancer

Initially identified as a result of their 'natural' cytotoxicity towards both syngeneic and allogeneic tumour cells, NK cells have widely demonstrated potent anti-tumoral cytotoxicity[36-38]. However, they are highly heterogeneous both between cancer types and intra-tumourally[36]. Moreover, the variable functional status of NK cells is seen to greatly impact their anti-tumoral efficacy[36]. Upregulation of the inhibitory receptor NKG2A has been associated with NK cell exhaustion and poorer prognosis in patients with liver cancer[39], whilst PD-1 engagement of NK cells has been shown to block the polarisation of lytic granules and impair outside-in integrin signalling[40]. Increased expression of PD-1 on NK cells has also been associated with poorer overall survival in patients with hepatocellular carcinoma[41]. Conversely, high proportions of functionally active NK cells result in favourable outcomes in many cancer types [42]. Retrospective flow cytometric assessment of peripheral blood samples from metastatic prostate cancer patients (Gleason scores between 6-9) followed by univariate Cox regression analysis demonstrated a significant correlation between expression of the activating receptors NKp46 and NKp30, and longer overall survival [42]. Similarly, immunostaining of tissue samples from 98 patients with gastric cancer (stage 1-4) combined with multivariate analysis revealed a positive correlation between NKG2D expression in tumour infiltrating lymphocytes and prolonged OS (hazard ratio 0.34)[43]. With the propensity to exhibit potent anti-tumoral activity, NK cell-based immunotherapy research has flourished in the past few years[36].

NK cells as prognostic markers in PDAC

NK cell number has been found to convey prognostic significance. Through flow cytometric analysis of PBMCs from resectable PDAC patients (stage Ib-III) both pre- and post-surgery, Hoshikawa *et al*[44] demonstrated a positive correlation between the percentage of NK cells in peripheral blood and recurrence-free survival, with patients who exhibited high NK levels expressing later disease recurrence. Moreover, univariate and multivariate analysis using the Cox proportional hazard regression model demonstrated NK cell frequency to be the only favourable prognostic factor for recurrence-free survival. Additional factors tested included tumour stage, N status, radicality, age and gender. Importantly, no additional circulating mononuclear cells were included in this analysis. NK cell infiltrate within the TME (assessed by gene expression profile) was also found to be associated with later disease recurrence, however, this was not statistically significant[44]. Assessment of a larger cohort of patients would improve the power of this assessment and may provide further insight into the role of tumour infiltrating NK cells in PDAC.

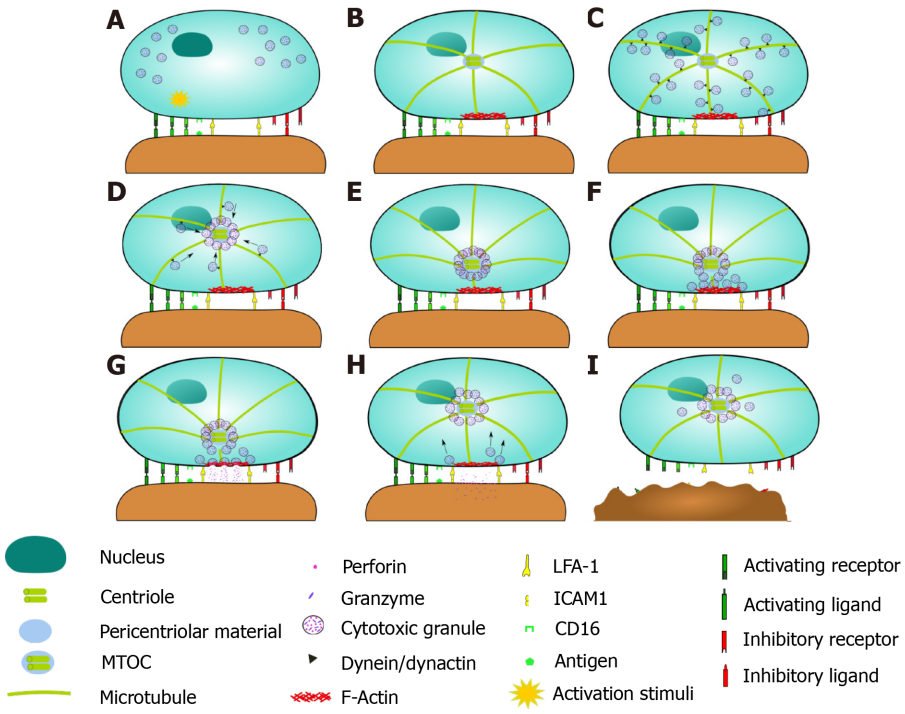

Figure 4 Cytotoxic granule convergence and microtubule organising centre polarisation. A: Following receptor stimulation leading to natural killer (NK) cell activation, leukocyte function-associated antigen 1 engages with its ligand ICAM1 on the malignant cell, forming a stable immunological synapse; B: F-actin accumulates and polymerises at the immune synapse, forming a filamentous mesh which modulates the release of cytolytic granules. Tubulin microtubules then form from the microtubule organising centre (MTOC); C and D: Cytotoxic granules converge on the microtubules (C) and are polarised towards the MTOC where they converge (D); E: This granule movement is dependent on dynein/dynactin motor function. Dynamic rearrangement of the microtubules facilitates polarisation of MTOC towards the immunological synapse; F and G: This polarisation is stimulated *via* Integrin-linked kinase, paxillin, Pyk2 and RhoGEF7 signalling. Following polarisation to the immunological synapse, a subsection of cytotoxic granules fuse with the plasma membrane (F) (a process largely regulated by Munc 13-4 and Rab27a) and undergo degranulation *via* either complete or incomplete fusion (G); H: Cytotoxic granules which do not degranulate are recycled and are hypothesised to remain converged at the MTOC to facilitate serial NK cell killing. Granules which undergo incomplete fusion are rapidly recycled through clathrin mediated endocytosis of granule membrane proteins, further facilitating serial killing; I: Finally, the malignant cell undergoes perforin induced necrosis or granule dependent apoptosis. NK cells detach from the malignant cell and move on to the next target.

Finally, gene set enrichment analysis revealed that patients with enriched type I and II IFN signatures within tumour tissues had later disease recurrence. Type I and II IFNs are closely associated with NK cell function. Type I IFNs induce NK cell activation both directly, through binding to type I IFN receptor, or indirectly, through stimulating dendritic cell release of IL-15, whilst type II IFNs (namely IFN-γ) are produced by activated NK cells[45,46]. Moreover, IFNs can induce CXCL10 release, leading to the further recruitment of NK cells to the tumour tissue[44]. Thus, enriched IFN signatures were also concluded to be a positive prognostic biomarker in PDAC [44].

Yang *et al*[47] also suggest prognostic implication of NK cells in PDAC. High densities of NK cells in peripheral blood samples (analysed by flow cytometry) were found to correlate with poor overall survival in patients with advanced PDAC (stage III/IV) when accompanied by a high neutrophil: lymphocyte ratio (obtained from routine hospital data). NK cell number (HR 1.45), as well as patient age (HR 1.34), neutrophil to lymphocyte ratio (HR 1.48) and absence of metastasis (HR 0.72) were found to be independent prognostic markers following univariate and multivariate Cox regression analysis. It is perhaps prudent to note that subsequent ELISAs to measure release of IFN-γ and TNF-α from NK cells as well as serum IL-2 Levels, a known activator of NK cells, demonstrated lower levels of all three markers in patients with high NK cell densities, although only IFN-γ reached significance. These results suggest a distinct subtype of NK cells with impaired function in PDAC patients and thus prognostic significance may rely not just on NK cell numbers, but on functional subtypes[47]. The results obtained within this study focus solely on peripheral blood circulating NK cells. Further work to classify functional NK cell subtypes within PDAC tumour tissue would provide more conclusive insights into the prognostic significance of these effector cells in PDAC.

NK cells in murine models of PDAC

NK cells have demonstrated potent anti-tumoral efficacy in murine models of PDAC. Using a transgenic mouse model in which oncogenic transposons for KrasG12V and myristoylated Akt2 were introduced into p53fl/fl mice *via* intra-pancreatic injection and electroporation, along with plasmids for Cre recombinase and sleeping beauty transposase, Brooks *et al*[48] demonstrated that neo-adjuvant PD-1 blockade plus adjuvant CD96 inhibition in combination with gemcitabine prevented relapse and facilitated long term remission following resection surgery. To further investigate the role of immune cells in neo-adjuvant and adjuvant treatment of PDAC, mice were injected (intraperitoneal) with anti-CD8 and anti-NK.1.1 depletion antibodies. Pre-operatively, depletion of both $CD8^+$ T cells and NK cells significantly reduced survival when compared to the control. Furthermore, adjuvant NK but not $CD8^+$ depletion was found to impair survival and resulted in an increase in local disease recurrence. *In vitro* luciferase cytotoxicity assays confirmed NK cell cytolytic efficacy against tumour derived cancer cells, a finding consistent with the results obtained for depletion experiments. Thus, targeting both T and NK cells through immune checkpoint inhibition may confer long term survival benefits in metastatic cases of PDAC[48]. Similarly, in a $Kras^{LSL\text{-}G12D}$ $p53^{LSL\text{-}R172H}$ *Pdx1-Cre* (KPC) model of PDAC, NK cell-based adoptive transfer immunotherapy was found to significantly delay tumour growth [49]. In addition, immunostaining of KPC tumours at end time-points revealed significantly elevated areas of necrosis in mice treated with adoptive transfer of NK cells compared to control mice, demonstrating the cytotoxic efficacy of NK cell treatment, a finding replicated through *in vitro* flow cytometry cytotoxicity assays[49].

Xenograft models of PDAC have also been used to demonstrate the efficacy of *ex vivo* expansion of NK cells. NOD *scid* gamma (NSG) mice injected subcutaneously with MiaPaca2 cells demonstrated a significant reduction in tumour growth when treated with adoptive transfer of *ex vivo* expanded NK cells (versus control group)[50]. This finding demonstrated both successful trafficking of NK cells to the tumour site and tumour control following intravenous injection, suggesting that NK cells may prove an effective systemic treatment in xenograft models of PDAC[50]. It is prudent to note that the nature of this model requires the mice included within the study to be immuno-compromised, and this must be taken into consideration when reviewing the data presented. Employing additional models would add further validity to the therapeutic impact of *ex vivo* expanded NK cells in murine models of PDAC[50].

Notorious for their resistance to typical anti-proliferative/cytotoxic therapies, cancer stem cells are an important subpopulation involved in cancer progression, metastasis and recurrence. Despite their notoriety, CSCs are preferentially targeted by NK cells in both the autologous and allogeneic setting; an effect which was found to be NKG2D dependent[51]. Metastatic, intra-pancreatic and subcutaneous models of PDAC were established in NSG mice using the PANC-1 cancer cell line. Mice treated with adoptively transferred NK cells were found to have substantial reductions in tumour volume when compared to untreated mice. Flow cytometric analysis also demonstrated a significant reduction in CSC populations in mice treated with adoptive NK cell transfer (denoted by aldehyde and CD24 expression). Moreover, immunostaining revealed co-localisation of NK cells and CSCs within tumour tissues. Ames *et al*[51] conclude that NK cells possess the ability to identify and preferentially target CSCs in solid tumours. As such, further work investigating the impact of systemic adoptive transfer of NK cells on CSC populations may yield exciting new therapeutic insights into the treatment of this dismal prognosis disease.

Tumour induced NK cell dysfunction and immune evasion in PDAC

Pancreatic tumour cells have developed several methods of NK-cell immune evasion. Murine models of pancreatic cancer specifically designed to express MYC at the Rosa26 Locus, either with or without the *Lsl-Kras*^{G12D} allele, were used to determine the role of MYC in pancreatic tumour progression. MYC expression was found to drive pancreatic cancer development and accelerate disease progression in precursor lesions initiated by KRAS. Moreover, bulk RNA sequencing of end stage tumours revealed reduced expression of T-cell, B-cell and NK cell markers in tumour tissue, suggesting that immune cell infiltration may be regulated by MYC. GeneGo analysis revealed that allelic activation of both KRAS and MYC resulted in significant downregulation of type I IFN, an effect that was found to be dependent on repressional binding of MYC-interacting zinc finger protein, MIZ1. Deletion of MIZ1 resulted in restoration of NK and B cell infiltration into tumour tissues, an effect ablated upon antibody dependent blockade of type I IFN[52]. Similarly, the oncoprotein Sloan-Kettering Institute has been shown to inhibit SMAD association with the acetyltransferases CBP and p300,

which are key regulators of inducible expression of NKG2D ligands on cancer cells, and facilitate repression of gene transcription *via* histone deacetylases. This results in downregulation of NKG2D ligands on tumoral cells, reducing NKG2D dependent cytotoxicity and facilitating immune evasion[53]. In addition, tumoral cells are seen to exhibit intricate crosstalk with NK effector cells and have been shown to induce functional deregulation[54]. Co-culture with the pancreatic cancer cell line MiaPaca2 was found to induce NK cell anergy *via* fibrinogen-like protein 2 and was characterised through decreased expression of DNAM-1, IFN-γ and CD107a and increased expression of PD-1[55]. Importantly, when co-cultured, anergic NK cells were seen to induce anergy in naïve NK cells, reducing IFN-γ and CD107a expression[55]. Further work investigating the reversal of NK cell anergy, both tumour-dependent and bystander anergy may lead to novel therapeutic insights, and provide a baseline from which to further challenge PDAC immune evasion.

MHC class-I chain-related molecules A/B (MICA/B) are crucial ligands for the activating receptor NKG2D. Several studies have demonstrated immune evasion as a result of MICA/B shedding by tumour cells. Duan *et al*[56] demonstrated that high glucose levels, which are widely correlated with pancreatic cancer, could facilitate immune evasion. Specifically, *in vitro* assays demonstrated a decrease in NK cell induced lysis of pancreatic cancer cell lines (demonstrated by lactate dehydrogenase release assays) when cultured in high glucose conditions. Moreover, western blot and quantitative real-time polymerase chain reaction (PCR) analysis revealed that this decrease in function was a result of reduced expression of MICA/B in cancer cell lines at both the protein and mRNA level. Mechanistically, high glucose was found to inhibit AMP-activated protein kinase signalling, leading to upregulation of the polycomb group protein (PcG) Bmi1, and subsequent promotion of GATA2 expression. This augmentation inhibited expression of MICA/B on pancreatic cancer cells and led to their immune evasion[56]. Similar shedding of the NKG2D ligand has been observed in response to hypoxia. High levels of HIF1α have been correlated with decreased expression of MICA/B on pancreatic tumour cells and also with increased internalisation of the activating receptor NKG2D, suggesting a dual role for the hypoxic TME in NK cell dysfunction[57]. Specifically, immune-histochemical analysis of PDAC patient tumour tissues revealed a significant correlation between MICA surface expression and high HIF1α. Moreover, immune-fluorescent staining of NK cells isolated from patient PBMCs demonstrated clear internalisation of the activating NKG2D receptors as well as MICA/B[57].

Receptor expression is found to be largely augmented in pancreatic cancer patients. Flow cytometric analysis of participant blood samples revealed that expression of the activating receptors DNAM-1 (CD226) and CD96 were significantly reduced in pancreatic cancer patients (stage I-IV) when compared to healthy controls. This downregulation was suggested to lead to NK cell dysfunction and tumour evasion.[58] Downregulation of NKG2D in pancreatic cancer patients has also been correlated to reduced cytotoxicity of the effector cells. *In vitro* blockade of NKG2D using neutralising antibodies was found to significantly decrease killing of MiaPaca2, BxPC3 and Capan2 cell lines by IL-15 stimulated NK cells[59]. This finding highlights the functional importance of NKG2D expression on NK cell function and may prove to be a crucial marker of NK cell efficacy against malignancy.

Additionally, flow cytometric analysis of cell surface markers following *in vitro* co-cultures of NK cells derived from healthy PBMCs and pancreatic cancer cell lines revealed downregulation of the NK activating receptors NKG2D, NKp30, NKp46 and DNAM-1. ELISA analysis suggested that this dysfunction was induced as a result of matrix metalloproteinase 9 (MMP9) and Indoleamine 2,3 dioxygenase (IDO) signalling cascades.[60] In addition to downregulation of activating receptors, exposure to MMP9 and IDO also led to decreased TNF-α and IFN-γ production by NK cells, an effect that was reversed upon blockade of MMP9 and IDO using tissue inhibitor of metalloproteinases 1 and 1-Methyl-DL-tryptopan, respectively[60]. This finding was replicated in a comprehensive study of surface receptor expression and cytotoxic granule positive cells in pancreatic (stage II and IV), gastric (stage 0-IV) and colorectal cancer (stage I-IV) patients. Using flow cytometry, Peng *et al*[61] identified significant downregulation of the activating receptors NKG2D, NKp30, NKp46 and DNAM-1 on NK cells identified in peripheral blood samples from pancreatic cancer patients when compared to healthy controls, whilst expression of the inhibitory receptor KIR3DL1 was significantly upregulated. Moreover, the percentage of perforin positive circulating NK cells was found to be significantly reduced in pancreatic cancer patients. Taken together, these alterations evidence the dysfunction of NK cells observed in malignancy.

Impairment in degranulation was also identified by Jun *et al*[62]. In an *in vitro* flow cytometry-based degranulation assay, PBMCs derived from malignant patients, non-malignant patients or healthy controls were mixed with target cells before staining with CD107a. NK cells derived from pancreatic cancer patients showed significantly impaired degranulation when compared to non-malignant and/or healthy control samples. Despite demonstrating impaired cytotoxic capabilities, no significant difference in NK cell IFN-γ production was observed between cancer patients and healthy controls. Importantly, multivariate analysis revealed that tumour-induced NK cell dysfunction correlated with disease stage, suggesting progressive impairment of NK cells with advanced-stage disease. Thus, as previously suggested, NK functional status may prove an attractive prognostic marker in PDAC.

NK cell interaction with stromal cells

NK cell-stromal cell interactions have been shown to result in significant cellular dysfunction and exclusion of NK cells from tumour tissues, suggesting that NK cells can be educated by the TME[47,63]. As crucial players in the development of the pre-metastatic niche, extracellular vesicles are key to the progression of PDAC and contain large numbers of immune regulatory factors including TGF-β, nectin-2 and PVR. Flow cytometric analysis following *in vitro* co-culture of NK cells and extracellular vesicles demonstrated downregulation of multiple NK cell receptors and cytokines, specifically, IFN-γ, TNFα, CD107a and NKG2D, resulting in gross cytotoxic impairment (demonstrated through tumour sphere cytotoxicity assays) and NK cell dysfunction. This impairment was further associated with the activation of the TGF-β-Smad3/4 signalling pathway[64]. It should also be noted that NK cell dysfunction is heavily regulated by the soluble factors and cytokines secreted by both tumoural and stromal cells, with TGF-β, IDO, MMPs and interleukins proving largely responsible for this impairment (Figure 5)[50,60,65-67].

NK cell interaction with suppressive immunoregulatory cells has a significant impact on cytotoxic effector function. Both MDSCs and TAMs produce IL-23, IL-6, IL10 and TGF-β which results in the downregulation of IFN-γ, perforin and IL-12 production by NK cells, decreasing cytotoxicity and NK cell proliferation within the TME[65]. TAMs also inhibit NK cell function through cell-cell interactions. M2 macrophages express PDL-1 which binds to PD-1 expressed on NK cells. This checkpoint protein prevents NK cell engagement and induces downregulation of the activating receptors NKG2D, natural cytotoxicity receptors and DNAM1, leading to reduced cytotoxicity[67]. Immunosuppressive Tregs release IL-10 and TGF-β into the TME. This cytokine release decreases NK cell cytotoxicity through the downregulation of activating receptors and decreased production of anti-tumour cytokines such as IFN-γ[65].

Moreover, MDSCs have also been shown to augment FcR mediate NK cell functions. Adoptive transfer of MDSC in a Panc02-EGFR$^+$ murine model of pancreatic cancer significantly inhibited the efficacy of monoclonal antibody therapy, with mice receiving Cetuximab + MDSCs expressing significantly larger tumour volumes than did mice treated with Cetuximab + splenocytes. Furthermore, NK cells co-cultured with tumour derived (melanoma) MDSCs were found to express significantly reduced phospho-ERK than did though cultured alone (as measured by flow cytometry). Thus, it was concluded that MDSCs inhibit FcR mediated signal transduction, resulting in impaired cytokine production, ADCC dysfunction and reduced anti-tumour activity. An effect that was found to be, at least in part, in response to MDSC nitric oxide production[68].

Likewise, tumour-associated neutrophils have been shown to impair NK cell function through the release of arginase and ROS[67]. *In vitro* co-culture assays using PBMCs derived from healthy donors demonstrated that activated granulocytes (stimulated with Phorbol 12-myristate 13-acetate) produced ROS including hydrogen peroxide, which was found to be cytotoxic to CD56^{dim} CD16$^+$ NK cells, but not CD56^{bright} CD16$^-$ subsets, suggesting that interactions with stromal cells may result in differential effects on cytotoxic effector subset[69].

Tumour-associated macrophages and cancer-associated fibroblasts have also been shown to produce growth arrest-specific gene 6 (Gas6) in the pancreatic TME. As a negative regulator of the immune system, the Gas6-AXL pathway is seen to prevent NK cell activation. Orthotopic syngeneic models of pancreatic cancer in which tumour cells derived from KPC mice were transduced with zsGreen/Luciferase and orthotopically injected into the pancreas of immune-compromised mice were used to investigate the role of Gas6 in NK cell activation and pancreatic cancer development. Pharmacological blockade of Gas6 signalling using neutralising antibodies was found to inhibit pancreatic cancer metastasis. Moreover, immunohistochemical analysis of

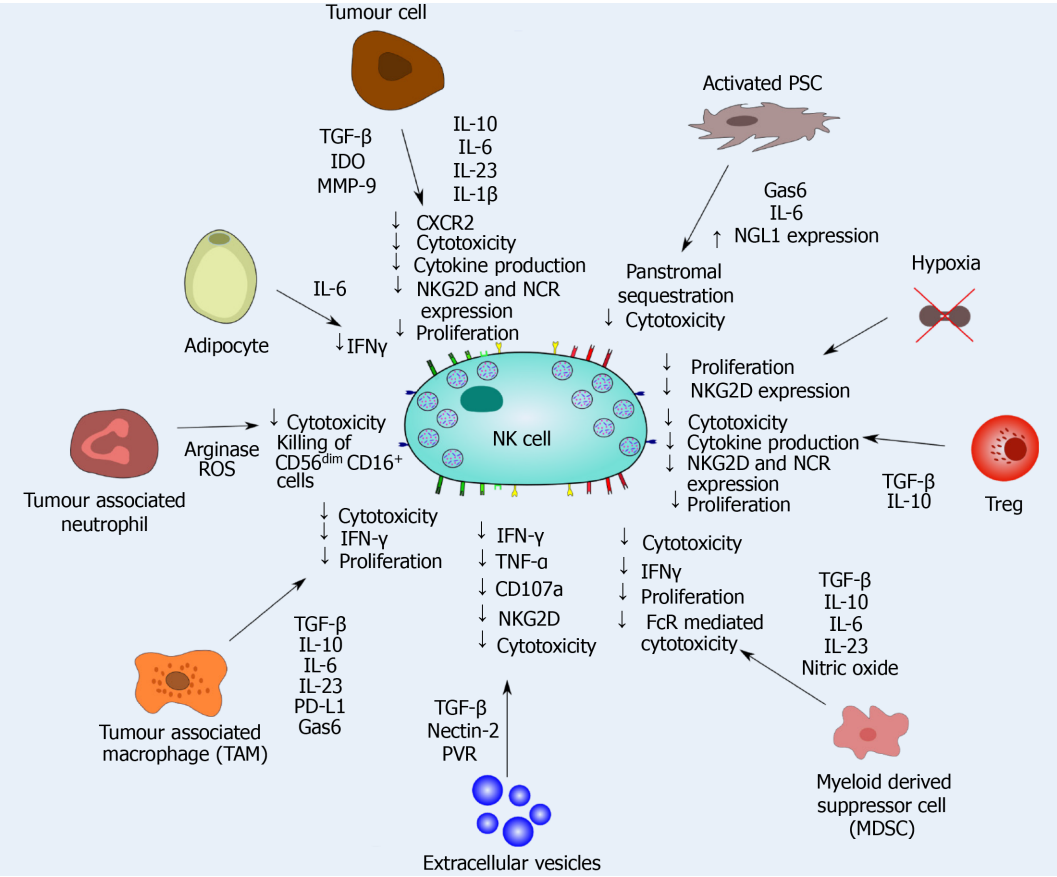

Figure 5 Natural killer cell dysfunction caused by tumoral and stromal cells in pancreatic ductal adenocarcinoma. Natural killer (NK) cell interactions with multiple stromal and tumour cells significantly impacts the cytotoxic efficacy of NK cells in pancreatic ductal adenocarcinoma. Transforming growth factor -β, interleukin (IL)-10, IL-6, IL-23 and IL-1β release significantly dampens NK cell cytotoxicity and function, and inhibits intratumoural proliferation of NK cells. NK cell mediated cytokine release is also inhibited within the immunosuppressive tumour microenvironment. Finally, chemokine release may also sequester NK cells in the panstromal compartment, preventing engagement with tumour cells.

NK cell infiltrates revealed a significant increase in the number of NKp46^{+} NK cells in lung metastasis in mice treated with anti-Gas6 therapy when compared to the control. This finding was also observed in the tumour draining lymph nodes; however no significant difference was observed in NK cell infiltration in primary tumour sites between treatment groups[70]. As such, Gas6 blockade may prove a promising new therapeutic target for the treatment of metastatic lesions in pancreatic cancer.

NK cells have been shown to be excluded from tumour tissue as a result of NK-stromal cell interactions. Through *ex vivo* immunostaining and Ariol image analysis of tissue micro-arrays, Ene-Obong *et al*[4] demonstrated that infiltrates of CD56^{+} natural killer cells were significantly lower in the juxtatumoural compartment of PDAC tissues (stage I-III) when compared to panstroma. This finding suggests PSCs sequester NK cells in the panstromal compartment of the TME, preventing NK induced cancer cell death. Similarly, Lim *et al*[50] demonstrated that NK cell frequencies in malignant tissue from PDAC patients (stage I-IV) were as low as < 0.5% (as assessed by flow cytometry). This low infiltration was attributed to reduced expression of the CXCR2 receptor on NK cells which resulted in poor chemotaxis into tumour tissues.

Known orchestrators of the TME, pancreatic stellate cells are crucial regulators of immune cell infiltrates in pancreatic cancer and have been shown to promote tumour progression through the development of an immunosuppressive environment[5,71-73]. In an orthotopic mouse model of pancreatic cancer in which either Panc02 cells alone or in combination with activated PSCs were injected into the pancreas of C57BL/6 mice, Li *et al*[74] report a significant reduction in the number of NK cells in tumour tissue in mice co-transplanted with PSCs when compared to mice injected with Panc02 cells alone. This finding is consistent with *ex vivo* analysis of human PDAC samples[4]. Further work is needed to demonstrate the mechanistic link between PSCs and NK cell tumoural exclusion.

In a recent study, Francescone *et al*[75] demonstrated dynamic crosstalk between PDAC cancer cells, CAFs and immune cells. Specifically, in *in vitro* co-culture assays

CAFs were found to ectopically express the neural presynaptic protein NetrinG1 (NG1) whilst its binding partner, NGL1 was identified on PDAC cells. This interaction was found to convey protection on cancer cells from NK cell-driven elimination,a fact quantified through CRISPR/Cas9 NG1 knockout which resulted in decreased expression of the immunosuppressive cytokines TGF-β, IL-8 and IL-6, and restored NK cell anti-tumour activation. This finding was reproduced in murine orthotopic models of PDAC in which NGL1 was knocked out of syngeneic mouse cells. This work provides a potential novel target in PDAC in which the immunosuppressive nature of PDAC induce by CAF cells can be reverted.

Finally, *in vitro* and xenograft models of PDAC in NOD SCID mice demonstrated that stromal TGF-β signalling stimulates CAF cells to secrete IL-6, which in turn suppresses NK cell function as assessed through NK cell killing assays. Concomitantly, stromal TGF-β limits NK cell function itself[76]. *In vivo* TGFβ blockade using the anti-mouse TGFβR2 mAb, 2G8, was found to reduce tumour progression, an effect that was reversed upon NK cell depletion[76]. Furthermore, a conditional KRAS (G12D) model of pancreatic cancer combined with a high-fat calorie diet demonstrated that stromal adipocytes produce IL-6, reducing NK cell function through IL-6 mediated downregulation of IFN-γ production. Thus, it has been suggested that adipocytes and PSCs may provide tumour sanctuaries within the TME through the immunosuppression of NK cells[77,78].

Taken together these findings demonstrate the complex interactions between NK cells and the PDAC microenvironment. Despite their cytotoxic efficacy against malignancy, the dysfunction induced by stromal cells greatly augments the natural anti-tumoral activity of NK cells. As such, a deeper understanding of these interactions is crucial to fully unlock the potential of NK cells in the treatment of PDAC.

NK CELLS AS THERAPY IN PDAC

Despite the known dysfunction of NK cells associated with PDAC, these cytotoxic effector units display evident cytotoxic capabilities and as such have received much attention as a potential immunotherapeutic tool in the treatment of PDAC. One of the immunotherapeutic routes explored is the use of NK cells as cellular immunotherapy [79]. When compared to T cells, the use of NK cells as cellular immune-therapeutics express several advantages. Firstly, NK cells present a much lower risk of on-target/off-tumour toxicity, rendering NK cells a relatively safe treatment option. Moreover, neurotoxicity, cytokine release syndrome and graft-versus-host disease are much less likely to occur in chimeric antigen receptor (CAR)-NK treatment than observed with the use of CAR-T therapy. Secondly, due to this reduced risk of graft-versus-host disease from allogenic NK sources, NK cells have the potential to be derived from multiple sources, including cell lines such as NK92. This facilitates the development of a truly 'off-the-shelf' cellular immunotherapeutic, eliminating the need for personalised therapeutics and the subsequent challenges these treatments invoke[79]. Thirdly, CAR-NK cells retain their natural cytotoxic capabilities and consequently can eliminate malignant cells in both a CAR dependent and independent manner[79,80].

Several studies have focused on harnessing the anti-tumoral efficacy of NK cells against PDAC. Lee *et al*[81], rationally designed a CAR-NK to specifically target folate receptor alpha (FRα) and death receptor 4 (DR4) which were found to be highly expressed on tumour cells. In addition, the CAR-NK was loaded with an apoptosis-inducing death ligand to further induce anti-tumoral cytotoxicity. Treatment with the FRα/DR4 targeting NK cells significantly increased tumour-selective apoptosis and NK cell infiltration in tumour tissue in sub-cutaneous models of pancreatic cancer. Similarly, Xia *et al*[82] investigated the efficacy of a Robo1 bi-specific CAR-NK cell treatment in combination with ^{125}I seed radiotherapy in orthotopic murine models of pancreatic cancer. Expressed on pancreatic cancer cells, Robo-1 is a member of the axon guidance receptor family which has been found modulate T-cell chemotaxis into the TME. Tumour size was significantly reduced in mice treated with combination therapy when compared to ^{125}I seed treatment alone. Moreover, tumours of mice receiving combination therapy displayed a significantly higher greyscale value than did ^{125}I treatment alone. Thus, it was concluded that bi-specific CAR-NK cells may prove a promising immunotherapeutic when combined with ^{125}I seed therapy in treatment of pancreatic cancer. Identification of novel antigens for therapy are useful strategies for cell-based therapy[83,84].

Table 1 Current clinical trials employing natural killer cells therapy in pancreatic cancer

Primary therapy	Additional intervention	Tumour type	Phase	NCT number	Status	Ref.
NK cell infusions	Irreversible electroporation	Advanced pancreatic cancer	I/II	NCT02718859	Completed	[90, 91]
Dendritic cell activated, cytokine induced killer treatment	S-1 (drug)	Advanced pancreatic cancer	I/II	NCT01781520	Completed	[87, 92]
BiCAR NK cells (ROBO1 CAR-NK cells)		Pancreatic cancer	I/II	NCT03941457	Recruiting	[93]
Ex-vivo expanded autologous NK cells (SNK01)	Trastuzumab; Cetuximab	Advanced solid tumour; metastatic cancer; HER-2+ breast cancer; HER-2 positive gastric cancer; HER-2 protein overexpression; oesophageal cancer; ovarian cancer; endometrium cancer; bladder cancer; pancreatic cancer; colorectal cancer; NSCLC; EGFR+ NSCLC; head and neck squamous cell carcinoma; triple-negative breast cancer; cervical cancer; sarcoma	I/IIa	NCT04464967	Not yet recruiting	[94]
High activity NK cells		Pancreatic cancer	I/II	NCT03008304	Completed	[95]
Activated NK cells		Lung cancer; breast cancer; colon cancer; pancreatic cancer; ovarian cancer	I/II	NCT03634501	Recruiting	[96]
FT500-an allogenic, iPSC derived NK cell immunotherapy	Nivolumab; pembrolizumab; atezolizumab; cyclophosphamide; fludarabine; IL-2	Advanced solid tumours; lymphoma; gastric cancer; colorectal cancer; head and neck cancer; squamous cell carcinoma; EGFR positive solid tumour; HER2 positive breast cancer; hepatocellular carcinoma; small-cell lung cancer; renal cell carcinoma; pancreas cancer; melanoma; NSCLC; urothelial carcinoma, cervical cancer; microsatellite instability; merkel cell carcinoma	I	NCT03841110	Recruiting	[97]
FT500-101 allogenic NK cell immunotherapy		Advanced solid tumours; lymphoma; gastric cancer; colorectal cancer; head and neck cancer; squamous cell carcinoma; EGFR positive solid tumour; HER2 positive breast cancer; hepatocellular carcinoma; small-cell lung cancer; renal cell carcinoma; pancreas cancer; melanoma; NSCLC; urothelial carcinoma, cervical cancer; microsatellite instability; merkel cell carcinoma	Observational study	NCT04106167	Recruiting	[98]
FATE-NK100 (donor-derived *ex-vivo* activated immunotherapy)	Trastuzumab; Cetuximab	HER2+ gastric cancer; colorectal cancer; head and neck squamous cell carcinoma; $EGFR^+$ solid tumours; advanced solid tumours; HER2 postie breast cancer; hepatocellular carcinoma; NSCLC; renal cell carcinoma; pancreatic cancer; melanoma	I	NCT03319459	Active, not recruiting	[99]
Autologous dendritic cell vaccine loaded with personalised peptides to stimulate innate and adaptive immune response *via* activating T and NK cells	Standard of care; Nivolumab	Pancreatic adenocarcinoma	Ib	NCT04627246	Recruiting	[100]
ACE1702 cellular therapy (anti-HER2 NK cells)	Cyclophosphamide; Fludarabine	Locally advanced solid tumours; metastatic cancer; solid tumour; HER-2+ gastric cancer; HER-2 + metastatic breast cancer	I	NCT04319757	Recruiting	[101]
NK cells	Bortezomib	CML; pancreatic cancer; colon/rectal cancer; multiple myeloma; carcinoma-NSCLC	I	NCT00720785	Recruiting	[102]
Cytokine-induced killer cells	Tegafur; Gimeracil; Oteracil potassium	Advanced cancer	II	NCT03002831	Terminated	[103, 104]
Anti-MUC1 CAR-pNK cells		Hepatocellular carcinoma; NSCLC; pancreatic cancer; triple negative invasive breast carcinoma; malignant glioma of the brain; colorectal carcinoma; gastric carcinoma	I/II	NCT02839954	Unknown	[105]

Autologous NK/NK T cell immunotherapy	Breast cancer; glioma; hepatocellular carcinoma; squamous cell lung cancer; pancreatic cancer; colon cancer; prostate cancer	I	NCT00909558	Suspended	[106]

NK cell: Natural killer cell; CAR: Chimeric antigen receptor; NSCLC: Non-small cell lung cancer; CML: Chronic myeloid leukemia; iPSC: Induced pluripotent stem cells.

In addition to CAR therapies, cytokine supplement either alone or in combination with additional therapeutics has demonstrated significant benefit in both preclinical studies and clinical trials. In combination with the immune priming CD40 agonist, supplementation of IL-15 in orthotopic and KPC mouse models of pancreatic cancer resulted in a significant increase in immune cell infiltration into tumour tissue (assessed through multi-colour flow cytometry), particularly of NK and $CD8^+$ T cells. This infiltration resulted in enhanced anti-tumour effect and significantly improved long term survival (log-rank tests; $P \leq 0.0001$)[85]. Furthermore, Lin *et al*[86] demonstrated that allogeneic NK cell transfer in combination with irreversible electroporation significantly increased median progression-free and overall survival in stage III PDAC patients and increased median overall survival in stage IV patients. Moreover, multiple allogenic transfers correlated with better prognosis in stage III patients. Similarly, treatment with a dendritic cell/cytokine-induced killer cell vaccine was found increase both overall and progression-free survival in advanced pancreatic cancer patients[87]. Taken together these results highlight the therapeutic potential of NK cell-based therapies. Current clinical trials employing NK cell therapy in pancreatic cancer (identified through entering the search terms 'natural killer cells' and 'pan-creatic cancer' at clinicaltrials.gov) are highlighted in Table 1.

CONCLUSION

NK cells are powerful effector units, which, when harnessed, can confer striking therapeutic benefit. With the innate effector units demonstrating strong efficacy against malignancy, both in preclinical studies, and clinical trials, the potential for NK cell therapy in PDAC is only just beginning to come to light. Additional research is needed to fully elucidate the role of NK cells in pancreatic cancer and deconvolute the intricate relationships between NK and stromal cells within the TME, facilitating co-targeting of tumour stroma[73,88]. Through defining these relationships, novel functional and mechanistic insights into this devastating disease can be achieved[89] and the full therapeutic potential of NK cells can be harnessed.

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REVIEW

COVID-19 and its effects on the digestive system

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Abstract

Coronavirus disease 2019 (COVID-19) is caused by infection of the coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) with typical respiratory symptoms. SARS-CoV-2 invades not only the respiratory system, but also other organs expressing the cell surface receptor angiotensin converting enzyme 2. In particular, the digestive system is a susceptible target of SARS-CoV-2. Gastrointestinal symptoms of COVID-19 include anorexia, nausea, vomiting, diarrhea, abdominal pain, and liver damage. Patients with digestive damage have a greater chance of progressing to severe or critical illness, a poorer prognosis, and a higher risk of death. This paper aims to summarize the digestive system symptoms of COVID-19 and discuss fecal-oral contagion of SARS-CoV-2. It also describes the characteristics of inflammatory bowel disease patients with SARS-CoV-2 infection and discusses precautions for preventing SARS-CoV-2 infection during gastrointestinal endoscopy procedures. Improved attention to digestive system abnormalities and gastrointestinal symptoms of COVID-19 patients may aid health care providers in the process of clinical diagnosis, treatment, and epidemic prevention and control.

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Core Tip: Coronavirus infections can cause a series of digestive diseases and may also be accompanied by digestive manifestations. Furthermore, the potential mechanisms of coronavirus disease 2019 (COVID-19) on the digestive system, the fecal-oral contagion of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the characteristics of inflammatory bowel disease patients with SARS-CoV-2 infection, and the management during gastrointestinal endoscopy procedures are also discussed. This review provides a new perspective to clinicians for the prevention and treatment of COVID-19.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a neo-type respiratory infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; previously known as 2019-nCoV). SARS-CoV-2 emerged in Wuhan, Hubei Province in late December 2019[1] and quickly spread throughout China and subsequently throughout over 213 countries, evolving into a pandemic and threatening global public health by human-to-human transmission. By December 29, 2020, over 79 million reported cases and over 1.7 million deaths have been confirmed globally since the start of the pandemic[2].

SARS-CoV-2 is a single-stranded positive-sense RNA virus belonging to the β-coronavirus family. SARS-CoV-2 shows over 88% homology with two bat-derived severe acute respiratory syndrome (SARS)-related coronaviruses[3] and is identified as the eighth coronavirus with human infection capacity[4]. Other similar coronaviruses with this capacity include SARS-CoV, which causes severe acute respiratory syndrome, and Middle East respiratory syndrome (MERS-CoV), which causes Middle East respiratory syndrome. In contrast to SARS-CoV and MERS-CoV, the new virus is highly transmissible between individuals even during the pre-clinical phase. It has higher transmission and infection potentiality but a reported lower mortality rate as compared to SARS-CoV and MERS-CoV[5]. Although respiratory compromise with dominant symptoms of fever and cough is the cardinal feature of the disease, involvement of the gastrointestinal (GI) tract and the hepatic system has been increasingly reported. In this review article, we discuss in detail GI symptoms and the role of liver involvement in COVID-19. We also discuss the possible effects of COVID-19 in inflammatory bowel disease (IBD) patients and precautions to be taken during GI endoscopy procedures.

DIGESTIVE TRANSMISSION OF COVID-19 OUTBREAKS

SARS-CoV-2 is spread and transmitted mainly through direct or indirect droplet exposure. The finding of SARS-CoV-2 nucleic acid in patients' feces indicates that SARS-CoV-2 has the potential to be transmitted through the fecal-oral route. Several studies have reported the presence of viral RNA in feces or anal/rectal swabs of patients with COVID-19[6-9]. In a study about SARS-CoV-2 detection in the specimens of 205 COVID-19 patients, the live virus was detected in 29% of fecal specimens, implying that SARS-CoV-2 may be transmitted by the fecal route[10]. Guan *et al*[11] found that SARS-CoV-2 RNA was detected in four (6.5%) of 62 stool specimens, and four rectal swabs were positive for SARS-CoV-2 RNA. The percentage of positive stool samples has been reported up to 53.42% among hospitalized patients confirmed with

COVID-19[12]. Chen *et al*[13] reported a special case of an infected COVID-19 patient with a positive virus nucleic acid result in a fecal specimen and negative findings on several pharyngeal and sputum samples. This case report contributes to the understanding of the infection route of SARS-CoV-2 by demonstrating that the virus can grow in the digestive tract and may be capable of spreading through fecal-oral transmission[13]. The duration time of positive stool results ranged from 1 to 12 d and 17 patients continued to have positive results in stool after showing negative results in respiratory samples[12]. Another systematic review and meta-analysis found that viral RNA was detected in stool samples from 48.1% of patients in the study sample, even in stool collected after respiratory samples had produced negative test results[14]. These findings indicate that viral gastrointestinal tract infections and potential fecal transmission may have persisted even after the virus was cleared in the respiratory tract. This positive detection of SARS-CoV-2 in stool specimens was a breakthrough because it demonstrated that the virus could replicate and exist in the digestive tract. The duration of viral nucleic acid in feces is longer than that in respiratory specimens, and the peak of viral load is later. Therefore, clinicians should consider the possibility of viral transmission through the fecal-oral route in the management of COVID-19. The importance of the high detection rate of viral RNA in fecal samples needs to be more carefully considered so that fecal-oral transmission of SARS-CoV-2 can be better controlled and prevented.

GASTROINTESTINAL SYMPTOMS OF COVID-19 PATIENTS

The most prominent clinical presentations of COVID-19 in the existing literature are respiratory symptoms such as fever, cough and sputum, and dyspnea. Digestive system symptoms in COVID-19 patients have been increasingly reported with the accumulation of case data as the pandemic continues to evolve[1,15-21] (Table 1). The most commonly reported gastrointestinal symptoms of the disease are diarrhea, anorexia, nausea, vomiting, abdominal discomfort, and gastrointestinal bleeding. Loss of appetite, diarrhea, and vomiting were the three most frequent digestive symptoms in patients with COVID-19. There have been some reports of a small number of patients presenting only with diarrhea and vomiting without fever or cough[22,23]. In a meta-analysis of 60 studies including 4243 patients, the pooled prevalence of all gastrointestinal symptoms was 17.6%[14]. Eleven studies in the meta-analysis compared the prevalence of gastrointestinal symptoms to COVID-19 disease severity; 11.8% of patients with non-severe COVID-19 and 17.1% of patients with severe COVID-19 had gastrointestinal symptoms, indicating that the prevalence of severe disease was more common in patients with gastrointestinal symptoms[14]. Redd *et al*[24] similarly found that gastrointestinal symptoms occurred in 61.3% of patients included in the study, and they were the predominant presenting complaint amongst 20.3% of patients. Importantly, gastrointestinal manifestations may be the only initial symptoms in some patients with COVID-19. In the study by An *et al*[25], 54 patients reported only gastrointestinal symptoms without fever or respiratory symptoms. Six (66.7%) patients had anorexia. None of the cases presented with fever at onset, and all cases presented with digestive symptoms occurring 1-3 d prior to admission[25]. Another study involving 1141 COVID-19 patients found that 183/1141 (16%) presented with GI symptoms only[18]. In addition, digestive symptoms appeared to be associated with worse prognoses. Multiple studies have reported a higher incidence of diarrhea, nausea, or vomiting in patients with severe disease as compared to those with non-severe disease. In addition, there is a connection between the presence of diarrhea and respiratory symptom severity; Li *et al*[26] reported that more patients with diarrhea required ventilator support and were admitted to the intensive care unit (ICU) than those without diarrhea. Pan *et al*[27] found that patients with digestive symptoms had a longer time from onset to hospital admission as compared to patients without digestive symptoms. Sixty percent of patients without digestive symptoms recovered and were discharged, while only 34.3% of patients with digestive symptoms recovered[27]. Digestive symptoms appeared to be tied to worse COVID-19 outcomes.

This bank of published literature provides robust evidence for GI symptoms as common clinical manifestations of COVID-19. GI symptoms should attract the attention of both patients and doctors. It is crucial for clinicians to be on the alert for atypical symptoms to avoid missed COVID-19 diagnosis.

Table 1 Summary of clinical features of coronavirus disease 2019 patients with digestive symptoms, *n* (%)

Ref.	Total patients	Diarrhea	Anorexia	Nausea	Vomiting	Abdominal pain
Huang *et al*[1], 2020	38	1 (2.6)				
Chen *et al*[15], 2020	99	2 (2.0)		1 (1.0)	1 (1.0)	
Zhou *et al*[105], 2020	141	9 (4.7)		7 (3.7)	7 (3.7)	
Mao *et al*[19], 2020	6686	601 (9)	1404 (21)	401 (6)	401 (6)	200 (3)
Guan *et al*[11], 2020	1999	42 (3.8)		55 (5.0)	55 (5.0)	
Pan *et al*[27], 2020	204	35 (17.2)	4 (2.0)		81 (39.7)	2 (0.98)
Holshue *et al*[20], 2020	1 (first case)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Luo *et al*[18], 2020	1141	68 (6.0)	180 (15.8)	134 (11.7)	119 (10.4)	45 (3.9)
Han *et al*[9], 2020	206	67 (32.5)	112 (49.5)		24 (11.7)	9 (4.4)
Lin *et al*[106], 2020	95	23 (24.2)	17 (17.9)	17 (17.9)	4 (4.2)	2 (2.1)
Wang *et al*[32], 2020	138	55 (39.9)	14 (10.1)	14 (10.1)	5 (3.6)	3 (2.2)
Zhang *et al*[107], 2020	140	17 (12.2)	18 (12.9)	24 (17.3)	7 (5)	8 (5.8)
Liu *et al*[23], 2020	137	11 (8.0)				

MECHANISMS OF GASTROINTESTINAL TRACT INVOLVEMENT

Intestinal damage caused by SARS-CoV-2 infection has been verified by autopsy and biopsy. A recent report described the intestinal autopsy from a COVID-19 patient who developed alternating segmental dilatation and stenosis of the small intestine[28]. However, the mechanism by which SARS-CoV-2 causes gastrointestinal symptoms remains unclear. The pathogenicity of COVID-19 is thought to be related to the angiotensin converting enzyme 2 (ACE2) receptor[29-32]. The SARS-CoV-2 virus consists of four main structural proteins: The spike (S) protein, membrane (M) proteins, nucleocapsid (N) proteins, envelope (E) proteins[33,34]. The S protein is the key component that mediates the entry of the virus into the host cell[35,36]. In the process of infecting cells, the S proteins of most coronaviruses are cleaved into S1 receptor-binding subunit and S2 fusion subunit by host cell Furin-like protease. S1 contains a receptor-binding domain (RBD) that plays an important role in recognizing and binding to the ACE2. Epigallocatechin gallate (EGCG), an active constituent of green tea, has been identified as a potential inhibitor of the RBD domain and other proteins of SARS-CoV-2[37]. The effect of green tea consumption may be explored for inhibition of S protein domains to prevent its binding with ACE2[38]. This may be particularly effective in the gastrointestinal tract, as the oral route implies maximum availability in the digestive tract. The S2 subunit is associated with the fusion of the viral membrane and the host cell membrane. The entry of coronavirus into susceptible cells is a complex process that requires ACE2 receptor binding and the enhancement of proteolytic distribution of protein S[36]. ACE2 is also closely related to the physiological processes of virus infection, virus killing, and natural immunity in the immune system (Figure 1). Transmembrane serine proteinase 2 (TMPRSS2) plays an indispensable role in the invasion and transmission of the virus for S protein priming. ACE2 is distributed in many tissues and organs of the human body. It is not only expressed in the heart, lung, kidney, and blood vessels but also in the digestive system, such as in the duodenum, jejunum, and liver[26]. Hoffmann *et al*[35] confirmed that the host cell entry of SARS-CoV-2 depends on the SARS-CoV receptor ACE2 and that the entry can be blocked by a clinically proven inhibitor of the cellular serine protease TMPRSS2. Xiao *et al*[12] observed the staining for viral nucleocapsid protein in the cytoplasm of gastric, duodenal, and rectum glandular epithelial cells and the positive staining for ACE2 and SARS-CoV-2 in the gastrointestinal epithelium from patients who tested positive for SARS-CoV-2 RNA in feces. Another study by Qian *et al*[39] also observed typical SARS-CoV-2 virus particles in the intestinal epithelial cells of a patient under electron microscopy and obtained direct evidence of active SARS-CoV-2 viral replication in the intestine. Virus particles were found in the cytoplasm of intestinal epithelial cells in a rectal adenocarcinoma patient with co-existing COVID-19 and the virions showed a typical morphology of coronavirus under electron microscopy[39]. These findings indicate that SARS-CoV-2 could directly target

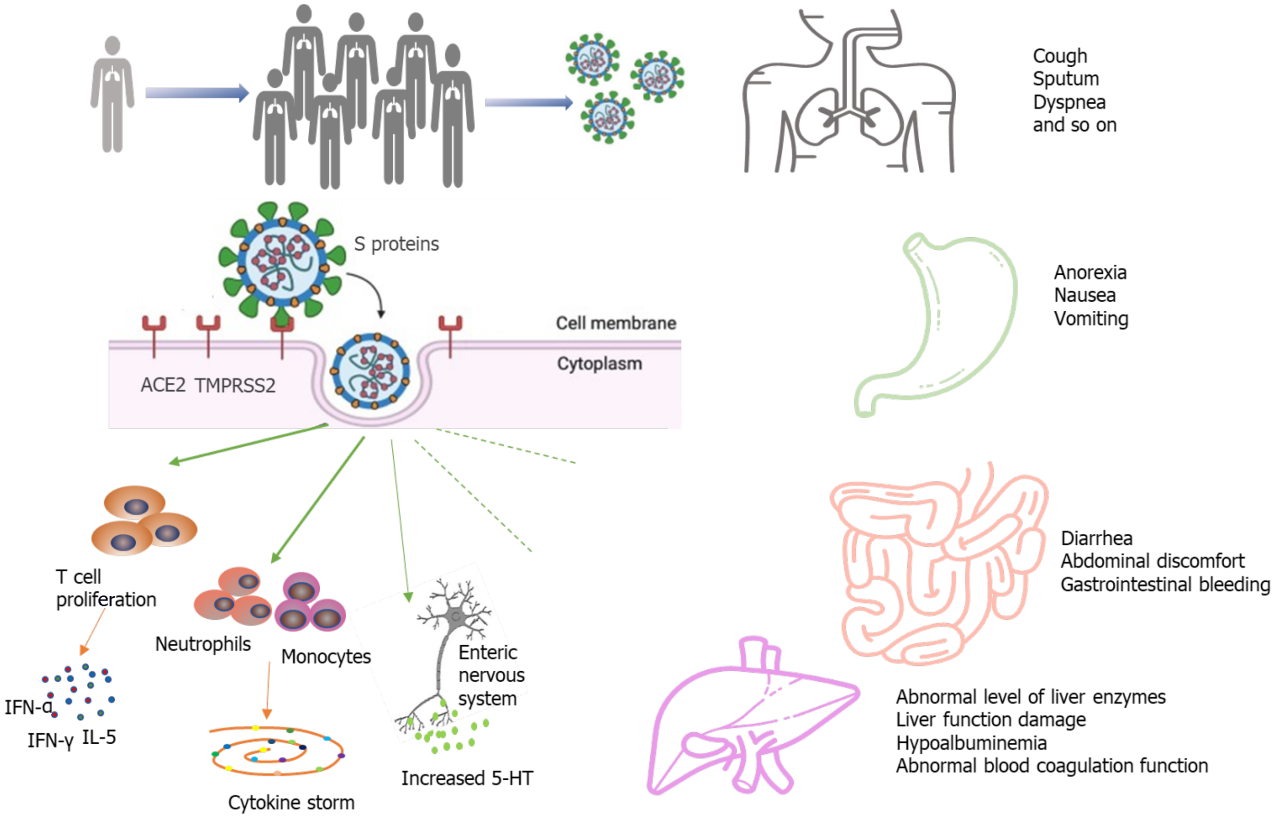

Figure 1 A simplified diagram of the potential pathological mechanisms for gastrointestinal symptoms in severe acute respiratory syndrome coronavirus 2 infection. ACE2: Angiotensin converting enzyme-2; TMPRSS2: Transmembrane serine protease-2.

gastrointestinal cells, especially gastric and intestinal epithelial cells, leading to inflammatory reactions.

The "cytokine storm" has been considered as an essential mechanism of multiple organ dysfunction in COVID-19 patients. High expression of pro-inflammatory cytokines was detected in the serum of most COVID-19 patients, including interferon-γ, interferon-α, and interleukin (IL)-5[1]. The cytokine storm in COVID-19 infection is mainly caused by inducing varieties of pro-inflammatory signals through antigen-presenting cells and T cells against viral infection, and activated macrophages and dendritic cells[40-42]. These cells release chemokines and cytokines to attract more inflammatory cells, such as neutrophils, monocytes, and dendritic cells, which aggregate to the site of inflammation and activate the cascade of inflammatory response. The activated inflammatory cells release more cytokines, further worsening injury to the organs. The fierce cytokine-induced immunopathological attack contributes to the aggravation of patient's condition with a poorer prognosis.

LIVER INJURY IN COVID-19 PATIENTS

An increasing number of studies have reported liver damage in patients with COVID-19 and several have reported COVID-19 patients to have an increased risk of liver dysfunction[1,15,22,43,44]. COVID-19 patients may incur different degrees of liver function damage with elevated aspartate amino transaminase (AST), glutamate moderately amino transaminase (ALT), and total bilirubin (TBil)[45,46]. The risk of liver damage in severe and critically ill patients was higher than in mild patients in most studies. However, there was a subtle difference in the prevalence of lung injury and COVID-19 disease severity across studies, and the exact extent of liver involvement in the COVID-19 disease course remains uncertain[19,46]. In a meta-analysis of 12 studies comprised of 1267 patients, the pooled prevalence of liver injury was 19%, the prevalence of ALT elevation was 18%, the prevalence of AST elevation was 21%, and the prevalence of total bilirubin elevation was 6%[47]. Another cohort study of 1992 patients observed that 215 (11%) patients had an abnormal level of ALT or TBil[41]. Among patients with an abnormal ALT or TBil test result, 77% had a mild

increase, 17% had a moderate increase, and 6% had a severe increase[48]. Additionally, the degree of liver function damage appears to correlate with the occurrence of gastrointestinal symptoms. Jin *et al*[47] reported that the incidence rate of elevated AST was significantly higher in patients with GI symptoms than in those without. Xu *et al*[49] observed moderate microvesicular steatosis and mild lobular and portal activity in the liver biopsy specimens of the patient with COVID-19, which provided evidence of liver injury. It is worth noting that the elevated prothrombin time among COVID-19 patients with digestive symptoms is common, and several studies have reported thromboembolism as a presenting clinical feature of COVID-19[50-53]. Therefore, liver function and the level of liver enzymes should be monitored early in COVID-19 patients with digestive symptoms (Figure 1).

COVID-19 may promote deterioration of liver function in patients who had been diagnosed with chronic liver disease previously and predict an increased risk for severe illness. Several studies have demonstrated that baseline liver disease severity is strongly associated with COVID-19-related morbidity and mortality; additionally, decompensated cirrhosis, hepatocellular carcinoma, and alcohol-related liver disease are risk factors for adverse outcomes from COVID-19[54-58]. A multi-center study involving 867 patients with chronic liver disease and COVID-19 reported that 14.0% of patients died, 60.4% were hospitalized, 23% were admitted to the ICU, and 7.7% developed hepatic decompensation[55]. Another study indicated that mortality was 32% in COVID-19 patients with a previous history of cirrhosis compared to 8% in those without ($P < 0.001$)[59]. Moon *et al*[60] found that 23.3% of patients with cirrhosis and COVID-19 were admitted to the ICU, 17.5% were treated with invasive ventilation, 18.6% were given non-invasive ventilatory support, 4.9% were given renal replacement therapy, and 39.8% died. Nowadays, accumulated data suggest that SARS-CoV-2 infection in patients with cirrhosis appears to be a particularly lethal combination. Compared to the patients without baseline liver disease, the patients with baseline liver disease are prone to unfavorable prognoses.

The mechanisms of liver injury in COVID-19 patients are complex. The higher overall mortality among patients with CLD and COVID-19 may be due to cirrhosis-associated immune dysfunction and metabolic syndrome[61,62], while it needs more research to confirm and explore.

IBD PATIENTS WITH SARS-COV-2 INFECTION

IBD, which mainly includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, nonspecific inflammatory disease with unclear etiology and pathogenesis. At present, intestinal microbial growth disorder, intestinal mucosal barrier injury, abnormal immune response in intestinal mucosal tissue, genetic susceptibility, and environmental factors are considered to be involved in the occurrence of intestinal inflammation. Patients with IBD are prone to frequent and severe infections and are often treated with corticosteroids, which are immunosuppressive agents. Whether the clinical presentation in patients with IBD differs from that of non-IBD people and the risk of SARS-CoV-2 infection or development of COVID-19 in patients with active IBD are uncertain[63]. GI symptoms including diarrhea and abdominal pain appear to be more frequent in COVID-19 IBD patients than in the COVID-19 non-IBD group. Aziz *et al*[64] reported an incidence of 0.3% for COVID-19 in their cohort of 9177 patients with IBD, an ICU admission rate of 8.6% in IBD patients with COVID-19, and a mortality rate of 6.3% in IBD patients with COVID-19. In a multicenter study from eight major gastrointestinal centers in Lombardy, Italy, IBD patients were not reported to have an increased risk of COVID-19 specific symptoms or more severe disease as compared with a control group of gastroenterology patients[65]. Another observational study confirmed that patients with IBD had a higher risk of COVID-19 incidence as compared to the general population and that tumor necrosis factor (TNF) antagonists may reduce the severity of COVID-19[66]. Singh *et al*[67] indicated no significant differences in the composite outcome of hospitalization or mortality between COVID-19 patients with IBD and those without. In addition, IBD patients with COVID-19 on long-term biologics or non-steroid immunomodulatory therapies did not have a higher risk of worse COVID-19 outcomes[67]. As the pandemic continues, it is essential to continue the collection of descriptive data to determine if patients with IBD are more vulnerable to SARS-CoV-2 infection.

Due to the frequent use of biologics and immune suppressors in patients with IBD, questions arose on whether IBD treatment can be continued during SARS-CoV-2 infection and whether a potential threat from the application of biologics and immune

suppressors would be present for patients. Several studies implied that thiopurines, steroids, and oral salicylates could be associated with an increased risk of developing severe COVID-19[68,69]. Feldmann *et al*[70] indicated that anti-TNF antibodies might reduce some of the processes that occur during COVID-19 lung inflammation. As well, Brenner *et al*[68] found that tumor necrosis factor antagonists did not appear to be associated with severe COVID-19. Future studies are required to investigate the impact of anti-TNF antibodies. The American Gastroenterological Association (AGA) recommended that patients with IBD who have been infected with SARS-CoV-2 but have not developed COVID-19 should hold thiopurines, methotrexate, and tofacitinib and should delay the biological therapies; on the other hand, patients with IBD who develop COVID-19 should hold thiopurines, methotrexate, tofacitinib, and biological therapies during the viral illness as well and restart previous treatment after complete symptom resolution or negative PCR tests for SARS-CoV-2[63]. Olendzki *et al*[71] propounded that the Anti-Inflammatory Diet (IBD-AID) was effective in some IBD patients as an adjunct dietary therapy. In the absence of biological therapies, IBD-AID and some identified anti-inflammatory aliments such as ginger, turmeric, and cinnamon may be provided to IBD patients due to their potential inhibitory effects on key pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6[72].

MALNUTRITIONAL RISK AND NUTRITIONAL INTERVENTION IN COVID-19 PATIENTS

Malnutrition has been associated with outcomes of COVID-19 patients and is correlated with a higher risk of mortality[73]. In a study of 348 severe patients with COVID-19, 161 patients had mild malnutrition and 139 suffered moderate-severe malnutrition. The patients with moderate-severe malnutrition had a lower survival rate and higher mortality compared to those with normal or mild malnutrition[74]. One of the reasons is that COVID-19 induces an acute inflammatory response, which accelerates the consumption of nutrients, such as protein, micronutrients, and glucose [75,76]. Gastrointestinal symptoms caused by SARS-CoV-2 further impacted the nutrition absorption and exacerbated malnutrition. In addition, patients' anxiety and poor appetite were also potential contributors to malnutrition[11,77,78]. Some studies show that elderly patients with COVID-19 have a higher risk of developing moderate-severe malnutrition and multi-system organ dysfunction due to their weaker immune function and chronic comorbidities[79,80].

The albumin levels are also decreased in COVID-19 patients and are positively correlated with the infection severity[81]. Zhang *et al*[82] found that 54.78% of COVID-19 patients (63/115) present hypoalbuminemia and among the severe COVID-19 patients, the percentage is 90.32%. Decreased protein synthesis, inadequate nutrition intake, increased tissue catabolism, and nutrient overconsumption may be potential contributors to hypoalbuminemia. Albumin is synthesized in the liver and has several key physiologic functions, such as maintaining colloid oncotic pressure, binding and transporting substances, and sustaining acid-base equilibrium[83]. During the acute stress response, inflammatory mediators such as C-reactive protein, fibrinogen, and alpha 1-antitrypsin are increased, whereas albumin synthesis is decreased[84,85]. Due to the inflammation-induced higher endothelial permeability, albumin can escape to the extravascular space through capillaries, leading to lower serum albumin levels. Hypoalbuminemia may be related to a worse clinical outcomes for COVID-19 patients [73,86-89]. In a retrospective analysis of 181 patients with COVID-19, patients with higher albumin levels on admission were associated with a lower risk of developing ARDS, admission to the ICU, and for every 1 g/dL increase of albumin, there is a 72% decreased risk of developing venous thromboembolism[90]. In another retrospective analysis, the researchers showed significant differences in the rates of hypoalbuminemia (odds ratio = 5.68) between deceased and recovered patients[91].

Previous studies also show that micronutrients play important roles in boosting the immune system[92-94]. Several studies have demonstrated that low levels or decreased intake of micronutrients such as vitamins A, E, B6, B12, Zn, and Se were associated with adverse clinical outcomes of COVID-19[95-99]. Im *et al*[100] reported that 24.0% of the patients with COVID-19 had severe vitamin D deficiency, and among 12 patients with respiratory distress, 91.7% were deficient in at least one nutrient. They speculated that vitamin D deficiency might be an important risk factor for the poor prognosis of COVID-19. In order to maximize anti-infection nutritional defense, we suggest the daily provision of vitamins and trace elements to malnourished patients with COVID-19. The European Society for Clinical Nutrition and Metabolism (ESPEN)

recommended reasonable provision of vitamins and trace elements to benefit anti-infection nutritional defense[101].

Plenty of scientists have emphasized the significance of various nutritional interventions to regulate immune function[102]. Nutrition support is of great importance for patients with severe COVID-19, which can elevate immune function, decrease the incidence of multiple organ failure, and improve the prognosis of the disease. Therefore, the nutritional status of COVID-19 patients should be taken into consideration after admission.

PRECAUTIONS FOR PREVENTING SARS-COV-2 INFECTION DURING GI ENDOSCOPY PROCEDURES

With the growing spread of COVID-19, concerns should be raised with respect to guaranteeing safety for endoscopy operators. Exposure of the respiratory tract and/or digestive tract during endoscopic examination inevitably provides the risk for patients' secretions and excretions to become potential sources of infection, which will significantly increase the probability of nosocomial cross-infection during the pandemic. Therefore, it is important that endoscopy centers pay great attention to indications that most vitally warrant digestive endoscopy procedures. Iacucci *et al*[103] recommended four different urgent scenarios that could necessitate endoscopy: Confirmation of a new diagnosis, especially in a moderate-to-severe scenario; a severe acute flare-up in patients with ulcerative colitis; partial bowel obstruction in patients with IBD, which could be secondary to neoplasia or ileocolonic anastomotic stricture; and cholangitis and jaundice in patients with known primary sclerosing cholangitis with dominant bile duct stricture. Further, endoscopists and assistants must strictly execute hospital infection control requirements and take corresponding classified protective measures according to the actual situation of patients. The AGA recommended the use of N95 (or N99 or PAPR) masks instead of surgical masks as part of appropriate personal protective equipment[104]. It is essential to evaluate the risk of patients with suspected or confirmed COVID-19 before endoscopy, in order to defer unnecessary endoscopies to minimize concomitant exposure.

CONCLUSION

Patients with COVID-19 may also develop various gastrointestinal symptoms, which may be pre-existing or not accompanied by respiratory symptoms. The importance of recognizing and diagnosing the occurrence of gastrointestinal symptoms is immeasurable. Clinicians should be cautious in the management of this highly infectious disease and recognize that gastrointestinal symptoms may be characteristic of COVID-19. Careful consideration of gastrointestinal symptoms may enable early COVID-19 detection, diagnosis, isolation, and intervention. Evidence on fecal-oral contagion of SARS-CoV-2 continues to increase. It is therefore important to step up infection control measures to avoid fecal-oral transmission and standardize health care operational processes.

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REVIEW

Neuropilin-1: A feasible link between liver pathologies and COVID-19

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Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has a tremendous impact on the health of millions of people worldwide. Unfortunately, those suffering from previous pathological conditions are more vulnerable and tend to develop more severe disease upon infection with the new SARS-CoV-2. This coronavirus interacts with the angiotensin-converting enzyme 2 receptor to invade the cells. Recently, another receptor, neuropilin-1 (NRP-1), has been reported to amplify the viral infection. Interestingly, NRP-1 is expressed in nonparenchymal liver cells and is related to and upregulated in a wide variety of liver-related pathologies. It has been observed that SARS-CoV-2 infection promotes liver injury through several pathways that may be influenced by the previous pathological status of the patient and liver expression of NRP-1. Moreover, coronavirus disease 2019 causes an inflammatory cascade called cytokine storm in patients with severe disease. This cytokine storm may influence liver sinusoidal-cell phenotype, facilitating viral invasion. In this review, the shreds of evidence linking NRP-1 with liver pathologies such as hepatocellular carcinoma, liver fibrosis, nonalcoholic fatty liver disease and inflammatory disorders are discussed in the context of SARS-CoV-2 infection. In addition, the involvement of the infection-related cytokine storm in NRP-1 overexpression and the subsequent increased risk of SARS-CoV-2 infection are also analyzed. This review aims to shed some light on the involvement of liver NRP-1 during SARS-CoV-2 infection and emphasizes the possible involvement this receptor with the observed liver damage.

Key Words: Liver; Liver sinusoidal endothelial cells; Hepatic stellate cells; SARS-CoV-2; COVID-19; Pathology

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Core Tip: Severe acute respiratory syndrome coronavirus 2 uses angiotensin-converting enzyme 2 and neuropilin-1 (NRP-1) receptors to infect cells. NRP-1 expression is upregulated in several liver pathologies, which may facilitate viral infection. Moreover, the cytokine storm might increase liver permeability and NRP-1 expression, giving rise to an increased severity of infection and a worse prognosis.

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INTRODUCTION

The identification of a new coronavirus from patients suffering from an outbreak of pneumonia of unknown origin in the city of Wuhan, China, in December 2019[1], alarmed the scientific and medical community. This coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), spread all over the globe, becoming a public health emergency and a pandemic that has paralyzed the world for a year. Moreover, the high incidence of newly infected individuals has pushed health systems worldwide to the limit, with a continuous influx of patients requiring hospitalization because of complications related to this new coronavirus. After the chaotic initial months of the pandemic, an increasing number of studies have shown that the severity of SARS-CoV-2 is directly related to the health status of the infected individuals[2] and also with gender[3]. Severe complications and an increased risk of mortality have been linked with various pathologies[2,4] including obesity, diabetes, lung disease, and hypertension[2,4-7]. SARS-CoV-2 invades the mucosal cells of the host, mainly through the nose and mouth. Once in the body, SARS-CoV-2 infects the epithelial cells of the nasal cavity using a specific receptor present in those cells[8]. In detail, angiotensin-converting enzyme 2 (ACE2) protein expressed in the membrane of the epithelial cell surface serves as the entry for the spike protein of the coronavirus, facilitating the infection[2,8]. Once in the cell, SARS-CoV-2 kidnaps the genetic machinery of the host cell to increase its copy number, leading to virus amplification.

Interestingly, there is recent evidence of another SARS-CoV-2 receptor expressed on the surface of host cells during infection, neuropilin-1 (NRP-1)[9,10]. NRP-1 is a non-tyrosine kinase receptor isoform of the NRP protein family, which also includes NRP2. These transmembrane glycoproteins consist of a common short cytoplasmic domain and a large extracellular domain of 840 amino acid residues[11]. NRPs are present in all vertebrates and initially found in neurons, where they function as adhesion molecules[12]. NRPs lack catalytic activity, but they closely interact with the cytosolic adaptor protein synectin or GIPC1[13] to participate in signaling events. NRPs play a critical role during vasculogenesis[14,15] because of their ability to interact with vascular endothelial growth factor (VEGF)[16]. NRP-1 also interacts with transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF)[17] to regulate a broad spectrum of processes under both normal physiological conditions and pathological responses. The findings led to an increasing number of studies of NRPs in health and disease that have shown their involvement in diverse diseases, including pathologic angiogenesis, fibrosis, cirrhosis, and cancer[18-22]. The expression of NRPs is upregulated in those diseases, which suggests that they are potential therapeutic targets.

NRP-1 was initially found in the nervous system, but is expressed in many cell types in tissues of the heart, lung, pancreas, skeletal muscle, and liver. This widespread expression has put NRP-1 under the spotlight because of evidence that it facilitates the entry of SARS-CoV-2 into host cells[9,10] and increases the extent of infection.

The pathologies associated with NRP-1 are common to several organs, and many involve the liver, including fibrosis, cirrhosis, malignancies (hepatocellular carcinoma, liver metastasis, cholangiocarcinoma, and others), and angiogenesis. NRP-1 is expressed in liver-resident cells, especially nonparenchymal cells, such as liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSC). Some NRP-1 ligands are involved in liver pathologies. For example, VEGF mediates the angiogenic

response of LSECs[23] and is associated with metastatic growth[24]. Another NRP-1 ligand, TGF-β, mediates the activation of HSCs during fibrogenesis[25,26], leading to liver fibrosis and extracellular matrix (ECM) remodeling during liver metastasis and the creation of a premetastatic niche[27]. Platelet-derived growth factor (PDGF) is also involved in HSC activation, a required step in the pathogenesis of liver fibrosis, cholangiocarcinoma, liver metastasis, and HCC[28-31]. It is tempting to hypothesize that the increased expression of NRP-1 in the liver under both physiological conditions and in patients with liver diseases modulates coronavirus disease 2019 (COVID-19) infection. This review summarizes the potential implications of liver expression of NRP-1 during SARS-CoV-2 infection and its possible role in COVID-19 disease progres -sion and severity.

NRP-1 AND THE LIVER: FOCUS ON NONPARENCHYMAL CELLS

The liver is a functionally complex organ that not only maintains metabolic homeostasis but also has immune functions, such as the elimination of pathogens. Recently, NRP-1 has been identified as a facilitating receptor for SARS-CoV-2 infection. As it is expressed in some types of liver cells, it may affect the status of liver disease in COVID-19 patients[10]. Liver functions are carried out by a number of different cell populations, including hepatocytes, which make up about 92.5% of the liver volume[32], and nonparenchymal cells that include LSECs, Kupffer cells (KCs) [33], and HSCs[34]. Small, but important percentages of leukocytes, such as natural killer (NK) cells, natural killer T (NKT) cells, myeloid-derived suppressor cells, and T cells[35,36].

NRP-1 expression has been detected in LSECs[37] and HSCs[38] in the adult liver. Although the expression of NRP-1 is weak in HSCs, it increases following activation associated with diseases with various etiologies. NRP-1 expression in HSCs will be discussed in later sections. Bergé *et al*[39] reported that NRP-1 was not expressed in the hepatocytes of healthy adult livers[39]. Aung *et al*[40] reported weak expression in the cytoplasm of adult hepatocytes but no expression in KCs[40]. There is no doubt of the NRP-1 expression in fetal hepatic monocytes observed by Rantakari *et al*[41] and the absence of NRP-1 in adult hepatic macrophages.

Hepatic sinusoidal endothelium is characterized by the presence of fenestrae and the absence of a true basement membrane. This characteristic of LSECs allows direct contact between blood components and other hepatic cell types[42]. In the fetal liver, NRP-1 is expressed in LSECs in close association with plasmalemma vesicle associated protein (also known as PV-1 and MECA32) during biogenesis of the fenestra, an association that is lost in the adult liver[43]. In fetal LSECs, plasmalemma vesicle associated protein forms additional associations with components of the VEGF signaling pathway. Despite the unknown functional implication of this association, mice deficient in this protein present with significant leukocyte infiltration and an evident steatosis[44].

NRP-1 is expressed in HSCs, but its expression is largely confined to LSECs and co-distributed with that of VEGFR in normal liver tissue. NRP-1 regulates the expression of VEGFR2 at both the transcriptional and post-translational levels by the activation of focal adhesion kinase. The activation of NRP-1 in LSECs thus initiates multiple intracellular signal transduction pathways that regulate cell proliferation, survival, and migration, which are essential for angiogenesis[20].

During physiological aging, the expression of NRP-1 increases in LSECs and is associated with factors present in the lumens of the sinusoids. NRP-1 interacts with hypoxic inducing factor (HIF)-2α to suppress anti-thrombotic and anti-inflammatory pathways that are correlated with profibrotic aggregation of macrophages and platelets. The inhibition of NRP-1 or its association with HIF-2α normalizes the profibrotic niche, with restores the regenerative ability of the liver[45]. It is interesting to note that during aging, LSECs undergo a pseudo capillarization associated with a decrease in endocytic capacity and an increase in leukocyte adhesion, with reduced liver perfusion[46]. However, whether there is a direct relationship between increased expression of NRP-1 in LSECs or pseudo capillarization with a decrease in endocytic capacity is unknown at this time.

The presence of multiple ligands for NRP-1 underscores the importance of this receptor in the liver environment[47]. During adulthood, liver vascularization is stimulated by low blood flow associated with an increase in VEGF and the consequent proliferation of LSECs[48]. Under physiological conditions, hepatic angiogenesis that takes place during regeneration contributes to the formation of new functional

sinusoids. As a VEGF coreceptor NRP-1 is the main mediator of angiogenesis[48], although the role of this coreceptor in intrahepatic angiogenesis is currently unclear. NRP-1 may regulate the action of VEGF on vascular permeability, the proliferation of endothelial cells, and leukocyte adhesion. Some studies suggest that NRP-1 acts independently of VEGFR2 or modulates its activity by stimulating cell migration and adhesion, which are essential for the development of an angiogenic response. In addition to its direct action on VEGFR phosphorylation, NRP-1 indirectly stimulates the VEGFR-dependent angiogenic pathway by preventing the binding of VEGF and its decoy receptor, placental growth factor[16,49].

The interaction of NRP-1 with VEGFR and VEGF is dependent on heparin, which can alter VEGF signaling in endothelial cells to either stimulate or inhibit angiogenesis [50]. Heparin is an anticoagulant that is synthesized in the body, eliminated by receptor-mediated endocytosis in LSECs, and accumulates in the liver. The formation of complexes consisting of heparin and other proteins has important clinical implications[51]. Heparin increases the binding of VEGF with the VEGFR/NRP-1[52] complex and the binding of NRP-1 to placental growth factor-2[53,54] by increasing the number of binding sites without affecting the affinity itself.

As mentioned previously, hepatic macrophages in the adult liver do not express NRP-1. However, they do produce a wide range of angiogenic factors that interact with NRP-1, including HIF-2α and VEGF in addition to TGFβ[55], which requires extracellular activation by NRP-1 to increase its affinity for its receptors. The effects of TGFβ on angiogenesis depend on the receptor with which it interacts. Both receptors are expressed in LSECs, suggesting a balancing function in the angiogenic process. The signaling pathways initiated after the interaction of TGFβ with its receptor are highly dependent on the specific microenvironment in the liver at the time. Indeed, signaling *via* TFGβ receptor binding of anaplastic lymphoma kinase promotes angiogenesis; while the interaction with activin receptor-like kinase 5 inhibits angiogenesis. Although this factor has long been considered profibrogenic in cooperation with other NRP-1 ligands such as PDGF-B, its inhibition causes undesired effects. Expression of both NRP-1 and TGF-β is low in quiescent HSCs in the normal liver and both increase immediately upon liver damage[56]. That suggests a complex scenario for NRP-1 as a coreceptor with angiogenic and profibrogenic receptors.

We must not forget the functions of the liver as an immune organ, in which NRP-1 participates. In addition to the liver lymphocyte population, which is selectively enriched with NK and NKT cells, circulating lymphocytes interact closely with LSECs, KCs, and dendritic cells present in liver sinusoids[57]. NRP-1 has been associated with inhibition of immune responses[58], which may also occur in the liver, an organ that is part of the innate immune system.

FEASIBLE INVOLVEMENT OF NRP-1 DURING LIVER PATHOLOGIES AND COVID-19

COVID-19 affects mainly the upper airways. In some cases, involvement of the lower airways, gives rise to pneumonia[59]. Along with the ability to infect other tissues in addition to the airways, the impact of SARS-CoV-2 infection is detectable in various organs even without local viral invasion[59]. Liver injury occurs in a large proportion of COVID-19 patients and is characterized by elevated levels of gamma-glutamyl transferase, alanine aminotransferase, and/or aspartate aminotransferase enzymes; and occasionally, moderate hyperbilirubinemia[60-63]. The persistence and degree of liver damage caused by COVID-19 seem to vary. In most cases, liver function recovers soon after viral infection, but patients with severe disease may develop irreversible hepatic injury[64-66]. In line with that observation, increased severity of SARS-CoV-2 infection is related to reduced hepatic function[62,67]. A recent postmortem analysis found microvesicular steatosis along with lobular and portal activity in a CODIV-19 patient[68].

To date, apart from immune-related inflammation and hypoxia generated by airway malfunction, the mechanisms proposed to explain the reported liver damage were drug toxicity, inflammation, and hypoxia resulting from lung malfunction[60,69]. The expression of ACE2 in cholangiocytes has also been proposed as a possible mechanism of liver injury. NRP-1 expression in LSECs and HSCs may thus act to amplify the liver damage as a consequence of SARS-CoV-2 infection (Figure 1).

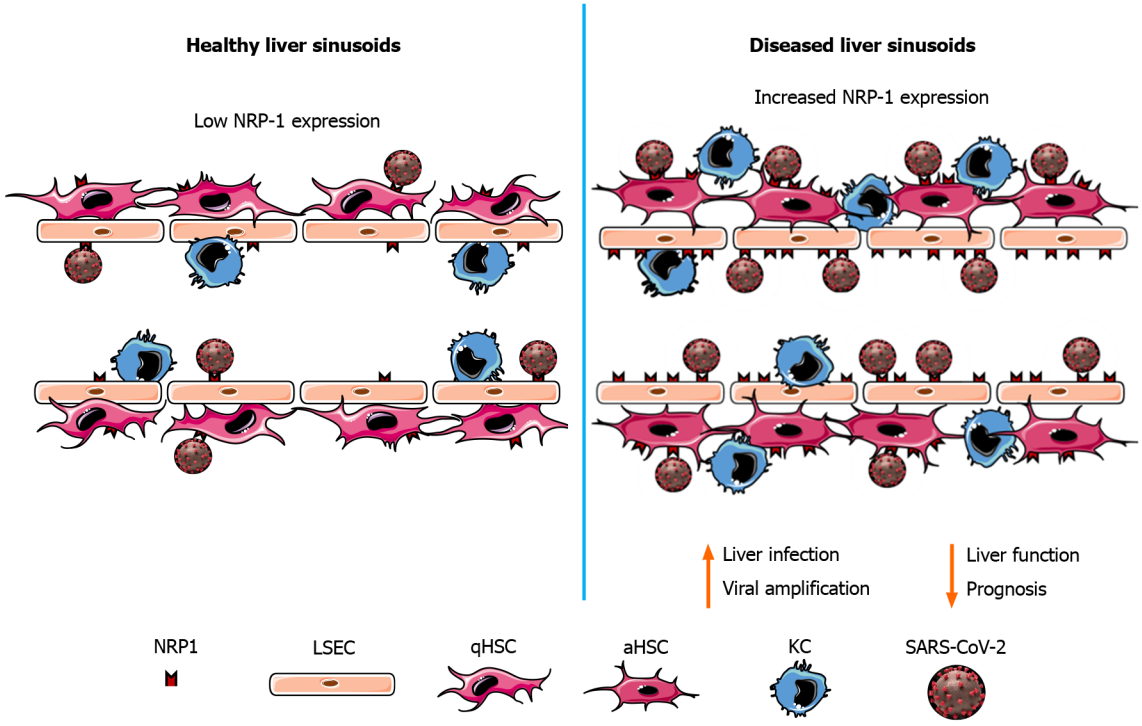

Figure 1 Neuropilin-1 expression in healthy and injured liver. Neuropilin-1 (NRP-1) is expressed at low levels in liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs) in healthy liver sinusoids. However, NRP-1 is upregulated in activated LSECs and HSCs present in several liver pathologies. The increase in NRP-1 during liver disease may facilitate and amplify infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), driving to liver failure and a worse prognosis because of the development of severe COVID-19 disease. SARS-CoV-2 image adapted from NIAID. aHSC: Activated hepatic stellate cell; KC: Kupffer cell; qHSC: Quiescent hepatic stellate cell.

LIVER FIBROTIC RESPONSE

Hepatic fibrosis is a scarring response involving various types of cells affected by liver diseases of different etiologies[70], including metabolic diseases, exacerbated immune responses, and viral infections. Hepatitis C virus (HCV) infection has some similarities to infections of the SARS-CoV-2 virus family[71]. In SARS, the defects observed in the liver are more the result of viral infection than other factors, such as drug toxicity or systemic inflammatory responses[71]. However, the cause of the liver disease observed in a high percentage of COVID-19 patients following SARS-CoV-2 infection is not clear, but steatosis and fibrosis are observed in liver biopsies.

Wang *et al*[72] pointed to direct infection of the liver by SARS-CoV-2 as the cause of the steatosis, lobular inflammation, endothelitis, and fibrosis in patients with COVID-19. Even though the ACE2 receptor has been identified as the main mediator of virus entry[73], the probability that SARS-CoV-2 infects the liver by that route is low given its low or null expression in hepatocytes, HSCs, and LSECs[74]. The development of the fibrotic response in COVID-19 patients may then depend on other routes. It is interesting to note that high levels of other molecules involved in the infectivity of SARS-CoV-2 such as furin, TMPRSS11a, and NRP-1 were detected in infected cells[75] with and without ACE2 receptors. Apart from its involvement in the entry of SARS-CoV-2, the role of NRP-1 as a signaling platform has been established. Cao *et al*[38] found that NRP-1 was a signaling element in HSCs during fibrotic processes caused by viral infections. NRP-1 could thus be the link between infection with SARS-CoV-2 and the development of hepatic steatosis with the presence of a fibrotic response.

HSCs play a central role during liver fibrosis. Following activation, HSCs undergo a change from a quiescent phenotype to a myofibroblastic phenotype characterized by increased proliferation, motility, and accumulation of extracellular matrix. They also contribute to angiogenesis and vascular remodeling processes[70]. HSC activation is initiated by the binding of PDGF-B to its receptor along with a temporal increase in NRP-1. NRP-1 also promotes signaling *via* other key growth factors involved the development of liver fibrosis, such as TGF-β and VEGF[38]. The overexpression of NRP-1 in patients with cirrhosis caused by HCV infection contributes to the progression of liver fibrosis either by influencing the angiogenic response or effects on the PDGF and TGF-β signaling pathways. NRP-1 regulates not only the motility of

HSCs but also collagen deposition, and the severity of fibrosis associated with steatohepatitis and HCV infection are related to the expression of NRP-1. To date, no studies have linked NRP-1 expression with liver involvement in COVID-19 patients. However, it is tempting to hypothesize that the fibrotic response is related to the expression of NRP-1 as an extracellular coreceptor. In other scenarios, such as acute lung SARS or Middle East respiratory syndrome (MERS) infection, cell entry is mediated by TGF-β [76], a mechanism possibly relevant to SARS-CoV-2 and its coreceptor, NRP-1. In the acute phase of COVID-19, the levels of TGF-β are directly related to the development of pulmonary fibrosis[77] and liver fibrosis in pathologies derived from different etiologies[78]. Currently, no studies have found a similar relationship of TGF-β to the fibrotic processes observed in the livers in patients with COVID-19.

Additionally, the mechanical ventilation required by many COVID-19 patients alters hepatic hemodynamics, with reduced portal flow that can result in acute liver damage and activation of HSCs[79]. Although it has not been possible to relate the presence of metabolic fatty liver diseases with the increased risk of hospitalization, nor to the outcomes of hospitalized patients with both diseases, Campos-Murguía *et al*[80] observed an increased risk of the need for mechanical ventilation with the development of liver fibrosis, among other symptoms. However, other studies observed an increase in the severity of the disease in the presence of a fibrotic development. Even today, it is not clear whether SARS-CoV-2 is solely responsible for the development of liver damage or if the damage is a consequence of systemic inflammation caused by the virus or its treatment[81]. Regardless of the cause of liver damage, conditions such as hypoxia, inflammation, and fibrotic responses that develop as a result of the viral infection are related to elevated levels of NRP-1 in both HSCs and LSECs. Indeed, in hypoxic states, such as those observed during COVID-19 progression, HSCs respond through an increase in VEGF. As a result, there is a concomitant increase in the expression of NRP-1 in LSECs, HSC motility, and TGF-β-dependent collagen production[47]. In the presence of TGF-β the glycome of activated HSCs favors the binding of galectins to NRP-1, which promotes migration and further activation[19,82]. It is interesting to note that the levels of both galectin-1 and -3 are increased in COVID-19 patients and have been significantly associated with the severity of the disease[83].

In addition to all the above, the activation of HSCs, which drives liver fibrosis, is induced by profibrotic and proinflammatory cytokines. The resulting inflammatory environment during the development and progression of COVID-19 may be one of the causes of the reported liver damage and a cause of HSC activation, with the consequent induction of the fibrotic response. In fact, during the development of liver fibrosis, infiltration of a subgroup of macrophages enriched in genes associated with tissue repair has been observed[84,85]. The genes encode coreceptors of NRP-1 and the inflammatory cytokines that regulate its expression[86]. One of those cytokines is IL-6, which results from activation of the immune system by SARS-CoV-2 in COVID-19 patients and is associated with altered levels of liver enzymes[87]. In various viral infections, IL-6 is associated with the development of liver fibrosis[88] and with an increase in the expression of NRP-1[89]. In addition, the level of galectin-3, which binds to NRP-1 and has a structure similar to a part of the SARS-CoV-2 spike protein [90], leads to dysregulated expression of proinflammatory cytokines[83,91]. Its expression is increased secondary to diverse types of injury mediated by viral diseases including hepatitis B, hepatitis C, and, SARS-CoV-2[92]. The findings suggest a nexus between systemic inflammation and subsequent liver fibrosis in patients with COVID-19 through NRP-1.

LIVER CANCER

Hepatocellular carcinoma

Viral infection of the liver is the leading cause of hepatocellular carcinoma (HCC) worldwide[93]. Changes in signaling pathways during viral infection promote inflammation that contributes to the development and progression of chronic liver disease beginning with hepatic cirrhosis and finally, HCC[94]. Interestingly, HCV elimination improves the overall survival of HCC patients, indicating that viral infections complicate the outcome[95]. Similarly, SARS-CoV-2 viral infection may facilitate HCC or complicate the outcome of the disease. In the context of the SARS-CoV-2 pandemic, several reports have linked the presence of cancer with an increase of a worse outcome in COVID-19 patients[96,97]. Furthermore, a prospective nationwide cohort study carried out in China revealed that 1% of COVID-19 patients had a history of cancer.

Those patients had an increased risk of complications, ICU admission, and a fatal outcome compared with patients without cancer. Interestingly, cancer survivors had more likely to suffer disease complications than healthy people but less likely than cancer patients[98]. Recent studies describe a possible link between SARS-CoV-2 receptor ACE2 in cancer tissues and increased risk of infection[99]. In liver cancer, Dai *et al*[100] found that upregulated expression of ACE2, the primary SARS-CoV-2 ligand in infected cells, was related to improved survival in HCC patients. However, an increase in ACE2 expression might make such patients more vulnerable to SARS-CoV-2 infection. The link between NRP-1 and liver cancer was discovered some years ago, with increased NRP-1 expression in hepatocellular carcinoma[39]. NRP-1 expression was higher in LSECs from HCC biopsies than from healthy liver biopsies. Hepatocyte expression of NRP-1 correlated with primary HCC and increased with tumor progression. Blocking NRP-1 protein led to impaired tumor growth and vascular remodeling[39]. Interestingly, NRP-1 expression stimulated the activation of HSCs [101], liver-resident myofibroblast-like cells that contribute to the malignant growth of liver metastasis[23,24,102]. The NRP-1-dependent activation boosted tumor proliferation, cell migration, and invasiveness[101]. Therefore, it is tempting to hypothesize that HCC patients with increased NRP-1 expression in LSECs, tumor cells, and HSCs may have an increased risk of SARS-CoV-2 infection.

Cholangiocarcinoma

Cholangiocarcinoma (CCA) is a relatively uncommon liver malignancy, accounting for about 10% of liver cancers[103]. The risk of CCA appears to be increased by both HCV and hepatitis B virus (HBV) infection[104], which may also account for increased SARS-CoV-2 risk. Little is known about the involvement of NRP-1 in CCA. There is evidence that NRP-1 expression is elevated in intrahepatic CCA tissue compared with normal biliary tissue. Moreover, the association of NRP-1 and CCA development has been confirmed by NRP-1 knockdown leading to impaired cancer cell proliferation, blocked cell cycle, reduced cell migration, and reduced focal adhesion kinase expression[105]. In line with that finding, NRP-1 overexpression in intrahepatic CCA cells that was associated with miR-320a downregulation boosted cell proliferation and epithelial to mesenchymal transition and stimulated tumor angiogenesis[106]. Recently, high NRP-1 expression was correlated with poor prognosis and reduced overall survival of intrahepatic CCA patients[107]. Intrahepatic CCA may favor development of an inflammatory milieu driving to vascular permeabilization and increased expression of NRP-1 upon activation of liver-resident cells. The inflammation may be mediated by IL-13, which significantly increased in CCA patients and is known to promote the expression of adhesion molecules in endothelial cells[108,109]. Therefore, CCA could indirectly promote NRP-1 expression in both LSECs and HSCs and facilitate liver damage by immune infiltration. Based on the data, SARS-CoV-2 infection may be facilitated by CCA through NRP-1 overexpression. HCV, HBV, or SARS-CoV-2 infection could drive to liver inflammation and LSEC and HSC activation. The process would lead to increased recruitment of inflammatory cells, NRP-1 overexpression, and assisted viral infection accompanied by liver injury.

CYTOKINE STORM MAY INCREASE LIVER DAMAGE AND NRP-1 EXPRES-SION

Cytokine storm is a potentially fatal consequence of SARS-CoV-2 infection. The release of inflammatory cytokines into the blood of infected individuals is a major turning point in the prognosis and survival of patients with severe disease[110]. The liver filters and detoxifies the blood supply coming from the portal vein and hepatic artery. During COVID-19, both amplifying viruses and cytokines released during the cytokine storm enter the liver and flow through the liver sinusoids and small diameter capillaries in contact with nonparenchymal liver cells, LSECs, KCs, and HSCs[51]. These small vessels have specific properties adapted for their function, such as fenestrations in the endothelial layer, tightly controlled immune tolerance, close contact with nonparenchymal-cell subsets and hepatocytes, and a wide array of cell adhesion molecules and receptors[111,112].

As mentioned previously, the cytokine storm is induced by the release of inflammatory mediators such as IL-6, IL-1β, IL-10, TNF-α, interferon-γ, macrophage inflammatory protein (MIP) 1α and 1β, and VEGF[67,113] and complicates the course of the disease, driving to multiorgan failure and coagulation. The role of these inflammatory mediators in the recruitment of immune and nonimmune cells into the infectious foci,

mainly the lungs, is widely recognized and well characterized. However, these soluble proteins have the intrinsic ability to switch on a wide spectrum of cellular responses in both immune cells and nonimmune cells in epithelial, endothelial, and other tissues [114]. Some of the soluble mediators released during the cytokine storm may increase liver permeability. The effects of TNF-α, IL-6, and IL-1β increase the expression of intercellular adhesion molecule 1 (ICAM-1) in endothelial cells[115-117], which may take place during SARS-CoV-2 infection. LSEC ICAM-1 mediates the adhesion and infiltration of immune cells in the liver[118], which may further increase the number of inflammatory cells and increase the extent of liver damage.

VEGF mediates the recruitment of several immune populations[119] and is considered the main stimulus for the proliferation and migration of endothelial cells [120]. It is also a ligand of NRP-1[53]. Interestingly, VEGF acts on endothelial cells not only as a proliferative and promigratory signal, but it can stimulate the expression of NRP-1[121,122], creating a feedback loop. Consequently, VEGF may facilitate SARS-CoV-2 infection in the liver through the upregulation of the local expression of NRP-1, the recently discovered SARS-CoV-2 receptor. It is tempting to hypothesize that this process may take place in other tissues such as the lung epithelium and that the involvement of IL-6 in promoting increased NRP-1 expression might be underestimated. Previous reports have described NRP-1 upregulation in pancreatic cancer[89, 123], which was stimulated by IL-6 and mediated by STAT3 transcription activity[89]. Interestingly, the IL-6/STAT3 pathway plays a significant role during HSC activation [124] and might boost the expression of NRP-1 in these liver-specific cells.

CONCLUSION

The severity of SARS-CoV-2 infection is increased in patients with previous pathologies. A high proportion of COVID-19 patients experience liver damage. NRP-1 is a recently discovered coreceptor for SARS-CoV-2 virus, and is overexpressed in the injured and pathologic liver. Patients with SARS-CoV-2 infection and liver diseases should be followed to monitor the liver response and overall health status. The increased expression in NRP-1 in the pathologic liver of patients suffering from COVID-19 may represent an amplifying pathway to further complicate the infection and worsen the prognosis and severity of the disease. There is evidence of a link between liver NRP-1 and SARS-CoV-2 infection, but further study is needed to determine whether previous liver disease and NRP-1 influence COVID-19 progression, severity and mortality. Even though the presence of liver disease can promote the increased severity of COVID-19 disease, direct infection and liver injury by SARS-COV-2 virus cannot be ruled out. Liver diseases, such as fibrosis and different types of liver cancers, share active mediators that are directly linked to NRP-1, indicating a feasible and direct relationship of NRP-1, liver disease with COVID-19.

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MINIREVIEWS

Hepatitis delta virus: From infection to new therapeutic strategies

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Abstract

The hepatitis delta virus (HDV) is a small RNA virus that encodes a single protein and which requires the hepatitis B virus (HBV)-encoded hepatitis B surface antigen (HBsAg) for its assembly and transmission. HBV/HDV co-infections exist worldwide and show a higher prevalence among selected groups of HBV-infected populations, specifically intravenous drug users, practitioners of high-risk sexual behaviours, and patients with cirrhosis and hepatocellular carcinoma. The chronic form of HDV-related hepatitis is usually severe and rapidly progressive. Patterns of the viral infection itself, including the status of co-infection or super-infection, virus genotypes (both for HBV and HDV), and persistence of the virus' replication, influence the outcome of the accompanying and manifested liver disease. Unfortunately, disease severity is burdened by the lack of an effective cure for either virus type. For decades, the main treatment option has been interferon, administered as mono-therapy or in combination with nucleos(t)ide analogues. While its efficacy has been reported for different doses, durations and courses, only a minority of patients achieve a sustained response, which is the foundation of eventual improvement in related liver fibrosis. The need for an efficient therapeutic alternative remains. Research efforts towards this end have led to new treatment options that target specific steps in the HDV life cycle; the most promising among these are myrcludex B, which inhibits virus entry into hepatocytes, lonafarnib, which inhibits farnesylation of the viral-encoded L-HDAg large hepatitis D antigen, and REP-2139, which interferes with HBsAg release and assembly.

Key Words: Hepatitis delta virus; Hepatitis B virus; Myrcludex; Lonafarnib; REP 2139

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Core Tip: The hepatitis delta virus (HDV) is a small defective virus, of interest to scientists for its replicative reliance on and ability to inhibit the common hepatitis B virus (HBV). HDV infection occurs worldwide but shows some geographic variation and its prevalence is generally underestimated. HDV/HBV co-infection causes severe liver disease and the persistence of viral replication is associated with poor prognosis, although interferon is effective in around a quarter of patients. Improved understanding of the HDV life cycle has led to identification of specific antiviral targets and new drugs, including inhibitors of the sodium tauro-cholate cotransporting polypeptide receptor and farnesyltransferase.

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INTRODUCTION

The hepatitis delta virus (HDV) is a defective virus that survives in the host by enveloping itself with the hepatitis B surface antigen (HBsAg) provided by the hepatitis B virus (HBV)[1]. This unique virus was discovered in 1977 by Rizzetto *et al* [2] following description of the delta antigen in HBsAg carriers. The subsequent research uncovering HDV modes of replication and its interactions with HBV have provided information with clinical implications[3]. HDV is highly infectious and induces a heterogeneous liver disease that rapidly progresses to cirrhosis; the outcome itself is influenced by several viral and host factors, including virus genotypes and level of viremia (for both HDV and HBV) as well as the presence of any concomitant causes of liver damage[3]. According to recent reports, 10.58% of general HBV-positive individuals have co-infection with HDV, excluding the intravenous drug users (IVDUs) and practitioners of high-risk sexual behaviours (HRSBs)[4].

Collectively, these data highlight the need for HDV screening and control strategies, which must be developed rationally. In the industrialized world, the decline of new infections has regrettably reduced awareness and testing for HDV[1,5]. Moreover, there is a significant lacuna in the literature, with limited data reported from the Americas, Southern Africa and Asia[4], although reliable data from across the globe are required to overcome this pathogenic threat. Once HDV infection is diagnosed, the greatest challenge to clinical care is related to the treatment of associated liver disease. Interferon (IFN) treatment has been the only pharmacological option for decades but its efficacy remains unsatisfactory[6]. The perseverance of HDV researchers is beginning to pay off, however, with enhanced knowledge on the viral life cycle leading to development of drugs targeting specific steps in such, several of which are now under advanced clinical evaluation[6-8].

VIROLOGY

HDV is similar to viroids and virusoids in its structure and replication process but it presents a distinctive identity. Accordingly, HDV was classified as the sole member of the *Deltaviridae* genus[1,9]. The virus contains about 1700 nucleotides and has a circular structure that is formed by base-pairing involving 74% of its genomic RNA. It encodes a single protein - the delta antigen (HDAg) — which combines with the HDV genome to form a ribonucleoprotein complex (referred to as an RNP). The RNP becomes enveloped upon interaction with HBV, which serves as a helper virus, providing the HBsAg component. The latter is necessary for HDV assembly, release and transmission, but it is not required for any of the other steps in replication[3,10]. HDAg is present in two isoforms: small (S-HDAg) and large (L-HDAg), each carrying out a specific role in the HDV life cycle. S-HDAg is composed of 195 amino-acids and promotes RNA replication, while L-HDAg is composed of 214 amino-acids and is essential for the virion packaging. The isoforms differ by 19 amino-acids located in the C-terminal region of L-HDAg exclusively, the presence of which results from an

editing process that is regulated by the adenosine deaminase enzyme ADAR1[11]. HDAg modification occurs at the post-transcriptional level, similar to the well-known processes of phosphorylation, acetylation, methylation and isoprenylation, all of which regulate the hepatitis viral life cycles (Figure 1).

HDV replicates exclusively in the liver, inside hepatocyte nuclei. The virus uses the same entry mechanism as HBV, being enveloped by HBsAg, differentiated in the small (S-HBsAg), large (L-HBsAg) and medium (also known as M-HBsAg) forms. After ligating the hepatocyte's surface heparan sulphate proteoglycans (commonly referred to as HSPGs), the pre-S1 domain of L-HBsAg binds to the receptor for HBV, identified as the sodium tauro-cholate cotransporting polypeptide (NTCP)[12]. The viral RNP then translocates into the nucleus, where its genomic RNA is transcribed first into the complementary antigenomic RNA, then into new genomic RNA and mRNA by the host's RNA polymerase II enzyme. The fact that HDV does not encode its own RNA polymerase enzyme but redirects the host's is a further peculiarity of this hepatitis virus.

Once linear polymers are produced in the HDV-infected hepatocyte, they self-cleave *via* a ribozyme activity of the virus and then circularize. The viral mRNA then migrates from the nucleus to the cytoplasm, for subsequent synthesis of either S-HDAg or L-HDAg. The protein products associate with the viral genomic RNA to form new ribonucleoproteins, each of approximately 20 nm in diameter. This complex relies on HBV surface antigens for complete formation and subsequent secretion[13]. The latter process occurs only after a farnesylation process[14], which makes molecules more lipophilic, whereby L-HDAg interacts with HBsAg at the endoplasmic reticulum.

While at first evaluation this entire process appears rudimentary, HDV has evolved a complex and unusual replicative mechanism, intriguing for researchers but proving a challenge to fully elucidate. It has been proposed, in the field of innovative research, that viruses other than HBV, such as flavivirus and hepacivirus, could package the HDV ribonucleoprotein[15]. This unconventional transmission of HDV certainly requires confirmation in a human system; however, the newest knowledge on its interaction with the NTCP receptor[12] and the farnesylation process of L-HDAg[14] has led to new therapeutic proposals.

EPIDEMIOLOGY

Sero-epidemiological studies performed between the 1980s and 1990s estimated the HDV prevalence among HBsAg-positive patients to be 5%, equating to about 20 million people worldwide[3,16]. Regional prevalence of HDV infection was higher in South Europe, Middle East, East Africa and Asia, with relatively lower rates in Northern Europe, South Africa and North America[17]. During the more recent 2000s and 2010s, the introduction of HBV vaccination, institution of preventive measures and improvements in hygiene habits have led to decreases in HDV prevalence rates in multiple regions. In Taiwan, for example, HDV prevalence among HBsAg-positive subjects declined substantially, from 24% in 1983 to 4% in 1995[18], while in Italy, it declined from 23% in 1987 to 14% in 1992 to 8% in 1997[19]. However, in parallel with the decreased prevalence rates in previously endemic areas, other HDV hot zones emerged, including South Eastern Russia, Northern India, Vietnam and Albania.

Moreover, starting in the early 2000s, immigration activities have prompted a new rise in HDV prevalence in some European States, following a large influx of immigrants from endemic areas, such as Romania, the ex-Soviet Union and North Africa[20]. France also experienced a remarkable increase in cases, reaching a rate of 6.5% in 2010 after remaining stable at around 1% from 1997 to 2005[21]. Similarly, Germany showed an initial decline during the 1990s (from 19% to 7%) but then experienced an upswing trend, with rates fluctuating between 8% and 14%, in the 2000s[22].

Nowadays, HDV infection maintains its worldwide distribution. According to two recent systematic reviews, global prevalence is around 0.16%-0.98% among the general population and 4.5%-14.6% among HBsAg-positive subjects[4,23]. Geographic variations are still present, considering both the overall population and HBV carriers. Among the general population worldwide, Mongolia has reported the highest HDV prevalence[4], with primacy among HBsAg-positive people (prevalence rate of 36.9%). However, prevalence rates greater than 10% are also reported by Moldova and Western and Middle African countries[23]. According to demographical characteristics, men are slightly more affected than women[4] and the elderly are at greater risk

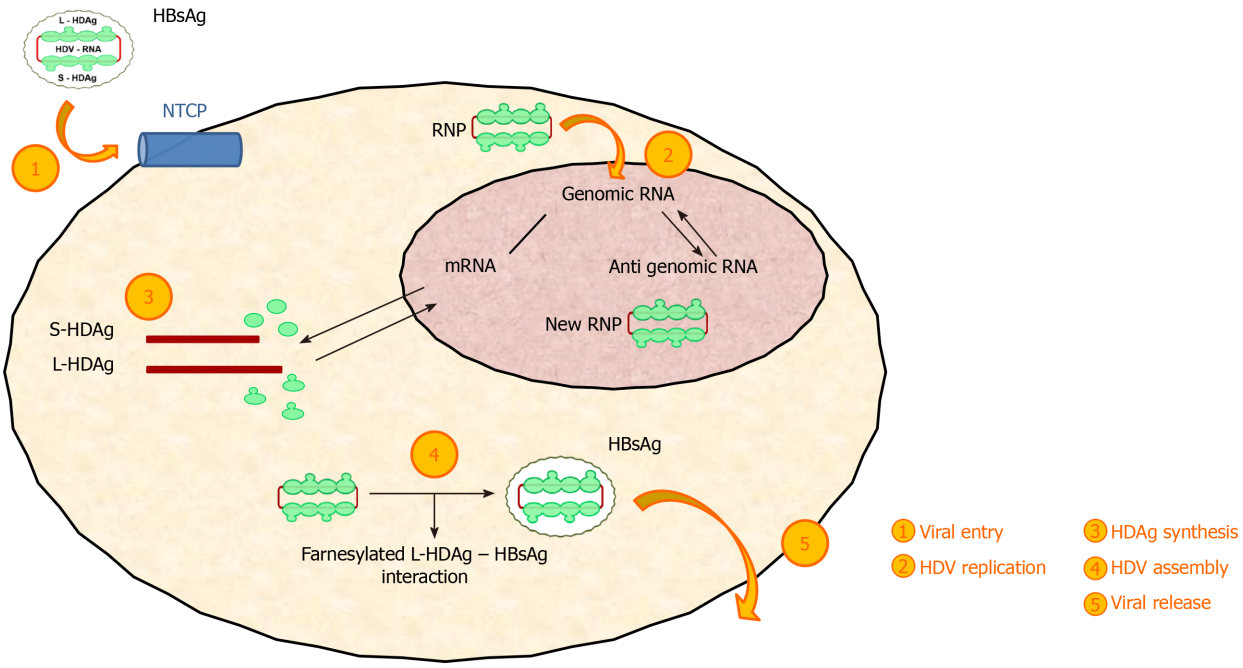

Figure 1 Schematic representation of the hepatitis D virus life cycle. New drugs interfere with the hepatitis B surface antigen at the viral entry or viral assembly level. HBsAg: Hepatitis B surface antigen; HDAg: Hepatitis D antigen; HDV: Hepatitis delta virus; L-HDAg: Large hepatitis D antigen; NTCP: Sodium taurocholate cotransporting polypeptide; RNP: Ribonucleoprotein complex; S-HDAg: Small hepatitis D antigen.

than the young (80% among those ≥ 50-years-old *vs* 3% among those < 30-years-old) [24].

Among the eight recognized HDV genotypes (identified as 1-8), genotype 1 is the most common and it is present worldwide[23,25]. The other seven genotypes are more localized, with genotypes 2 and 4 mainly confined to Asia, genotype 3 to Latin America and genotypes 5-8 to Africa[23].

As exposure to blood of infected subjects is the predominant route of HDV transmission, some groups of high-risk people have been identified. Most HDV-infected patients are IVDUs, who had been infected upon needle-stick injury or use of contaminated syringes[26]. The reported prevalence rates among this subgroup range from 21%[27] to 36%[28], with a pooled odds ratio (OR) of 19.0[23]. Another group of high-risk individuals are practitioners of HRSBs, including commercial sex workers and promiscuous homosexuals[26], among whom the reported prevalence reaches up to 11%[29], with a pooled OR of 18.7[23]. The risk of infection is also higher among the human immunodeficiency virus-positive population (pooled OR: 6.6) and haemodialytic patients (pooled OR: 3.4)[23]. Nowadays, nosocomial infection and transfusion transmission occur less frequently than in the past. However, some cultural practices, such as tattoos and piercing, are quickly becoming an appreciable route of transmission[30].

Taken together, these data show that the global presence of HDV infection has not decreased and it is probably still underestimated.

CLINICAL MANIFESTATIONS AND DIAGNOSIS

Viremia, genotypes and liver disease

The replicative activity of HDV can influence the course of liver disease[31].

A multicentre study found that subjects with persistent HDV replication had worse prognosis in terms of liver failure, need for liver transplantation and/or death than those with undetectable HDV-RNA[32]. On the other hand, the few patients in whom HDV-RNA had become undetectable during their follow-up were determined to be less likely to develop liver cirrhosis than those with persistently positive HDV-RNA levels[32].

To date, little is known about the determinants of spontaneous clearance of HDV-RNA, despite the early clearance of HDV infection being one of the main parameters determining prognosis of the related liver disease[10,33].

Although HBV replication is usually suppressed in the presence of HDV infection, high levels of HBV viremia are associated with more severe liver damage[34]. In addition, HBV genotypes may also play a role in the course of chronic hepatitis D (CHD), which is considered the most severe and rapidly progressive form of chronic viral hepatitis[3].

Besides host-related factors, other viral factors might be involved in clearance of the virus. One such factor is genotypic variability. Among the eight known genotypes, HDV-1 has the highest pathogenic potential[31,33,35], while HDV-2 and HDV-4 are associated with milder forms of the disease[35]. According to a study conducted in Taiwan, patients infected with HDV-2 develop liver failure less frequently than those with genotype 1[34]. HDV-3 has been associated with an increased risk of fulminant hepatitis[3,36], whereas HDV-5 has a better prognosis than HDV-1[33]. The pathogenic properties of HDV 5-8 are not well characterized[7,36]. Among the spectrum of host and viral factors that may underlie the different outcomes of infection with these various genotypes, we can include variability in virus replication and virion assembly efficacy, both of which contribute to the rate of HDV virion secretion, and such host factors as race or presence of single-nucleotide polymorphisms[35].

Clinical manifestation and outcomes of the hepatitis D disease differ according to the HDV acquisition modality, itself depending on HBsAg status of the infected individual. In HBV/HDV co-infections, the presence of both viruses causes wide hepatic necrosis, bringing about severe or occasionally fulminant hepatitis[37]. Acute hepatitis can occur either with a single peak of disease (mono-phasic) or with two distinct peaks (biphasic). In the latter case, the first peak matches to an initial HBV spread, and the second peak to HDV propagation[7,37]. The HBV/HDV co-infection usually leads to a self-limited acute hepatitis; indeed, only 2% of patients with co-infection progress to cirrhosis[3,38-41]. The diagnosis of co-infection is confirmed by the simultaneous presence of serological markers for primary HBV and HDV infections[37]. Generally, the first of these to appear are the host's antibody to hepatitis B core protein (anti-HBc IgM), while that to hepatitis D (anti-HD IgM) appears within 2 wk from clinical onset, usually remaining detectable for up to 5-6 wk afterwards. The anti-HD IgG antibody reaches a detectable level after the IgM antibodies disappear, and may persist for months or even years. A failure in anti-HD IgM clearance predicts the chronicity of hepatitis[26,35,42].

Conversely, in HDV super-infection, the virus infects individuals with chronic HBV infection, and it typically leads to HDV persistence with development of cirrhosis[35]. It may present either as an exacerbation of a known chronic hepatitis B status accompanied by hepatic decompensation, or as a new acute hepatitis status in asymptomatic HBsAg carriers[35]. Super-infection usually leads to a chronic infection (in > 90% of cases), with rapid progression to cirrhosis[23,38-40]. Diagnosis of the super-infection is based upon a positive test for anti-HD IgM and a negative test for anti-HBc IgM in HBsAg carriers[35,37]. The super-infection itself is characterized by early presence of HDV-RNA and HDV antigen, followed by increase in anti-HD IgM that remains persistently detectable, in parallel with development of chronicity. In addition, during the acute phase, there is a suppression of HBV replication[35].

CHD

Among chronic viral hepatitis, CHD is distinguished by its severity and higher risk of evolution. It involves a 3-fold increase in the risk of cirrhosis and a 2-fold increase in the risk of death, as compared to HBV and hepatitis C virus infection alone[3,7,35,39,43]. Half of the patients with CHD have experienced a previous acute hepatitis attack, which often represents the time of super-infection with HDV. Clinically, CHD can range from asymptomatic forms, which are discovered incidentally, to symptomatic ones, manifested as fatigue, malaise, and anorexia[3,35]. Diagnosis is achieved upon detection of high titres of anti-HD IgG and IgM in serum, whereas the HD antigen remains persistently detectable in the liver[35]. Typically, alanine aminotransferase (ALT) levels remain stably elevated or fluctuate, reflecting the destruction of hepatic cells. The lowering of such levels is usually meaningful for tracking disease progression to cirrhosis[35,44].

Cirrhosis

The main complication of chronic hepatitis is the development of cirrhosis. Cirrhosis occurs in 70% of cases within 5 to 10 years after hepatitis development, but in 15% of patients it may occur within 1 year to 2 years[7,37]. While the precise mechanism of this progression remains unknown, it has been proposed that the long form of HDAg may stimulate liver fibrosis through interaction with transforming growth factor-β-induced signal activation[35].

Despite liver biopsy remaining the gold standard procedure for diagnosis and staging of cirrhosis, there are validated (and less invasive) predictive scoring systems applicable to patients with HDV disease. The Delta-4 fibrosis score uses gamma-glutamyl transferase (commonly referred to as GGT), ALT, platelet count, and liver stiffness as parameters[45]. A similar score called the "delta Fibrosis Score" considers GGT along with age, albumin and serum cholinesterase[46]. Abbas *et al*[40] proposed to simply use spleen size and platelet count as the predictive parameters of cirrhosis. Other non-invasive markers of fibrosis that are in use clinically include components of the extracellular matrix, namely procollagen III N-peptide, collagen IV, and hyaluronic acid; however, these factors have lower diagnostic accuracy than in other forms of chronic hepatitis[35].

Once HDV cirrhosis is established, it can remain asymptomatic or induce non-specific symptoms, such as muscle weakness and jaundice. In the advanced stage, it can also evolve complications[37]. In half of the cases, these complications occur within 8 years and include portal hypertension, abdominal ascites, gastrointestinal bleeding and hepatic encephalopathy[31,44]. On average, the annual incidence of liver decompensation in cirrhosis ranges from 2.6% to 3.6%, being more than doubled compared to HDV-negative hepatitis B cases[3,44,47]. Among HDV-positive patients, mortality from cirrhosis and hepatocellular carcinoma (HCC) is higher than that for patients with HBV infection alone[23,44,48].

HCC

HCC is a frequent cause of death from cancer worldwide, and its incidence and mortality rates remain on an upward trajectory. The estimated annual incidence of HCC ranges from 2.6% to 2.8%[26,47]. Chronic viral hepatitis, particularly related to hepatitis B, C and D viruses, is responsible for more than 80% of all cases of HCC[43,49]. While both HBV and hepatitis C virus have already been classified by the World Health Organization as oncogenic viruses, the role of HDV infection in HCC development is still under debate[43,48].

Previous studies have shown that the incidence of HCC was similar among patients with HDV and HBV chronic infections, suggesting that HDV is not a contributory factor, *per se*, to the carcinogenic process. Contrariwise, recent lines of evidence from cohort studies have shown a significantly increased risk of HCC among patients with HDV liver disease, which suggests a greater extent of correlation to HDV than to HBV replication[26,43,48,50,51].

Some studies have estimated that between 16.7% and 20% of cirrhosis or HCC cases among people with hepatitis B worldwide are attributable to HDV infection[23]. Nevertheless, the mechanisms of HCC development in HDV-infected patients remain debated[37]. Bockmann *et al*[48] suggest that the high rate of HCC was caused by the rapid progression to liver cirrhosis and not by viral activity at baseline. In addition, experiments in transgenic mice expressing HDAg proteins seem to rule out a direct carcinogenic role of HDV[3]; however, the L-HDAg may promote oxidative stress through activation of signal transducer and transcription-3 and nuclear factor-kappa B, factors vital to cancer cell communication and carcinogenesis. Moreover, it may also promote cancer cell survival through epigenetic mechanisms, such as DNA methylation of the tumour suppressor gene and acetylation of histone H3 of the clustering promoter[40].

TREATMENT

HDV infection with related liver disease is difficult to treat. Drugs for HBV cure are generally not effective on HDV, and the absence of its own polymerase for the latter complicates attempts to identify suitable therapeutic targets. The ever popular IFN-based therapy was introduced about 35 years ago. Pegylated (Peg)-IFN-α is now preferred over the standard formulation, according to its comparatively improved efficacy and safety. This underlies the biochemical and virological responses that are achieved in about 25% of treated patients[47], with response usually being evaluated clinically at 6 mo post-treatment. Guidelines from the European Association for Study of the Liver recommend Peg-IFN for 48 wk (Table 1) in patients with compensated liver disease and nucleos(t)ide analogues for HBV/HDV patients if HBV replication is above 2000 IU/mL[52]. In the last decade, articles published on the HDV have addressed numerous questions related to the role of combination therapy, to the optimal dose and duration of PEG-IFN administration, and to the real end-points of HDV treatment as well as the clinical benefit.

Table 1 Established and undergoing drugs in the management of hepatitis delta virus chronic hepatitis

Molecule	Drug	Mechanism of action	Drug administration	Duration of therapy	Current therapy/clinical trials	Most frequent side effects
Interferon	Pegylated Interferon-α	Immunomodulatory and antiviral activity	Subcutaneously Weekly	Recommended at least 48 wk	Established Therapy; Long-term follow up, after discontinuation of therapy, available	Flu-like symptoms; Headache; Myalgias; Arthralgias; Anemia; Leukopenia; Thrombocytopenia
Myristoylated Lipopeptide	Myrcludex B; Bulevirtide	Interference with HDV viral entry through NTCP	SubcutaneouslyDaily; ± Peg-IFN; ± Tenofovir	At present maximum 48 wk	Ongoing Phase 3 clinical trial	Thromocytopenia; Neutropenia; Lymphopenia; Eosinophilia; Bile Acids elevation
Farnesyl-transferase Inhibitor	Lonafarnib	Inhibition of HDV viral assembly	Orally; Daily; ± Ritonavir; ± Peg- IFN	At present maximum 48 wk	Ongoing Phase 3 clinical trial	Nausea; Diarrhea; Loss of appetite; Weight loss; Abdominal Bloating Increased ALT levels
Phosphorothioate nucleic acid polymer	Rep 2139	Post-entry inhibition of HBsAg secretion; Possible interference with viral entry	Intravenously; Weekly; ± Peg-IFN	At present maximum 30 wk	Phase 2 clinical trial	Thromocytopenia; Neutropenia; Anaemia; Increased ALT levels

HDV: Hepatitis delta virus; NTCP: Sodium tauro-cholate cotransporting polypeptide; Peg-IFN: Pegylated interferon; ALT: Alanine aminotransferase; HBsAg: Hepatitis B surface antigen.

Strategies to improve the efficacy of Peg-IFN-α

In a large randomized controlled study - the Hep-Net-International Delta Hepatitis Intervention Trial (also referred to as the HIDIT-1) - performed by the German Network, 90 patients were assigned to receive weekly treatment with either 180 μg of Peg-IFN-α plus 10 mg of adefovir (31 patients), 180 μg of Peg-IFN-α plus placebo (29 patients), or 10 mg of adefovir alone (30 patients) for 48 wk[53]. Twenty-four weeks after the treatment initiation, a sustained HDV-RNA clearance was observed in 28% of the patients who had been treated with Peg-IFN-α either as mono-therapy or in combination with adefovir; no therapeutic effect was observed in the group treated with adefovir alone. The HIDIT-2 study[54] embodied a second attempt to clarify if combination therapy was superior to Peg-IFN-α alone in treatment of HDV patients, but this time examining tenofovir use. In that trial[54], 120 patients with HDV-RNA positivity were randomized to receive either Peg-IFN-α plus tenofovir or Peg-IFN-α plus placebo for 96 wk. At the end of therapy, viremia was negative in 48% of patients on combination therapy and in 33% of those on the Peg-IFN-α mono-therapy[54]. At week 24 post-therapy, virological response was observed in less than 30% of patients. The combination therapy with tenofovir did not provide benefits in HDV patients with low HBV-DNA levels, not even when 96 wk of the Peg-IFN-α regimen was compared to 48 wk. However, more side-effects were associated to the prolonged therapy, having consequence for the most advanced form of liver disease.

While other studies have also suggested no clear benefit in extending Peg-IFN-α treatment, positive results were obtained from a retrospective study that included 99 patients, with a median treatment duration of 24 mo and post-treatment follow-up of 55 mo[55]. Altogether, however, the previous studies have recognized the efficacy of IFN in about one-third of treated patients[56]. Yet, the optimal treatment duration remains to be defined and the addition of nucleos(t)ide analogues does not seem to improve the result[57,58].

Predictors of long-term response to IFN

Fifty-six percent of patients showing negativity for HDV-RNA (*i.e.* sustained viral response) in the HIDIT-1 trial experienced a late relapse[59]. These data prompted questions regarding the role of the 6-mo post-therapy negative viremia, considering its potential roles as a surrogate marker of HDV cure and sustained viral response[60]. New suggestions arose from a further sub-analysis of the HIDIT-1 trial to investigate positive and negative predictors of response[60]; in particular, the negative level of HDV-RNA at week 24 of treatment or at the end of treatment was found to identify responders with a prediction value of 71% or 100% respectively. Moreover, HBsAg kinetic parameters during treatment were proposed for monitoring response to

therapy, in association to the HDV-RNA. According to an Italian study, a cut-off of HBsAg < 1000 at month 6 of Peg-IFN therapy is able to discriminate responders and partial responders from non-responders[61]. Furthermore, the association of HBsAg reduction (0.105 Log) and HDV-RNA decrease (1.610 Log) from baseline to month 6 was found to be predictive of HBsAg clearance.

Clinical impact of IFN-based therapy

An important end-point of HDV therapy is HDV-RNA clearance, as determined by sensitive assays[62]. Unfortunately, the presence of minimal residual virus that falls below the threshold of detection of an assay could explain reactivation in the presence of HBsAg. The ideal goal of therapy in HBV/HDV co-infection is the clearance of HBsAg; although, this is rarely achieved[63]. In any case, IFN treatment was reported effective in altering the disease progression-associated events[64]. The loss of HDV-RNA during follow-up was a more frequent occurrence among the IFN-α treated patients and it was linked to long-term survival without complications[55,65]. Based on this observation, a durable undetectability of HDV viremia was proposed as a surrogate and more realistic end-point[64].

New therapeutic targets in HDV

Some new promising drugs have advanced in phase II clinical trials. Specifically, these represent inhibitors of the NTCP receptor[66] and farnesyltransferase[67] and the group of nucleic acid polymers[68] (Table 1).

Myrcludex B: Myrcludex B (MyrB, subsequently named bulevirtide) is a myristoylated synthetic peptide, containing 47 amino acids of the preS1 domain of the HBV L-surface protein. It is able to bind to the NTCP, which has been identified as a receptor for HBV/HDV entry (Figure 1)[12]. The concentration of this drug required to inhibit the virus is 100 times lower than that required for bile salt transport inhibition[69].

After a preliminary study[70], Bogomolov *et al*[71] assessed the efficacy and safety of MyrB in a total of 24 patients. The study design assigned 7 patients to receive the 48-wk Peg-IFN-α treatment, 7 patients to receive 2 mg MyrB administered by subcutaneous injection for 24 wk followed by Peg-IFN for 48 wk, and 7 patients to receive MyrB and Peg-IFN for 24 wk plus an additional 24 wk of Peg-IFN. Notably, the HDV-RNA levels decreased by at least one log in the MyrB cohort at week 24 and ALT normalized in 6 patients. While the decline of viremia was observed in all three treatment groups, HBsAg level showed no change in any.

A subsequent phase II trial enrolled 120 patients with CHD, and randomized them into four treatment groups. Three groups were treated with three different doses of MyrB (2 mg, 5 mg and 10 mg) in combination with tenofovir (245 mg/d), while one group was administered tenofovir alone[72]. At the end of treatment, the best result (a -2.7 Log decline of HDV-RNA) was found in patients who received the higher dose of MyrB plus tenofovir. However, for all groups, about two-thirds of the patients who were responders experienced relapse during the follow-up.

A new phase II study was designed to evaluate the efficacy of 2 mg and 5 mg MyrB combination therapy with Peg-IFN-α, and compared to Peg-IFN-α and MyrB mono-therapies[73]. The MyrB and Peg-IFN combination yielded high rates of off-treatment HDV-RNA suppression and reduction in HBsAg. Recently, MyrB was authorized as an orphan medicine by the European Medicine Agency, making it available for usage in patients with HDV compensated liver disease and active viral replication.

Lonafarnib: Lonafarnib (LNF) is a farnesyl-transferase inhibitor, originally developed in cancer treatment. In the HDV setting, the drug inhibits the farnesylation of L-HDAg, which is essential for interaction with HBsAg in the assembly of new viral particles. In a first, short-term controlled study, patients received oral LNF at 100 mg or 200 mg twice daily. While the drug was effective in reducing virus levels, it was mostly associated with gastro-intestinal adverse events and weight loss[74].

Four different subsequent trials were designed and identified as 'LOWR-HDV'. In studies 1 and 2, the LNF was administered at different doses, either alone or in combination with ritonavir or Peg-IFN. Overall, it induced a significant viral decline [75], with a mean log reduction of -5.57 Log^{10} U/mL achieved in the triple combination group[76]. In study 3, patients on nucleos(t)ide analogues were treated with LNF at 50 mg/d, 75 mg/d, 100 mg/d plus ritonavir for 12 wk or 24 wk[77]. The regimen of LNF 50 mg plus ritonavir once daily was superior, when compared to the higher doses of the drug. Finally, the regimen of dose escalation was tested in study 4, the LOWR-HDV-4[78].

Altogether, the findings from these different LOWR-HDV studies supported the antiviral effect of LNF. Moreover, its combination with ritonavir allowed for the use of lower doses and produced more manageable side effects. At present, a phase 3 clinical study (D-LIVR) is ongoing. This is a randomized placebo-controlled trial comparing 50 mg LNF plus ritonavir twice per day with or without Peg-IFN-α in patients maintained on nucleos(t)ide analogues.

Nucleic acid polymers: Nucleic acid polymers are phosphorothioate oligonucleotides with antimicrobial activity, some of which have shown more specific HBV inhibiting properties (*e.g.*, REP 2139). The antiviral effect seems to be related to various mechanisms, including the inhibition of HBsAg release and assembly and the interaction with both S-HDAg and L-HDAg[79,80]. REP 2139 was tested in 12 patients with CHD at a dose of 500 mg administered intravenously once a week for 15 wk, followed by a 250 mg dose combined with subcutaneous injection of 180 μg Peg-IFN for 15 wk and finally Peg-IFN mono-therapy given for 33 wk[81]. Nine patients, who became HDV-RNA-negative during treatment, remained non-viremic at the end of treatment and showed a mean HDV-RNA decline of 5.34 Log^{10} IU/L. REP 2139 induced a remarkable HBsAg decline (3.5 Log^{10} IU/mL) compared to baseline. The most common side effects experienced by patients during treatment were thrombocytopenia, neutropenia and increased ALT levels. Eleven participants were followed for 3.5 years in the REP 301-LTF study[82] and they showed a long-term safety profile as well as persistent virological control and functional cure.

CONCLUSION

HDV remains an important health problem worldwide. Although the infection map is subject to change due to diffusion of the HBV vaccine, some geographic areas are currently a virus reservoir. Moreover, the real burden of infection appears to be underestimated, both in the general and at-risk populations. It is known that HDV-related liver disease is influenced by replicative activity of the virus, which is crucial in inducing cirrhosis and promoting hepatic decompensation. For this reason, the research for new therapeutic strategies has focused on molecules interfering with the replication cycle of HDV.

These novel drugs have demonstrated antiviral efficacy and tolerability, but there remain open questions to be answered and clarified in long-term treatment and follow-up. MyrB is administered *via* subcutaneous injection. Although it induced a decrease in viral loads, it did not reduce the HBsAg level. On the other hand, MyrB showed a good safety profile, even in some patients with compensated cirrhosis. LNF, on the other hand, has the advantage of oral intake. Moreover, when administered in combination with ritonavir, it was the determinant for reduction of gastrointestinal side-effects. For both drugs, viral reactivation and ALT flares were reported at the end of treatment. REP 2139 requires an intravenous administration. It produced a rapid reduction in HBsAg levels, and not only in HDV viremia, in a lasting way; however, this effect needs to be tested on larger numbers of patients.

In conclusion, MyrB, LNF and REP 2139 represent expected and promising therapeutic options for HDV infection. Further studies are needed to define the utility of combination therapy with IFN.

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MINIREVIEWS

Usefulness of artificial intelligence in gastric neoplasms

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Abstract

Recently, studies in many medical fields have reported that image analysis based on artificial intelligence (AI) can be used to analyze structures or features that are difficult to identify with human eyes. To diagnose early gastric cancer, related efforts such as narrow-band imaging technology are on-going. However, diagnosis is often difficult. Therefore, a diagnostic method based on AI for endoscopic imaging was developed and its effectiveness was confirmed in many studies. The gastric cancer diagnostic program based on AI showed relatively high diagnostic accuracy and could differentially diagnose non-neoplastic lesions including benign gastric ulcers and dysplasia. An AI system has also been developed that helps to predict the invasion depth of gastric cancer through endoscopic images and observe the stomach during endoscopy without blind spots. Therefore, if AI is used in the field of endoscopy, it is expected to aid in the diagnosis of gastric neoplasms and determine the application of endoscopic therapy by predicting the invasion depth.

Key Words: Artificial intelligence; Convolutional neural network; Gastric neoplasm; Esophagogastroduodenoscopy; Diagnosis; Invasion depth

Core Tip: Recently, image analysis based on artificial intelligence (AI) has been applied in the field of diagnostic endoscopy in gastroenterology, and active research is also being conducted on gastric neoplasms. Several studies reported that AI-based early gastric cancer diagnosis and the prediction of invasion depth showed excellent performance and that the differential diagnosis from non-neoplastic lesions including benign gastric ulcers was possible. Therefore, if AI is used in clinical practice, it can be expected to help diagnose gastric neoplasms and determine treatment methods.

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Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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INTRODUCTION

Gastric cancer is the fifth most common malignant neoplasm in the world and the third most common cause of cancer-related death[1,2]. Although advanced gastric cancer (AGC) is associated with poor outcomes, the detection of early gastric cancer (EGC) can improve survival up to 90%[1,3]. Endoscopy is the most important tool for detecting and diagnosing gastric cancer. However, the accuracy of detection relies upon the expertise and experience of the endoscopist and complex factors of the gastrointestinal (GI) tract. Accordingly, endoscopy techniques and related fields such as image-enhanced endoscopy have been developed to improve the diagnosis of EGC. Since its introduction in the 1950s, artificial intelligence (AI) such as deep learning (DL) has experienced remarkable progress in the last decade, and many researchers have studied the application of AI not only in the field of medical imaging but also in predicting patient prognosis based on medical records[4,5]. Many studies have utilized AI in endoscopic diagnosis. The application of AI in colonoscopy has significantly improved the adenoma detection rate (29.1% *vs* 20.3%, $P < 0.001$), and can even differentiate whether a detected polyp is non-neoplastic or neoplastic[6,7]. Based on such advancements, companies have already adapted AI for use in colonoscopy. Medtronic developed the GI Genius™ Intelligent Endoscopy Module that utilizes AI for the detection of colon polyps in real-time colonoscopy, while Olympus developed the EndoBRAIN-EYE[8]. In addition, Pentax and Fuji released the PENTAX medical Discovery™ and computer-assisted diagnosis (CAD)-EYE, respectively. Many studies have also been conducted in the field of AI in esophagogastroduodenoscopy (EGD). Thus, this article aimed to review recent developments and the use of AI in gastric neoplasms focusing on EGC, which has its unique characteristics among various GI diseases.

AI TECHNOLOGY

AI refers to machines that can do complex tasks like humans by imitating the cognitive functions of human intelligence such as learning and problem-solving (Figure 1). It was first introduced in 1955 and has been rapidly integrated into modern technologies and medicine[9]. Five subfields are included in AI, machine learning (ML), artificial neural network (ANN), natural language processing, DL, and computer vision[10]. ML is a field of AI where large amounts of data and algorithms are incorporated into the machine, and the machine automatically learns the input data by analyzing its patterns. Although the machine is capable of learning data patterns, the process still requires a certain amount of human instruction. DL is an important technique among many methods of ML, which is a process where the machine collects, analyzes, and processes data without receiving human instructions. Using massive amounts of data, the machine creates a learning model by extracting the key features of the given data. ANNs are the core technology of DL, and just as the human brain structure is formed by groups of neurons, the learning model of ML connects several computational nodes into several layers composed of an input layer, an output layer, and one or more hidden layers between them (Figure 2). The simplest type of neural network is called a perceptron, which consists of one input layer and one output node. The weight is a concept that gives a certain amount of importance to each input. The perceptron creates an output using inputs and weights. When an input is received, a weighted sum is calculated according to the weight, and when the value satisfies a specific criterion (activation function), the result is returned as 1 or 0. Convolutional neural network (CNN) is a kind of ANN, an algorithm that automatically learns features from the data, used mainly for image recognition[11]. It is an advanced ML model designed to think similarly to the human brain using large image datasets to learn patterns in correlating images. CNN is typically composed of three types of layers that extract features of the image and those that classify the data[12]. The convolution and pooling layers extract features of the image, while the fully connected layer is responsible for

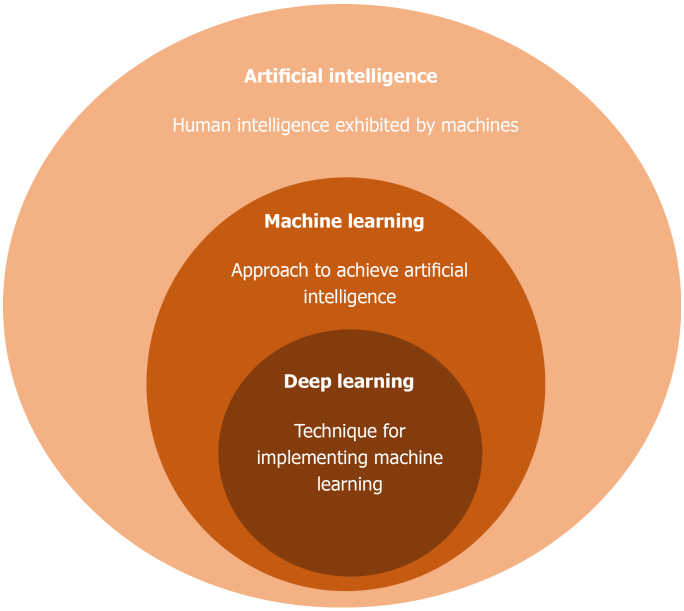

Figure 1 Overview of artificial intelligence, machine learning, and deep learning. Artificial intelligence refers to machines that can do complex tasks like humans by imitating human intelligence. One of the most important ways to achieve artificial intelligence is machine learning. Machine can learn by itself from the data provided to make accurate decisions. Deep learning is an important technique among many methods of machine learning. It is a kind of artificial neural network and learns data through an information input/output layer similar to neurons in the brain.

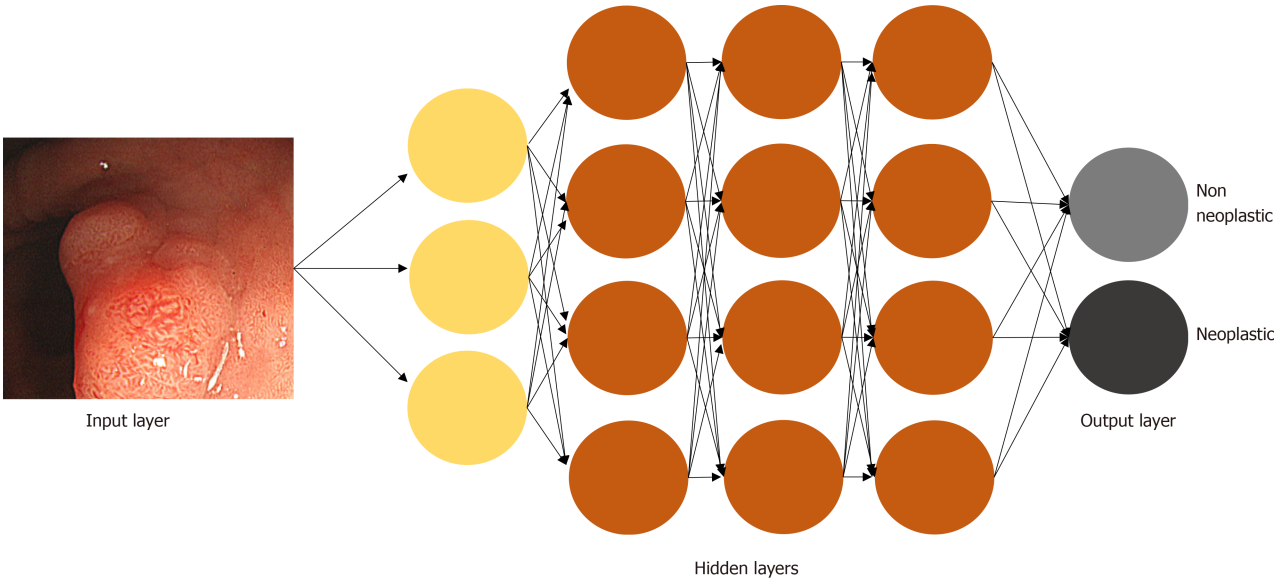

Figure 2 Illustrative model of artificial neural network. Once endoscopic image is selected as input layer, hidden layers are connected to next layer. Through this network, the input image is classified into output layer.

mapping them into output. The convolutional layer is a key in CNN, typically composed of a filter and an activation function. Using the image as input data, the filter extracts features of the image, and the activation function converts the value to a non-linear value. The CNN has multiple network layers of consecutive convolutional layers after pooling layers, and many filters are used as the input image is processed into consecutive convolutional layers. The extracted features are accumulated and become more complex to determine the characteristics of the input image. Subsequently, classification is performed through the fully connected layers, which are the last layers of CNN (Figure 3). As terms appearing in CNNs, one epoch refers to one forward and backward passes of the entire dataset to update the weight. The batch size is the number of training examples processed at one forward and backward pass, and iteration refers to the number of batches to complete one epoch.

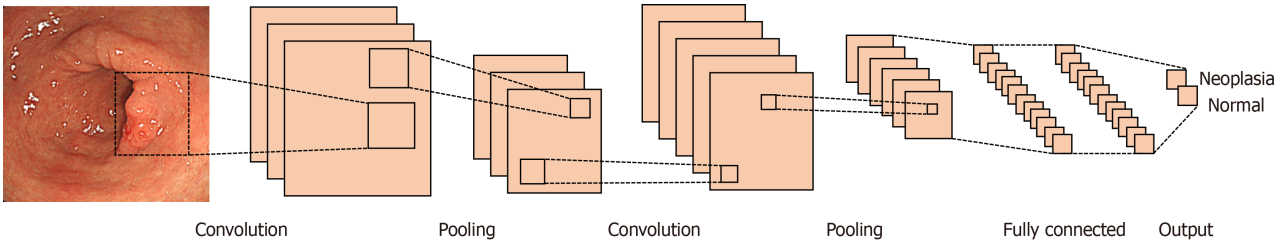

Figure 3 Overview of convolutional neural network. It is composed of stacks of convolutional layers, pooling layers, and fully connected layers. Convolutional and pooling layer extract features of input images, while fully connected layers make output based on classification.

Most examinations and diagnosis of GI tract diseases are performed through endoscopy and endoscopic imaging is one of the most effective applications of AI-based analytics in the field of medicine[9]. The use of CNN is ideal for endoscopic image recognition to detect and localize GI neoplasms. An AI algorithm learns what a neoplasm looks like in an endoscopic image using an image labeled by an endoscopist. After training, the CNN is tested on non-labeled new images to which it has not been previously exposed to and it is validated that the model can correctly identify previously unseen neoplasms. As a result, the algorithm can identify what it believes is a neoplasm in a real-time endoscopic video feed.

AI IN GASTRIC NEOPLASMS

Detection of gastric neoplasms

The detection of early-stage stomach cancer and precancerous lesions is essential to improving survival. Endoscopy is the most important and widely used detection tool for gastric cancer screening but since it is a manual procedure performed by an endoscopist, it is prone to technical and cognitive errors depending upon the endoscopist. EGC lesions usually show subtle changes of mucosa, such as elevation, depression, and redness. Moreover, they are surrounded by chronic inflammation or intestinal metaplasia. Therefore, there is a possibility of missing the subtle changes seen in the early forms of gastric cancer, especially in countries where the incidence of gastric cancer is low and where training is limited. Previous studies reported false-negative rates for detecting gastric cancer ranging between 4.6% and 25.8%[13-17]. A method to improve diagnostic accuracy involves the use of image-enhanced endoscopy such as narrow-band imaging (NBI) and blue laser imaging, which are more effective than conventional white light imaging alone[18,19]. However, such an optical diagnosis requires substantial expertise and experience[20], hindering its general use in gastroscopy. The 5-year survival rate of gastric cancer patients is highly correlated with the stage of gastric cancer at the time of diagnosis. Thus, it is paramount to improve the detection rates of EGC. Many groups have already started integrating AI into their routine practice to improve the overall detection rates of gastric cancer. AI-assisted evaluation can provide a better objective approach to improving diagnostic accuracy and avoiding unnecessary biopsies[10]. A list of studies using AI in gastric neoplasms is summarized in Table 1.

To evaluate the diagnostic accuracy of AI in the detection of gastric cancer, Hirasawa *et al*[21] used a CNN-based algorithm called the Single Shot MultiBox Detector to train using 13584 endoscopic images of gastric cancer, then tested using 2296 images (714 with confirmed gastric cancer) from 69 patients[21]. The overall sensitivity was 92.2% in the detection of gastric cancer, and the process took 47 s to analyze 2296 test images. The CNN accurately detected all invasive gastric cancer images. The detection rate for lesions larger than 6 mm was 98.6% while invasive cancers were all identified by AI. However, in the case of minute cancers that are less than 5 mm, 1 out of 6 (16.7 %) was detected, and 161 non-neoplastic lesions were included in the total 232 lesions that were machine-identified as gastric cancer, which produced a lower positive predictive value (PPV) of 30.6%[21]. The most common cause of false-positive lesions was gastritis with a change in color tone or irregular mucosal surface, which are sometimes difficult to distinguish even by endoscopists, and the next most common cause was normal structures such as cardia, pylorus, and angle. Ishioka *et al*[22] applied the same algorithm to video images collected from 62 patients who underwent endoscopic submucosal dissection (ESD) for EGC[22]. When

Table 1 Recently published articles on application of artificial intelligence in gastric neoplasms

Ref.	Purpose	AI type	Endoscopy type	Subjects	Outcomes
Detection of gastric neoplasms					
Hirasawa *et al* [21], 2018	Detect EGC	CNN (SSD)	Conventional endoscopy	Training: 13584 images; Test: 2296 images from 69 patients.	Sensitivity 92.2%, PPV 30.6%
Ishioka *et al*[22], 2019	Real time detection of EGC	CNN (SSD)	Conventional endoscopy	Live video of 62 patients	Accuracy 94.1%, median time 1 s (range: 0-44 s)
Sakai *et al*[23], 2018	Detect EGC	CNN	Conventional endoscopy	Training: 348943 images; Test: 9650 images	Accuracy 82.8%
Kanesaka *et al* [24], 2018	Detect EGC	SVM	M-NBI	Training: 126 images; Test: 81 images	Accuracy 96.3%, sensitivity 96.7%, specificity 95%
Li *et al*[25], 2020	Detect EGC	CNN (Inception-v3)	M-NBI	Training: 2088 images; Test: 341 images	Accuracy 91.2%, sensitivity 90.6%, specificity 90.9%
Horiuchi *et al* [26], 2020	Classifying EGC from gastritis	CNN (GoogLeNet)	M-NBI	Training: 2570 images; Test: 258 images.	Accuracy 85.3%, sensitivity 95.4%, specificity 71.0%, test speed 51.83 images/s (0.02 s/image)
Horiuchi *et al* [27], 2020	Detect EGC	CNN (GoogLeNet)	M-NBI	174 videos	Accuracy 85.1%, AUC 0.8684, sensitivity 87.4%, specificity 82.8%, PPV 83.5%, NPV 86.7%
Luo *et al*[28], 2019	Real time detection of EGC	GRAIDS	Conventional endoscopy	1036496 images from 84424 patients	Sensitivity (0.942) similar to the expert (0.945), superior to the competent (0.858) and the trainee (0.722) endoscopist
Ikenoyama *et al* [29], 2021	Detect EGC	CNN (SSD)	WLI, NBI chromoendoscopy	Training: 13584 images; Test: 2940 images.	Sensitivity 58.4%, specificity 87.3%, PPV 26.0%, NPV 96.5%
Classification of gastric neoplasms					
Sun *et al*[30], 2018	Classify ulcers	DCNN	Conventional endoscopy	854 images	Accuracy 86.6%, sensitivity 90.8%, specificity 83.5%
Lee *et al*[31], 2019	Detect EGC and benign ulcer	CNN (ResNet50, Inception-v3, VGG16)	Conventional endoscopy	Training: 717 images; Test: 70 images	AUC 0.95, 0.97, and 0.85 in Inception, ResNet50, and VGG16
Cho *et al*[32], 2019	Detect AGC, EGC, dysplasia	CNN (Inception-v4, ResNet152, Inception-Resnet-v2)	Conventional endoscopy	5217 images from 1469 patients	Gastric cancer: accuracy 81.9%, AUC 0.877; Gastric neoplasm: accuracy 85.5%, AUC 0.927
Kim *et al*[33], 2020	Classify gastric mesenchymal tumors	CNN	Endoscopic ultrasonography	Training: 905 images; Test: 212 images.	Accuracy 79.2%, sensitivity 83.0%. specificity 75.5%
Prediction of invasion depth					
Kubota *et al*[39], 2012	Predict invasion depth	Back propagation	Conventional endoscopy	Training: 800 images; Test: 90 images	Accuracy 77.9%, 29.1%, 51.0% and 55.3% in T1, T2, T3, and T4 stage; Accuracy 68.9% and 63.6% in T1a and T1b stage
Zhu *et al*[40], 2019	Predict invasion depth	CNN (ResNet50)	Conventional endoscopy	Training: 790 images; Test: 203 images	AUC 0.94, overall accuracy 89.2%, sensitivity 76.5%, specificity 95.6%
Yoon *et al*[41], 2019	Detect cancer, and predict invasion depth	CNN (VGG16, Grad-CAM)	Conventional endoscopy	11539 images	Detection AUC 0.981, depth prediction AUC 0.851 (undifferentiated type histology with a lower accuracy)
Cho *et al*[43], 2020	Predict invasion depth	CNN (Inception-ResNet-v2, DenseNet-161)	Conventional endoscopy	Training: 2899 images, test: 206 images	Internal validation: accuracy 84.1%, AUC 0.887; External validation: accuracy 77.3%, AUC 0.887
Nagao *et al*[44], 2020	Predict invasion depth	CNN (ResNet50)	WLI, NBI, indigo-carmine	16557 images from 1084 cases of gastric cancer	WLI: AUC 0.9590, sensitivity 89.2%, specificity 98.7%, accuracy 94.4%, PPV 98.3%, NPV 91.7%; NBI: AUC

					0.9048; Indigo-carmine: AUC 0.9191
Blind-spot monitoring					
Wu *et al*[48], 2019	Detect blind spot	DCNN	Conventional endoscopy	34513 images	Accuracy of detecting blind spot: 90.0%; Blind spot rate: 5.9%
Wu *et al*[49], 2019	Detect EGC and blind spot	DCNN	Conventional endoscopy	24549 images	Accuracy 92.5%, sensitivity 94.0%, specificity 91.0%, PPV 91.3%, NPV 93.8%
Chen *et al*[50], 2020	Detect blind spot	DCNN	Conventional endoscopy, U-TOE	Live video of 437 patients	Blind spot rate with AI: Sedated C-EGD, 3.4%; unsedated U-TOE, 21.8%; unsedated C- EGD, 31.2%

AI: Artificial intelligence; EGC: Early gastric cancer; CNN: Convolutional neural network; SSD: Single Shot MultiBox Detector; PPV: Positive predict value; SVM: Support vector machine; M-NBI: Magnified narrow-band imaging; AUC: Area under curve; GRAIDS: Gastrointestinal Artificial Intelligence Diagnostic System; WLI: White light imaging; NPV: Negative predict value; DCNN: Deep convolutional neural network; VGG: Visual Geometry Group; AGC: Advanced gastric cancer; Grad-CAM: Gradient-weighted class activation mapping; U-TOE: ultrathin transoral endoscopy; C-EGD: conventional esophagogastroduodenoscopy.

applied to the live video images, the diagnostic accuracy was 94.1%, and the median time for lesion detection was one second. Although the accuracy was low in minute cancers, AI showed great performance in lesions larger than 6 mm which looked very promising. In another study, Sakai *et al*[23] trained a CNN-based system with 348943 images (with data augmentation) obtained from 58 patients and tested 9650 images [23]. The accuracy of detecting gastric cancer by AI was 82.8%, and the image processing time was 4 ms *per* image.

Gastric cancer has many visual features that are challenging for endoscopists to describe. To improve diagnostic accuracy during endoscopy, several techniques have been developed to assist the gastroenterologist. Magnified NBI (M-NBI) has been shown to have higher detection rates for EGC, however, many endoscopists are not trained to confidently use M-NBI. To facilitate detection using M-NBI, Kanesaka *et al* [24] developed a CAD system to help diagnose EGC using only M-NBI images[24]. They used support vector machine to train with 66 EGC images and 60 non-cancer images, then tested detection and delineation of gastric cancer with 61 EGC and 20 non-cancer images. They reported an accuracy of 96.3%, a PPV of 98.3%, a sensitivity of 96.7%, and a specificity of 95%. Their CAD processed each image in 0.41 s[24]. In a related study, Li *et al*[25] used 386 non-cancerous M-NBI images and 1702 M-NBI images of EGC to train the Inception-v3 CNN model and tested 341 endoscopic images [25]. The sensitivity, specificity, and accuracy for the detection of EGC were 91.2%, 90.6%, and 90.9% respectively[25]. In another study by Horiuchi *et al*[26], the 22-layer GoogLeNet CNN model was trained using 1492 M-NBI images of EGC and 1078 M-NBI images of gastritis, then tested on 258 images (151 images of EGC)[26]. Further, the authors tried to determine if the differentiation between gastritis and cancer was possible. The reported accuracy for the detection of cancer was 85.3%. The sensitivity was 95.4%, the specificity was 71.0%, the PPV was 82.3%, and the negative predictive value (NPV) was 91.7%. The CNN falsely diagnosed 31 gastritis images as cancers, which were reported to have localized atrophy, atrophy of the fundic gland, and intestinal metaplasia[26]. The diagnostic performance of the same model was evaluated using 174 endoscopic videos (87 cancers and 87 non-cancers)[27]. The area under the curve (AUC) was 0.8684 and the accuracy, sensitivity, specificity, PPV, and NPV were 85.1%, 87.4%, 82.8%, 83.5%, and 86.7%, respectively. When compared to 11 experts, CAD was significantly more accurate than two experts, and not significantly different from eight experts[27].

As other studies were single-center results, or limited in the number of included cases, Luo *et al*[28] conducted a multi-center, case-controlled study of real-world endoscopic imaging to evaluate the accurate diagnosis of upper GI cancer with a CNN [28]. Using 157207 images obtained from 18765 participants from one university cancer center, the authors developed and validated the Gastrointestinal AI Diagnostic System (GRAIDS) algorithm through training, intrinsic verification, and internal validation. Then, they tested the performance of GRAIDS using a prospective validation dataset and additional external validation datasets obtained from five other hospitals, which included 879289 images from 65659 participants. The AUC in the external validation of the five participating hospitals ranged from 0.966 (95%CI: 0.965-0.967) to 0.990 (95%CI: 0.990-0.991)[28]. When compared to the diagnostic accuracy of the endoscopists, the diagnostic accuracy of the GRAIDS was 0.928 (95%CI: 0.919-0.937), which was

significantly lower than the diagnostic accuracy of 0.967 (95%CI: 0.961-0.973; $P < 0.0001$) of the expert endoscopist (professor with more than 10 years of endoscopic experience) and 0.956 (95%CI: 0.949-0.963; $P < 0.0001$) of the competent endoscopist (attending doctor with more than five years of endoscopic experience), but significantly higher than the diagnostic accuracy of 0.886 (95%CI: 0.875-0.897; $P < 0.0001$) of the trainee endoscopist (resident with two years of endoscopic experience). The sensitivity of the GRAIDS was not significantly different from the expert [0.942 (95%CI: 0.924-0.957) *vs* 0.945 (95%CI: 0.927-0.959); $P = 0.692$]. When compared to the competent expert [0.858 (95%CI: 0.832-0.880), $P < 0.0001$] and the trainee endoscopist [0.722 (95%CI: 0.691-0.752), $P < 0.0001$], the sensitivity of the GRAIDS was confirmed to be superior. The PPV of the GRAIDS was 0.814 (95%CI: 0.788-0.838), the expert endoscopist was 0.932 (95%CI: 0.913-0.948), the competent endoscopist was 0.974 (95%CI: 0.960-0.984), and the trainee endoscopist was 0.824 (95%CI: 0.795-0.850). The PPV of the GRAIDS was lower than that of the expert and the competent endoscopist but was similar to that of the trainee. These problems are mainly because the GRAIDS misinterprets normal structures (the pylorus, angle, mucus, gastric wall elevation during peristalsis, *etc.*) as lesions, and validation was conducted with data that had a low prevalence (3.8%-9.5%) in upper GI cancer. However, it seems that normal structures can be easily distinguished by the endoscopist and confirmed as false positives. This was a notable study that used more than one million images obtained from more than 80000 patients from different centers in China. A study by Ikenoyama *et al*[29] also compared the diagnostic accuracy of AI to that of endoscopists[29]. The AI model from the previous study by Hirasawa *et al*[21]. was tested on images obtained from 75 patients with gastric cancer [66 with mucosal cancer (T1a), and nine with submucosal cancer], and the diagnostic accuracy was compared to that of 67 endoscopists (33 board-certified endoscopists with more than 18 years of experience, and 34 uncertified endoscopists with about eight years of experience). The sensitivity, specificity, PPV, and NPV of the CNN were 58.4%, 87.3%, 26.0%, and 96.5% respectively. Compared to the CNN, the endoscopists showed a sensitivity, specificity, PPV, and NPV of 31.9%, 97.2%, 46.2%, and 94.9%, respectively, which showed that the CNN had significantly higher sensitivity than the endoscopists. Also, the average time it took for the CNN to evaluate an image was 45.5 ± 1.8 s, which was much faster than the 173.0 ± 66.0 min taken by an endoscopist and suggested that AI accurately diagnosed EGC at a much higher speed[29].

Classification of gastric neoplasms

While many studies have tested the diagnostic accuracy of AI in differentiating cancerous lesions from normal mucosa, several attempts at classifying other non-cancerous lesions have been made (Table 1). Sun *et al*[30] created a network-based model that could classify ulcers into different types (benign ulcers or malignant ulcers) with a performance comparable to that of endoscopists[30]. The study reported that the DL model was able to identify and classify ulcers with a total accuracy of 86.6%, which was comparable to that of the endoscopist with the highest accuracy (86.3%) and higher than that of the endoscopist with the lowest accuracy (62.5%)[30]. Lee *et al*[31] developed a model that could distinguish gastric ulcers and malignancy[31]. Using the Inception-v3 network, ResNet50 and the Visual Geometry Group (VGG) Net to classify normal *vs* cancer, normal *vs* benign ulcers, and cancer *vs* benign ulcers, 180 normal images, 200 ulcer images, and 337 cancer images were used for training. When tested on 20 normal, 20 ulcers, and 30 cancer images, the best performance was observed in ResNet50, with a diagnostic accuracy of 0.9649 for differentiating between normal *vs* cancer, 0.9262 for differentiating between normal *vs* ulcers, and 0.7712 for differentiating between cancer *vs* ulcers. Based on such findings, AI was proposed as an efficient means for the classification of endoscopic images[23]. Cho *et al*[32] made a novel attempt at developing a DL model that could automatically classify gastric neoplasms using conventional endoscopic images[32]. Using 5017 images from 1269 participants, three CNN architectures (Inception-v4, ResNet-152, and Inception-ResNet-v2) were used to train and validate the classification of conventional endoscopic images. The images were classified into two categories from two perspectives, which were cancer *vs* non-cancer, and neoplasm *vs* non-neoplasm. All images were grouped into five categories, AGC, EGC, high-grade dysplasia (HGD), low-grade dysplasia (LGD), and non-neoplasm. To compare the diagnostic accuracy, six endoscopists with experience with more than 6000 endoscopies also viewed and classified the endoscopic images. The Inception-ResNet-v2 model was reported to have the best performance at classifying the images into the five categories, with an accuracy of 84.6% (95%CI: 83.69-85.5) and a mean classification time of 0.0264 s. The AUC was highest for the detection of AGC (range: 0.802-0.855) and the lowest for

HGD (range: 0.491-0.522). In prospective validation, the performance of Inception-ResNet-v2 was not significantly inferior to that of the endoscopist with the worst performance. However, the endoscopist with the highest performance showed significantly better performance with a diagnostic accuracy of 87.6% (95%CI: 84.3-90.9) compared to 76.4% (95%CI: 72.1-80.7) for Inception-ResNet-v2. This suggested that AI could have the potential for classifying endoscopic lesions into several categories[32]. A recent study by Kim *et al*[33] assessed the ability of the CNN model to classify gastric mesenchymal tumors using endoscopic ultrasonography (EUS) images[33]. Using 905 EUS images from gastric mesenchymal tumors that were histologically confirmed by either resection or EUS-guided fine-needle biopsy, the CNN-CAD system was developed and validation was performed with 212 EUS images. The reported accuracy for detecting gastrointestinal stromal tumors was 79.2% using the CNN-CAD system, with a sensitivity and specificity of 83.0% and 75.5%, respectively. The performance was compared to that of six endoscopists (three experienced endoscopists who performed more than 500 EUS examinations, and three junior endoscopists who performed less than 200 EUS examinations). When compared to the diagnostic accuracy of the endoscopists, the sensitivity of CNN-CAD system was not significantly different from that of any of the endoscopists. The specificity and diagnostic accuracy of CNN-CAD system were significantly higher than that of two experienced endoscopists and one junior endoscopist[33], which suggested the potential application of AI in the classification of EUS images as well.

Prediction of invasion depth

The prediction of the invasion depth of gastric cancer (T-staging) is very important as it is an essential factor in determining the treatment method and prognosis of EGC. Tumors in the early stages that do not involve lymphovascular invasion and have an invasion depth no deeper than 500 μm of submucosa can be treated by endoscopic resection alone[34]. The gross findings of the tumor seen on endoscopy or EUS are used to determine the invasion depth of EGC. Some studies have reported that conventional endoscopy was comparable to EUS in predicting the invasion depth of EGC[35,36]. The reported overall accuracy of invasion depth using conventional endoscopy ranged between 69% and 79%[35,37]. In a study of depth prediction scores for differentiated EGCs, tumor sizes more than 30 mm, marked redness, an uneven surface, and marginal elevation were associated with deeper submucosal cancers[38]. However, gastric cancer depth can be difficult to determine by endoscopy alone and some patients may undergo surgery when endoscopic resection could have been an effective method of treatment. To overcome such problems, the utilization of AI to determine the depth of invasion has been studied (Table 1). Kubota *et al*[39] used retrospectively collected 902 conventional endoscopic gastric cancer images from 344 patients who underwent surgery or endoscopic resection to train and validate with a backpropagation algorithm for determining the depth of invasion[39]. The overall accuracy for detecting the depth of invasion was 64.7%, with 77.2% at the T1 stage (68.9% for T1a and 63.6% for T1b), 49.1% at the T2 stage, 51.0% at the T3 stage, and 55.3% at the T4 stage. This computer-aided system suggested a novel approach of using AI to determine cancer invasion depth by endoscopy[39]. Zhu *et al*[40] used 790 images from gastric cancer patients to train and another 203 images to validate ResNet50. The overall accuracy was reported to be 89.2%, which was significantly higher than the overall accuracy of 77.5% of the experienced endoscopists. The AUC for AI was 0.94 (95%CI: 0.90-0.97), and the sensitivity, specificity, PPV, and NPV were 76.5%, 95.6%, 89.7%, and 89.0%, respectively[40]. To test AI in the diagnostic accuracy for EGC stages, Yoon *et al*[41,42] included 800 patients, 428 patients with T1a and 372 patients with T1b histology-proven EGC, and selected 11539 images (896 T1a images, 809 T1b-EGC images, and 9834 non-cancer images) to train and validate the lesion-based VGG16-network and gradient-weighted class activation mapping (Grad-CAM) [41,42]. The overall AUC for EGC detection and invasion depth prediction was 0.981 and 0.851, respectively. Interestingly, the study also analyzed the factors affecting the AI prediction of invasion depth. The images of undifferentiated-type histology were associated with inaccurate predictions of invasion depth, especially in T1b cases[41]. As previous studies used already diagnosed gastric cancer images for training and testing, Cho *et al*[43] used Inception-ResNet-v2 and DenseNet-161 models to test the diagnostic accuracy of gastric neoplasms and invasion depth[43]. The authors used 2899 conventional endoscopic images obtained from 846 patients with confirmed pathology including LGD, HGD, EGC, and AGC. The AUC and diagnostic accuracy for determining the invasion depth were 0.887 and 77.3%, respectively, in the external validation set for the DenseNet-161 model. When applied to clinical simulation, the AI misdiagnosed only two cases that had submucosa invasion (misdiagnosed as mucosal

lesions), which were also misdiagnosed by the endoscopists. In 89 patients who underwent surgery, 11 cases were actually mucosal-confined lesions, among which AI correctly classified six cases as mucosal lesions. The authors developed an algorithm with substantial performance in predicting invasion depth from the endoscopic images of neoplasms[43]. As other studies used images obtained from conventional endoscopy, Nagao *et al*[44] retrospectively collected 16557 gastric cancer images from 1084 cases to train and validate ResNet50 for predicting invasion depth by conventional white light, non-magnifying NBI, and indigo-carmine stained images[44]. The AUC using white light imaging, NBI, and indigo-carmine stain imaging were reported to be 0.9590, 0.9048, and 0.9481 respectively, and the lesion-based accuracy for predicting invasion depth using white light imaging, NBI, and indigo-carmine were 94.5%, 94.3%, and 95.5%, respectively[44].

Blind-spot monitoring

Observing the whole stomach is a basic prerequisite for the diagnosis of gastric cancer at an early stage. To avoid blind spots, standardized procedures and guidelines have been made to map the entire stomach during gastroscopy. The European Society of Gastrointestinal Endoscopy published a protocol including 10 images of the stomach while the systematic screening protocol for the stomach published by Japanese researchers suggested 22 standard images of the stomach to avoid missing suspicious cancerous lesions[45,46]. However, insufficient supervision and the lack of practical tools make it difficult to follow protocols, which is related to the quality of endoscopic examinations[47]. To localize blind spots during EGD that may have been missed by an endoscopist, Wu *et al*[48] developed the WISENSE system, a real-time CNN to detect blind spots (Table 1)[48]. As the scope was inserted into the stomach, the deep CNN (DCNN) captured images and filled them into the corresponding part of the model, which enabled the endoscopist to identify the blind spots. These blind spots of the gastric mucosa, such as the lesser curvature of the antrum and the fundus, are areas that may hide lesions. If blind spots are not viewed during endoscopy, lesions could be missed. Trained on 34513 images of gastric locations agreed upon by at least four endoscopists, WISENSE was able to detect blind spots with an accuracy of 90.0% by identifying anatomic landmarks in EGD. In a single-center randomized control trial, 153 patients had their blind spots detected by WISENSE *vs* 150 in the control group without AI. The blind spot rate, defined as the proportion of the number of unobserved sites in 26 sites, was 5.9% in the WISENSE group which was significantly less than 22.5% in the control group ($P < 0.001$), suggesting that AI can also be used to improve the quality of EGD by identifying blind spots[48]. In another study by Wu *et al*[49], a DCNN was used for detecting gastric cancer and identifying blind spots. There were 24549 images used for training, and a grid model for the stomach was developed to generate a virtual stomach model[49]. The study reported a diagnostic accuracy of 92.5% for detecting malignancy, which was significantly higher than that of six experts. The reported sensitivity, specificity, PPV, and NPV were 94.0%, 91.0%, 91.3%, and 93.8%, respectively. The DCNN correctly identified the EGD images into 10 parts with an accuracy of 90.0% and into 26 parts with an accuracy of 65.9%, which was not significantly different from those of the endoscopists. When the model was tested on endoscopic videos, the DCNN accurately presented the covered parts synchronized with the process of EGD to verify that the entire stomach was mapped [49]. A related study by Chen *et al*[50] used ENDOANGEL (developed from WISENSE) to compare blind-spot monitoring in sedated conventional EGD (C-EGD), unsedated ultrathin transoral endoscopy (U-TOE), and unsedated C-EGD[50]. This prospective, 3-parallel-group, randomized study reported that the blind-spot rate with AI was significantly lower in sedated C-EGD compared with unsedated U-TOE and unsedated C-EGD (sedated C-EGD *vs* unsedated U-TOE *vs* unsedated CEGD: 3.4% *vs* 21.8% *vs* 31.2%, $P < 0.05$). Although the number of studies is limited, the application of AI in monitoring blind spots is very promising.

Prediction of curative endoscopic resection

Expanded indications for ESD in EGC include the undifferentiated type that is less than 2 cm and does not have ulcerations. However, observational studies have reported conflicting results. Thus, ESD in such groups has been considered an investigational treatment[34]. A meta-analysis of curative resection for EGC with undifferentiated type histology reported a rate of 61.4%, suggesting ESD as a feasible treatment for undifferentiated-type EGC[51]. To aid in the accurate prediction of curative resection in such cases, Bang *et al*[52] selected ML models that could predict curative resection in undifferentiated-type EGC. The XGBoost classifier presented the best performance with an accuracy of 81.5% in the first external validation and 89.8% in the

second external validation[52]. The size of the lesion was the most important feature that could be explained by AI analysis. As such, AI could aid in decisions for therapeutic management.

FUTURE PERSPECTIVES OF AI

The real-time application of AI in the field of medicine is within reach. Endoscopic models that automatically detect colon polyps or gastric cancers during endoscopy sessions and highlight them using segmentation box have already received approval for use in Europe, Japan, and other countries, while many systems are currently under development[8]. Many software codes have been provided as open-source codes, which can be freely utilized in research or actual practice. Architectures can be modified by fine-tuning an already established pre-trained model by adjusting layers of the ANN, increasing the learning epoch, adjusting the batch size, adjusting the iteration, or modifying hyperparameters such as the optimizer. Aside from adjusting the complex algorithms to optimize the model, recent developments have enabled the automatic optimization of hyperparameters in ML (*i.e.*, AutoGluon) that makes AI more user-friendly and easier to use for clinicians unfamiliar with AI[52]. Most research on AI for gastroenterology has focused on developing algorithms for the detection of lesions, the classification of images to improve diagnostic accuracy, predicting prognosis, and to improve the quality of screening endoscopy. In the near future, AI will most likely be applied to therapeutic management. Recently, AI-based treatment methods have been developed using technologies such as microendoscopy, decision support system-based treatment modalities, robot-assisted treatment, application, and digital therapeutics[53]. However, such development comes with social issues other than technology, such as patient safety, ethics, legal responsibility, government approval, and cost-effectiveness, which need to be addressed as well. Although studies have shown that accuracy of detecting gastric cancer by AI is comparable to some doctors, experienced doctors with expertise have shown better performance than AI. This means that there is limitation to relying solely on AI alone. However, beneficial factors from application of AI, such as improved efficacy and time spent on repetitive task, must be acknowledged as well. Accordingly, the most applicable field of AI would be medical image data processing that could aid in improved diagnostic performance of trainees and non-expert doctors. The AI algorithm, especially DL, is comparable to a black box that learns from training data. Using the patterns learned from the training data, the output values can be predicted from newly input data. This means that efficacy and accuracy are highly dependent upon the quality and quantity of the training data. Like any other clinical research, the quality and quantity of the usable data are undeniably essential in proving the quality of the evidence and the outcome. It is important to gather high-quality clinical data, while developing a model that accurately tests the data is equally important. To effectively utilize such an AI algorithm in clinical practice, further studies and discussions on the usefulness, profitability, possible risks, medicolegal responsibility, and regulatory measures of AI are needed.

CONCLUSION

AI in the field of endoscopy was first applied for the detection of colon polyps. As described in this review article, many studies have already been published as stepping-stones toward the application of AI in detecting gastric neoplasms such as EGC. As there is a lack of such prospective studies in the detection of EGC, randomized controlled studies are needed to advance the technique. It is expected that the application of AI would not only provide guidelines for the endoscopic treatment of EGC or avoid unnecessary surgery by predicting the invasion depth but also help improve the overall prognosis of patients with EGC. There is no doubt that the development of AI-based endoscopy would also help to alleviate physical fatigue that can be a burden to endoscopists. Such achievements can only be done when the application of AI can improve the quality of imaging diagnosis beyond that of human capability, and optical biopsy is possible. This is possible by improving AI performance using the specific characteristics of different organs and diseases. AI is being studied and developed by scientists all over the world in various fields with hopes of providing accuracy and convenience. In the field of medicine, medical records and imaging are becoming digitalized and a new phase in the history of

medicine is expected within five to 10 years. Accordingly, clinicians and researchers need to carefully approach and evaluate the results of further clinical studies using AI-based technology with great interest.

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MINIREVIEWS

Current impact of viral hepatitis on liver cancer development: The challenge remains

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Abstract

Chronic infections due to hepatitis B and hepatitis C viruses are responsible for most cases of hepatocellular carcinoma (HCC) worldwide, and this association is likely to remain during the next decade. Moreover, viral hepatitis-related HCC imposes an important burden on public health in terms of disability-adjusted life years. In order to reduce such a burden, some major challenges must be faced. Universal vaccination against hepatitis B virus, especially in the neonatal period, is probably the most relevant primary preventive measure against the develo-

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pment of HCC. Moreover, considering the large adult population already infected with hepatitis B and C viruses, it is also imperative to identify these individuals to ensure their access to treatment. Both hepatitis B and C currently have highly effective therapies, which are able to diminish the risk of development of liver cancer. Finally, it is essential for individuals at high-risk of HCC to be included in surveillance programs, so that tumors are detected at an early stage. Patients with hepatitis B or C and advanced liver fibrosis or cirrhosis benefit from being followed in a surveillance program. As hepatitis B virus is oncogenic and capable of leading to liver cancer even in individuals with early stages of liver fibrosis, other high-risk groups of patients with hepatitis B are also candidates for surveillance. Considerable effort is required concerning these strategies in order to decrease the incidence and the mortality of viral hepatitis-related HCC.

Key Words: Hepatitis B virus; Hepatitis C virus; Hepatocellular carcinoma; Epidemiology; Vaccination; Surveillance

Core Tip: Hepatitis B and C are associated with most cases of hepatocellular carcinoma, and it is estimated that this scenario will remain for the next decade. This review highlights the impact of viral hepatitis on the development of liver cancer, the characteristics of viral hepatitis-related hepatocellular carcinoma, and the challenges that must be faced in order to reduce their burden.

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INTRODUCTION

Viral infections are the leading cause of hepatitis, with hepatitis B virus (HBV) and hepatitis C virus (HCV) being the most important causes of chronic viral hepatitis worldwide. It was estimated that nearly 292 million individuals had chronic HBV infection in 2016 (prevalence of 3.9%) and 71 million people had chronic HCV infection in 2015 (prevalence of 1%)[1-3]. Hepatitis D virus (HDV) is a defective virus, requiring HBV coexistence in order to replicate. It was estimated that HDV caused chronic infection in approximately 12 million individuals in 2016[4]. Most HBV, HCV and HDV infections are concentrated in low- and lower middle-income countries[3].

The incidence of liver cancer is the sixth highest of all malignant neoplasms (905677 new cases in 2020, an incidence of 11.6/100000 inhabitants). Moreover, it is the second cause of cancer-related death in the world (830180 deaths in 2020, mortality of 10.7/100000 inhabitants)[5]. Hepatocellular carcinoma (HCC) accounts for approximately 75%-85% of primary liver cancers[6,7]. HCC frequently develops in patients with cirrhosis, with an annual incidence of 1%-4%[8]. HBV and HCV are currently the two most important risk factors for HCC development worldwide. This is reflected by the geographical distribution of liver cancer, which coincides with those of HBV and HCV, predominating in transitioning countries, particularly in Asia and Africa[6,7,9]. In a systematic review of 260 studies involving 119006 patients with HCC, the seroprevalence of HBV and/or HCV was over 60% in most of the 50 countries which contributed with data[10]. Furthermore, it is noteworthy that, according to the Global Burden of Disease Study, HBV-related liver cancer was associated with 5.80 million disability-adjusted life years, and HCV-related liver cancer, with 2.88 million disability-adjusted life years in 2019[11]. The impact of HBV may be further increased by the fact that HCC usually develops at an earlier age in patients infected with this virus[12].

Despite vaccination against HBV and effective treatments for HBV and HCV, it is estimated that their impact on liver health will remain for the next decade at least. Using data from 195 countries or territories between 1990 and 2017, a recent study proposed Bayesian models to project primary liver cancer incidence through 2030. The authors estimated that the age-standardized incidence rate of liver cancer will increase globally from 11.80 in 2017 to 14.08 per 100000 inhabitants in 2030. According to the etiology of liver disease, HBV was responsible for 46.5% of cases of liver cancer in 1990 and 42.4% in 2017, and it is estimated that it will be associated with 40.7% of cases in 2030. With regard to HCV, it was associated with 25.2% of cases of primary liver cancer in 1990 and 27.0% in 2017, and it is projected that it will be responsible for 26.8% of cases in 2030[13]. The aim of this article is to review the current impact of viral hepatitis on the development of liver cancer, the characteristics of viral hepatitis-related HCC and the challenges to reduce their burden (Figure 1).

IMMUNE ASPECTS OF VIRAL HEPATITIS IN HEPATOCARCINOGENESIS

In chronic viral hepatitis, the development of fibrosis, cirrhosis and HCC is linked to the activity of the immune response in the infected liver. Both HBV and HCV uniquely infect hepatocytes and replicate in a non-cytopathic manner. The ensuing liver damage that is observed during the acute and the chronic phase of infection is the consequence of virus-specific as well as non-specific immune activity within the inflamed liver. Histologically, active phases of chronic HBV and HCV infections are characterized by extensive infiltration around the portal tract areas consisting predominantly of CD4+ and CD8+ T cells, but these cells are incapable of clearing these viruses. Multiple mechanisms have been described that explain the weak activity of CD4+ and CD8+ T cells, and that are the main reasons for persistence of HBV and HCV in the liver, and the slowly progressing liver disease[14-16]. The mechanisms include impairment of dendritic cells and natural killer (NK) cells, increased production of immunosuppressive cytokines and an increase in the numbers of regulatory T cells. In addition, the continuous exposure of immune cells to high levels of viral antigens for many years further contributes and maintains the persistent infection due to exhaustion of HBV and HCV-specific T cells, which renders these cells functionally impaired mediated *via* triggering of exhaustion markers, such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and others[17].

In the liver, the balance between immune tolerance and immune activation is normally tightly controlled to ensure efficient elimination of pathogen products and transformed cells. Dysregulation of this balance due to viral infection has severe consequences for the process of immune surveillance that is highly efficient in the detection and elimination of transformed cells, and contributes to HCC development [18]. Also, alterations in lipid metabolism and the release of reactive oxygen species contribute to tumor initiation[19].

Interestingly, the status of tumor-infiltrating immune cells in HCC has a high degree of resemblance to that observed in an HBV or HCV inflamed liver: an increased T cell infiltrate is observed in HCC, but the CD8+ T cells are dysfunctional with low production of the cytolytic enzymes granzyme and perforin, low overall cytokine production, and relatively high expression of the exhaustion markers PD-1 and CTLA-4[20,21]. In addition, increased numbers of regulatory T cells and reduced numbers and functionality of NK cells have been reported by many groups[18,22,23]. These immune dysfunctions are generally more pronounced in the advanced HCC stages.

The importance of the immune system in controlling the development and progression to advanced stages of HCC is highlighted by observations that increased T cell infiltrates are associated with improved overall survival in HCC and lower tumor recurrence following resection[24-26]. Furthermore, the enrichment of exhausted T cells in HCC is associated with poorer survival, while higher numbers of infiltrating NK cells are associated with better survival. Using cytometry by time of flight and RNA sequencing, a recent study defined different immune subsets and showed that the tumor microenvironment in HBV-related HCC consisted of a more immunosuppressive and exhausted phenotype with an enrichment of regulatory T cells and resident memory T cells that differs from the non-viral-related HCC environment. The study further demonstrated that regulatory T cells are associated with poor prognosis, and memory T cells, with good prognosis[27].

The crucial importance of the patient's immune activity against the tumor has initiated the development of numerous immunotherapeutic approaches in recent years, all aimed at boosting anti-tumor immunity[28]. The approaches are diverse and

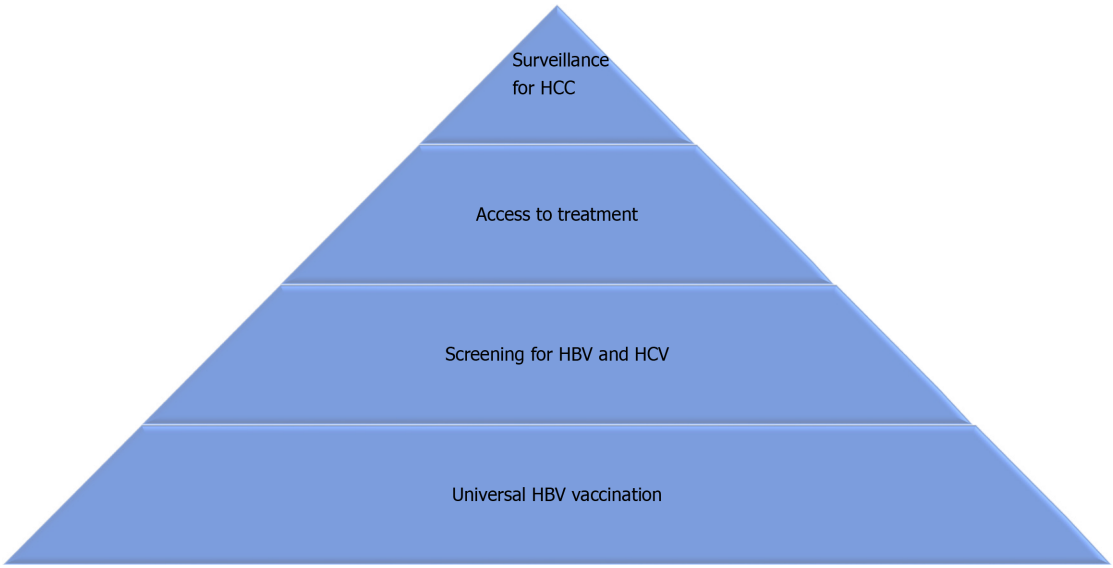

Figure 1 Major challenges to reduce the burden of viral hepatitis-related hepatocellular carcinoma. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

include strategies using peptide vaccines and cell-based therapies. Moreover, therapies aimed at neutralizing exhaustion markers using immune checkpoint inhibitors have shown promising results and PD-1 blockade has been approved as therapy for HCC. However, many studies are ongoing to improve therapy success by examining the therapeutic outcomes using different combinations of checkpoint inhibitors.

HEPATITIS B AND HCC

Implications on HCC

Advances in next-generation sequencing technology and integrative studies combining multiomic approaches, encompassing genomic and epigenomic characterization and transcriptome, proteomic, and metabolomic analysis of HCCs have shown that HCC is heterogeneous at the histomolecular level, with variable molecular features and clinical outcomes[29-31]. While ongoing hepatic inflammation, increased cell turnover, repeated cycles of cell death and regeneration, DNA instability leading to an alteration in DNA and dysregulation of CpG island methylation are common pathways leading to HCC regardless of the underlying etiology of liver disease[32], there are HBV specific pathogenesis pathways in HCC development. HBV promotes HCC through viral DNA integration into host genes that can provide a growth advantage to the host cell. HBV has been found to be integrated into or near cancer-related genes[33,34]. For example, promoter mutation of telomerase reverse transcriptase, which is often integrated by HBV DNA, is the most frequent genetic event in HBV-associated HCC. The translated protein products of the viral HBV genome have also been implicated in promoting carcinogenesis. For instance, HBx protein, a regulatory protein required for viral replication, has been shown to regulate hepatocyte proliferation, regeneration and apoptosis by modulating the PI3K/Akt-mTOR, STAT3/Nanog, and Wnt/β-catenin signaling pathways to promote the progression of HCC[35-37].

Occult HBV infection

Occult HBV infection is a condition where HBV DNA replication occurs in the liver in patients with negative serum HBsAg[38]. While detection of anti-HBc (hepatitis B core antibody) in the blood indicates prior HBV infection, 20% of patients with occult HBV infection are seronegative for anti-HBc. HBV DNA analysis should be further pursued to confirm the diagnosis[39]. Occult HBV infection increases the risk of HCC development in patients with or without chronic liver disease, which is thought to be mediated by HBV DNA integration into the host genome, the production of pro-oncogenic proteins such as HBx protein, and persistent low-grade necroinflammation leading to fibrosis progression[39]. Currently, HCC surveillance is not routinely

recommended in patients with occult HBV infection in the absence of advanced fibrosis/cirrhosis.

HCC surveillance in hepatitis B

HCC continues to be one of the leading causes of cancer-related death throughout the world and it frequently develops within the context of cirrhosis[40]. This has also led to the development of guidelines by all the major liver societies for HCC surveillance in patients with cirrhosis given its potential for early-stage cancer detection, which likely links to curative treatment[41,42]. Given its high oncogenic potential and its tendency to develop HCC in the absence of cirrhosis, screening for HCC is indicated in a broader population of HBV patients. All HBV patients with HBV cirrhosis (except for patients with severe liver dysfunction and not eligible for liver transplantation) are recommended to undergo HCC surveillance. Surveillance is also recommended in a selected high-risk population of non-cirrhotic HBV patients, as demonstrated in Table 1[43].

Eradication of hepatitis B through vaccination

HBV vaccination is an effective primary prevention method for HCC. The effectiveness of HBV vaccination in reducing HCC incidence was reported in a nationwide population-based study from Taiwan. The study used data from 2 Taiwanese HCC registry systems and showed that HCC incidence was four-fold higher in the HBV unvaccinated cohort than in the vaccinated at birth cohorts. Among the 1509 patients who were 6-26 years old diagnosed with HCC from 1983 through 2011, 1343 were born before, and 166 were born after the HBV vaccination program began. Moreover, the relative risks for HCC in patients vaccinated at 6-9 years old, 10-14 years old, 15-19 years old, and 20-26 years old in comparison to those who were not vaccinated were 0.26 [95% confidence interval (CI): 0.17-0.40], 0.34 (95%CI: 0.25-0.48), 0.37 (95%CI: 0.25-0.51), and 0.42 (95%CI: 0.32-0.56), respectively[44]. Although neonatal HBV vaccination is recommended in most countries, vaccine coverage is still less than 70% in most African countries where the incidence rates of HCC remain high[45], providing a clear window of opportunity for improvement in primary prevention of HCC[40].

Major challenges

Aside from identifying infected patients, offering effective treatment against HBV and surveying those at high risk for HCC, the major challenge in order to reduce HBV-related HCC burden is widening the vaccination coverage against the virus. Despite the increase in vaccination coverage between 2010 and 2018 in 97% of the 194 member states of the World Health Organization (WHO), with an increment from 73% to 84% in the completion of the three-dose vaccination schedule and with an increase from 28% to 42% in the neonatal vaccination coverage in the 108 countries in which newborns are vaccinated, improvements are still necessary[46]. For instance, vaccination coverage requires improvement in countries such as Nigeria, India and Indonesia, which together are responsible for 50% of HBV infections in children under 5 years of age[3].

HEPATITIS C AND HCC

Implications on HCC

HCV is one of the most important risk factors for HCC. As previously mentioned, HCV is a non-cytopathic virus, which leads to liver damage through immune-mediated mechanisms. However, the virus itself also plays a role in the development of HCC, which becomes clear when considering, for instance, that HCV genotype 3 is associated with a higher risk of HCC than other genotypes[47]. The mechanism through which HCV genotype 3 is involved in hepatocarcinogenesis is not yet completely understood, but the down-regulation of the phosphatase and tensin homolog (*PTEN*) gene, which has a tumor suppressor role, might be implicated[48].

Direct-acting antiviral (DAA) therapy in HCV-infected patients has been shown to reduce all-cause mortality (including liver-related and unrelated causes), as well as the incidence of HCC in patients with cirrhosis[49]. However, in the subgroup of patients with advanced fibrosis or cirrhosis, the risk of developing HCC after achieving sustained virologic response (SVR) remains high, ranging from 0.3% to 1.8% per year [50]. For this reason, different guidelines recommend lifelong surveillance for patients with advanced fibrosis or cirrhosis even after SVR[51]. Interestingly, recent studies have attempted to differentiate subgroups of patients with cirrhosis at the highest risk

Table 1 Indications for surveillance of hepatocellular carcinoma in patients with hepatitis B without cirrhosis

Indications for surveillance
Asian males > 40 yr
Asian females > 50 yr
African ancestry
First degree relative with HCC
HDV co-infection

HCC: Hepatocellular carcinoma; HDV: Hepatitis D virus.

for HCC after SVR. In an Italian study, the combination of clinical predictors (male sex and diabetes), albumin, and genetics identified patients at high risk for HCC[52]. Furthermore, it is suggested that changes in liver stiffness measured by transient elastography at 1 and 3 years after the end of HCV treatment could identify patients at low risk for HCC[53].

Population screening for hepatitis C

In 2017, the WHO Global Hepatitis Report stated that 71 million people worldwide were infected by HCV[54]. About 3.5 million to 5.0 million of these individuals are children and adolescents, and 2.3 million are co-infected with the human immunodeficiency virus (HIV)[51,54]. HCV infection causes approximately 400000 deaths annually, mainly from cirrhosis-related complications and HCC[54]. Despite the current availability of DAA therapies with high rates of SVR, the prevalence of HCV infection has not changed. One of the reasons that explain this situation is that only 20% of individuals with HCV infection worldwide are aware of their diagnosis. Another reason is that access to DAA treatments continues to be limited mainly by costs in several countries[54].

The WHO Global Health Sector Strategy on Viral Hepatitis recommended that an effort should be made in order to increase the HCV diagnosis rate to 30% by 2020 and to 90% by 2030[55]. Overall, HCV screening consists in a universal, one-time, and opt-out screening strategy in adults 18 years of age and older with an upper age limit of 79 years. However, periodic screening tests should be offered to individuals with behaviors, conditions or circumstances associated with an increased risk of exposure to HCV[51].

Vertical transmission could be present in about 5% of deliveries from mothers with HCV and accounts for the majority of HCV infections in the pediatric population[56]. Universal prenatal screening for HCV facilitates better identification of at-risk infants who require HCV testing[57]. This evaluation results in better detection of HCV infection in the pediatric population and enables early therapeutic interventions.

Eradication of hepatitis C

DAA regimens have a high cure rate for HCV infection[51]. Furthermore, new formulations have simplified the duration and administration of the treatment. However, in 2015, only 7% of people diagnosed with HCV had started antiviral treatment with DAA[54]. According to the strategy suggested by the WHO, access to DAA should reach 80% of eligible people by 2030[55]. After this declaration, access to treatment has improved in different countries. An example is the strategy in Australia, where more than 80% of the HCV-infected population was diagnosed during the last two decades and where there is currently an unrestricted DAA access program that permits prescriptions by any registered medical practitioner. This allowed for the initiation of DAA treatment in Australia for approximately 70% of the total population with HCV-related cirrhosis between 2014 and 2017[58].

Simultaneously with screening and therapy access, different preventive measures must be carried out in people capable of transmitting HCV (*e.g.*, people who inject drugs) in order to avoid new infections or reinfections in those who have been previously treated successfully. Global coverage of harm reduction programs for people who inject drugs, including needle and syringe programs, is currently less than 10%[54].

Major challenges

Currently, the major challenge in order to reduce HCV-related HCC burden is identifying infected patients. The silent course of the early stages of this disease makes this challenge even harder to overcome. Therefore, efforts should be made in order to improve awareness of the population and health care workers regarding the underdiagnosis of HCV[54]. It is also of great importance to shorten the pathway leading from the diagnosis to the extremely effective treatments against HCV, which could even allow for subgroups of patients to be treated in primary care[59].

Yet another challenge relates to the coronavirus disease 2019 pandemic. It is estimated that the impact of the pandemic on the global efforts towards HCV eradication could lead to an excess of 44800 cases of HCC[60].

CO-INFECTIONS AND HCC

As previously mentioned, HBV and HCV infections are the most common risk factors for HCC worldwide[61]. Individuals living with HIV are a high-risk population for developing HCC, mostly as a consequence of HCV and HBV co-infection[62,63]. Indeed, HCC has become a major clinical problem in HIV. Studies from Europe suggest that the incidence of HCC has risen in HIV-infected patients over the last 20 years, and in Spain alone HCC is the second cause of death in HIV-HCV co-infected patients with cirrhosis[64]. Studies from both the United States and Europe suggest that HIV may hasten the evolution of HBV-related HCC, resulting in an earlier age of HCC presentation in HIV co-infected patients[65,66].

Although the implementation of new treatments for HCV with DAA agents is expected to decrease HCC incidence, new cases will continue to emerge in the near-medium term[67,68]. Moreover, a recent study that reported an HCC risk increase of 1% every year in HIV-HCV co-infected individuals in the HEPAVIR cohort in Europe included in their cohort 61% of individuals with cured HCV, indicating that this population remains at risk for HCC[63].

In HIV-HBV co-infection, a recent study from the EuroSIDA cohort showed a stable incidence of HCC in non-cirrhotic individuals under HBV therapy (tenofovir specifically), while increasing rates of HCC were seen over time in those not on tenofovir. This suggests a potential threshold to not survey the former group for HCC. However, the same study did show a continued increase in HCC risk among those that had cirrhosis, indicating the importance of surveillance in this population[69].

Most of the data reported on HIV and HCC originate in resource-rich settings. These dynamics related to poor outcomes are likely to be increased in resource-limited settings, where most of HIV infections occur. A small African study of 60 patients found that those infected with HIV developed HCC at a younger age (32 years) compared to those without HIV (49 years)[70], and a more recent study from South Africa showed the age of HCC development to be lower in those co-infected with HBV and HIV (mean age of 36 years *vs* 46 years in HIV-uninfected). Interestingly, this study showed that females were more impacted by HCC when co-infected with HIV, and the age of HCC presentation was much lower in females with HIV (mean age of 36 years *vs* 50 years in HIV-uninfected)[71]. In South America and Asia overall, data are scarce on the interrelation between HCC and HIV infection. The specific mechanisms underlying younger HCC occurrence in co-infected individuals with viral hepatitis and HIV is unclear. However, a large Swiss study found a direct association between CD4+ T cells and the risk of developing HCC, suggesting that impairment of the immune system could be implicated[72].

Surveillance for HCC represents a major challenge in co-infected populations not only due to the complex nature of dual disease, but also because most guidelines are tailored towards mono-infected individuals at risk for HCC. In addition, recent studies show that ultrasound surveillance has a low performance for HCC in HBV or HCV individuals co-infected with HIV, with a suboptimal 43% rate of early-stage diagnosis, compared to 63%-71% found in studies on HIV-uninfected cohorts[73,74].

Triple co-infection of HIV, HBV and HDV is rather uncommon, ranging from 1% to 20% depending on the geographical area[75]. However, studies performed in small cohorts have shown that the main impact of HDV on HBV-HIV disease is an accelerated pace to cirrhosis and hepatic decompensation[76]. In this context, a Swiss HIV cohort reported a 9-fold increase in HCC in those triple co-infected with HIV-HBV-HDV compared to HDV-negative individuals[77].

CONCLUSION

Despite the advances in prevention and treatment of viral hepatitis, it is clear that many challenges remain in order to reduce the burden of viral hepatitis-related HCC. Universal neonatal vaccination against HBV, as well as vaccination of at-risk adult populations, screening for HBV and HCV, and access to highly-effective treatments against both viruses are instrumental measures that must be pursued with the objective of diminishing their impact on global health and particularly on the development of HCC. Finally, effective surveillance for HCC must be offered to patients who already have advanced fibrosis or cirrhosis and to high-risk HBV-infected individuals, so that HCC is detected at earlier stages, allowing for curative treatments and longer survival.

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MINIREVIEWS

Room for improvement in the treatment of pancreatic cancer: Novel opportunities from gene targeted therapy

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Abstract

Pancreatic cancer is one of the highest and in fact, unchanged mortality-associated tumor, with an exceptionally low survival rate due to its challenging diagnostic approach. So far, its treatment is based on a combination of approaches (such as surgical resection with or rarely without chemotherapeutic agents), but with finite limits. Thus, looking for additional space to improve pancreatic tumorigenesis therapeutic approach, research has focused on gene therapy with unexpectedly growing horizons not only for the treatment of inoperable pancreatic disease, but also for its early stages. *In vivo* gene delivery viral vectors, despite few disadvantages (possible immunogenicity, toxicity, mutagenicity, or high cost), could be one of the most efficient cancer gene therapeutic strategies for clinical application due to their superiority compared with other systems (*ex vivo* delivery strategies). Their dominance consists of simple preparation, easy operation and a wide range of functions. Adenoviruses are one of the most common used vectors, inducing strong immune as well as inflammatory reactions. Oncolytic virotherapy, using the above mentioned *in vivo* viral vectors, is one of the most promising non-pathogenic, highly-selective cytotoxic anti-cancer therapy using anti-cancer agents with high anti-tumor potency and strong oncolytic effect. There have been a variety of targeted therapeutic and pre-clinical strategies tested for gene therapy in pancreatic cancer such as gene-editing systems (*e.g.*, clustered regularly interspaced palindromic repeats-Cas9), RNA interference technology (*e.g.*, microRNAs, short hairpin RNA or small interfering RNA), adoptive immunotherapy and vaccination (*e.g.*, chimeric antigen receptor T-cell therapy) with

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encouraging results.

Key Words: Pancreatic cancer; Gene therapy; Viral vectors; Gene editing; miRNA; siRNA; Oncolytic virotherapy

Core Tip: Pancreatic cancer still remains one of the leading causes of cancer deaths worldwide. While there are various therapeutic approaches established, there is still a crucial need for improvement of conventional treatments and establishment of novel therapies to increase efficacy. This review exhibits an overview of the most promising present and future prospects regarding gene therapy which offers a new favorable opportunity not only to tackle with an inoperable pancreatic cancer, but also, to treat effectively its early stages.

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INTRODUCTION

Pancreatic cancer is an ever-worsening healthcare issue, predicted to ascend to the second place of highest mortality-associated neoplasias. The conundrum it represents for patients and physicians alike is aggravated by its challenging diagnosis, dismal prognosis, less than satisfactory treatment, and low survivability. Epidemiologically, the rate of new cases is approximately 13.1/100000 per year and the mean age of onset is 71 years for men and 75 for women, with the former slightly more affected[1]. The estimated 5-year survival is up to 8% and a mere 9.7% of patients are metastasis-free at diagnosis[2]. A mainstay of its globally increasing incidence is possibly the unequivocal relationship between its risk factors, mainly obesity, smoking, and alcohol, as well as the Western lifestyle[3]. Other contributing factors are age, gender, genetic and chronic inflammatory conditions.

There are numerous classifications of pancreatic cancer, such as macroscopic, pathological, molecular, and surgical, depending on the characteristic being observed. The most frequently recurring form of pancreatic neoplasia is the adenocarcinoma, a tumour of solid type and ductal origin. The most commonly affected site is the head of the pancreas, whereas roughly a fifth of the cancers concern the organ diffusely[4]. Pathophysiologically, there is a well-established sequelae, starting from a precursor lesion and culminating to proper neoplasia, through a series of oncogene and tumour-suppressive gene mutations, such as *KRAS*, *p53*, *p16*, *SMAD4* and *CDNK27*[5]. Precancerous lesions are further divided in pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasm and intraductal papillary mucinous neoplasm, with PanIN being the most frequent[4]. The two-tiered categorization of PanIN (low and high grade) bears great clinical significance, since the probability and duration of progression between low and high grade have been established and can be predicted [6,7].

Clinical presentation is non-specific and usually includes weight loss, abdominal pain, jaundice, malabsorption, and diabetes mellitus. This fact, together with the relative rarity of the disease, make screening ineffective, apart from patients with a positive family history of pancreatic neoplasia where screening is considered rationale [4,8]. Diagnostic process includes clinical check-up, routine blood work-up and imaging, along with the search for signs of malabsorption, diabetes, and hypoalbuminemia. Specifically, a combination of two imaging modalities, preferably endoscopic ultrasound and computerized tomography or magnetic resonance imaging/magnetic resonance cholangiopancreatography scan, is favored by the International Cancer of the Pancreas Screening Consortium[9,10].

As far as treatment is concerned, surgical resection and adjuvant or neoadjuvant chemotherapy is the best therapy so far[11]. The observed response to treatment is dependent on a plethora of factors, such as tumour stage at diagnosis, age, and comorbidities. However, 5-year survival remains low, ranging from 8%-27%, with the latter concerning complete resection of local non-metastatic disease. The surgical therapies of choice are pancreatoduodenectomy (Whipple's), distal or total pancreatectomy, coupled with potent chemotherapeutic agents, where applicable[12-14]. The most used regimens contain gemcitabine, nab-paclitaxel and mFOLFIRINOX[15,16].

METHODS AND RESULTS

We conducted a literature search in PubMed database, including most of the published studies in English language. The following keywords were used for literature search: "gene therapy", "gene therapy AND pancreatic cancer", "pancreatic cancer", "anti-angiogenesis AND pancreatic cancer", "viral vectors AND gene editing, "miRNA", "siRNA", "oncolytic virotherapy". Three and seven hundred forty-seven thousands publications fulfilled search requirements. Of these, 85 articles were chosen due to the appropriate content and the English language criteria . From the 85 articles, 35 were original articles and 50 were review articles, which included case series, case reports, clinical trials and clinical cohort studies. We reviewed all the abstracts and found 69 full-text articles appropriate for the current study (Figure 1). The three authors (DA, GM and GF) independently reviewed the abstracts and the full text of the related articles.

IS THERE ROOM FOR ANY IMPROVEMENT ON PANCREATIC CANCER TREATMENT?

As the mortality of pancreatic cancer has been unchanged the last decades, the pressure of discovery for a new therapeutic armamentarium is more pivotal than ever. Additionally, the fact that pancreatic ductal adenocarcinoma (PDAC) is expected to be levelled off at the second place of the most lethal neoplasias worldwide pushes the healthcare systems to investigate new treatment protocols. Over the last 20 years, promising results have been stated by the induction of gene-based therapies, shifting from the therapy of inherited diseases to acquired disorders, but most importantly cancer[17]. Gene therapy delivers genetic material to cells for the treatment of a particular disorder. Gene editing or gene transfer might be conducted *ex vivo* in isolated cells or *in vivo*, in cells located within their corresponding tissue environment [18]. The primary principles of gene therapy are to induce anti-tumor immune effects intervening with various signaling pathways. There are various types of gene-based therapy approaches that could be classified into three groups: (1) gene editing or addition; (2) genetically engineered cell therapy; and (3) oncolytic virotherapy (Figure 2)[19]. Gene delivery systems also play an important role in gene therapy affecting both the safety and efficiency of gene transfer. Furthermore, improved targeting relies upon the development of viral and non-viral methodologies of the therapeutic gene specific to the tumour[19].

GENE DELIVERY METHODS

Ex vivo delivery

In this particular system, sampling cells extracted from the patient (bone marrow or target tissue), are genetically modified (*in vitro* culture or proliferation) with a vector including the therapeutic gene of choice and subsequently reintroduced in the same individual after gene transfer[20]. In cancer treatment, cells explanted from cancer patients can be also cultured and genetically engineered *in vitro*, but normally they are utilised for the secretion of cytokines or vaccination[21]. In case of a tumor like PDAC characterized by invasive nature and accelerated growth, the *ex vivo* strategy is not well adjusted and a "ready to use" gene therapy product could be theoretically a better option[20].

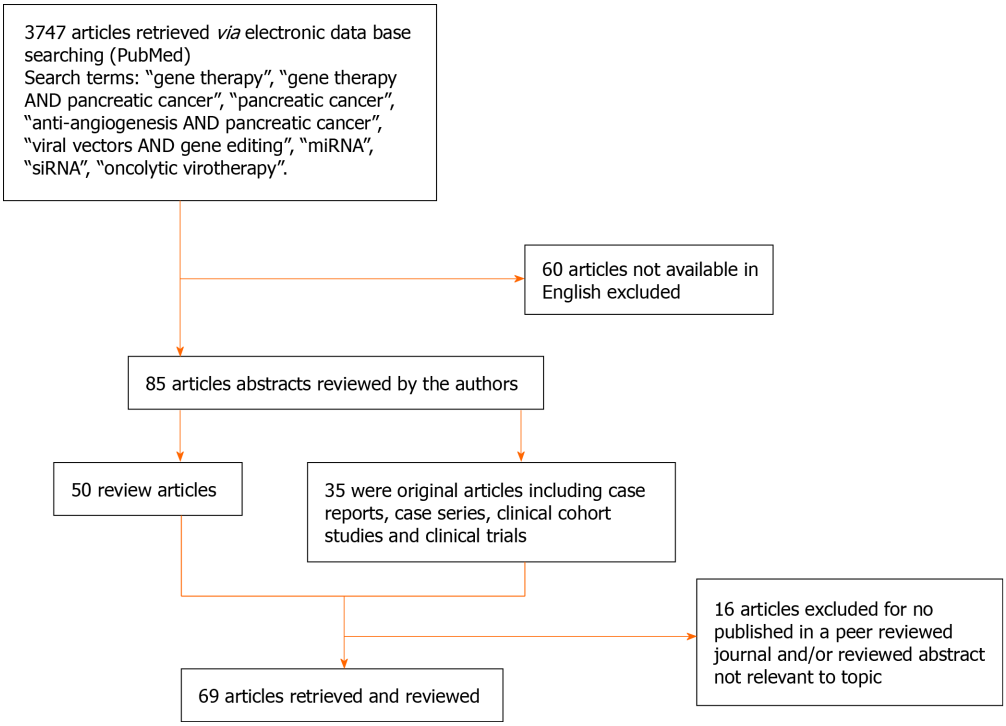

Figure 1 Decision tree for literature research strategy.

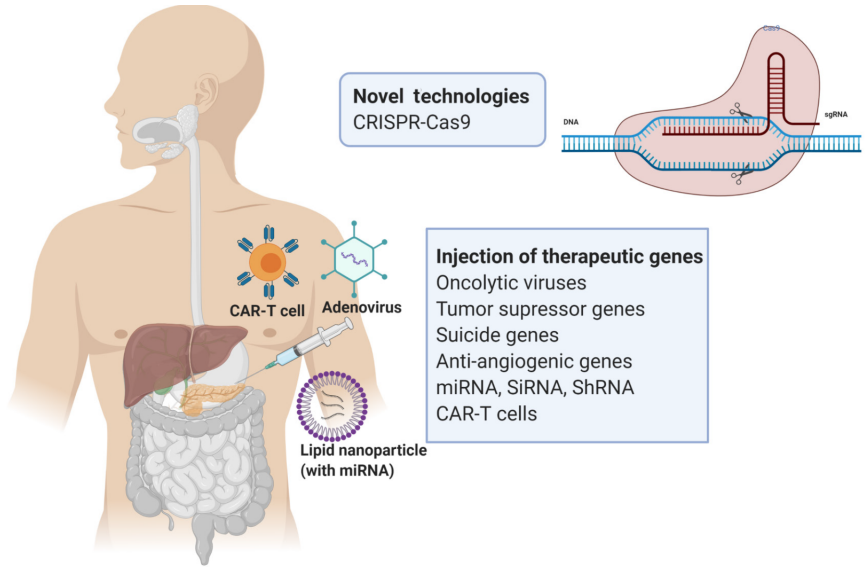

Figure 2 Summary of the therapeutic strategies of gene therapy for pancreatic cancer. The above diagram was created with Biorender.com. CAR-T: Chimeric antigen receptor T-cell; CRISPR-Cas9: Clustered regularly interspaced palindromic repeats-Cas9.

In vivo delivery

The second system of gene delivery is the *in vivo* delivery which includes injecting the gene vectors carrying the therapeutic genes into the bloodstream or the target tissue (tumor). In situ injections are used with imaging technologies and are tested in cases such as myopathy, cystic fibrosis and most importantly cancer[20]. Nowadays, the majority of clinical trials are being established following the methodology of intratumoral injection induced by ultrasound or computed tomography, gene-eluting stent implantation and vascular perfusion of tumor[21]. *In vivo* delivery could be one of the most efficient strategies for clinical application due to its superiority compared with other systems, based on its simple preparation, easy operation and broad range of functions. However, there still exist some disadvantages such as the lack of specificity on particular targets, short-term curative effect and immunologic complications[22].

GENE DELIVERY VECTORS

Presently there are two categories of gene delivery vectors: viral and non-viral vector systems[19]. The most widely used viral vectors are those that are mainly based on adenoviruses (ADs), retroviruses (RVs) and adeno-associated viruses (AAVs)[23]. Non-viral vector systems comprise of biological or chemical vectors, as well as physical approaches of gene delivery to present RNA molecules, naked DNA (plasmid DNA form) or oligonucleotides into the targeted cells[19,21]. Physical delivery methods primarily, consist of electroporation and microinjection. Nowadays, viral vectors represent the most efficient gene transfer method and thus, the most widely used gene delivery systems for cancer gene therapy[21,23].

Viral vectors

It is estimated that viral therapies represent more than two-thirds of clinical trials on gene therapy. More specifically, these viruses are capable of infecting and entering the cell *via* different mechanisms and transfer DNA into recipient cells without the need of any chemical or physical preparation. The gene of choice will then enter the nucleus resulting in host gene assimilation and expression[21].

The most common viral vectors in cancer gene therapy as previously described include RV, AD, AAV, herpes simplex virus (HSV), lentivirus, pox virus, Epstein-Bar virus and Newcastle disease virus[23]. Viral vectors usually offer long-term gene expression and better insertion capacity and efficiency, but might be linked with immunogenicity, toxicity, mutagenicity and high cost[24,25]. Thus, for the appropriate selection of viral vectors both advantages and disadvantages should be considered.

One of the most common class of viral vectors are ADs. ADs could infect a large variety of cells and for this reason, have been used for a long time for preclinical and clinical gene therapeutic strategies for PDAC. These vectors induce strong immune and inflammatory reactions. Nevertheless, no major incident has been observed during clinical trials with adenoviral vectors. Third generation AD vectors (no viral sequence) haven been developed to solve some issues of toxicity[22,25]. Another important class of viral vectors are AAV. They are capable of effectively transducing cells from liver and brain but have, rarely been examined in pancreatic cells[19,21,25]. The retroviridae family includes viral vectors such as lentiviral (derived from HIV-1) and retroviral (derived from murine leukemia virus) vectors. They are small RNA vectors with the advantage of low immunogenicity and ability of transducing many cells with high efficiency. However, these viral vectors have a high risk of immunogenicity. They are commonly used in *ex vivo* strategies but also in *in situ* administration, as they can transduce proliferating cells such as cancer cells[20,26]. A new class of lentiviral and retroviral known as self-activating vectors have been developed with specific modification allowing them to bypass the transactivation of oncogenes during their integration into the host cells[27]. Finally, many studies have been conducted for another class of viral vectors known as the HSVs. These viruses are highly immunogenic and can carry and incorporate huge amounts of DNA; therefore, compared with other viruses, they include a complex genome making it difficult to transfer genetic information to the target cell[20].

Oncolytic virotherapy

Oncolytic virotherapy is one of the most promising anti-cancer therapies using agents with high anti-tumor potency and strong oncolytic effect[2]. Pancreatic ductal carcinoma development is linked with a sequence of changes helping cancer cells to grow more rapidly. Nevertheless, these particular changes such as high metabolic activity, lack of interferon response and uncontrolled cell division, cause cancer cells to be more sensitive to viral infection[28]. These viruses are non-pathogenic and have a high selectivity and cytotoxicity to cancer cells[29]. In addition, they are able to stimulate their anticancer activity *via* various mechanisms such as targeting of specific pathways (dysfunction pathway TP53) and stimulation of specific and non-specific anti-tumor immunity[30].

Over the years, ADs have been assessed for their potential in PDAC therapy by different research programs. AV5, also known as Oncorine H101 was the first adenovirus to be approved for the treatment of nasopharyngeal cancer[29]. Oncorine H101 was chemically engineered to replicate and kill tumor cells demonstrating *p53* mutations. This specific oncovirus is closely related to the adenovirus Onyx-15[2]. Onyx-15, which replicates in cancer cells harbouring *p53* mutations, was not effective as a monotherapy. During phase II clinical trials it was demonstrated that combination of Onyx-15 with the anticancer drug gemcitabine was initially well tolerated in

pancreatic patients, but some issues appeared such as high titer neutralizing antibodies growth and low replication of the virus[29,31]. There has been a vast number of attempts to enhanced the efficacy of potential ADs for PDAC therapy *via* genetic engineering of the virus to induce the development of variants particular to the disease characteristics. One example is the adenovirus AxE1AdB-UPRT expressing uracil phosphoribosyltransferase, importing 5-FU resistance[32].

After the success of talimogene laherparepvec (T-VEC) approved for the treatment of nasopharyngeal malignancy, HSVs have been representing promising oncoviruses for cancer therapy[2,29]. T-VEC is currently being assessed in phase I clinical trials as monotherapy or in combination with radiotherapy for the treatment of Merkel cell carcinoma and melanoma[33]. Furthermore, it has also been assessed in clinical phase I trials for PDAC as monotherapy and displayed no clinical efficacy in 17 pancreatic cancer patients[31]. Another HSV with promising anti-cancer activity is Myb34.5, which has been assessed preclinically in pancreatic cancer models. The intratumoral injection of Myb34.5 variant induced apoptosis and inhibition of pancreatic tumors growth. This effect was further enhanced with the combination of gemcitabine[34].

TARGETED THERAPEUTIC AND PRE-CLINICAL STRATEGIES

The basic overall strategy differs upon the final objective either through introducing a therapeutic gene which is down-expressed or missing (*e.g.*, suicide gene) or through reintroducing a deficient gene (*e.g.*, tumor suppressor gene) or through inhibiting gene expression (*e.g.*, oncogene). There exist many barriers to the overall gene therapy delivery that could be overcome by intranuclear and intracellular penetration, protein and mature mRNA expression and entry to tumor cells[20]. There have been a variety of pre-clinical approaches for PDAC gene therapy and non-viral gene therapy applications such as gene-editing systems (*e.g.*, CRISP-CAS) and RNA interference technology (Table 1)[20,21].

Anti-angiogenic genes

Gene therapy serves as an effective strategy for therapeutic intervention to angiogenesis based on safety, specific targeting, and cost-effectiveness. Many strategies have been tested *in vitro* or *in vivo* by transfer of gene encoding anti-angiogenic molecules such as vasostatin, angiostatin, vascular endothelial growth factor (VEGF) and soluble fibroblast growth factor receptor[20,21]. Angiogenesis plays an important role in tumor growth. VEGF is a glycoprotein essential in angiogenesis, which is overexpressed in 90% of pancreatic cancers and is responsible for increased tumor progression and microvessel density, as well as poor prognosis. Clinical studies have demonstrated that serum levels of VEGF are much higher in metastatic prostate cancer patients than non-metastatic ones[35]. Various anti-VEGF therapies with molecular targeted agents and monoclonal antibodies have been developed for the therapy of different cancers. However, not substantial benefit and survival has been observed in pancreatic cancer clinical trials phase III with the use of anti-angiogenic agents, considering their high treatment costs as well[21,35,36].

Tumor suppressor genes

The main goal of this strategy is to induce the activation of a specific tumor suppressor gene, which is not expressed anymore during tumor growth[20]. The *p53* gene is one of the widely known tumor suppressor genes, which encodes TP53. TP53 protein is a particularly important tumor suppressor having a critical role in cancer development and therapy. It provides fundamental functions in cellular responses in various stressors and is responsible for the control of apoptotic cells entry (mutated in 50% of human tumors)[20,37]. Strategies based on gene transfer of TP53 gene have been utilized for therapy of various cancers (*e.g.* colon and liver cancer)[20]. Experimental studies have demonstrated that *p53* gene transfer *via* an AD vector, suppresses human pancreatic cancer cells *in vitro*[38]. Furthermore, intraperitoneal administration of the p53 retroviral vector in nude mice, inhibited significantly pancreatic tumor growth compared with the control group[39].

Suicide genes

Suicide gene therapy is based on the transduction of tumor cells with a gene encoding specific enzymes capable of converting nontoxic prodrugs into toxic metabolites[21]. One of the most-well known suicide gene strategies is the herpes simplex virus thymidine kinase gene (*HSV-TK* gene). This gene codes the thymidine kinase enzyme

Table 1 Targeted therapeutic and pre-clinical strategies tested for gene therapy in pancreatic cancer

Therapeutic strategy	Gene examples
Gene transfer	Anti-angiogenic genes (*VEGF*, *angiostatin*, *endostatin*, *thrombostatin etc.*)
	Tumor suppressor genes (*p53*)
	Suicide genes (*HSV-TK*, *Cytochrome P40*)
RNA therapy	miRNA (miR-21), ShRNA, siRNA and antisense oligonucleotides (ISIS-2503 and AEG35156)
Gene editing technology	CRISPR-Cas9
Active immunotherapy	Cytokine expression
	Interleukin expression
Adoptive immunotherapy	Peptide, pulsed dendritic cells DNA, bacteria and engineered cells
Vaccination	PLD-1 and CTLA-4 inhibitors
	CAR-T cells (targeting MUC-1)

VEGF: Vascular endothelial growth factor; *HSV-TK*: Herpes simplex virus thymidine kinase gene; CRISPR-Cas9: Clustered regularly interspaced palindromic repeats-Cas9; PLD-1: Phospholipase D1; CTLA-4: Cytotoxic T-lymphocyte antigen 4; CAR-T: chimeric antigen receptor T- cell; MUC-1: Mucin 1, cell surface associated.

responsible for the conversion of ganciclovir an antiviral drug with no antitumor toxicity to a toxic metabolite capable of interfering DNA replication and inhibiting DNA synthesis resulting in cell death[40,41]. Its therapeutic effect is based on a "by-standard effect" in which tumor cells transduced with HSV-TK causing toxicity to neighbouring tumor cells (unmodified)[21]. HSV-TK delivery *via* adenovirus and retrovirus have shown great anti-tumor efficiency in pancreatic cells both *in vitro* and *in vivo*[41,42]. Moreover, experimental studies have shown that the combination of retrovirus and adenovirus delivery of HSV-TK is more effective in tumor growth inhibition compared to single delivery *in vivo*[43].

Other examples of suicide gene strategies successfully tested *in vivo* (PDAC models) include: the nitroreductase gene responsible for the transformation of CB1954 into 4-hydroxylamine, cytosine deaminase gene responsible for the transformation 5-flurocytosine into 5-fluoroucacil and *cytochrome P40* gene responsible for the transformation of ifosfamide into acrolein[20,21,44]. The cytochrome P450/ifosfamide system proof of concept from pre-clinical studies has been used to conduct phase I and II clinical trials in pancreatic cancer patients with phenomenal success (improvement of 1-year survival by 3-fold and median survival doubled)[20,45].

RNA THERAPY

Another strategy besides the gene transfer with vectors is the RNA interference, which is based on post-transcriptional inhibition of gene expression *via* two groups of small non-coding RNAs: miRNAs and interfering RNAs including shRNAs and siRNAS[2,20]. miRNAs are a family of single stranded RNAs consisting of 21-24 ribonucleotides, which are usually transcribed *via* RNA polymerase II and act by repressing protein production by translational silencing[46]. Loss of their expression may result in important dysfunctions during carcinogenesis. Studies of mRNA expression in a large scale (microRNome) helped to better understand their role in cancer development and their respective mechanisms responsible for these dysfunctions. These miRNAs known as oncoMIR are able to be regulated through different gene therapy approaches (Figure 3)[20]. An experimental study has shown that targeting the oncogenic miRNA-21 could suppress tumor growth in pancreatic cancer *in vitro* and *in vivo*[47]. Many miRNAs have a critical role in PDAC growth process *via* the regulation of important pathways, such as targeting Kirsten rat sarcoma viral oncogene homolog mutation. However, no miRNA therapeutics have been tested clinically for pancreatic cancer treatment[48].

On the contrary to miRNAs, siRNAs are commonly used in a variety of clinical applications because of their lack of genomic integration and more simplistic sequencing. They are double-stranded RNA molecules (20-30 nucleotides in length),

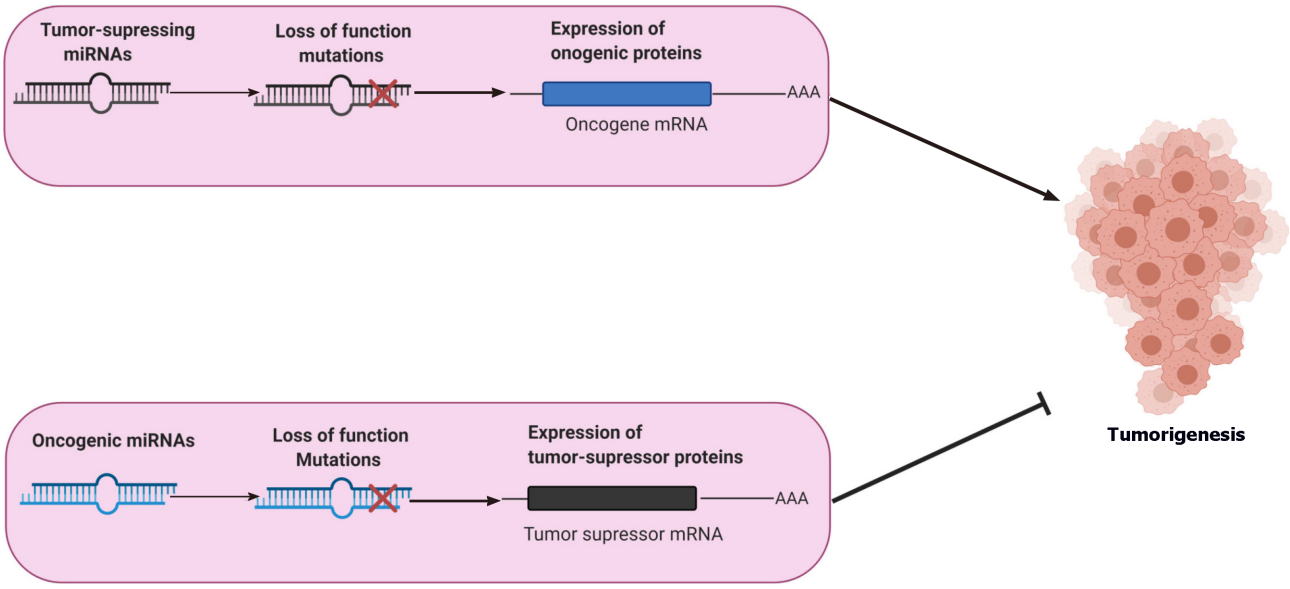

Figure 3 Roles of miRNAs in the regulation of tumorigenesis. miRNAs are essential for the regulation of tumorigenesis. The primary role of tumor-suppressing miRNAs is to suppress the expression of oncogenes, thus inhibiting tumorigenesis whereas oncogenic miRNAs (oncoMIR) block translation of tumor suppressor genes inducing tumorigenesis and, thus tumor formation. Loss of function (mutations) in tumor-suppressing miRNAs induce tumorigenesis (increased expression of oncogenic proteins). However, loss of function (mutations) in oncogenic miRNAs (increased expression of tumor-suppressor mRNA) results in the suppression of tumorigenesis. The above diagram was created with Biorender.com.

which like miRNAs inhibit translation resulting in gene silencing[49]. One of the most essential features of RNA interference (RNAi) technology is the small size of payload increasing the number of drugs delivered to the tumor of choice[50]. In an experimental study based on antitumor activity of drug-loaded polymeric micelles in pancreatic cancer, it was demonstrated that particles with a size of 30 nm or less, were able to penetrate poorly permeable pancreatic tumors, resulting in high antitumor activity[51]. In addition, conjugate siRNAs studies in pancreatic cancer have shown promising results in the clinic. One example is the liposomal conjugate of the drug Atu027 (siRNA-targeting protein kinase 3) which have enhanced tumor penetration [52,53].

In the same category of interfering RNA is the antisense strategy. This strategy is based on the synthesis of nucleic acid strands capable of interacting with the mRNA of the expressed target gene resulting in its inactivation. Furthermore, it is possible to affect the pre-RNA splicing, thus disrupting the mRNA exon content[20]. More specifically, antisense oligonucleotides (ASOs) are single-stranded nucleotide sequences that have been modified at their phosphodiester backbone allowing to protect them from degradation, thus facilitating cell entry[54]. ASOs have been tested clinically in targeting pancreatic cancer. One example is the ISIS-2503 and AEG35156 oligonucleotides. ISIS-2503 is an ASO targeting h-ras (important for pancreatic tumor progression) and AEG35156 synthesized for targeting X-linked inhibitor of apoptosis protein, an anti-apoptic protein overexpressed in pancreatic cancer. Both of these ASOs have been tested in clinical phase trials but with no real success due to their low clinical benefit[55-57].

GENE EDITING TECHNOLOGY

A recent and more sophisticated strategy is genome editing technology including a few techniques for genome manipulation by "rewriting genetic material". Clustered regularly interspaced palindromic repeats-Cas9 (CRISPR-Cas9) system has been given a lot of attention over the years due to its broad range of therapeutic applications. This system offers the ability to induce the cleavage of specific double-stranded DNA segments from the genome *via* the introduction of CRISPR sequences, marking cleavage sites through Cas9 protein endonucleases[58]. This strategy has been used for the therapeutic approach of pulmonary cancer, as well as HIV infection. Gene transfer of this system has also been investigated in pancreatic cancer models *in vivo*[59,60]. Current research is focused on utilizing CRISPR-Cas9 for screening of new gene

targets and to investigate different mechanisms of known genetic aberrations[61]. CRISPR-Cas9 cell therapies are currently tested in the clinical settings for various cancers and immunologic syndromes. CRISPR-Cas9 approach have been demonstrated to be also applicable to inflammatory conditions such as lupus nephritis [62]. However, significant challenges remain, including its efficiency of transduction, compared for example with oncolytic viruses[63].

IMMUNOTHERAPY AND VACCINATION

Tumor cells are able of escaping the recognition and elimination from the immune system resulting in aberrant tumor development and aggressiveness in certain cancers [20,21]. Even though immune cells are found in large numbers within the tumor stroma, they mostly correspond to immunosuppressive subsets such as T-helper 17 cells, regulatory T cells and tumor-associated macrophages (TAMs). Effectors T-cells located in the tumor are quite rare in respect with other tumors and express immune checkpoint receptors such as PD-1, at extremely high levels. PDAC induces a pro-tumoral inflammation and immunosuppressive environment. Furthermore, PDAC cells have a particular role due to paracrine signaling induced by cytokines such as IL-6 and GM-CSF[64,65]. Cancer cells also express checkpoint molecules such as programmed cell death 1 Ligand 1 (PD-L1). A small number of studies have been conducted in pancreatic cancer targeting these immune checkpoint inhibitors. Both PD-L1 and cytotoxic T-lymphocyte associated protein 4 inhibitors have been investigated in patients with metastatic or locally advanced pancreatic cancer in two clinical trials. However, the clinical outcomes were not as expected, with no clinical benefit[66, 67].

Cancer immunotherapy has been developed over the years to improve immune intolerance using active and passive immunity. The first approach is based on the stimulation of immune response to tumor-associated antigens through cancer vaccines. The second approach is based on administering activated effector cells, cytokines or monoclonal antibodies targeting specific tumor cells[21]. To prevent any side effects gene therapies have been evolved to promote local cytokine or interleukin either by *ex-vivo* approach or by the tumor cells themselves (Figure 4). Another promising approach for pancreatic cancer therapy is chimeric antigen receptor T-cell therapy. This approach uses patient's own T cells to target cancer cells *via* genetic engineering. The remarkable results observed in haematological malignancies have moved the attention further to solid tumors like pancreatic cancer[68]. In pancreatic cancer, antigens which are widely expressed are mesothelin and mucin 1 that are cell surface associated. Vaccine experiments have been conducted *in vivo* in various pancreatic cancer models using antigen-pulsed dendritic cells[69].

CONCLUSION

It is more than clear that pancreatic cancer is considered more than a challenging diagnosis, given the fact that the current treatment protocols provide a slight survival expectation. Nevertheless, research on gene therapy has been taking place unexpectedly increasingly, offering a new promising opportunity not only to tackle with an inoperable pancreatic disease, but also, to treat effectively early stages of PDAC. Pancreatic cancer gene therapy with oncolytic virotherapy and adoptive immunotherapy seem to be the cutting-edge therapeutic technology approach with exciting new breakthroughs in recent years. Focusing on vector development and methods to enhance the selectivity of either gene delivery or gene expression in combination with conventional chemotherapeutic agents (*e.g.* gemcitabine) would enhance the therapeutic benefit in human, either metastatic or non, pancreatic cancer model despite its high degree of complexity. Eventually, it seems that there is enough room for improvement in the treatment of pancreatic cancer. However, further clinical trials are considered of crucial importance testing the efficacy, efficiency, and safety of these new gene therapies.

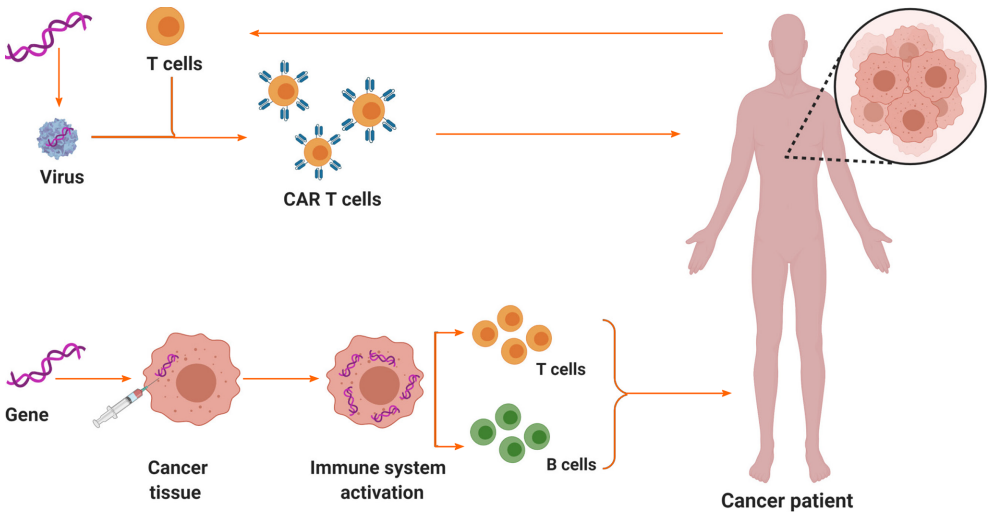

Figure 4 Schematic diagrams of two examples of immunotherapy for pancreatic cancer: chimeric antigen receptor T-cell therapy and immunogenic therapy (*in vivo* gene transfer). The above diagram was created with Biorender.com. CAR T-cell: Chimeric antigen receptor T-cell.

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ORIGINAL ARTICLE

Basic Study

Fasudil prevents liver fibrosis *via* activating natural killer cells and suppressing hepatic stellate cells

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Abstract

BACKGROUND

Fasudil, as a Ras homology family member A (RhoA) kinase inhibitor, is used to improve brain microcirculation and promote nerve regeneration clinically. Increasing evidence shows that Rho-kinase inhibition could improve liver fibrosis.

AIM

To evaluate the anti-fibrotic effects of Fasudil in a mouse model of liver fibrosis induced by thioacetamide (TAA).

METHODS

C57BL/6 mice were administered TAA once every 3 d for 12 times. At 1 wk after induction with TAA, Fasudil was intraperitoneally injected once a day for 3 wk, followed by hematoxylin and eosin staining, sirius red staining, western blotting, and quantitative polymerase chain reaction (qPCR), and immune cell activation was assayed by fluorescence-activated cell sorting. Furthermore, the effects of Fasudil on hepatic stellate cells and natural killer (NK) cells were assayed *in vitro*.

RESULTS

First, we found that TAA-induced liver injury was protected, and the positive area of sirius red staining and type I collagen deposition were significantly decreased by Fasudil treatment. Furthermore, western blot and qPCR assays showed that the levels of alpha smooth muscle actin (α-SMA), matrix metalloproteinase 2 (MMP-2), MMP-9, and transforming growth factor beta 1 (TGF-β1) were inhibited by Fasudil. Moreover, flow cytometry analysis revealed that NK cells were activated by Fasudil treatment in *vivo* and *in vitro*. Furthermore, Fasudil directly promoted the apoptosis and inhibited the proliferation of hepatic stellate

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cells by decreasing α-SMA and TGF-β1.

CONCLUSION
Fasudil inhibits liver fibrosis by activating NK cells and blocking hepatic stellate cell activation, thereby providing a feasible solution for the clinical treatment of liver fibrosis.

Key Words: Liver fibrosis; Natural killer cells; Fasudil; Hepatic stellate cells

Core Tip: Liver fibrosis is caused by inflammation and characterized by accumulation of the extracellular matrix; there is no clinically safe and efficient drug to treat this condition. Fasudil treatment inhibited liver injury and liver fibrosis *in vivo*, and prevented liver fibrosis *via* activating natural killer cells but suppressing hepatic stellate cells in a thioacetamide-induced model. As a drug used clinically, these results provide a feasible solution for the clinical treatment of liver fibrosis.

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INTRODUCTION

Liver fibrosis is caused by inflammation and characterized by the accumulation of extracellular matrix (ECM), which are major characteristics of chronic liver diseases such as cirrhosis and primary hepatic carcinoma[1]. The traditional view is that liver fibrosis is irreversible, but recent studies have shown that liver fibrosis can be partially reversed in experimental models of liver fibrosis[2]. Unfortunately, to date, there is no clinically safe and efficient drug to treat liver fibrosis in humans.

The mechanisms underlying hepatic fibrosis are inflammatory reaction, hepatic stellate cell (HSC) activation, and the biological function of various cytokines[3]. HSCs, as hepatic lipid-storing cells, are sensitive to hepatic injury and participate in the pathogenesis of liver fibrosis[4]. During chronic diseases, agents from damaged hepatocytes or other cells can promote the activation of HSCs. Then these activated HSCs become highly proliferative myofibroblast-like cells, exhibiting a migratory phenotype and producing large amounts of extracellular matrix proteins such as collagens and fibronectin, thereby leading to the development of hepatic fibrosis[5,6]. Therefore, HSC-specific targeted strategies are required for the effective treatment of liver fibrosis.

Immune cells in the liver exert different roles during the pathogenesis of liver fibrosis. Hepatic macrophages promote fibrosis by perpetuating an inflammatory phase, which leads to the release of pro-inflammatory cytokines and chemokines, and then the activation of HSCs[7]. T helper type 2 (Th2) cells can produce cytokines, including interleukin (IL)-13, IL-4, and IL-5, which are shown to promote fibrosis[8]. Natural killer (NK) cells suppress the pathogenesis of liver fibrosis by killing HSCs and producing interferon-γ (IFN-γ). Also, NK cells can induce apoptosis of HSCs and help clear senescent-activated HSCs, thereby facilitating the resolution of fibrosis, while the exact mechanisms remain unknown. NK cells are suppressed during the advanced stage of liver fibrosis in mice and patients[9,10]. Thus, properly regulating the activation of intrahepatic immune cells is essential for treating liver fibrosis. Practically, the restoration and promotion of NK cell activity might be an attractive strategy for the regression of liver fibrosis.

Ras homology family member A (RhoA) and its major downstream effector Rho-associated protein kinase (ROCK) play important roles in several downstream effects of the small GTP-binding protein Rho. Several cellular events including adhesion, motility, and contractility are regulated by Rho kinase[11]. Furthermore, increasing evidence has demonstrated that Rho kinase inhibition can improve liver fibrosis. The

Rho kinase, Y27632, decreases fibrotic parameters in models of liver fibrosis, and inhibits the activation status of the primary HSCs[12]. Additionally, RhoA-ROCK signaling pathway also plays a key role in regulating the function of immune cells. For example, the RhoA-ROCK pathway promotes the activation of downstream chemokine receptors. Therefore, T-cell activation, polarization, and migration are promoted by chemokines[13]. Fasudil, as a first-generation selective Rho/ROCK inhibitor in the clinic, is clinically used to improve brain microcirculation and promote nerve regeneration[14]. More importantly, Fasudil can prevent lung and skin fibroses in hypochlorous acid-injected mice[15]. Recently, Fasudil was shown to exert anti-inflammatory effects and markedly reduce the accumulation of ECM in type 1 diabetic rats[16]. However, the viability of Fasudil for liver fibrosis therapy and the associated mechanisms are still unclear. The regulatory effects of Fasudil on NK cells in liver fibrosis have not been fully explained.

In this study, we determined the effects of Fasudil on the progression of liver fibrosis and clarified the related mechanisms. We found that Fasudil performed anti-proliferative effects in a mouse model of thioacetamide (TAA)-induced liver fibrosis, providing a feasible solution for the clinical treatment of liver fibrosis.

MATERIALS AND METHODS

Animal model

C57BL/6 mice (male, 4-6 wk old) were provided by HuaFuKang Biological Technology Co., Ltd. (Beijing, China). The animals were caged under specific pathogen-free conditions, housed under a controlled temperature 23 ± 1°C and relative humidity 45%. The animal model of hepatic fibrosis was induced by administration of TAA at 200 mg/kg (T104039; Aladdin, Shanghai, China) once every 3 d for 12 times [17]. At 1 wk after induction with TAA, Fasudil (10 mg/kg) was intraperitoneally injected once a day for 3 wk. The procedures were approved by the Research Ethics Committee of Shandong University (Jinan, China).

Cell line and reagents

LX-2, an immortalized human hepatic stellate cell line preserved in our laboratory, was grown in Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Inc., Waltham, MA, United States). This cell line carries a typical phenotype and biochemical properties of activated HSCs, and was checked by referring to the International Cell Line Authentication Committee and National Center for Biotechnology Information databases[18]. Fasudil was purchased from Tianjin Chase Sun Pharmaceutical Co. Ltd. (Tianjin Shi, China).

Hematoxylin and eosin staining

In a TAA-induced hepatic fibrosis model, the mice were sacrificed under mild ether anesthesia after Fasudil treatment. Then mouse liver specimens were fixed overnight in 4% paraformaldehyde/phosphate-buffered saline (PBS) and embedded in paraffin. Then liver sections were examined and photographed under a light microscope after hematoxylin and eosin (Beyotime, Shanghai, China) staining. The criteria used for scoring fibrosis and inflammation were as follows: Score 0, normal (no visible fibrosis and inflammation); score 1, fibrosis and inflammation present (5%-30%); score 2, mild fibrosis and inflammation (31%-50%); and score 3, severe fibrosis and inflammation (51%-75%).

Sirius red staining

Liver specimens were stained with 0.4% sirius red in saturated picric acid for 0.5 h. The positively stained area was selected by threshold adjustment on a gray scale picture using Image J software (NIH, Bethesda, MD, United States) and the ratio of positively stained area/total area was then calculated. At least five different fields on each slide were measured. The stained area were determined using Image-pro Plus 6.0 software.

RNA extraction and real-time reverse transcription-polymerase chain reaction

The treated cells were added to TRIzol (Invitrogen, Thermo Fisher Scientific, Inc.), and the total RNA was extracted from cells following the manufacturer's instructions. Then cDNA was obtained and synthesized using M-MLV Reverse Transcriptase (Invitrogen; Thermo Fisher scientific, Inc.). The mRNA levels of matrix metalloproteinase 2 (MMP-

2), MMP-9, transforming growth factor beta (TGF-β), B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and Ki67 were detected according to the SYBR Green Master Mix kit instructions (Roche Diagnostics, Indianapolis, IN, United States). The primer sequences for quantitative polymerase chain reaction (qPCR) amplification are listed in Table 1. PCR was conducted using the iCycleriQ real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, United States).

Cell cycle analysis and apoptosis assay

After treatment, the cell cycle distribution of HSCs seeded was assayed by flow cytometry. Briefly, HSCs (1.5×10^5) were plated in 12-well plates and treated with Fasudil at the indicated concentrations (5 and 10 mM). After 24 h, cells were harvested and washed with ice-cold PBS. Then they were fixed in cold 70% ethanol and stored at 4°C overnight, and the fixed cells were washed and re-suspended in 1 mL staining solution containing 50 mg/mL propidium iodide (PI) (Beijing Solarbio Science & Technology, Co., Ltd.) and 100 mg/mL RNase for 30 min at 37°C. Then cell cycle analysis was conducted using a flow cytometer (BD Calibur).

FITC-Annexin V/PI (eBioscience; Thermo Fisher Scientific, Inc.) was utilized to determine rates of apoptosis. LX2 cells were plated and treated with Fasudil (5 mM, 10 mM) for 24 h. Cells were harvested and stained following the manufacturer's instructions. Finally, the flow cytometry data were analyzed using WinMDI.

Western blotting

Protein was extracted from LX-2 cells, NK-92 cells, or liver tissues with RIPA protein lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) (Beyotime, Shanghai, China). The protein concentration of each sample was determined by the BCA assay. Proteins (30 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel, and then transferred to PVDF membranes (Millipore, Billerica, MA, United States). The following primary antibodies were used: Anti-phosphorylated extracellular signal-related kinase (p-ERK) (#14227S; Cell Signaling Technology, Danvers, MA, USA), anti-ERK (#4348S, Cell signaling Technology), alpha smooth muscle actin (α-SMA) (#4668S; Cell signaling Technology), Collagen I (WL008; Wanleibio Co., Ltd., Shenyang, China), and β-actin (#3700; Cell signaling Technology). For ECL detection, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (#A0208; Beyotime, Shanghai, China) at a dilution of 1:10000 for 1 h at 37°C. ECL detection was performed according to the manufacturer's protocol (Millipore). Analysis of blots was performed using Image J.

Isolation of liver mononuclear cells and flow cytometry

Mice were euthanized and the liver tissues were harvested, and then intrahepatic liver mononuclear cells were isolated as indicated previously[19]. Antibodies used for fluorescence-activated cell sorting (FACS) analysis were PerCP-Cy5.5 anti-cluster of differentiation 3 (CD3) (#35-0031-82), FITC anti-NK1.1 (#11-5941-82), PE anti-CD69 (#12-0691-82), PE anti-NKG2A (#12-5897-81), PE anti-NKG2D (#12-5882-82), or isotype-matched controls (eBioscience, San Diego, CA, United States). Cells were analyzed on a FACSCalibur flow cytometer, and then analyzed using WinMDI analysis software.

Cytolysis assay

The cytolytic activity of NK-92 cells against LX2 was determined using the MTT assay. Briefly, 1×10^4 target cells (LX2 cells) were seeded into 96-well plates. NK-92 cells, pretreated with Fasudil (10 mM) overnight, were added to target cells at effector/target ratios of 1:20, 1:10, or 1:5. Then the effector and target cell were co-incubated for 12 h, and then 20 μL MTT (5 mg/mL) were added. After 4 h, the absorbance (A) at 490 nm was determined using a scanning multi-well spectrophotometer (Bio-Rad). The percent specific lysis was calculated using the following formula: % specific lysis $=1-(OD_{E+T}-OD_E)/OD_T \times 100\%$.

Statistical analyses

Data are shown as mean ± SD of three independent experiments. Significant differences were analyzed using the Student's *t*-test or one-way analysis of variance. Statistical analyses were conducted using GraphPad Prism (version 5.0a). $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

Table 1 Primers used in this study

Species	Gene	Primer (5' to 3')
Mouse	β-actin	F: ATCATGTTTGAGACCTTCAACA
		R: CATCTCTTGCTCGAAGTCCA
Mouse	MMP2	F: CTCCTGGTATGAGATAGCAAA
		R: TGCACCACCAACTGCTTAGC
Mouse	MMP9	F: TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC
		R: TGGACCTGTGGGTTGTTGACCTCAAACTTGGC
Mouse	α-SMA	F: CTGACAGAGGCACCACTGAA
		R: GAAGGAATAGCCACGCTCAG
Human	α-SMA	F: GATCTCAGTGCAGAGGCTCG
		R: TTTGCTTGTCCAGGTGGTCC
Human	Ki67	F: TCCTCCAGGGATCCAACGA
		R: GGCAGGCGGGAGGTCTT
Human	Bcl-2	F: CTTCCCTCATCCTCCTGCTAC
		R: ACAAACTGGGTAAAGGTGATGG
Human	Bax	F: CTCCTGGTATGAGATAGCAAA
		R: TGCACCACCAACTGCTTAGC
Human	Rho	F: TCCTCCAGGGATCCAACGA
		R: GGCAGGCGGGAGGTCTT
Human	β-actin	F: CACTGTGTTGGCGTACAGGT
		R: TCATCACCATTGGCAATGAG

Note: α-SMA, alpha smooth muscle actin; β-actin, beta actin; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; MMP, matrix metalloproteinase; Rho, Ras homology family member

RESULTS

Fasudil protects TAA-induced liver injury

To clarify whether Fasudil influences hepatic fibrosis, a fibrotic mouse model was induced by intraperitoneal injection of TAA (200 mg/mL) once time every 3 d[11]. At 7 d after induction with TAA, Fasudil (10 mg/kg) was injected once a day for 3 wk, and then mice were sacrificed (Figure 1A). As shown in Figure 1B, compared with the normal gross features of the livers observed in the control group, the liver surface from the TAA-treated group showed several spots of nodules, while the livers from the TAA + Fasudil group had much fewer nodular spots. There was no significant difference in liver/body ratio among these three groups. Meanwhile, the liver of the TAA-treated group exhibited a lobular structure destruction and inflammatory cell infiltration. Also, livers from TAA-treated mice gained loss of structural integrity, while TAA + Fasudil-treated mice revealed structural integrity of liver with no significant inflammatory cell infiltration (Figure 1C). Fasudil significantly lowered the indices of liver (Table 2). Furthermore, we observed a significant reduction of alanine aminotransferase and aspartate aminotransferase in serum after Fasudil treatment compared with TAA-treated mice ($P < 0.05$; Figure 1D). These data suggest that Fasudil might blunt TAA-induced liver fibrosis by preventing liver injury.

Fasudil prevents TAA-induced liver fibrosis in vivo

Hepatic collagen I deposition is an important characteristic of liver fibrosis level in mice[20]. As shown in Figure 2A, sirius red staining exhibited a significant increase in perisinusoidal collagen fibers in the TAA-treated group, which was significantly decreased by Fasudil treatment ($P < 0.05$). Furthermore, fibrosis-related genes such as α-SMA, MMP-2/9, and TGF-β1[21] were analyzed to evaluate the impact of Fasudil on hepatic fibrosis. As shown in Figure 2B, the expression of α-SMA and collagen I in

Table 2 Effect of Fasudil on the pathologic grading of hepatic fibrosis mice induce by thioacetamide

Group	Pathologic grading of hepatic fibrosis			
	0	I	II	III
Ctrl (n = 5)	15	0	0	0
TAA (n = 5)	0	0	6	7
TAA + Fasudil	0	7	6	2

Results are 15 fields of vision from five mice per group (three fields were chosen from one liver section). TAA: Thioacetamide.

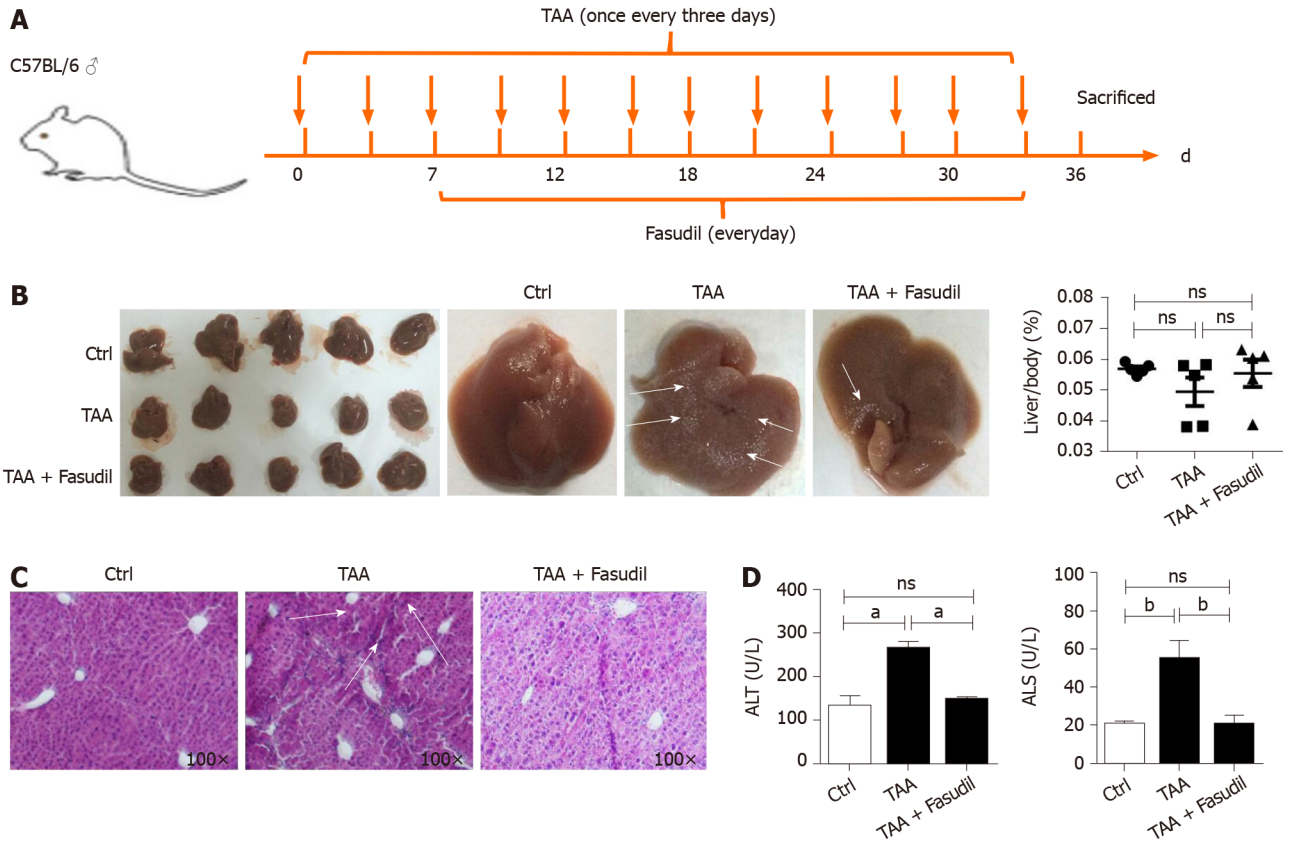

Figure 1 Fasudil protects thioacetamide-induced liver injury. A: Establishment of a liver fibrosis mouse model; B: Representative detection of liver images is shown. Gross pathological examination of liver taken from normal control, thioacetamide (TAA)-treated, and TAA + Fasudil-treated groups, and the liver/body ratio was assayed among these three groups; C: Histological observation of liver fibrosis by hematoxylin and eosin staining under light-field microscope with × 100 magnification; D: Enzyme-linked immunoassay was used to assess the level of serum alanine aminotransferase and aspartate aminotransferase. Data represent the mean ± SD from at least three independent experiments (n = 5/group). $^{a}P < 0.05$, $^{b}P < 0.01$.

TAA + Fasudil-treated mice was significantly lower than that in TAA-treated group. Meanwhile, mRNA levels of MMP-2 and MMP-9 were also downregulated by Fasudil treatment (Figure 2C). TGF-β1 induced by TAA was significantly decreased at both the mRNA and protein levels after Fasudil treatment ($P < 0.05$; Figure 2D). These data clearly demonstrate that TAA-induced liver fibrogenesis is markedly inhibited by Fasudil treatment.

NK cells are activated by Fasudil in a TAA-induced fibrotic mouse model

Immune cells in the liver play critical roles during the development of liver fibrosis [22]. As shown in Figure 3A, TAA treatment increased the total number of mononuclear cells in the liver compared to the normal control group, while no significant differences were observed between TAA-treated and TAA + Fasudil-treated groups. NK cells suppress liver fibrosis *via* strong cytolysis against HSCs[23, 24], and impaired NK cell may contribute to accelerated liver fibrosis progression in a TAA-induced fibrotic mouse model. Here, the proportion of NK cells was not

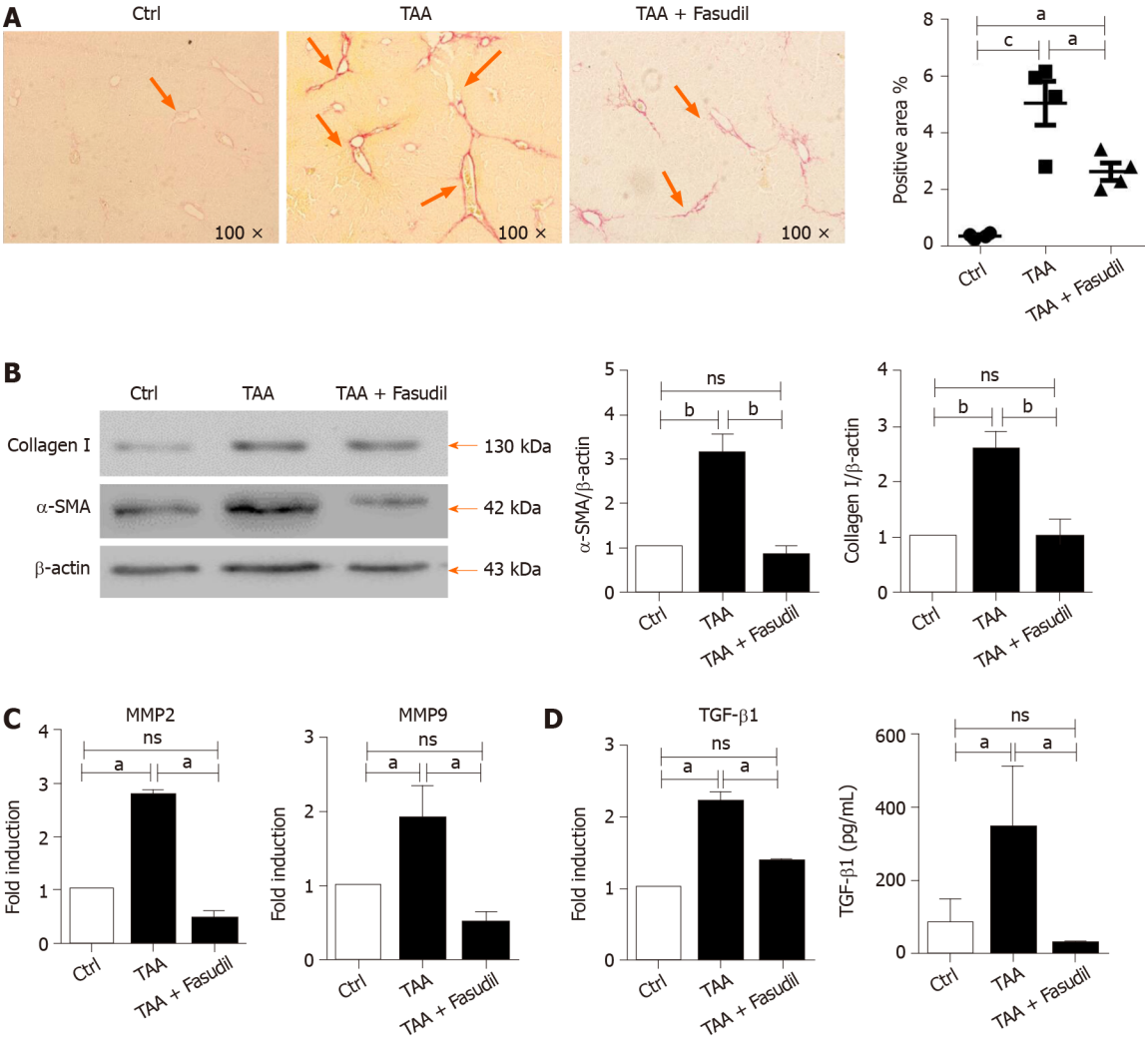

Figure 2 Fasudil prevents thioacetamide-induced liver fibrosis. A: Histological observation of collagen deposition by sirius red staining under a light-field microscope with × 100 magnification (left). Quantitative analysis of sirius red staining of each group (right); B: Expression of alpha smooth muscle actin (a-SMA) and collagen I in liver tissues was assayed by western blotting. Right panel shows the quantification of α-SMA and collagen I protein levels; C: Levels of matrix metalloproteinase 2 (MMP2) and MMP9 mRNA in liver tissues were detected by real time-reverse transcription polymerase chain reaction (RT-PCR); D: Hepatic expression of transforming growth factor beta 1 (TGF-β1) mRNA was detected by real time-RT-PCR, the secretion level of TGF-β1 in serum was assayed by enzyme-linked immunoassay. Data represent the mean ± SD from at least three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$.

significantly affected after Fasudil treatment (Figure 3B), while the level of the activation marker CD69 was markedly increased in NK cells from TAA + Fasudil-treated mice than that from TAA-treated mice ($P < 0.05$; Figure 3C), accompanied by the elevation of activation receptor NKG2D (Figure 3D). Meanwhile, the proportion of $CD69^{+}CD8^{+}$T cells (Figure 3E) and $CD69^{+}CD4^{+}$T cells (Figure 3F) in the liver was not significantly affected by Fasudil treatment. These data suggest that NK cells might be activated by Fasudil and exhibit anti-liver fibrosis effects in a TAA-induced fibrotic mouse model.

Fasudil promotes NK cell activation via ERK and nuclear factor kappa B signaling pathways

To investigate the mechanism by which Fasudil treatment suppresses the progression of liver fibrosis by activating NK cells, human NK-92 cells were treated with different concentrations of Fasudil (5 mM, 10 mM) for 24 h *in vitro*, and the concentration of 10 mM was equivalent to *in vivo* experiments. Then the levels of ERK, nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) were examined by western blotting. The levels of p-ERK and p-NF-kB were significantly increased in Fasudil-treated NK-92 cells, while activation of the STAT3 signaling pathway was not affected by Fasudil (Figure 4A). To determine the effects of NK cells activated by Fasudil on HSCs, we assayed the lysis of LX2 cells (human HSC cell line) by NK cells at different effector: target ratios. First, we treated NK-92 cells with Fasudil

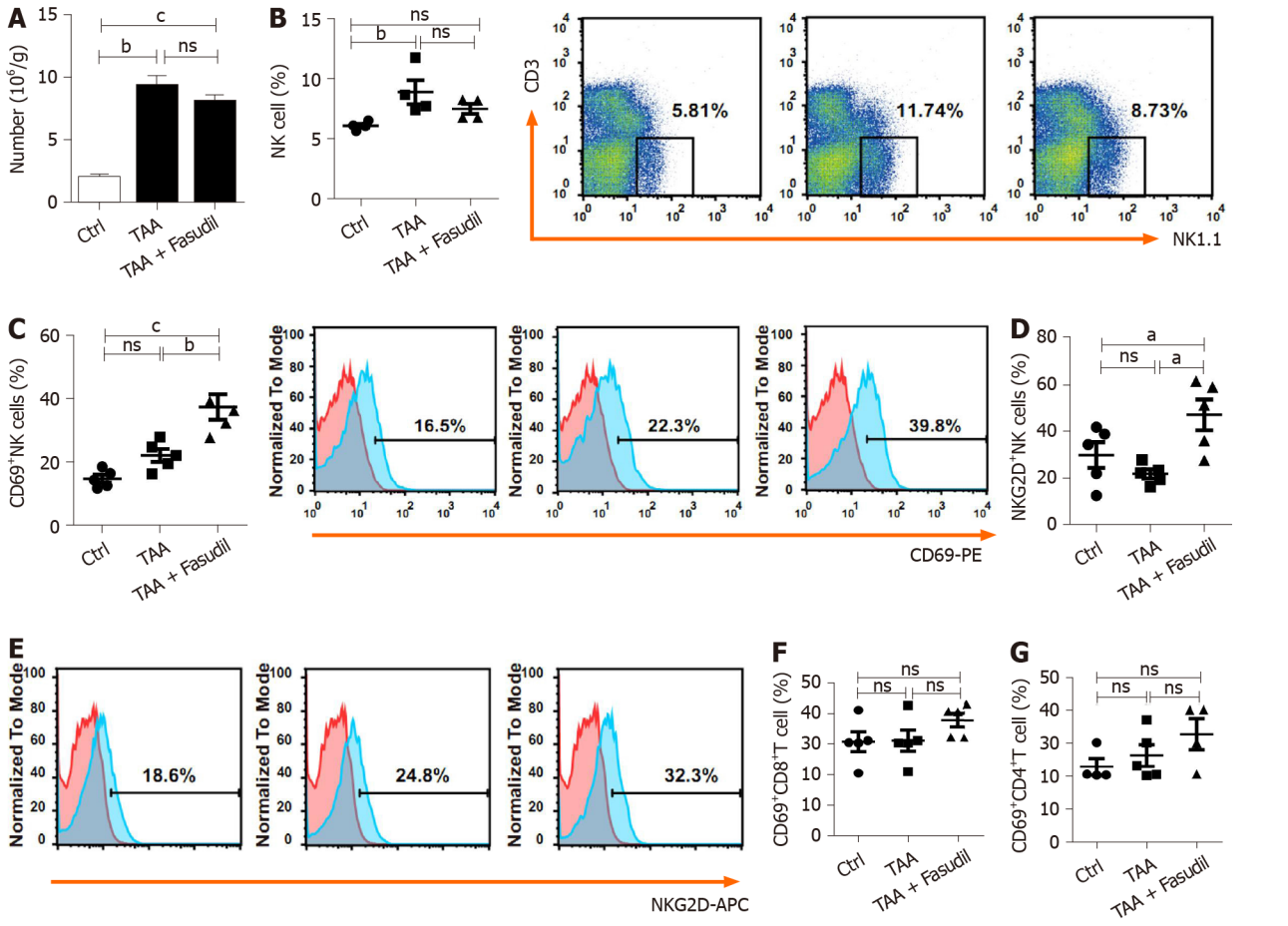

Figure 3 Natural killer cells are activated in a Fasudil-treated liver fibrotic mouse model. After the last treatment, the mice were sacrificed and the intrahepatic lymphocytes were isolated. A: Absolute number of mononuclear cells in the liver from the normal control, thioacetamide (TAA)-induced, and TAA + Fasudil groups was assessed; B-G: Then the percentages and representative fluorescence-activated cell sorting (FACS) plots of natural killer (NK) cells among lymphocytes (B), $CD69^+$ NK cells and representative FACS plots (C), quantification of $NKG2D^+$ NK cells (D), representative FACS plots of NKG2D on NK cells (E), $CD69^+CD8^+$ T cells (F) and $CD69^+CD4^+$ T cells (G) in liver from the above three groups were analyzed by FACS. Data represent the mean ± SD from at least three independent experiments (n = 5/group). $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

(10 mM) for 24 h, and assayed the cytotoxicity against cultured LX2 cells. We found that Fasudil treatment increased the lysis activity of NK cells against cultured LX2 cells (Figure 4B). Then, liver mononuclear cells from normal mice were isolated and treated with different concentrations of Fasudil for 24 h *in vitro*. FACS analysis showed the frequency of $CD69^+$ NK cells increased after Fasudil treatment (Figure 4C). Furthermore, the activation receptor NKG2D was enhanced in NK cells, while the inhibitory receptor NKG2A was not significantly influenced by Fasudil treatment (Figure 4D). In addition, the proportion of IFN-γ^+ NK cells was increased after Fasudil treatment (Figure 4E).

Taken together, these results demonstrate that Fasudil can promote NK cell activation and cytotoxicity by activating the ERK and NF-κB signaling pathways.

Fasudil inhibits proliferation but promotes the apoptosis of HSCs

The proliferation and activation of HSCs are one of the main triggers of liver fibrosis [25]. To observe the direct effects of Fasudil on HSCs, LX2 cells were treated with different concentrations of Fasudil (5 mM, 10 mM) for 24 h *in vitro*, and then the survival of these HSCs was observed. As shown in Figure 5A, the percentage of apoptotic HSCs was promoted by Fasudil treatment. Meanwhile, the level of the anti-apoptotic gene Bcl-2 was decreased, while the level of the pro-apoptotic gene Bax was induced in LX2 cells treated with Fasudil (Figure 5B), accompanied by an increase in p-ERK level (Figure 5C). Furthermore, Fasudil arrested the HSC cell cycle in the G1 phase (Figure 5D). Accordingly, the proliferation-related gene Ki67 in LX2 cells was downregulated (Figure 5E). Previous studies have reported that RhoA is involved in expression of the fibrosis-associated protein α-SMA[26]. Fasudil is a RhoA kinase inhibitor; we found that Fasudil decreased the expression of α-SMA (Figure 5E and F)

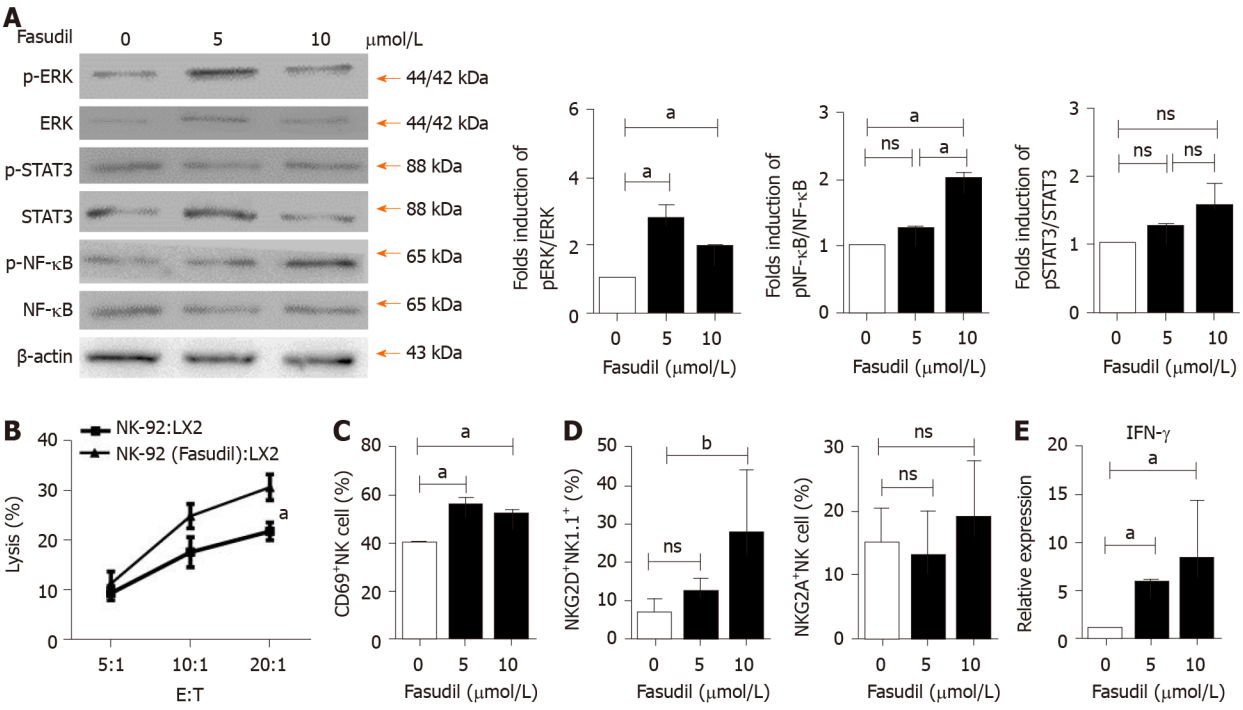

Figure 4 Fasudil can promote natural killer cell activation *via* extracellular signal-related kinase and nuclear factor kappa B signaling pathways. A: Natural killer (NK)-92 cells were treated with different concentrations of Fasudil (5 mM, 10 mM) for 24 h *in vitro*, and extracellular signal-related kinase (ERK), nuclear factor kappa B (NF-kB), and signal transducer and activator of transcription 3 (STAT3) levels were detected by western blotting. Then mononuclear cells from the livers of normal mice were isolated and then treated with Fasudil (5 mM, 10 mM) for 24 h *in vitro*; B: 1 × 10^4 LX2 cells were seeded into 96-well plates. NK-92 cells pretreated with Fasudil (10 mM) were added at different ratios (1:20, 1:10, or 1:5) for 12 h. Then the cytotoxicity was analyzed; C-E: Frequencies of $CD69^+$ NK cells (C), NKG2D NK cells, and NKG2A NK cells (D), as well as the proportion of IFN-γ-producing NK cells (D) were analyzed by FACS (E). Data represent the mean ± SD from at least three independent experiments. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

accompanied by the downregulation of RhoA (Figure 5G). These results indicate that Fasudil has a direct inhibitory effect on HSCs.

DISCUSSION

The current therapeutic interventions for liver fibrosis include removing the injurious stimuli, decreasing hepatic inflammation, suppressing HSC activation, and accelerating matrix degradation[27]. Repurposing old drugs for new clinical applications is a superior strategy for drug development. Recently, Rilpivirine, a widely used anti-HIV drug, was found to ameliorate liver fibrosis through selective STAT1-dependent induction of HSC apoptosis[28]. So, repositioning existing drugs might be an effective strategy for obtaining therapeutics against liver fibrosis.

Fasudil, a RhoA kinase inhibitor, is clinically applied for improving brain microcirculation and promoting nerve regeneration. Xie *et al*[16] found that RhoA/ROCK signaling pathway activation plays an important role in the development of diabetic hepatic fibrosis. Interestingly, the RhoA/ROCK-2 signaling pathway is necessary for activated HSC; the administration of RhoA/ROCK inhibitor can exert anti-inflammatory effects, markedly decrease collagen synthesis, and delay hepatic fibrosis[16, 29]. In accordance with this, we found that liver injury and liver fibrosis induced by repeated injection of TAA were prevented by Fasudil treatment (Figures 1 and 2). In addition, several studies have proved that RhoA/ROCK pathway activation promotes TGF-β1 secretion in several models[30]. As a RhoA/ROCK inhibitor, Fasudil inhibited the induction of TGF-β1 secretion in TAA-induced liver fibrosis, consistent with previous studies.

The immune response plays important roles in the development and treatment of liver fibrosis[31]. NK cells can promote the secretion of IFN-γ through the Janus kinase-STAT signaling pathway, thereby promoting the killing of HSCs[32]. The inhibition of NK cells cytotoxicity promotes the accumulation of LX2 cells[33]. In this study, with the elevation of CD69 and NKG2D, hepatic NK cell activation was promoted by Fasudil treatment (Figure 3). Additionally, NK cells were activated in

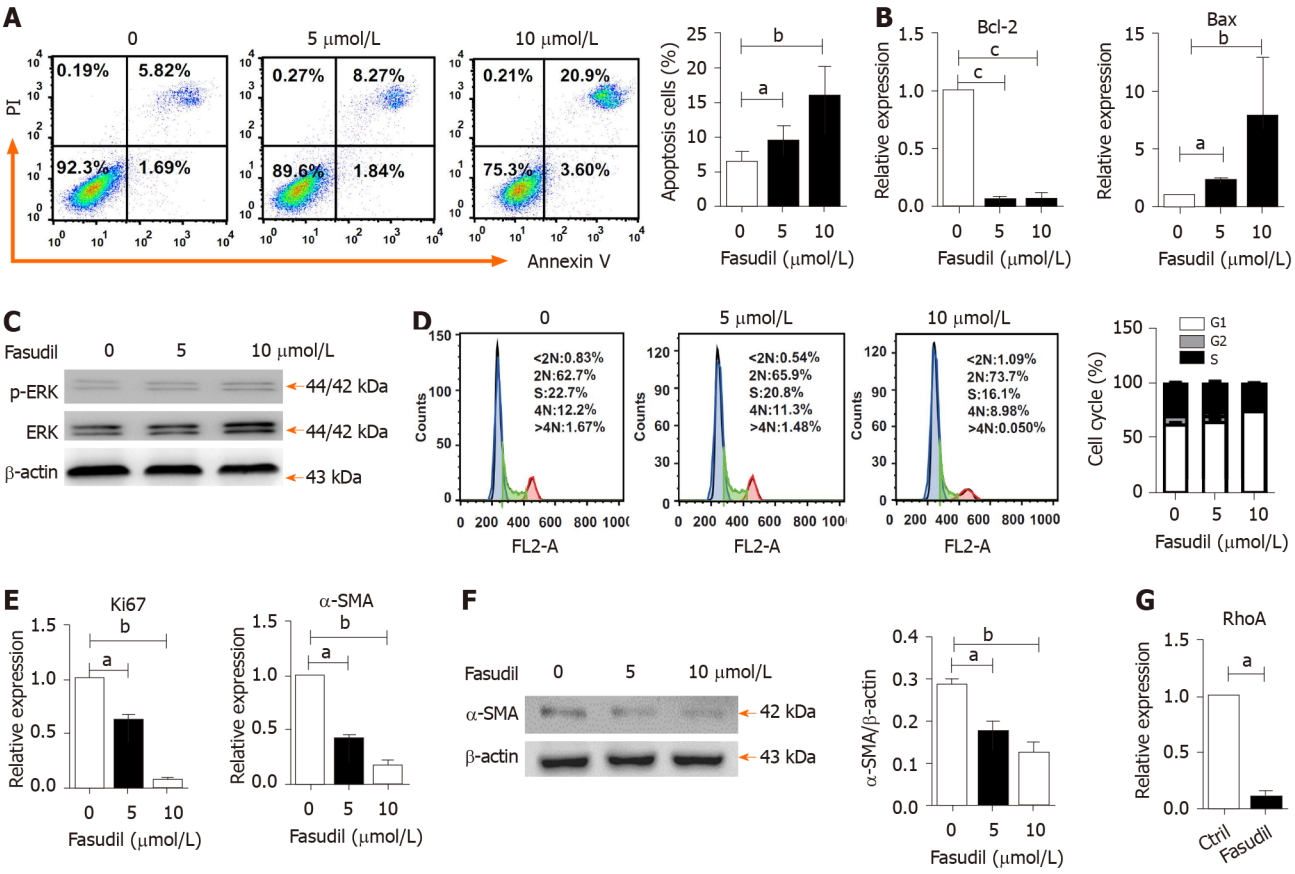

Figure 5 Fasudil inhibits the proliferation but promotes the apoptosis of hepatic stellate cells. A: LX2 cells were treated with different concentrations of Fasudil (5 mM, 10 mM) for 24 h *in vitro*; B: The apoptosis of LX2 cells was analyzed by fluorescence-activated cell sorting (FACS). mRNA levels of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) in LX2 cells were detected by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR); C: The phosphorylated extracellular signal-related kinase (ERK)/ERK levels in LX2 cells were detected by western blotting. The cell cycle of LX2 cells was analyzed by FACS; D: Left panel shows the representative FACS plots of cell cycle, and right panel shows the quantification of percentage of cells in G1, S and G2 phase; E: Relative expression level of Ki67 and alpha smooth muscle actin (α-SMA) mRNA in LX2 cells was analyzed by qRT-PCR; F: α-SMA level in LX2 cells was analyzed by western blotting; G: mRNA levels of Ras homology family member A in LX2 cells were analyzed by qRT-PCR. Data represent the mean ± SD from at least three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

spleen from mice with Fasudil treatment (data not shown). ERK and NF-κB signals are critical for NK cell-mediated cytolysis of target cells[34]. Here, we found that Fasudil drove ERK and NF-κB activation, and augmented the cytolysis activity of NK cells against HSCs. In addition, Fasudil promoted the secretion of IFN-γ in NK cells (Figure 4). Although STAT3 activation is essential for NK cells to exert anti-tumor effects[35], it was not significantly influenced by Fasudil treatment (Figure 4). These results demonstrate the impact of Fasudil on NK cell function in fibrotic mouse model.

In terms of T cells, different T-cell subpopulations play different roles in the process of liver fibrosis. Previous studies have reported that T-cell impairment generally does not alter fibrogenesis, while Th2 and Th17 $CD4^{+}$T cells are involved in the inflammatory process, promoting cytokine production and HSC activation[36]. Here, the ratio of $CD4^{+}$ T cells and $CD8^{+}$ T cells was upregulated in the liver and spleen (data not shown), but the activation of these T cells was not significantly affected by Fasudil treatment (Figure 3). The exact roles of T cell subtypes in Fasudil-treated liver fibrosis need to be further investigated.

HSCs play a vital role in the pathogenesis of liver fibrosis. They can secret fibrogenic factors and then promote several kinds of cell types including portal fibrocytes, fibroblasts, and bone marrow-derived myofibroblasts to produce collagen and thereby propagate fibrosis[4,37]. Interestingly, we found that Fasudil treatment significantly increased apoptosis and inhibited the proliferation of LX2 cells, indicating the direct effect of Fasudil on HSCs (Figure 5), consistent with previous studies[38]. Actually, the effect of ROCK signal pathway on apoptosis is complicated. For example, inhibition of the RhoA/ROCK signaling pathway promotes the apoptosis of human urethral scar fibroblasts[38], cardiac fibroblasts[39], and neutrophils[40]. However, the decrease of apoptosis by RhoA/ROCK inhibition is also found in several cell types, such as

nitrergic neurons[41] and progenitor cells[42]. RhoA is positively correlated with the expression of α-SMA, a gene involved in liver fibrosis[43]. Indeed, Fasudil, as an inhibitor targeting RhoA kinases, significantly inhibited both the expression of RhoA and α-SMA in HSCs. In addition, ERK is an important mediator of signal transduction in HSCs, which is involved in the growth, differentiation, survival, and apoptosis of HSCs[44]. In line with previous studies, we observed an increase in the p-ERK level by Fasudil treatment, which would promote HSC apoptosis. Thus, Fasudil directly disrupts HSC activation and survival in TAA-induced liver fibrosis.

CONCLUSION

In conclusion, our findings demonstrated that Fasudil prevented TAA-induced liver fibrosis by directly suppressing the cell cycle and activation of HSCs, and activating NK cells. Because Fasudil is a drug that has been used clinically, it has high safety and reliability and can provide a feasible solution for clinical treatment of liver fibrosis.

ARTICLE HIGHLIGHTS

Research background

Rho kinase inhibition reportedly improves liver fibrosis. Fasudil, as a RhoA kinase inhibitor, is used to improve brain microcirculation and promote nerve regeneration clinically. The viability of Fasudil in liver fibrosis is still unknown.

Research motivation

Repositioning existing drugs *e.g.,* Fasudil might be an effective strategy for obtaining therapeutics against liver fibrosis.

Research objectives

To evaluate the anti-fibrotic effects of Fasudil *in vitro* and in a mouse model of thioacetamide (TAA)-induced liver fibrosis.

Research methods

The anti-fibrotic effect of Fasudil was investigated in a TAA-induced mouse model. At 1 wk after induction with TAA, Fasudil was intraperitoneally injected once a day for 3 wk, hepatic pathological changes, liver fibrosis and immune cell activation were determined using hematoxylin and eosin staining, sirius red staining, western blotting and quantitative polymerase chain reaction and fluorescence-activated cell sorting. Furthermore, the effect of Fasudil on hepatic stellate cell (HSC) and natural killer (NK) cells was assayed *in vitro*.

Research results

Treatment with Fasudil alleviated hepatic pathological changes and reversed hepatic fibrosis in the TAA-chronic models with decreased deposition of collagen fibers, reduced expression of HSC activation marker (alpha smooth muscle actin), and reduced secretion of transforming growth factor beta 1 (TGF-β1), matrix metalloproteinase 2 (MMP-2), and MMP-9. Fasudil treatment increased NK cell activation and cytotoxicity by activating the extracellular signal-related kinase and nuclear factor kappa B signaling pathways. Fasudil directly promoted the apoptosis and inhibited the proliferation of HSC by decreasing α-SMA and TGF-β1.

Research conclusions

Fasudil inhibited liver fibrosis by activating NK cells and blocking HSC activation.

Research perspectives

Fasudil treatment prevents liver fibrosis *via* activating NK cells but suppressing HSCs. As a drug used clinically, these results provide a feasible solution for the clinical treatment of liver fibrosis.

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ORIGINAL ARTICLE

Basic Study

Early genetic diagnosis of clarithromycin resistance in *Helicobacter pylori*

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Abstract

BACKGROUND

The drug resistance rate of clinical *Helicobacter pylori* (*H. pylori*) isolates has increased. However, the mechanism of drug resistance remains unclear. In this study, drug-resistant *H. pylori* strains were isolated from different areas and different populations of Chinese for genomic analysis.

AIM

To investigate drug-resistant genes in *H. pylori* and find the genes for the early diagnosis of clarithromycin resistance.

METHODS

Three drug-resistant *H. pylori* strains were isolated from patients with gastritis in Bama County, China. Minimal inhibitory concentrations of clarithromycin, metronidazole, and levofloxacin were determined and complete genome sequencing was performed with annotation. *Hp1181* and *hp1184* genes were found in these strains and then detected by reverse transcription polymerase chain reaction. The relationships between *hp1181* or *hp1184* and clarithromycin resistance were ascertained with gene mutant and drug-resistant strains. The homology of the strains with *hp26695* was assessed through complete genome detection and identification. Differences in genome sequences, gene quantity, and

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gene characteristics were detected amongst the three strains. Prediction and analysis of the function of drug-resistant genes indicated that the RNA expression of *hp1181* and *hp1184* increased in the three strains, which was the same in the artificially induced clarithromycin-resistant bacteria. After gene knockout, the drug sensitivity of the strains was assessed.

RESULTS

The strains showing a high degree of homology with *hp26695*, *hp1181*, and *hp1184* genes were found in these strains; the expression of the genes *hp1184* and *hp1181* was associated with clarithromycin resistance.

CONCLUSION

Hp1181 and *hp1184* mutations may be the earliest and most persistent response to clarithromycin resistance, and they may be the potential target genes for the diagnosis, prevention, and treatment of clarithromycin resistance.

Key Words: *Helicobacter pylori*; Clarithromycin-resistance; Diagnostic gene; Early genetic diagnosis; *Helicobacter pylori* strains

Core Tip: The World Health Organization designated clarithromycin-resistant *Helicobacter pylori* (*H. pylori*) a high priority among bacteria for antibiotic research and development, but the clarithromycin resistance mechanism remains unclear. We isolated and cultured clinical *H. pylori* strains, determined their minimal inhibitory concentrations, completed genome sequencing of *hp1181* and *hp1184* genes, analyzed their mutations, and found that the expression of the genes *hp1184* and *hp1181* was associated with clarithromycin resistance, which suggested that they can be used as genes for early diagnosis. This research may prove useful in the diagnosis, prevention, and treatment of clarithromycin-resistant *H. pylori*.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is recognized as an important human pathogen that colonizes the gastric mucus, resulting in superficial gastritis, atrophic gastritis, and gastric cancer[1-3]. Present treatments for *H. pylori* infection include proton pump inhibitors, bismuth in combination with amoxicillin, metronidazole and clarithromycin [4,5]. The rate of drug resistance is increasing because of the wide use of antibiotics and high resistance rates to clarithromycin, metronidazole, and levofloxacin are associated with the failure of *H. pylori* eradication[6-8]. The World Health Organization designated clarithromycin-resistant *H. pylori* a high priority bacterium for antibiotic research and development[9].

At present, the mechanism of antibiotic resistance of *H. pylori* is not completely understood[10,11]. It is widely accepted that the resistance to these antimicrobials is related to mutations in *H. pylori* genes, and clarithromycin-resistant strains present three point mutations in the region of domain V of 23S ribosomal RNA (rRNA): A2142G, A2142C, and A2143G[12,13]. In addition to the mutations, the efflux pump cluster is also involved in the development of resistance to clarithromycin[14,15]. However, there may be gene mutation sites that are not yet known, and the mechanism of drug resistance warrants further study.

We isolated and cultured *H. pylori* from the population in Bama County, which is a township known for the longevity of its residents in Guangxi, and randomly selected three strains of multiple drug-resistant *H. pylori* with resistance to clarithromycin.

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Complete genome sequences were analyzed to study the genomic characteristics of the strains and to elucidate the underlying mechanism of drug resistance in *H. pylori*.

MATERIALS AND METHODS

Isolation and culture of H. pylori

This study had received a strict medical ethics review from Youjiang Medical University for Nationalities. Written informed consent was obtained from each patient. Gastric mucosa tissue samples were collected from the People's Hospital of Bama Yao Autonomous County in patients' gastric body and pylorus with gastritis or gastric ulcers. Isolation and culture of *H. pylori* were performed at the Research Center for the Prevention and Treatment of Drug Resistant Microbial Infection, Youjiang Medical University for Nationalities. Patients investigated had not taken any antibiotics for at least 4 wk before examination. The isolation and identification of *H. pylori* were performed as previously described[16,17]. The bacteria were cultured on Columbia agar plates containing 5% fresh defibrinated sheep blood. The microaerophilic conditions included 5% O_2, 10% CO_2, and 85% N_2 at 37 °C for 3 to 5 days. Suspicious colonies were confirmed by Gram staining, urease, oxidase, and catalase activity testing, and urease gene polymerase chain reaction (PCR).

Antibiotic susceptibility testing

The antibiotic resistance of *H. pylori* was measured by dilution methods with reference to the protocols of the Clinical and Laboratory Standards Institute (Wayne, PA, United States)[18]. Briefly, the density of *H. pylori* was adjusted to be 1 × 10^6 CFU/mL and incubated at 37 °C for 3 to 5 d under microaerophilic conditions. After incubation, the plates were visually examined and the minimal inhibitory concentration (MIC) was determined to be the lowest concentration that resulted in no turbidity. Metronidazole (Aladdin, d1707126), amoxicillin (Xiansheng pharmaceutical, Co., Ltd, China), levofloxacin (Shandong Lukang Pharmaceutical Group Saite Co., Ltd, China), and clarithromycin (Yangzi River Pharmaceutical Group Co., Ltd, China) were also used.

Complete genome sequencing and analysis

Drug-resistant strains were selected and sent to the Shenzhen Huada Gene Co., Ltd (China) for complete genome analysis. After the DNA samples were delivered, the quality of the samples was tested and then used to construct a BSlibrary. The purified genomic DNA samples including genomic DNA, bacterial artificial chromosomes, or long-length PCR products were sheared into smaller fragments by CovarisS/E210 or using a Bioruptor. The overhangs resulting from fragmentation were converted into blunt ends using T4 DNA polymerase, Klenow fragment, and T4 polynucleotide kinase. After adding an 'A' base to the 3' end of the blunt phosphorylated DNA fragments, adapters were ligated to the ends of the DNA fragments. The desired fragments were purified though gel-electrophoresis, selectively enriched, and amplified by PCR. The index tag was introduced into the adapter at the PCR stage as appropriate and a library quality test was conducted. Finally, the qualified BSlibrary was used for sequencing. Genomic component and gene function analyses were performed, including gene prediction, tRNA, sRNA, and gene annotation, and prediction of open reading frames by GO.

Drug-resistant gene detection

Drug-resistant genes were predicted based on the results of the complete genome sequence analysis and selected for detection by reverse transcription PCR (RT-PCR). The reaction for cDNA synthesis was held at 25 °C for 10 min, 42 °C for 60 min, and then 99 °C for 5 min. The reaction consisted of 32 cycles with each cycle composed of 1 min at 95 °C, 4 min at 56 °C, and 7 min at 70 °C. After a final extension of 15 min at 72 °C, the RT-PCR products were visualized by electrophoresis on 1% agarose gel and 15% acrylamide gel with a 200-bp ladder size marker.

Knockout of mutant genes

Hp1181 and *hp1184* knockout mutants were constructed by insertion of the KAN resistance cassette. Double-knockout mutants were made by natural transformation of the KAN resistance cassette with pBSII KS (as presented by Bi HK, Laboratory of Nanjing Medical University, China) containing an internal fragment interrupted with a cat cassette from pAV35, with selection for both KAN- and CHL-resistant colonies.

Table 1 Drug resistance characteristics of three drug-resistant strains (minimal inhibitory concentration: μg/mL)

Strain	Metronidazole	Clarithromycin	Levofloxacin	Amoxicillin
Hpbs1	32	8	8	0.125
Hpbs2	16	8	0.125	0.125
Hpbs3	0.125	8	8	0.125

Table 2 Sequence information of three drug-resistant strains

Sample name	ID name	Sequence type	Sequence topology	Sequence number	Total length (bp)	GC content
Hpbs1	Chromosome1	Chromosome	Circular	1	1563701	38.90
	All	All	-	1	1563701	38.90
Hpbs2	Chromosome1	Chromosome	Circular	1	1534481	38.87
	All	All	-	1	1534481	38.87
Hpbs3	Chromosome1	Chromosome	Circular	1	1534930	38.90
	All	All	-	1	1534930	38.90

Sequence type: Chromosome or plasmid; Sequence topology: Circular or linear.

Table 3 Gene information of three drug-resistant strains

Sample name (#)	Genome size (#)	Total number (#)	Total length (bp)	Average length (#)	Length/genome length (%)	GC content (%)
Hpbs1	1563701	1571	1434202	912.92	91.72	39.49
Hpbs2	1534481	1792	1395399	778.68	90.94	39.44
Hpbs3	1534930	1732	1407495	812.64	91.70	39.49

Total number denotes the count of genes. Total length represents the total length of all genes. Average length refers to the average length of all genes. GC content is the content of G and C in a gene. Length/genome length is the proportion of gene length in the genome.

Table 4 Gene annotation statistics A

Sample name (#)	Total	P450 (#) (%)	VFDB (#) (%)	ARDB (#) (%)	CAZY (#)	SWISSPROT (#) (%)	NOG (#) (%)	COG (#) (%)	CARD (#) (%)	NR (#)
Hpbs1	1571	22 (1.4)	196 (12.47)	0 (0)	14 (0.89)	742 (47.23)	67 (4.26)	1084 (69)	14 (0.89)	1599 (99.23)
Hpbs2	1792	21 (1.17)	177 (9.87)	0 (0)	14 (0.78)	751 (41.9)	125 (6.97)	1111 (61.99)	13 (0.72)	1723 (96.14)
Hpbs3	1732	22 (1.27)	174 (10.04)	0 (0)	14 (0.75)	750 (43.3)	97 (5.6)	1113 (64.26)	15 (0.86)	1698 (98.03)

Insertion of the KAN and cat resistance cassette at the desired locations in the *H. pylori* putative efflux genes was validated by PCR.

Induction of drug resistance

The MIC of clarithromycin to hp26695 was detected. Drug resistance was induced by 1/4 MIC. The culture medium was changed every 2 d and MIC was detected every 4 d. The concentration of induced drug was changed with MIC.

Table 5 Gene annotation statistics B

Sample name (#)	DBCAN (#) (%)	T3SS (#) (%)	TREMBL (#) (%)	IPR (#)	PHI (#) (%)	KEGG (#) (%)	GO (#) (%)	KOG (#) (%)	Over all (#) (%)
Hpbs1	30 (1.9)	175 (11.13)	1557 (99.1)	1234 (78.54)	54 (3.43)	1026 (65.3)	957 (60.91)	142 (9.03)	1563 (99.49)
Hpbs2	29 (1.61)	197 (10.99)	1706 (95.2)	1372 (76.56)	52 (2.9)	1078 (60.15)	1056 (58.92)	144 (8.03)	1750 (97.65)
Hpbs3	30 (1.73)	209 (12.06)	1688 (97.45)	1340 (77.36)	51 (2.94)	1067 (61.6)	1030 (59.4)	139 (8.02)	1711 (98.78)

RESULTS

Bacterial resistance

Three drug-resistant strains were isolated and identified by Gram staining, urease, oxidase, catalase activity testing, and urease gene PCR. The drug resistance information of these strains is summarized in Table 1.

Bacterial sequence information

Based on the valid data from the previous sequencing platform, the CleanData could be assembled for each sample and the optimal assembly results were obtained after multiple adjustments. The assembly sequence was analyzed by correcting single base, circular judgment, and plasmid comparison. The results of the genome assembly statistics of each sample are displayed in Table 2. These three strains have been uploaded to the NCBI Biosample database: Hpbs1 (https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN10461767). Hpbs2 (https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN10663081), and Hpbs3 (https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN10663175).

Gene information

Gene prediction was applied to determine gene composition. The statistics are shown in Table 3.

Circular genome analysis

GC skew analysis was carried out using (G-C)/(G+C) calculations based on genomic sequences of strains. The results of gene distribution, ncRNA distribution, and gene annotation are demonstrated in Figure 1. Hpbs1 had 835 genes, 26 tRNAs, 6 rRNAs, and 2 sRNAs in the positive chain. It also had 736 genes, 10 tRNAs, 0 rRNAs, and 5 sRNAs in the negative chain and 157 repeats without positive or negative chain. There are 943 genes, 26 tRNAs, 6 rRNAs, 3 sRNAs, 849 genes, 10 tRNAs, 0 rRNA, 3 sRNAs, and 153 repeats in Hpbs2; there are 869 genes, 26 tRNAs, 6 rRNAs, 3 sRNAs, 863 genes, 10 tRNAs, 0 rRNA, 3 sRNAs, and 155 repeats in Hpbs3.

Gene annotation

Functional annotation was accomplished by analysis of protein sequences. We aligned genes with databases to obtain their corresponding annotations. To demonstrate the biological meaning, the highest quality alignment result was chosen as a gene annotation. Functional annotation was completed by blast resistance genes with different databases. In this project we have finished annotations using 17 databases, including P450, VFDB, ARDB, CAZY, SWISSPROT, NOG, COG, CARD, NR, DBCAN, T3SS, TREMBL, IPR, PHI, KEGG, GO, and KOG. The annotation results are shown in Tables 4 and 5.

Analysis of drug-resistant gene database

The drug resistance gene numbers of three strains were different in the CARD (Comprehensive Antibiotic Resistance Database), which are 14, 13, and 15 genes, respectively. However, after sorting, it was found that some genes were repetitive. The specific numbers and characteristics of genes are presented in the Tables 6 and 7. NP_207975.1 and NP_207972.1 were efflux pump genes of 26695 strain, *i.e.*, *hp1181* and *hp1184* genes. Their drug resistance was verified by RT-PCR, as illustrated in Figure 2. After knocking out the drug-resistant genes, drug sensitivity was significantly improved, as shown in Figure 3.

Table 6 Analysis of gene resistance in CARD

Gene ID	Subject ID	Align length	Mismatch	Gap	Gene start	Gene end	Subject start	Subject end	*E* value
GL000175	YP_208874.1	97	39	0	2	98	4	100	6.00E-40
GL000286	YP_006374661.1	398	88	2	1	397	29	421	0
GL000295	NP_312937.1	1389	658	21	8	1371	8	1339	0
GL000296	AAK44936.1	124	35	0	1	124	1	124	4.00E-63
GL000306	NP_207975.1	459	16	0	1	459	1	459	0
GL000309	NP_207972.1	443	10	0	1	443	1	443	0
GL000772	AIL15701	421	220	3	1	420	1	417	4.00E-126
GL000822	YP_002344422.1	853	293	6	3	818	2	851	0
GL000911	NP_415611.1	247	130	2	1	247	1	243	2.00E-66
GL000972	WP_005768149.1	810	390	18	3	773	12	809	0
GL001063	AJF83452.1	287	164	2	1	283	2	288	1.00E-71
GL001265	NP_415804.1	262	141	1	1	261	1	262	2.00E-80
GL001295	YP_001332362.1	222	123	4	1	221	1	216	7.00E-51
GL001455	AJF82049.1	254	141	2	4	255	7	260	2.00E-62

Identification of 23S rRNA gene mutations

As three strains were resistant to clarithromycin, so we analyzed and identified the sites of clarithromycin-resistant mutations. We found that three strains had mutations in A2142G, A2143G, G2144T, and some had mutations in other sites, as shown in Table 8.

Gene mutation induced in drug-resistant strains

After induction with clarithromycin, hp26695 drug resistance was enhanced on the 12th day, reached the highest level on day 16, and increased to 8 μg/mL on the 24th day. The expression of *hp1181* and *hp1184* was also increased with increasing clarithromycin resistance, especially *hp1184*, as shown in Figure 4. Only A2142G and A 2143G mutations were detected in 23S RNA, with no other mutation sites being found, as shown in Table 9. These data indicated that these two genes may be involved early in the regulation of clarithromycin resistance.

DISCUSSION

The treatment of *H. pylori* infection remains reliant on bismuth tetralogy at present. *H. pylori* is eradicated clinically using common antibiotics including clarithromycin, amoxicillin, metronidazole, tetracycline and levofloxacin. However, in recent years, the growing rate of antibiotic resistance has resulted in the failure of *H. pylori* eradication[19,20]. The most serious resistance has developed to drugs including metronidazole, clarithromycin, and levofloxacin star. The common mechanisms of bacterial resistance involve the production of inactivated enzymes, change in the target position of antibacterial drugs, change in the permeability of bacterial outer membrane, effects on the active outflow system, and formation of bacterial biofilm and cross resistance[21-23]. There are some differences in the mechanisms of drug resistance of each kind of bacteria; however, the same kind of bacteria still have different resistances to the same antibiotic in different areas[24]. The mechanism of drug resistance of *H. pylori* remains unclear and needs further study.

We selected drug-resistant strains using metronidazole, clarithromycin, and levofloxacin for genome sequencing analysis. We found that there were no significant differences in the number of drug-resistant genes in the CARD database. This may be because two kinds of antibiotic resistance can develop and the drug-resistant genes in *H. pylori* are mainly *hp1181* and *hp1184*. *Hp1181* encodes a putative NDA translocase that is related to the major facilitator superfamily and is an integral membrane protein; *hp1184* encodes another translocase that belongs to the MATE family, resulting in the aforementioned susceptibility. These can contribute to resistance *via* a multidrug-

Table 7 Characteristics of drug-resistant genes in CARD

Subject ID	ARO number	Definition of term
YP_208874.1	*Neisseria gonorrhoeae* FA 1090	rpsJ is a tetracycline resistance protein identified in *Neisseria gonorrhoeae*. Tetracycline resistance is conferred by binding to the ribosome as a 30S ribosomal protection protein[27]
YP_006374661.1	*Enterococcus faecium* DO	Sequence variants of *Enterococcus faecium* elongation factor Tu that can confer resistance to GE2270A[28]
NP_312937.1	*Escherichia coli* O157•H7 str. Sakai	Point mutation that occurs in *Escherichia coli* rpoB resulting in resistance to rifampicin[29]
AAK44936.1	*Mycobacterium tuberculosis* CDC1551	Ribosomal protein S12 stabilizes the highly conserved pseudoknot structure formed by 16S rRNA. Amino acid substitutions in RpsL affect the higher-order structure of 16S rRNA and confer streptomycin resistance by disrupting interactions between 16S rRNA and streptomycin[30-35]
NP_207975.1	*Helicobacter pylori* 26695	hp1184 is a translocase that belongs to the MATE efflux pump family. It is found in *H. pylori* and is involved in the active efflux of antibiotics[25,26]
NP_207972.1	*Helicobacter pylori* 26695	hp1181 is a translocase that is part of the MFS efflux pump family. It is found in *H. pylori* and plays a role in the active efflux of antibiotics[25]
AIL15701	*Escherichia coli* ATCC25922	murA or UDP-N-acetylglucosamine enolpyruvy1 transferase catalyzes the initial step in peptidoglycan biosynthesis and is inhibited by Fosfomycin. Over-expression of murA through mutations such as Asp369Asn and Leu370I1e confers fosfomycin resistance. Extensive evidence has shown the significance of C115 mutations in conferring fosfomycin resistance since this residue represents a primary binding site for the antibiotic across many species[36-39]
YP_002344422.1	*Campylobacter jejuni* subsp. *jejuni* NCTC 11168	*Campylobacter jejuni* is a major bacterial infectious agent associated with gastroenteritis. Quinolone resistance is reportedly conferred by a single C-257-T nucleotide substitution in the gyrA gene[40]
NP_415611.1	*Escherichia coli* str. K-12 substr. MG1655	Fab G is a 3-oxoacyl-acyl carrier protein reductase involved in lipid metabolism and fatty acid biosynthesis. The bacterial biocide Triclosan blocks the final reduction step in fatty acid elongation, inhibiting biosynthesis. Point mutations in fabG can confer resistance to Triclosan[41]
WP_005768149.1	*Bartonella bacilliformis* KC583	Point mutation in *Bartonella bacilliformis* results in amino coumarin resistance[42]
AJF83452.1	*Acinetobacter baumannii*	The LpxC gene is widely known to be involved in the biosynthesis of lipid A in Gram-negative bacteria and mutations to this gene may cause resistance to antimicrobial peptides that target the outer membrane[43,44]
NP_415804.1	*Escherichia coli* str. K-12 substr. MG1655	fabI is an enoyl-acy1 carrier reductase used in lipid metabolism and fatty acid biosynthesis. The bacterial biocide Triclosan blocks the final reduction step in fatty acid elongation, inhibiting biosynthesis. Point mutations in fabI can confer resistance to Triclosan and Isoniazid[41]
YP_001332362.1	*Staphylococcus aureus* subsp. *aureus* str. Newman	Ar1R is a response regulator that binds to the norA promoter to activate expression. Ar1R must first be phosphorylated by Ar1S[45]
AJF82049.1	*Acinetobacter baumannii*	The *LpxA* gene is widely known to be involved in the biosynthesis of lipid A in Gram-negative bacteria and mutations to this gene may cause resistance to antimicrobial peptides that target the outer membrane[43,44]

H. pylori: *Helicobacter pylori*.

resistant efflux protein, active-efflux of antibiotics, and other efflux pump genes, such as *HefA*. After knockout of these two genes, the MICs of the drugs were significantly decreased and the sensitivity was increased. It is noteworthy that in addition to these two genes, the *GE2270A* gene of *Enterococcus* and *MurA* gene of *Escherichia coli* also show a correlation. It is likely that the drug-resistant plasmids of other strains invade *H. pylori* through transformation or other mechanisms. Bacteria other than *H. pylori* in

Table 8 Mutations in the 23S rRNA genes of *Helicobacter pylori* strains

Nucleotide position	Ref	Mutation	Hpbs1	Hpbs2	Hpbs3
2143	A	G	+	+	+
2142	A	G	+	+	+
2144	G	T	+	+	+
2302	A	G	-	-	+
2182	T	C	-	+	-
2173	C	T	+	+	+
1513	G	A	-	+	+
2196	C	T	+	-	-
1280	A	G	+	-	-
1023	G	A	-	-	+

Table 9 23S rRNA mutations of *Helicobacter pylori* strains

Nucleotide position	Ref	Mutation	26695(S)	26695(R)
2142	A	G	-	+
2143	A	G	-	+

the gastric mucosa of patients can indirectly confirm this view. The main reason for this may be long-term acid resistant treatment, gastric erosion, or intestinal bacterial reflux. This will lead to drug resistance becoming more difficult to prevent and control. In addition, all three strains have clarithromycin resistance. The mechanism of resistance to clarithromycin is mainly reflected in the mutations A2142G, A2143G, and G2144T. In addition, it is common that there are several mutations in the same strain.

Hp1181 and *hp1184* are related to multidrug resistance and to clarithromycin resistance, which has been previously reported in the literature[25,26]. The RNA expression of *hp1181* and *hp1184* were increased with the emergence of clarithromycin resistance, with *hp1184* showing the fastest increase. Therefore, these genes are also involved in the regulation of drug resistance and may be one of the mechanisms of *H. pylori* resistance to clarithromycin. Compared with the clinical isolates, 23S RNA mutation sites of *H. pylori* were less frequent in artificially induced strains that had only A2142G and A2143G mutations. These may be attributed to the single factor of artificial induction that is not as complex as human stomach environment. More importantly, *hp1181* and *hp1184* mutations may be the earliest and most persistent response to clarithromycin resistance, and they may be the main target genes for the diagnosis, prevention, and treatment of clarithromycin resistance.

The genetic characteristics of multidrug-resistant strains in this area were preliminarily identified: The relationship between *hp1181* or *hp1184* and clarithromycin resistance was ascertained through genome sequencing analysis and gene function identification of drug-resistant *H. pylori* from Bama County, Guangxi Province. Our study further provided an improved experimental basis for the prevention and treatment of drug resistance of *H. pylori*.

CONCLUSION

Hp1181 and *hp1184* mutations may be the earliest and most persistent response to clarithromycin resistance, and they may be the main target genes for the diagnosis, prevention, and treatment of clarithromycin resistance.

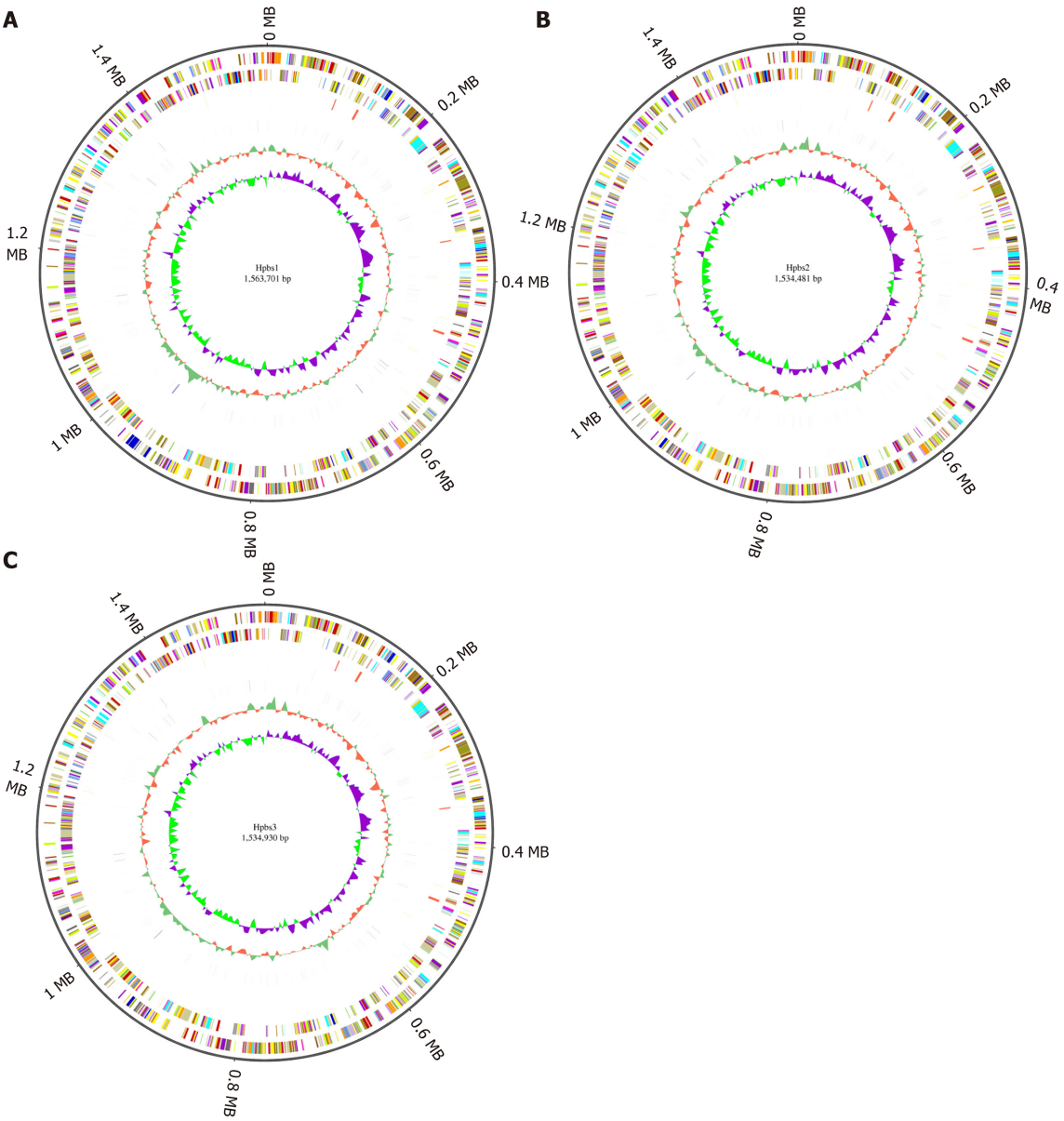

Figure 1 Circular genome analysis of three drug-resistant strains. A: Hpbs1; B: Hpbs2; C: Hpbs3.

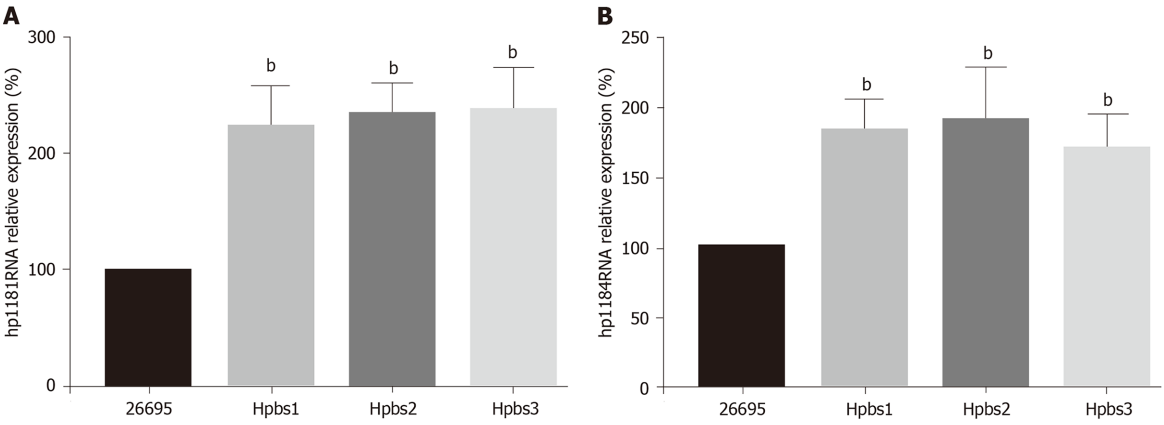

Figure 2 *Hp1181* and *hp1184* gene expression in drug-resistant strains. A: *Hp1181*; B: *Hp1184*. $^{b}P < 0.01$.

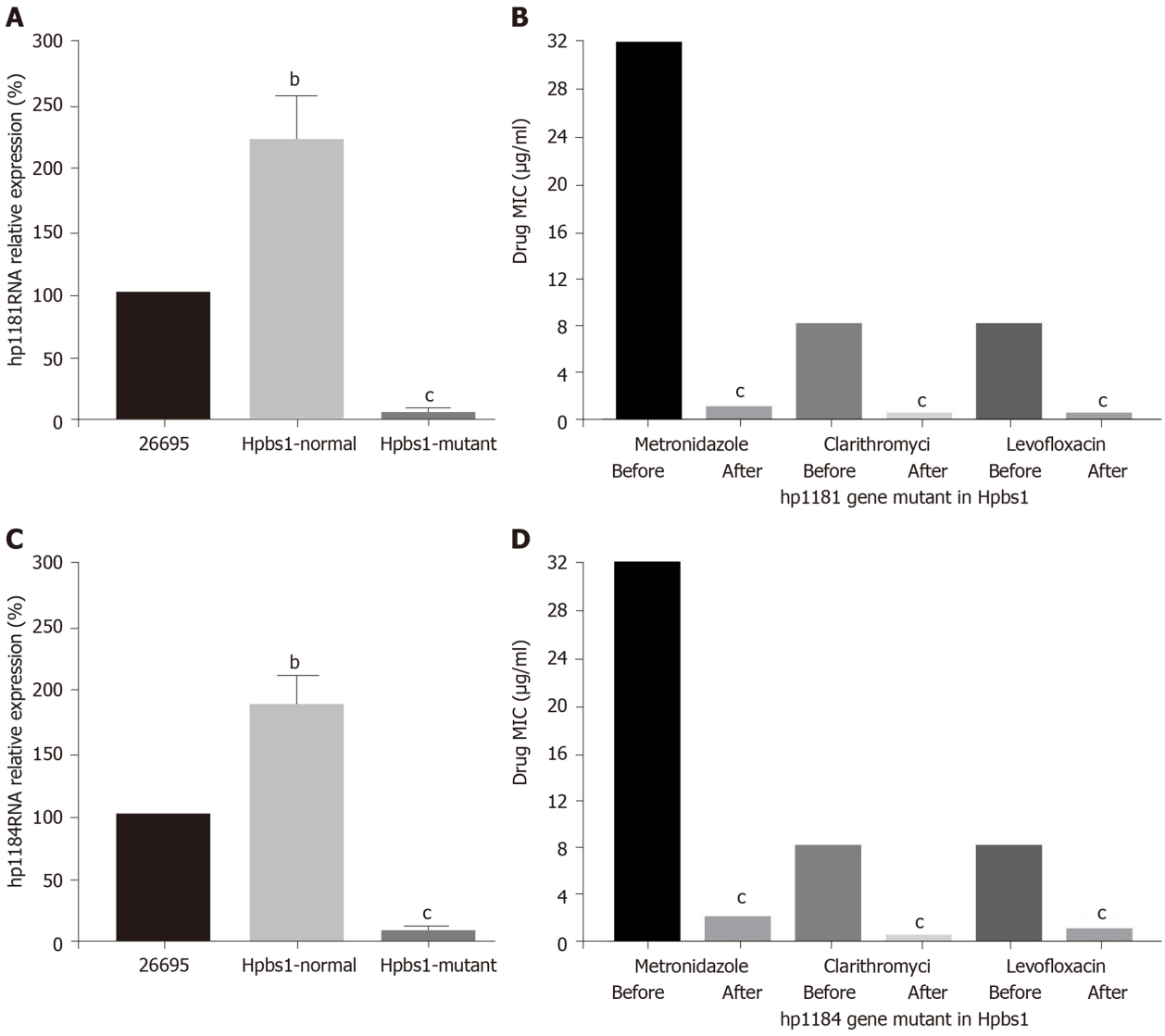

Figure 3 Drug sensitivity is improved after knockout of the drug-resistant genes. A: *Hp1181* knockout; B: Minimal inhibitory concentration (MIC) after *hp1181* knockout; C: *Hp1184* knockout; D: MIC after *hp1184* knockout. MIC: Minimal inhibitory concentration. $^{b}P < 0.01$; $^{c}P < 0.001$.

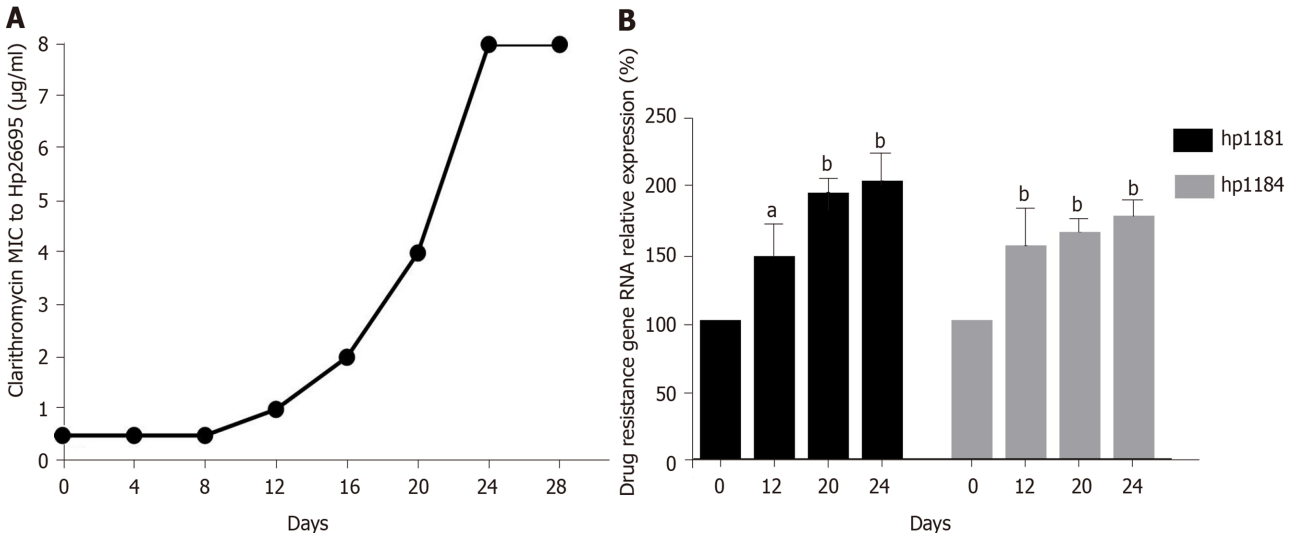

Figure 4 Induction of resistance to clarithromycin and expression of drug-resistant genes in *Helicobacter pylori*. A: Induction of clarithromycin resistance; B: Expression of drug-resistant genes. $^{a}P < 0.05$; $^{b}P < 0.01$.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) is recognized as an important human pathogen associated with superficial gastritis, atrophic gastritis, gastric cancer, *etc*., each of which has become a serious threat to human health and survival. The rate of drug resistance is increasing due to the wide use of antibiotics and high rates of resistance to clarithromycin, metronidazole, and levofloxacin are associated with the failure of *H. pylori* eradication. At present, the mechanism of antibiotic resistance of *H. pylori* is not completely understood. It is very difficult to prevent drug resistance and improve the rate of eradication of the target, thus warranting exploration of the mechanism of drug resistance to *H. pylori*, and provision of an experimental basis for the prevention and treatment of drug resistance.

Research motivation

Clarithromycin-resistant *H. pylori* urgently needs new antibiotics; however, antibiotic research and development are very difficult. If we can detect drug resistance by detecting drug-resistant genes in a timeous manner, this may help to alleviate the problem of clarithromycin resistance.

Research objectives

The objectives of this study were to investigate drug-resistant genes in *H. pylori*, and find a gene suited to early diagnosis of clarithromycin resistance, thereby rationalizing the rate of use of the drug.

Research methods

H. pylori strains were isolated and cultured, minimal inhibitory concentrations were measured, and complete genome sequence was determined. Prediction and analysis of the function of drug-resistant genes indicated that the RNA expression of *hp1181* and *hp1184* increased in the *H. pylori* strains, which was the same in the artificially induced clarithromycin-resistant bacteria. The relationships between *hp1181* or *hp1184* and clarithromycin resistance were confirmed with gene mutant and drug-resistant strains.

Research results

Hp1181 and *hp1184* genes were found in these *H. pylori* strains. Their expression was associated with clarithromycin resistance.

Research conclusions

Hp1181 and *hp1184* mutations may be the earliest and most persistent response to clarithromycin resistance, and they may be the main target genes for the diagnosis, prevention, and treatment of clarithromycin resistance.

Research perspectives

The relationship between *hp1181* or *hp1184* and clarithromycin resistance was demonstrated, providing an improved experimental basis for early diagnosis of clarithromycin resistance in *H. pylori*.

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ORIGINAL ARTICLE

Case Control Study

Altered profiles of fecal bile acids correlate with gut microbiota and inflammatory responses in patients with ulcerative colitis

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Author contributions: Yang ZH designed and performed the study, analyzed the data, and drafted the manuscript; Liu F and Jia ZJ collected the samples and clinical data of the patients; Zhu XR and Suo FY took part in designing the study and analyzing the data; Yao SK designed the study, supervised the study performance, revised the manuscript, and obtained the funding.

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Abstract

BACKGROUND

Gut microbiota and its metabolites may be involved in the pathogenesis of inflammatory bowel disease. Several clinical studies have recently shown that patients with ulcerative colitis (UC) have altered profiles of fecal bile acids (BAs). It was observed that BA receptors Takeda G-protein-coupled receptor 5 (TGR5) and vitamin D receptor (VDR) participate in intestinal inflammatory responses by regulating NF-κB signaling. We hypothesized that altered profiles of fecal BAs might be correlated with gut microbiota and inflammatory responses in patients with UC.

AIM

To investigate the changes in fecal BAs and analyze the relationship of BAs with gut microbiota and inflammation in patients with UC.

METHODS

The present study used 16S rDNA sequencing technology to detect the differences in the intestinal flora between UC patients and healthy controls (HCs). Fecal BAs were measured by targeted metabolomics approaches. Mucosal TGR5 and VDR expression was analyzed using immunohistochemistry, and serum inflammatory cytokine levels were detected by ELISA.

RESULTS

Thirty-two UC patients and twenty-three HCs were enrolled in this study. It was found that the diversity of gut microbiota in UC patients was reduced compared with that in HCs. *Firmicutes*, *Clostridium IV*, *Butyricicoccus*, *Clostridium XlVa*, *Faecalibacterium*, and *Roseburia* were significantly decreased in patients with UC (P = 3.75E-05, P = 8.28E-07, P = 0.0002, P = 0.003, P = 0.0003, and P = 0.0004,

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respectively). *Proteobacteria*, *Escherichia*, *Enterococcus*, *Klebsiella*, and *Streptococcus* were significantly enriched in the UC group (P = 2.99E-09, P = 3.63E-05, P = 8.59E-05, P = 0.003, and P = 0.016, respectively). The concentrations of fecal secondary BAs, such as lithocholic acid, deoxycholic acid, glycodeoxycholic acid, glycolithocholic acid, and taurolithocholate, in UC patients were significantly lower than those in HCs (P = 8.1E-08, P = 1.2E-07, P = 3.5E-04, P = 1.9E-03, and P = 1.8E-02, respectively) and were positively correlated with *Butyricicoccus*, *Roseburia*, *Clostridium IV*, *Faecalibacterium*, and *Clostridium XlVb* (P < 0.01). The concentrations of primary BAs, such as taurocholic acid, cholic acid, taurochenodeoxycholate, and glycochenodeoxycholate, in UC patients were significantly higher than those in HCs (P = 5.3E-03, P = 4E-02, P = 0.042, and P = 0.045, respectively) and were positively related to *Enterococcus*, *Klebsiella*, *Streptococcus*, *Lactobacillus*, and pro-inflammatory cytokines (P < 0.01). The expression of TGR5 was significantly elevated in UC patients (0.019 ± 0.013 *vs* 0.006 ± 0.003, P = 0.0003). VDR expression in colonic mucosal specimens was significantly decreased in UC patients (0.011 ± 0.007 *vs* 0.016 ± 0.004, P = 0.033).

CONCLUSION

Fecal BA profiles are closely related to the gut microbiota and serum inflammatory cytokines. Dysregulation of the gut microbiota and altered constitution of fecal BAs may participate in regulating inflammatory responses *via* the BA receptors TGR5 and VDR.

Key Words: Ulcerative colitis; Gut microbiota; Bile acids; Takeda G-protein-coupled receptor 5; Vitamin D receptor

Core Tip: This study comprehensively investigated the changes in gut microbiota and fecal bile acid profiles and analyzed the relationship of bile acids with gut microbiota and inflammation in patients with ulcerative colitis. It was demonstrated that fecal bile acid profiles are closely related to gut microbiota and serum inflammatory cytokines. Dysregulation of gut microbiota and altered constitution of fecal bile acids may participate in regulating inflammatory responses *via* the bile acid receptors Takeda G-protein-coupled receptor 5 and vitamin D receptor.

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INTRODUCTION

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD). It is characterized by continuous and diffuse inflammation starting in the rectum that can extend to proximal segments of the colon. The typical symptoms of UC include bloody diarrhea, abdominal pain, fecal urgency, and tenesmus. According to relevant epidemiological studies, the prevalence rates are high in Western developed countries, particularly in Europe (505 per 100000 in Norway) and North America (286 per 100000 in the United States)[1]. With the popularization of colonoscopy screening and changes in lifestyle and diet, the incidence and prevalence of UC have been increasing over time worldwide[2].

The natural history of UC includes periods of remission and flare-ups, and the goal of therapy is to induce and maintain clinical remission free of corticosteroids, thus minimizing the impact on quality of life[3]. Currently, treatments for UC include 5-aminosalicylates[4], corticosteroids[5], antitumor necrosis factor alpha drugs[6], antibiotics[7], probiotics[8], and immunosuppressants[9]. Studies indicate that a

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substantial proportion of patients who fail to respond to mesalamine for remission induction often rely on corticosteroids and/or immunomodulators to control the disease[10]. Corticosteroid resistance/refractoriness rates range from 8.9% to 25% in individuals with IBD[11,12]. Patients with long-standing IBD involving at least 1/3 of the colon are at an increased risk for colorectal cancer [13]. Colectomy is needed in up to 15% of patients with UC[14]. Therefore, it is necessary to explore the pathogenesis of UC, and effective therapies that can induce and maintain remission in UC without serious side effects are forthcoming.

The etiology and pathogenesis of IBD are still unclear. It is generally believed that various factors, such as the environment, genetics, immunity, and intestinal microbes, play a key role in the occurrence and development of IBD[15]. The human gut microbiota is a dynamic and diverse community of commensal bacteria, fungi, and viruses; among them, bacteria, of which there are over 1000 different species, constitute the majority[16,17]. Microorganisms regulate multiple aspects of host functions, including fermentation of dietary fibers[18], pathogen defense[19], metabolism, and immune maturation[20]. Multiple studies have indicated differences in the composition of the gut microbiota in UC, including reduction in diversity, decreased abundances of bacterial taxa within the *Firmicutes* and *Bacteroides*, and increases in the members of the *Proteobacteria phylum*, such as *Enterobacteriaceae*[21-23].

One of the primary modes by which the gut microbiota interacts with the host is by means of metabolites. Bile acid (BA) metabolites have recently drawn much attention in UC. BAs can be divided into two categories based on structure: Free BAs, including cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithochalic acid (LCA); and conjugated BAs, which are a combination of the abovementioned free BAs and glycine or taurine. BAs can also be divided into primary BAs and secondary BAs based on the source. The intestinal microbiota converts the primary BAs synthesized by host cells to secondary BAs. A few clinical studies have shown that there is a disorder of BA metabolism in patients with UC, which is characterized by enrichment of primary BAs and conjugated BAs and reduction of secondary BAs such as LCA and DCA[24-26]. Nevertheless, previous studies have only measured various fecal BAs but have not extensively investigated their relationships with the gut microbiota, its metabolites, and inflammatory cytokines in patients with UC.

BA receptors mediate the effects of BAs in the intestine, including nuclear receptors and membrane receptors. Nuclear receptors include farnesoid X receptor (FXR), pregnane X receptor, and vitamin D receptor (VDR). Membrane receptor refers to G protein-coupled bile acid receptor 1, also known as Takeda G protein-coupled receptor 5 (TGR5)[27,28]. The TGR5 and VDR signaling pathways play an important role in regulating inflammatory responses, cell proliferation, and apoptosis and controlling glycolipid and energy metabolism. Animal studies have shown that TGR5 and VDR participate in intestinal immune regulation and barrier function and reduce inflammatory responses[29]. However, the expression of the BA receptors VDR and TGR5 in colonic mucosal specimens from UC patients is still unclear.

Therefore, we hypothesized that dysregulation of the gut microbiota and altered constitution of fecal BAs may participate in regulating inflammatory responses *via* the BA receptors TGR5 and VDR. This study focused on the changes in the gut microbiota, fecal BA profiles, and BA receptor expression in the colonic mucosa. Correlations between these parameters were also analyzed. The findings may provide new insights into the pathogenesis of UC and the development of effective therapeutic methods for UC.

MATERIALS AND METHODS

Recruitment of subjects and sample collection

Based on the sample size of other studies[26,30-32], a total of 32 UC patients and 23 age- and sex-matched healthy controls (HCs) were recruited in the study. All patients were treated at the gastroenterological department of China-Japan Friendship Hospital from April 2019 to January 2020. Ulcerative colitis usually presents with bloody diarrhea and is diagnosed by colonoscopy and histological findings, as well as the exclusion of infectious and noninfectious colitis. The Mayo score of disease activity of the enrolled patients was required to be 4-12 to ensure that the clinical symptoms were significant at the study entry.

The exclusion criteria for UC patients were as follows: (1) Below the age of 18 or above the age of 65 years; (2) Use of probiotics or prokinetics, antispasmodics, and analgesics within 2 wk, or antibiotics, corticosteroids, immunosuppressants, BA

sequestrants, and lipid-lowering agents within 3 mo; (3) Previous major abdominal surgery or organic diseases such as celiac disease; (4) Severe hypertension, diabetes, coronary heart disease, psychiatric disorders, or biliary or liver comorbidities; (5) Pregnancy, lactation, or planned pregnancy; and (6) Uncooperativeness. All subjects gave written informed consent before participation. The study protocol was approved by the Ethics Committee of the China-Japan Friendship Hospital (No. 2019-K16).

After enrollment, the clinical status of each subject was first assessed using validated questionnaires. Fasting blood specimens were collected from all subjects, and serum samples were obtained and stored at -80 °C until analysis. Stool samples were collected as soon as possible within 1 d of UC patients' visit to prevent initiation of medical treatment from changing the composition of the intestinal flora. The pharmacological agents aforementioned were not allowed throughout the study period. Each fecal sample was divided into two parts with sterile plastic tubes after defecation. Samples were frozen in liquid nitrogen immediately and stored at -80 °C. All subjects were required to maintain their daily dietary habits at least 1 wk before the collection of the stool samples and until all of the assessments were finished. The next day, subjects underwent colonoscopy after standard bowel preparation with polyethylene glycol electrolyte solution, and one mucosal pinch biopsy was taken from the colorectal lesion. The specimen was immediately fixed in 10% formalin for at least 72 h, embedded in paraffin, and sectioned (4 μm) for immunohistochemistry.

Clinical assessments

Clinical assessments were first conducted using standardized questionnaires. The severity indexes of UC were assessed using the previously validated Mayo score[33]. The scoring system determines the severity of UC based on the patient's bloody diarrhea, doctor's assessment, and colonoscopy. Montreal classification was used to assess the extent of UC[34]. The Bristol Stool Form Scale (BSFS), a 7-point scale, was used to measure stool form.

DNA extraction, 16S rDNA amplification, and Illumina sequencing

Total DNA extraction was performed according to the instructions of the E.Z.N.A. soil kit (Omega Biotek, Norcross, GA, United States). The DNA concentration and purity were determined using a Thermo NanoDrop2000, and the quality of DNA extraction was validated by 1% agarose gel electrophoresis; 341F (5′-CCTACGGGRSGCAGCAG-3′) and 806R (5′-GGACTACVVGGGTATCTAATC-3′) primers were used for PCR amplification of the V3-V4 variable region using the following amplification procedure: Predena-turation at 95 °C for 3 min, amplification for 27 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s), and final extension at 72 °C for 10 min (PCR instrument: ABI GeneAmp 9700). The 20 μL reaction mixture included 4 μL of 5 × FastPfu buffer, 2 μL of 2.5 mmol/L dNTPs, 0.8 μL of primer (5 μmol/L), 0.4 μL of FastPfu polymerase, and 10 ng of DNA template.

The PCR products were recovered using a 2% agarose gel, purified with an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States), eluted with Tris-HCl, and detected by 2% agarose electrophoresis. Quantification was performed using QuantiFluor-ST (Promega, United States). Sequencing was performed using Illumina's MiSeq PE250 platform (Illumina, San Diego, United States).

Bile acid quantitation

BAs in feces were measured according to previously reported methods[35,36]. A Waters ACQUITY ultra-performance LC system coupled with a Waters XEVO TQ-S mass spectrometer with an ESI source controlled by MassLynx 4.1 software (Waters, Milford, MA) was used for all analyses. Chromatographic separations were performed with an ACQUITY BEH C18 column (1.7 μm, 100 mm × 2.1 mm internal dimensions; Waters, Milford, MA). UPLC-MS raw data obtained in negative mode were analyzed using TargetLynx Applications Manager version 4.1 (Waters Corp., Milford, MA) to obtain calibration equations and the concentration of each BA in the samples.

Immunohistochemistry

Paraffin sections were processed for immunohistochemistry. Following deparaffinization, antigen repair, endogenous peroxidase inhibition, and nonspecific antigen blocking, the sections were incubated with primary antibodies (rabbit monoclonal anti-TGR5 antibody, 1:100; rabbit monoclonal anti-VDR antibody, 1:100; Abcam, Cambridge, United Kingdom) overnight at 4 °C. Following thorough washing with

PBS, slides were incubated at room temperature for 1 h with horseradish peroxidase-conjugated anti-mouse rabbit secondary antibody (Zhongshan Gold Bridge, Beijing, China) and then visualized using diaminobenzidine. Finally, slides were counter-stained with hematoxylin and viewed under a light microscope.

For each section, five nonoverlapping fields at 400 × magnification were randomly selected and scanned under an OLYMPUS microscope. Images were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, United States). The mean optical density of the mucosal staining area was used to measure the expression of TGR5 and VDR. All sections were inspected independently by two blinded observers, and the mean values of the readings were used for final analysis.

Statistical analysis

Statistical analyses were performed using SPSS software, version 24.0 (SPSS Inc, Chicago, IL, United States). The normality of the distribution of the variables was tested using the Shapiro-Wilk test. Normally distributed data are presented as the mean ± SD, and abnormally distributed data are expressed as the median [interquartile range (IQR)]. Comparisons between groups were performed using independent sample *t*-tests for normally distributed data with homogeneous variances or nonparametric Mann-Whitney *U* tests. The χ^2 test or Fisher's exact test was used to analyze qualitative data. Correlations between BA metabolites and other parameters were explored using Pearson's correlation analysis for normally distributed data or Spearman's correlation analysis for nonnormally distributed data or ranked data. *P* values were two-sided, and differences were considered significant at $P < 0.05$. Statistical charts were generated with GraphPad Prism 5.0 software (GraphPad Software Inc, La Jolla, CA, United States).

RESULTS

Characteristics of study subjects

The demographics and clinical characteristics of UC patients and HCs are presented in Table 1. Thirty-two UC patients (17 males and 15 females; median age 37.0 years, IQR: 32.00-49.75) and twenty-three HCs (13 males and 10 females; median age 32.0 years, IQR: 27.00-51.00) were enrolled in this study. There were no significant differences between the groups in age ($P = 0.570$), sex ($P = 0.803$), or body mass index ($P = 0.337$). In UC patients, the duration of disease ranged from 0.5 to 25 years (median 2 years). According to the Mayo scores (7.8 ± 1.9), 5 (15.6%) patients had mild UC, and 25 (78.1%) had moderate UC. The BSFS score [UC: 6.0 (6.0, 6.0) *vs* HC: 4.0 (4.0, 4.0)] was significantly higher in UC patients than in HCs.

Structural characteristics of gut microbiota in the UC and control groups

Among the 594 operational taxonomic units (OTUs) detected, a total of 317 OTUs were identified in the two groups, including 86 unique OTUs in the UC group and 191 unique OTUs in the control group (Figure 1A). The dilution curve analysis based on the Sobs index for community richness and the Shannon index for community diversity showed that the sequencing volume covered all the microorganisms in the samples and met the data analysis requirements (Figure 1B). The species accumulation curve based on whether the sample size is sufficient and the estimated species richness showed that the sequencing sample size was sufficient, which can reflect most of the microbial information in the sample (Figure 1C). Principal coordinate analysis was performed to assess the similarity of the bacterial communities, which clearly differentiated the intestinal flora of the UC group from the control group (Figures 1D). Chao community richness index or the Shannon and Simpson community alpha diversity indexes of the UC group were significantly lower than those of the control group, indicating that the diversity of flora was reduced (Figure 1E). The community compositions of the intestinal microbes in the UC group and control group were analyzed at the phylum and genus levels. At the phylum level, the dominant phyla found in both groups were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* (the proportions in the two groups were 46.06%, 28.69%, 20.60%, and 3.61% *vs* 68.75%, 26.32%, 2.28%, and 1.66%, respectively), while at the genus level, *Bacteroides*, *Faecalibacterium*, *Escherichia*, *Prevotella*, and *Roseburia* were the top five genera (the proportions of which were 24.64%, 10.88%, 15.03%, 6.50, and 4.96% *vs* 21.05%, 24.59%, 0.63%, 7.12% and 8.32%, respectively) (Figure 1F).

Table 1 Demographics and clinical characteristics of the study subjects

Feature	UC patients	Controls	*P* value
n	32	23	NA
Age (yr)	37.0 (32.00, 49.75)	32.0 (27.00, 51.00)	0.570
Gender (male:female)	17:15	13:10	0.803
Body mass index (kg/m^2)	21.85 ± 3.64	22.75 ± 2.95	0.337
Duration of disease (yr)	2 (0.5, 8)	NA	NA
Mayo score	7.8 ± 1.9[b]	NA	< 0.001
BSFS score	6.0 (6.0, 6.0)[b]	4.0 (4.0, 4.0)	< 0.001
Inflammation location			
Rectum	9	NA	NA
Left colon	11	NA	NA
Whole colon	12	NA	NA
Disease activity		NA	NA
Mild activity	5	NA	NA
Moderate activity	25	NA	NA
Severe activity	2	NA	NA

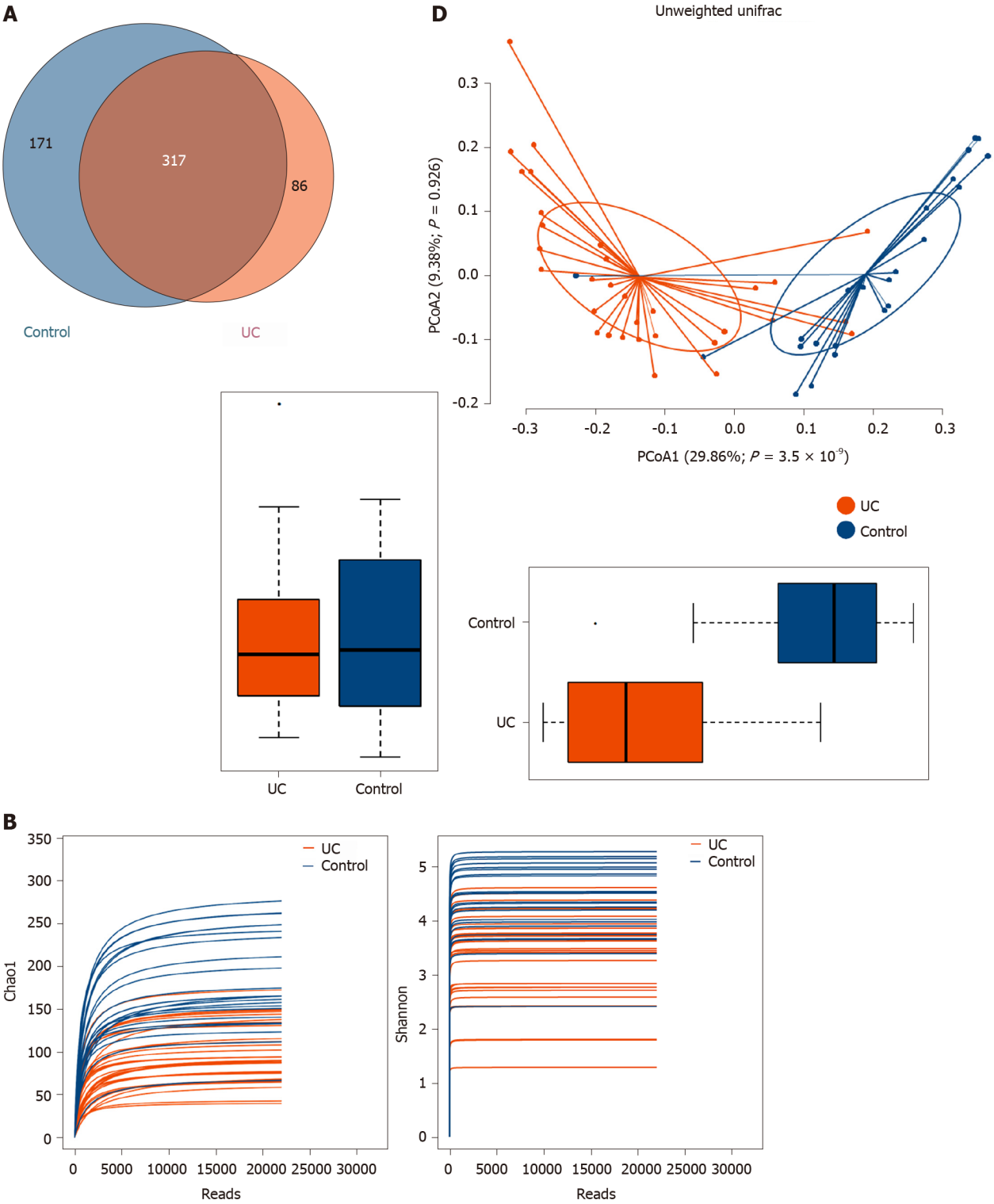
[b]$P < 0.01$ *vs* controls. The data are presented as the mean ± SD or median (interquartile range). UC: Ulcerative colitis; BSFS: Bristol stool form scale; NA: Not applicable.

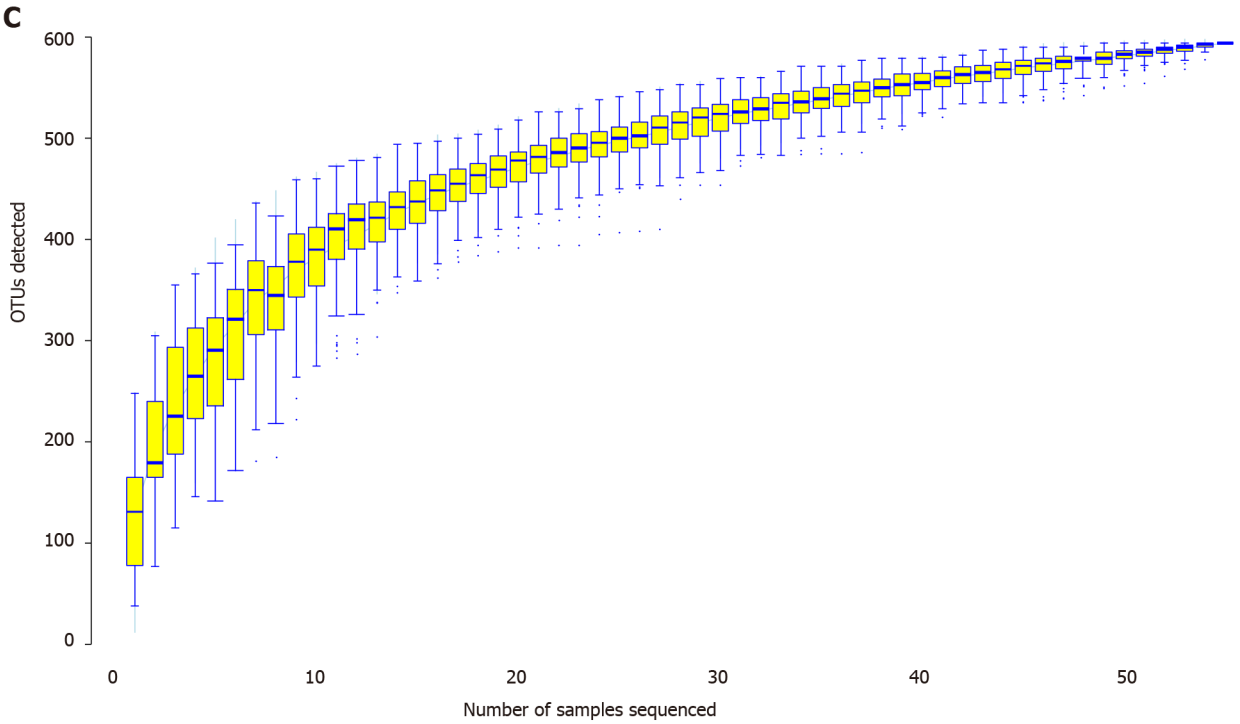
Screening of different key gut microbiota between the UC and control groups

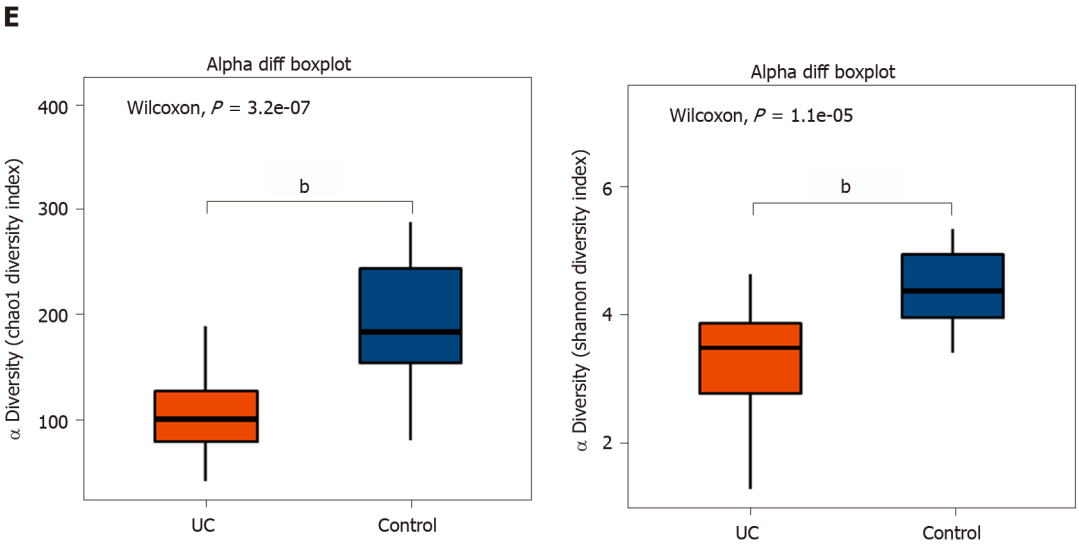
The Wilcoxon rank sum test of differential species between the two groups showed significant changes in the intestinal microbes between the UC and control groups (Figure 2A). At the phylum level, *Firmicutes* and *Proteobacteria* in the UC group were significantly different from those in the control group ($P = 3.75 \times 10^5$, $P = 2.99 \times 10^9$). At the genus level, the percentages of *Clostridium IV*, *Butyricicoccus*, *Clostridium XlVa*, *Faecalibacterium*, *Roseburia*, and *Coprococcus* in the UC group were significantly lower than those in the control group ($P = 8.28 \times 10^7$, $P = 0.0002$, $P = 0.003$, $P = 0.0003$, $P = 0.0004$, and $P = 7.38 \times 10^6$, respectively), and the percentages of *Escherichia*, *Enterococcus*, *Klebsiella*, and *Streptococcus* were significantly higher than those in the control group ($P = 3.63 \times 10^5$, $P = 8.59 \times 10^5$, $P = 0.003$, and $P = 0.016$, respectively). LEfSe analysis identified (threshold 2) the differential intestinal microbial communities between the two groups (Figure 2B). *Clostridia*, *Clostridiales*, *Firmicutes*, *Ruminococcaceae* and *Faecalibacterium* were significantly enriched in the control group. *Proteobacteria*, *Gammaproteobacteria*, *Enterobacteriaceae*, *Enterobacteriales*, and *Escherichia_Shigella* were significantly enriched in the UC group.

Analysis of difference in fecal BAs between the UC and control groups

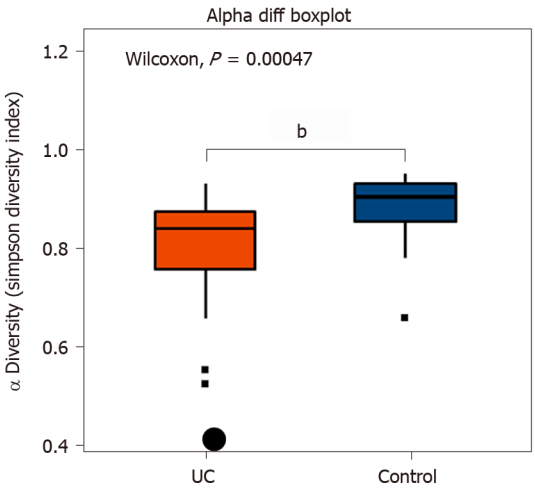
PCA was performed to evaluate the similarity of the fecal BAs of the two groups. Twenty-four BAs clearly distinguished the UC group from the control group (Figure 3). Fecal secondary BAs were significantly decreased in UC patients compared with healthy controls (Figure 3B). The concentrations of fecal secondary BAs such as LCA, DCA, 12_ketoLCA, glycol-deoxycholic acid (GDCA), glycol-lithocholic acid (GLCA), and tauro-lithocholic acid (TLCA) were significantly lower than those in healthy controls ($P = 8.1 \times 10^8$, $P = 1.2 \times 10^7$, $P = 6.3 \times 10^7$, $P = 3.5 \times 10^4$, $P = 1.9 \times 10^3$, and $P = 1.8 \times 10^2$, respectively) (Figure 3C-H). The concentrations of primary BAs such as taurocholic acid (TCA), CA, tauro-chenodeoxycholic acid (TCDCA), and glycol-chenodeoxycholic acid (GCDCA) were significantly higher than those in HCs ($P = 5.3 \times 10^3$, $P = 4 \times 10^2$, $P = 0.042$, and $P = 0.045$, respectively) (Figure 3I and M). The concentrations of CDCA and glycol-cholic acid (GCA) showed a tendency to increase in UC patients but failed to reach a significant level ($P = 0.138$ and $P = 0.074$, respectively) (Figure 3J and N).


A
171
317
86
Control
UC
D
Unweighted unifrac
PCoA2 (9.38%; P = 0.926)
PCoA1 (29.86%; P = 3.5 × 10-9)
UC
Control
Control
UC
UC
Control
B
Chao1
Reads
Shannon
Reads
UC
Control


C
OTUs detected
Number of samples sequenced
0
100
200
300
400
500
600
0
10
20
30
40
50


E
Alpha diff boxplot
Wilcoxon, P = 3.2e-07
α Diversity (chao1 diversity index)
b
UC
Control

Alpha diff boxplot
Wilcoxon, P = 1.1e-05
α Diversity (shannon diversity index)
b
UC
Control


Alpha diff boxplot
Wilcoxon, P = 0.00047
α Diversity (simpson diversity index)
b
UC
Control

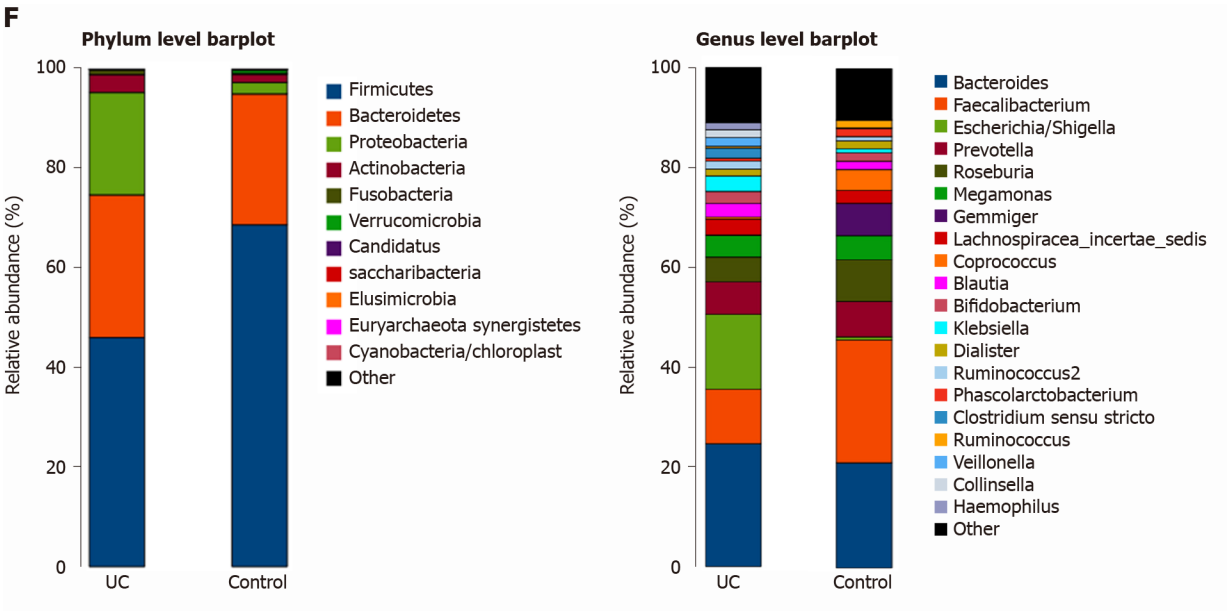


Figure 1 Structural characteristics of intestinal microbes in the ulcerative colitis and control groups. A: Among the 594 operational taxonomic units (OTUs) detected, a total of 317 OTUs were identified in the two groups, including 86 unique OTUs in the UC group and 191 unique OTUs in the control group. B: The dilution curve analysis based on the Chao1 index for community richness and the Shannon index for community diversity showed that the sequencing volume had covered all the microorganisms in the samples. C: The species accumulation curve shows that the sequencing sample size was sufficient, which reflected most of the microbial information in the sample. D: Principal coordinate analysis clearly differentiated the intestinal flora of the ulcerative colitis (UC) group from the control group. E: Chao community richness index or the Shannon and Simpson community α diversity indexes of the UC group were significantly lower than those of the control group. F: The community compositions of the intestinal microbes in the UC group and the control group were analyzed at the phylum and genus levels. UC: Ulcerative colitis; OTUs: Operational taxonomic units; PCoA: Principal coordinate analysis.

Correlations between fecal BAs and intestinal microbes in all subjects

Correlative assessments were made on the fecal BAs and intestinal microbes (Figure 4). The results showed that DCA, LCA, and 12_ketoLCA were negatively correlated with *Enterococcus*, *Klebsiella*, *Streptococcus*, and *Lactobacillus*. CA, CDCA, TCA, TCDCA, GCA, and GCDCA were positively related with *Enterococcus*, *Klebsiella*, *Streptococcus*, and *Lactobacillus*. *Butyricicoccus*, *Roseburia*, *Clostridium IV*, *Faecalibacterium Ruminococcus*, *Clostridium XlVb*, *Coprococcus*, and *Alistipes* were negatively correlated with the concentrations of CA, CDCA, TCA, TCDCA, GCA, and GCDCA and positively correlated with the concentrations of DCA, LCA, 12_ketoLCA, GLCA, and GDCA.

Mucosal immunohistochemistry

Representative photomicrographs of the immunoreactivity of TGR5 and VDR in the mucosa of UC patients and HCs are shown in Figure 5A-D (× 400 magnification). The level of TGR5 in mucosal biopsies was significantly higher in UC patients than in HCs (0.019 ± 0.013 *vs* 0.006 ± 0.003, P=0.0003) (Figure 5E). VDR expression in colonic mucosal specimens decreased significantly in UC patients (0.011 ± 0.007 *vs* 0.016 ± 0.004, P = 0.033) (Figure 5F).

Serum inflammatory cytokine levels and correlations between fecal BAs and serum inflammatory cytokines

The levels of inflammatory cytokines in the serum of UC patients and HCs were quantified by ELISA. The levels of IL-1α, IL-1β, TNF-α, IL-2, and IL-6 were significantly higher in UC patients (P < 0.0001) (Figure 6A-E). TCA, GCA, and GCDCA were positively correlated with IL-1α (P < 0.05); TCA and TNF-α were positively correlated (P < 0.05); LCA, DCA, 12-KetoLCA, TLCA, GDCA, and 6-Keto-LCA were negatively correlated with the levels of IL-1α, IL-1β, TNF-α, and IL-6 (P < 0.01) (Figure 6F and G).

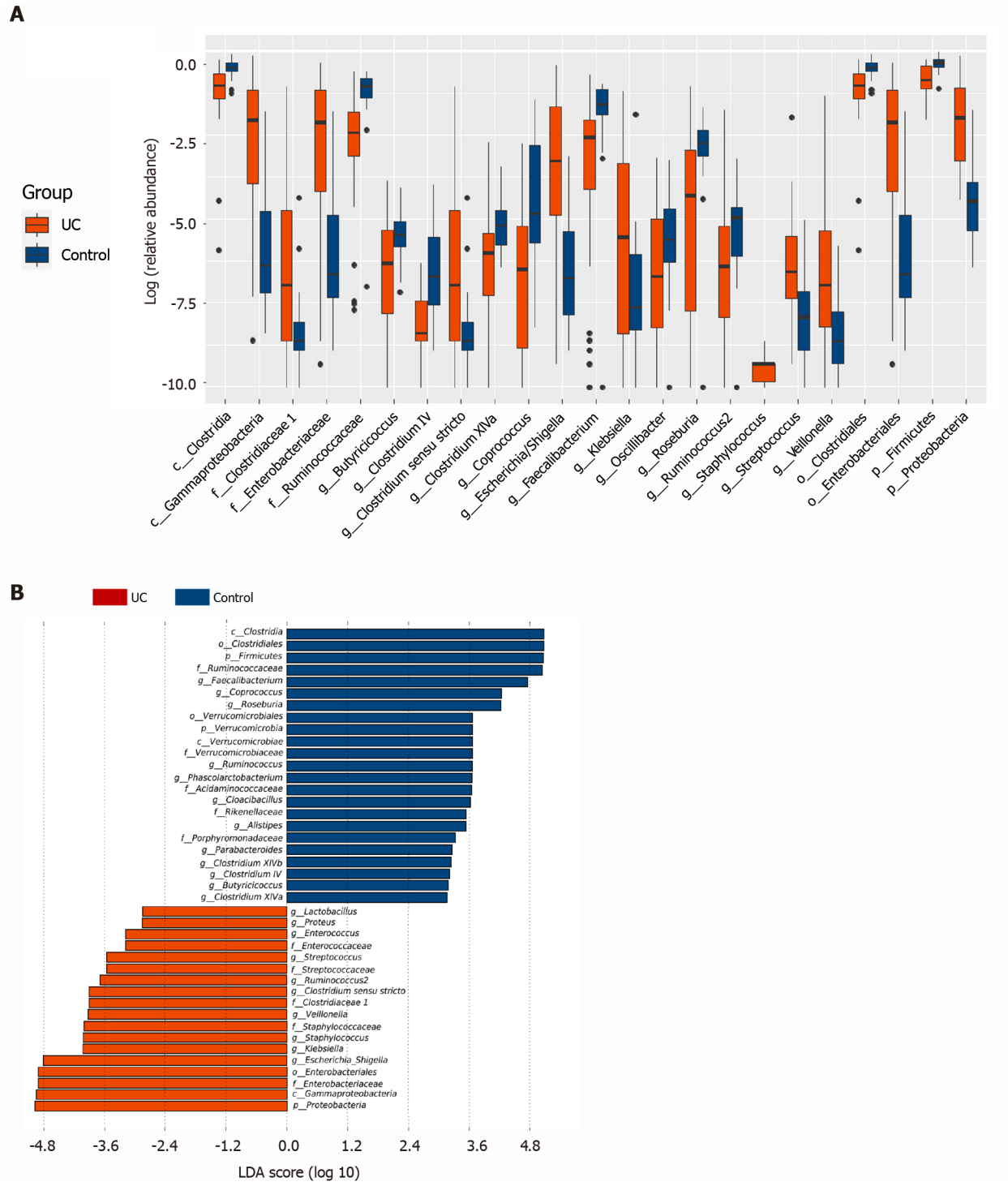
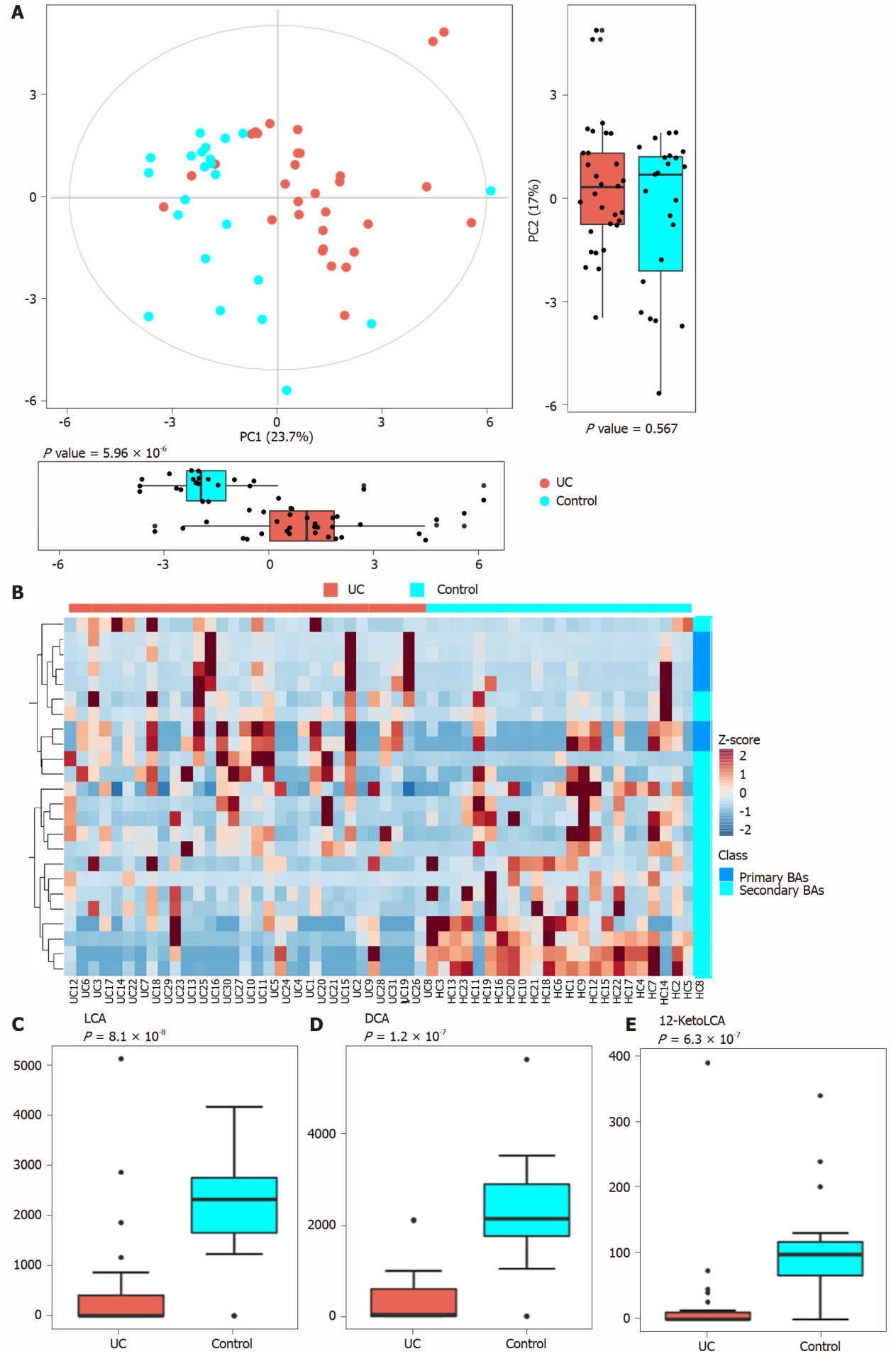


Figure 2 Screening of different key microorganisms between the ulcerative colitis and control groups. A: The Wilcoxon rank sum test of differential species between the two groups showed significant changes in the intestinal microbes between the ulcerative colitis and control groups; B: LEfSe analysis identified (threshold 2) the differential intestinal microbial communities between the two groups. UC: Ulcerative colitis; LDA: Linear discriminant analysis.

DISCUSSION

This study comprehensively investigated the changes in fecal BA profiles and analyzed the associations of BAs with the gut microbiota and inflammation in patients with UC. As expected, these data confirmed the differences in fecal BA compositions between UC patients and HCs, and the concentrations of some BAs were significantly correlated with the gut microbiota and serum inflammatory cytokines. Specifically, the concentrations of fecal secondary BAs such as LCA, DCA, GDCA, GLCA, and TLCA in UC patients were significantly lower than those in HCs and were positively correlated with *Butyricicoccus*, *Roseburia*, *Clostridium IV*, *Faecalibacterium*, and


A
PC1 (23.7%)
PC2 (17%)
P value = 0.567
P value = 5.96 × 10-6
UC
Control
B
UC
Control
Z-score
2
1
0
-1
-2
Class
Primary BAs
Secondary BAs
C
LCA
P = 8.1 × 10-8
D
DCA
P = 1.2 × 10-7
E
12-KetoLCA
P = 6.3 × 10-7

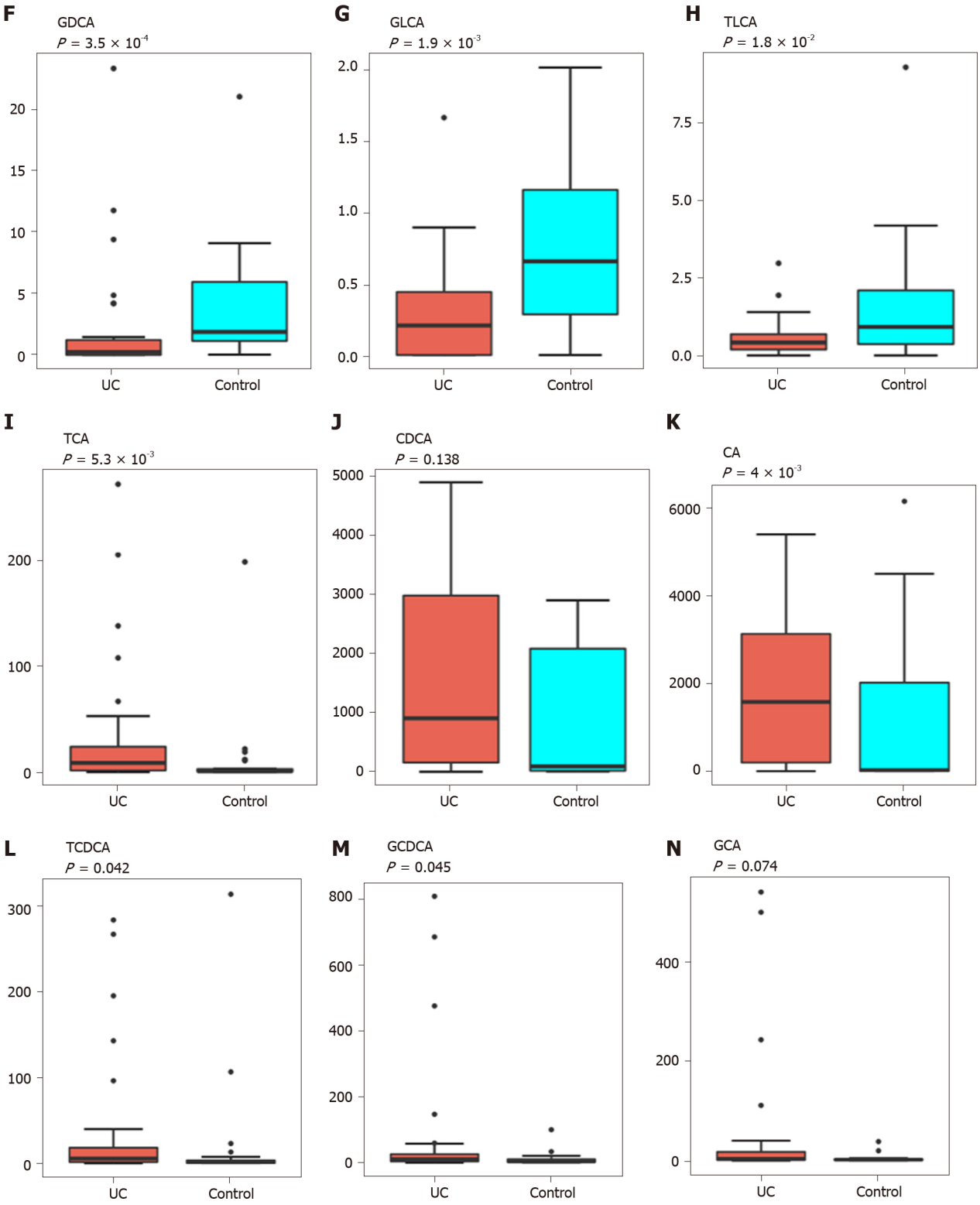


Figure 3 Analysis of difference in fecal bile acids between the ulcerative colitis and control groups. A: Principal component analysis was performed to evaluate the similarity of the fecal BAs of the two groups. Twenty-four BAs clearly distinguished the ulcerative colitis (UC) group from the control group; B: Heatmap showing the individual BA concentrations in the samples (log-transformed). Shades of red and blue represent high and low BA concentrations, respectively (see color scale); C-H: Fecal secondary BAs in UC patients, such as lithocholic acid, deoxycholic acid, glyco-deoxycholic acid, glyco-lithocholic acid, and tauro-lithocholate, were significantly lower than those in healthy controls; I and K-M: The primary BAs such as tauro-cholic acid, cholic acid, tauro-chenodeoxycholic acid, and glyco-chenodeoxycholic acid were significantly higher than those in healthy controls; J and N: The concentrations of chenodeoxycholic acid and glyco-cholic acid showed a tendency to increase in UC patients but the increases were not significant. UC: Ulcerative colitis; PCA: Principal component analysis; BAs: Bile acids; LCA: Lithocholic acid; DCA: Deoxycholic acid; GDCA: Glyco-deoxycholic acid; GLCA: Glyco-lithocholic acid; TLCA: Tauro-lithocholate; TCA: Tauro-cholic acid; CA: Cholic acid; TCDCA: Tauro-chenodeoxycholic acid; GCDCA: Glyco-chenodeoxycholic acid; CDCA: Chenodeoxycholic acid; GCA: Glyco-cholic acid.

Clostridium XlVb. The concentrations of primary BAs such as TCA, CA, TCDCA, and

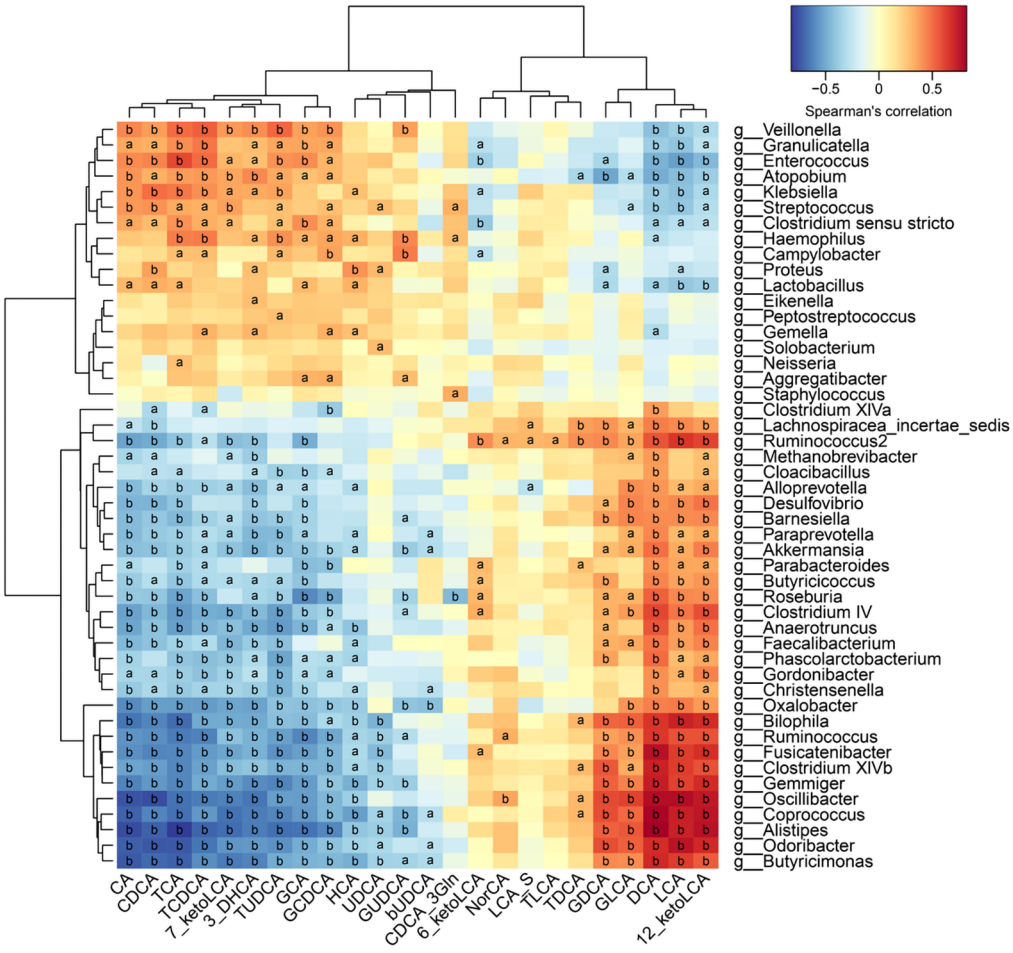


Figure 4 Correlations between fecal bile acids and intestinal microbes. A heatmap of correlative assessments was made on the fecal bile acid metabolites and intestinal microbes ($^{a}P < 0.05$, $^{b}P < 0.01$). LCA: Lithocholic acid; DCA: Deoxycholic acid; GDCA: Glyco-deoxycholic acid; GLCA: Glyco-lithocholic acid; TLCA: Tauro-lithocholate; TDCA: Tauro-deoxycholic acid; UDCA: Ursodeoxycholic acid; TUDCA: Tauro-ursodeoxycholic acid; GUDCA: Glyco-ursodeoxycholic acid; TCA: Tauro-cholic acid; CA: Cholic acid; TCDCA: Tauro-chenodeoxycholic acid; GCDCA: Glyco-chenodeoxycholic acid; CDCA: Chenodeoxycholic acid; GCA: Glyco-cholic acid.

GCDCA in UC patients were significantly higher than those in HCs and positively correlated with *Enterococcus, Klebsiella, Streptococcus, Lactobacillus*, and proinflammatory cytokines. The mucosal expression of the BA membrane receptor TGR5 was significantly elevated in UC patients. Additionally, BA nuclear receptor VDR expression in colonic mucosal specimens was significantly decreased in UC patients. Based on these findings, we concluded that dysregulation of the gut microbiota and altered constitution of fecal BAs may participate in regulating inflammatory responses *via* the BA receptors TGR5 and VDR.

For demographics, 32 UC patients (17 males and 15 females; median age 37.0 years, IQR: 32.00-49.75) were enrolled in this study. Similar to previous studies, no sex predominance existed in UC, and the peak age of disease onset was between ages 30 years and 40 years[37]. In order to prevent initiation of medical treatment from changing the composition of the intestinal flora of UC patients, we collected stool samples as soon as possible within 1 d of UC patients' visit to ensure that the clinical symptoms were significant at the study entry. Except for five patients who took mesalazine for a short period of time, the remaining active (relapse) patients included in our study did not receive any treatment before collecting stool and serum samples. Although the time of active disease is not specified, the Mayo score of disease activity of the enrolled patients was required to be 4-12.

Gut microbes were demonstrated to be an essential factor in intestinal inflammation in UC. It has been consistently shown that there is a decrease in biodiversity and species richness in UC[38]. Changes in the composition of the gut microbiota led to metabolite alterations that are likely to have a role in UC pathogenesis[39]. The intestinal microbiota converts ingested food or host products into metabolites that target either the intestinal microbial population or host cells. Hence, the presence of metabolites depends on microbial metabolic activity[40,41]. It is estimated that more

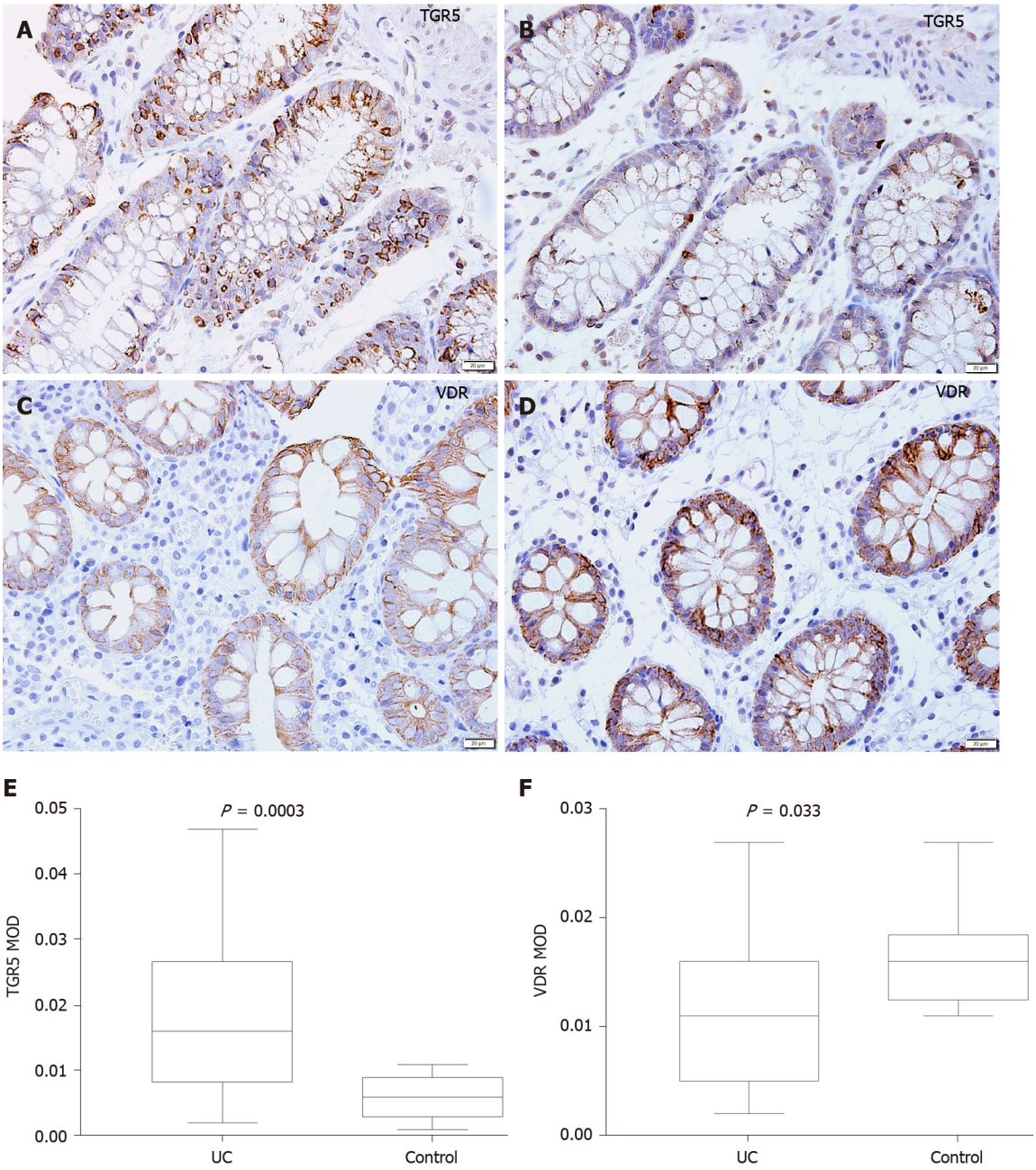


Figure 5 Mucosal immunohistochemistry in patients with ulcerative colitis and healthy controls. A and B: Takeda G protein-coupled receptor 5 (TGR5) immunoreactivity was mainly scattered in the epithelium in ulcerative colitis (UC) patients (scale bar = 20 μm); C and D: Vitamin D receptor (VDR) immunoreactivity was distributed in the epithelium and lamina propria in UC patients and healthy controls (Scale bar = 20 μm); E: The mean optical density of TGR5 in the colonic mucosa in UC patients was significantly higher than that in healthy controls (P = 0.0003); F: The mean optical density of VDR in the colonic mucosa decreased significantly in patients (P = 0.033). UC: Ulcerative colitis; TGR5: Takeda G protein-coupled receptor 5; VDR: Vitamin D receptor; MOD: Mean optical density.

than 50% of metabolites found in fecal matter and urine are derived from or modified by the intestinal microbiota[42]. It is particularly noteworthy that the intestinal flora has an important influence on the composition of BA metabolites. The BAs in feces are mainly secondary BAs but also contain a small amount of primary BAs, trace conjugated BAs, and ursodeoxycholic acid (UDCA)[43]. The conversion of conjugated BAs to free BAs depends on the bile salt hydrolase that exists in the intestinal flora [44], which has been identified in *Bacteroides fragilis, Clostridium, Lactobacillus,* and *Bifidobacterium*. Thus, we infer that the imbalance of the intestinal flora affects the deconjugation of BAs, leading to an increase in the concentration of conjugated BAs. In agreement with these studies, we found that the concentrations of conjugated BAs such as TCA, TCDCA, and GCDCA in UC patients were significantly higher than those in HCs and negatively related to *Clostridium IV*, *Faecalibacterium*, *Ruminococcus*, and *Clostridium XlVb*.

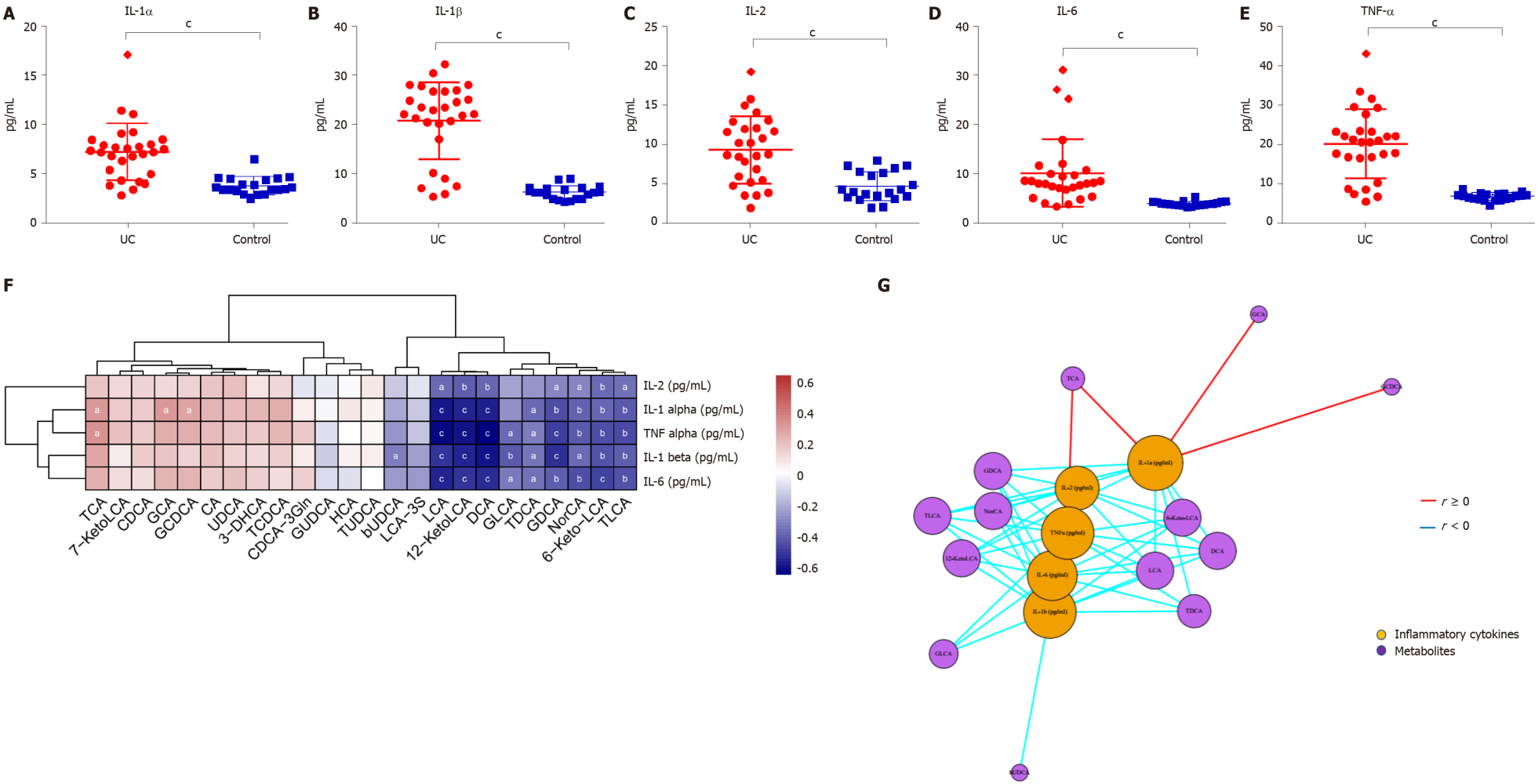


Figure 6 Serum inflammatory cytokine levels and correlations between fecal bile acids and serum inflammatory cytokines in all subjects. A-E: The levels of IL-1α, IL-1β, TNF-α, IL-2, and IL-6 were significantly higher in UC patients ($P < 0.0001$); F: A heatmap of correlative assessments was made of the fecal bile acids and serum inflammatory cytokines ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$); G: A network diagram of correlative assessments was made on the fecal bile acid metabolites and serum inflammatory cytokines (purple nodes represent bile acid metabolites and orange nodes represent inflammatory cytokines; red lines represent positive correlations and blue lines represent negative correlations). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. LCA: Lithocholic acid; DCA: Deoxycholic acid; GDCA: Glyco-deoxycholic acid; GLCA: Glyco-lithocholic acid; TLCA: Tauro-lithocholate; TDCA: Tauro-deoxycholic acid; UDCA: Ursodeoxycholic acid; TUDCA: Tauro-ursodeoxycholic acid; GUDCA: Glyco-ursodeoxycholic acid; TCA: Tauro-cholic acid; CA: Cholic acid; TCDCA: Tauro-chenodeoxycholic acid; GCDCA: Glyco-chenodeoxycholic acid; CDCA: Chenodeoxycholic acid; GCA: Glyco-cholic acid.

A sequence of enzymatic reactions in the liver converts cholesterol to primary BAs in humans. The intestinal microbiota converts primary BAs to secondary BAs by various reactions, including deconjugation, dehydroxylation, esterification, and desulfatation[29,45-47]. Dehydroxylation only occurs after deconjugation and is catalyzed by the Firmicutes phylum, including *Clostridium* and *Eubacterium*. Desulfatation driven by BA sulfatase is catalyzed by *Clostridium*, *Peptococcus*, *Fusobacterium*, and *Pseudomonas*[47,48]. Therefore, dysregulation of gut microbiota impairs the deconjugation, dehydroxylation, and desulfatation of BAs. As a result, patients with UC have increased secondary BAs and decreased primary BAs. In accordance with these studies, we also found that the concentrations of fecal secondary BAs such as LCA, DCA, GDCA, GLCA, and TLCA in UC patients were significantly lower than those in HCs and were positively correlated with *Butyricicoccus*, *Roseburia*, *Clostridium IV*, *Faecalibacterium*, and *Clostridium XlVb*.

Moreover, we demonstrated that altered constitution of fecal BAs may participate in regulating inflammatory responses *via* BA receptors. TGR5 is a BA reactive receptor expressed in various cell types and is widely distributed throughout the gastrointestinal tract[49]. Different types of BAs have different agonistic effects on TGR5: LCA > DCA > CDCA > UDCA > CA[50]. TGR5 also plays a role in inflammation, energy balance, and insulin signaling[51]. Studies have shown that TGR5 negatively regulates liver inflammation in mice by antagonizing NF-κB signaling. Compared with WT mice, mice with *TGR5* gene deficiency have significantly higher levels of serum inflammation markers after induction with LPS, but treating WT mice with TGR5 agonists can reduce inflammatory responses[52]. Nevertheless, TGR5 is increased in experimental colitis, and the mRNA expression of *TGR5* is upregulated in patients with Crohn's disease[53,54]. The anti-inflammatory properties of TGR5 indicate that TGR5 activation may be beneficial to IBD, which might be a compensatory mechanism to counterbalance the vicious cycle of inflammation in IBD.

In addition, this study found that BA metabolites can regulate the immune response in a VDR-dependent fashion. The nuclear receptor VDR is highly expressed in the small intestine and colon and is an essential regulator of intestinal cell proliferation, barrier function, and immunity[55,56]. Evidence strongly supports a protective effect of VDR in UC, and the underlying mechanism may be that VDR can ameliorate intestinal inflammation by downregulating NF-κB signaling and activating autophagy [57,58]. In experimental models of colitis, *Vdr* whole-body knockout mice are known to develop severe colitis[59]. The secondary BA LCA, as a VDR ligand that is produced by *Clostridium* bacteria in the gut lumen, controls Th1 immune responses and suppresses the production of the Th1 cytokines IFNγ and TNFα by activating VDR [60]. *Vdr* knockout mice have lower *Clostridium* in the gut, illustrating the influence of crosstalk between the microbiome and VDR signaling in immunity[61]. Consistent with previous studies[62], the current study showed that the low expression of VDR in the intestine of patients with UC may be related to the imbalance of the flora and the decrease of secondary BAs such as DCA and LCA.

There were several limitations in this study. First, due to limited time and conditions, the sample size was relatively small in this study, and subgroup analysis of the microbiota composition and BA profiles with different disease activities and stages of UC patients has not been performed yet. Previous studies have shown that there are differences in the intestinal flora of UC patients during active and remission periods [63,64]. Longitudinal analyses revealed reduced temporal microbiota stability in UC, particularly in patients with changes in disease activity[65,66]. As the number of subjects increases, we will collect stool samples from a large population of patients with UC at different time points during periods of active and remission disease and rank the contribution of variables to microbiota composition and BA profiles. Second, our conclusions are based on observational research, and such cross-sectional studies do not provide information about the timing of dysbiosis relative to disease onset and, therefore, should be interpreted with caution particularly with regards to cause–effect relationships[39]. We will later conduct intervention studies and animal experiments to verify their relationship. Third, considering that the short-term modification of a diet can rapidly disturb the gut microbiota[67], all subjects were required to maintain their daily dietary habits before the collection of the stool samples. However, dietary constituents have been shown to affect the inflammatory status, in great part mediated through the modulation of the microbiota[68,69], so it is better to supply a standardized diet for subjects. The standardized diet minimizes diet-induced deviations in the gut microbiota and BA metabolites, but masks the gut microbiota under usual dietary habits. Therefore, the measures of gut microbiota and BAs before and after a standardized diet combined with a detailed assessment of the usual dietary habits of patients are necessary for a future study. Finally, since the nuclear FXR is mostly

distributed in hepatocytes, the small intestine, and macrophages[50], this study did not detect the expression of FXR in the colonic mucosa.

CONCLUSION

In conclusion, this study provides new evidence that fecal BAs are closely related to the gut microbiota and serum inflammatory cytokines. Dysregulation of the gut microbiota and altered constitution of fecal BAs may participate in regulating inflammatory responses *via* the BA receptors TGR5 and VDR. This study provides a preliminary exploration for possible involvement of the gut microbiota and BA metabolites in the inflammatory responses of UC in humans.

ARTICLE HIGHLIGHTS

Research background

The gut microbiota and its metabolites are involved in the pathogenesis of inflammatory bowel disease. Bile acid (BA) metabolites have recently drawn much attention in ulcerative colitis (UC). Animal studies have shown that secondary BAs participate in intestinal inflammatory responses *via* the BA receptors Takeda G-protein-coupled receptor 5 (TGR5) and vitamin D receptor (VDR). However, there are few studies about the quantitative analysis of fecal BAs and intestinal TGR5 and VDR expression in patients with UC. The relationship between BAs and inflammatory cytokines has not been investigated in UC patients. It was hypothesized that BA metabolites may play a role in the pathogenesis of UC.

Research motivation

The main topics of this study included clinical assessments, screening different key gut microbiota and BAs, examining BA receptor expression in UC patients and healthy controls (HCs), performing correlation analyses between these parameters, and clarifying whether there were similar mechanisms to those of animal studies in UC patients. The findings suggested a mechanism by which the gut microbiota and BAs may participate in the pathophysiology of UC and may provide new insights into the management of UC.

Research objectives

The aims of this study were to compare differences in the gut microbiota, fecal BAs, and BA receptor expression in the intestinal mucosa between UC patients and HVs and to analyze the relationship of BAs with the gut microbiota and inflammatory cytokines.

Research methods

The present study used 16S rDNA sequencing technology to detect the differences in the intestinal flora between UC patients and HCs. Fecal BAs were measured by targeted metabolomics approaches. Mucosal TGR5 and VDR expression was analyzed using immunohistochemistry, and serum inflammatory cytokine levels were detected by ELISA.

Research results

It was found that the diversity of gut microbes in UC patients was reduced compared with that in HCs. The concentrations of fecal secondary BAs such as lithochalic acid, deoxycholic acid, glycodeoxycholic acid, glycolithocholic acid, and taurolithocholic acid in UC patients were significantly lower than those in HCs and were positively correlated with *Butyricicoccus*, *Roseburia*, *Clostridium IV*, *Faecalibacterium*, and *Clostridium XlVb*. The concentrations of primary BAs such as taurocholic acid, cholic acid, taurochenodeoxycholic acid, and glycochenodeoxycholic acid in UC patients were significantly higher than those in HCs and positively correlated with *Enterococcus*, *Klebsiella*, *Streptococcus*, *Lactobacillus* and proinflammatory cytokines. The mucosal expression of TGR5 was significantly elevated in UC patients. VDR expression in colonic mucosal specimens was significantly decreased in UC patients.

Research conclusions

Fecal BAs are closely related to the gut microbiota and serum inflammatory cytokines. Dysregulation of the gut microbiota and altered constitution of fecal BAs may participate in regulating inflammatory responses *via* the BA receptors TGR5 and VDR. These findings not only contribute to the understanding of the role of the gut microbiota and metabolites in UC pathogenesis but also offer a valuable reference for future research and more effective therapies.

Research perspectives

This preliminary study investigated the changes in the fecal BA metabolite profile and analyzed the relationship between metabolites, the gut microbiota, and inflammation in patients with UC. In the future, we will focus on the following aspects. First, due to limited time and conditions, the sample size was relatively small, which may impact the reliability of the conclusion. Second, we cannot draw causal inferences in this cross-sectional study. Therefore, conclusions need to be further verified by well-designed large-sample clinical studies and basic studies. Third, diet was not standardized during the study period. It is necessary to standardize diet in future studies to avoid the influence of diet on the intestinal flora and metabolites.

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ORIGINAL ARTICLE

Retrospective Study

High rate of complete histopathological response in hepatocellular carcinoma patients after combined transarterial chemoembolization and stereotactic body radiation therapy

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Institutional review board statement: This study was reviewed and approved by the local Ethics Committee of each participating center.

Informed consent statement: We performed a retrospective analysis and all data were completely anonymized for analysis and storage. In addition, there was no risk for the subjects as our study was a retrospective analysis of patients treated with standard of care procedures. According to local ethics committees there is no informed consent required given the retrospective study design, anonymous data analysis, and lack of any risks for the subjects in this study.

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Abstract

BACKGROUND

Liver transplantation (LT) presents a curative treatment option in patients with early stage hepatocellular carcinoma (HCC) who are not eligible for resection or ablation therapy. Due to a risk of up 30% for waitlist drop-out upon tumor progression, bridging therapies are used to halt tumor growth. Transarterial chemoembolization (TACE) and less commonly stereotactic body radiation therapy (SBRT) or a combination of TACE and SBRT, are used as bridging therapies in LT. However, it remains unclear if one of those treatment options is superior. The analysis of explant livers after transplantation provides the unique opportunity to investigate treatment response by histopathology.

AIM

To analyze histopathological response to a combination of TACE and SBRT in HCC in comparison to TACE or SBRT alone.

METHODS

In this multicenter retrospective study, 27 patients who received liver transplantation for HCC were analyzed. Patients received either TACE or SBRT alone, or a combination of TACE and SBRT as bridging therapy to liver transplantation. Liver explants of all patients who received at least one TACE and/or SBRT were analyzed for the presence of residual vital tumor tissue by histopathology to assess differences in treatment response to bridging therapies. Statistical analysis was performed using Fisher-Freeman-Halton exact test, Kruskal-Wallis and Mann-Whitney-*U* tests.

RESULTS

Fourteen patients received TACE only, four patients SBRT only, and nine patients a combination therapy of TACE and SBRT. There were no significant differences between groups regarding age, sex, etiology of underlying liver disease or number and size of tumor lesions. Strikingly, analysis of liver explants revealed that almost all patients in the TACE and SBRT combination group (8/9, 89%) showed no residual vital tumor tissue by histopathology, whereas TACE or SBRT alone resulted in significantly lower rates of complete histopathological response (0/14, 0% and 1/4, 25%, respectively, P value < 0.001).

CONCLUSION

Our data suggests that a combination of TACE and SBRT increases the rate of complete histopathological response compared to TACE or SBRT alone in bridging to liver transplantation.

Key Words: Hepatocellular carcinoma; Transarterial chemoembolization; Stereotactic body radiation therapy; Bridging therapy; Liver transplantation

Core Tip: In patients with early-stage hepatocellular carcinoma (HCC) who are not eligible for resection or ablation, liver transplantation presents a curative treatment option. To halt tumor growth during waiting time, bridging therapies such as transar-

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terial chemoembolization (TACE), ablation, and stereotactic body radiation therapy (SBRT) are used prior to liver transplantation. In a multicenter retrospective trial with 27 HCC patients who received either TACE or SBRT alone, or a combination of TACE and SBRT, explant histopathology was analyzed to assess treatment response. Strikingly, almost all patients in the combination group exhibited no residual vital tumor by histopathology, whereas TACE or SBRT alone resulted in significantly lower rates of complete histopathological response.

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INTRODUCTION

Hepatocellular carcinoma (HCC) ranks among the leading causes of cancer-associated deaths worldwide. In very early [1 tumor < 2 cm, Barcelona clinic liver cancer (BCLC) 0, United Network for Organ Sharing (UNOS) T1] and early (1 tumor 2-5 cm or 2-3 tumors ≤ 3 cm, BCLC A, UNOS T2) stage HCC, surgical resection or local ablation is the treatment of choice. However, accompanying cirrhosis and tumor location often preclude these curative treatment approaches. Additionally, recurrence rates after resection of early HCC (BCLC A) are high with up to 70% after 2 years[1]. In contrast, liver transplantation (LT) is a curative treatment option not only for the tumor but also for the underlying precancerous condition (*i.e.* liver cirrhosis, chronic HBV infection, non-alcoholic fatty liver disease) with excellent 5-year survival (65%-78%) and low recurrence rates (11%-18%) if Milan criteria (MC, 1 tumor ≤ 5 cm or 3 tumors ≤ 3 cm, without vascular invasion) are fulfilled[2,3]. Acceptable outcomes after LT are even achieved in patients outside MC, though 5-year survival rates are noticeably lower (46%-60%), depending on size and number of tumor lesions, alpha-fetoprotein (AFP), and treatment response[2,4,5]. Therefore, MC are widely accepted to identify HCC patients who will benefit from LT.

Currently about 30%-35% of patients on the waiting list for LT in Europe suffer from HCC[3]. In the US, the percentage is lower, but has been steadily rising over the recent years[6]. Due to organ shortage, waiting periods are long with a high risk for tumor progression and therefore drop-out from the waiting list. Without any bridging therapy, tumor progression beyond MC has been reported in up to 30% of cases[7]. To avoid tumor progression, locoregional therapies are used as bridging to LT and several countries have now implemented response to locoregional therapies into their transplant allocation systems[8,9].

The most commonly used locoregional bridging therapies are transarterial chemoembolization (TACE) and thermal ablation, such as radio frequency ablation (RFA) and microwave ablation (MWA). These therapies are also recommended in current guidelines for the treatment of BCLC A or B stage HCC[3,9]. However, in patients with tumors not suitable for these standard treatment modalities, individual treatment approached such as 90Y radioembolization or stereotactic body radiation therapy (SBRT) have been used to control tumor growth[10-12].

RFA or MWA are established in the treatment of early and very early stage HCC (BCLC A and 0, respectively) as a curative approach or as bridging with excellent long term outcomes after LT[13-16]. However, thermal ablation is not technically feasible in all patients. Tumor location can preclude safe and successful treatment, for example in subcapsular HCC close to the diaphragm or in lesions close to the liver hilum[17]. If these lesions are not amenable for resection, TACE and less commonly alternative treatment options such as SBRT or radioembolization are used to achieve tumor control in a pre-transplant setting[12].

TACE, which also presents the standard of care in patients with intermediate stage HCC, is a widely used bridging therapy that can efficiently halt tumor growth in the pre-transplant setting[2,3,9,18-21]. Even in patients initially outside MC, who achieve

down-staging to fulfill MC after TACE, overall survival rates are comparable to patients that were never outside MC [5,22]. On the other hand, insufficient response to TACE is a predictor of post-transplant HCC recurrence[20]. With longer waiting times due to organ shortage, the risk of tumor progression after treatment with TACE remains a substantial concern.

SBRT is a local ablative treatment option for patients not suitable for resection or thermal ablation. In particular, SBRT can also be applied to tumors close to large blood vessels or wherever tumor location precludes RFA or MWA[17]. Even with excellent local control of tumor lesions and a good safety profile, current guidelines do not regard SBRT as primary treatment option due to a lack of large randomized trials[23-26]. SBRT is therefore mainly performed as an individualized treatment approach in selected cases. In a pre-transplant setting, complete histopathological response after SBRT in 3 of 11 tumor lesions (27%) in a small cohort of 10 patients has been reported [27]. In a larger retrospective analysis of 30 patients treated with SBRT prior to transplantation, drop-out rate (16.7%) and 5-year survival (61%) were not different from patients treated with TACE or RFA[10].

A combination of TACE and SBRT is an alternative local treatment option with therapeutic benefits and a good safety profile, though data from large randomized controlled trials is still missing[28,29]. A recent retrospective analysis of SBRT and TACE (n = 49) compared to TACE alone (n = 98) showed significantly better disease control, progression free survival, and 3-year overall survival for the TACE and SBRT combination group[30]. In a larger study of 199 patients with tumors ≤ 5 cm, combination therapy lead to improved local control rates, but did not have any effect on overall survival[31]. To date, TACE and SBRT combination therapy has been mainly used as a palliative treatment approach and only rarely as bridging to LT. Therefore, data on histopathologic response is limited with only one study reporting on two tumors which showed near complete tumor response after treatment[12]. Three ongoing prospective trials are currently recruiting patients to evaluate TACE and SBRT combination therapy in comparison to TACE (NCT01918683; NCT02513199; NCT03895359).

Given the promising results achieved with TACE and SBRT combination therapy, we aimed to analyze treatment response prior to liver transplantation in patients within MC who could not be treated with resection or ablation and were treated with TACE and SBRT.

MATERIALS AND METHODS

Patients

This multi-center retrospective trial was conducted to specify treatment options that may improve prognosis in patients with HCC and possible LT. Three German transplant centers, University Hospital rechts der Isar, Munich, University Hospital of Munich, and Hannover Medical School participated in the study. Protocols for patient analysis were reviewed and approved by the local ethics committee of each participating center. Decisions for tumor treatment were discussed in a multidisciplinary tumor board. Patients received treatment as standard of care and data were collected retrospectively.

For this study, medical records of all patients with liver cirrhosis and HCC within MC who underwent LT between 2007 and 2019 were retrospectively reviewed. From Hannover Medical School, only patients who received TACE and SBRT or SBRT alone prior to LT were screened. Patients who received at least one TACE with or without SBRT (TACE only: 8 patients at University Hospital rechts der Isar, Munich; 6 patients at University Hospital of Munich; TACE + SBRT: 4 patients at University Hospital rechts der Isar, Munich; 5 patients at University Hospital of Munich), or at least one SBRT alone (2 patients at University Hospital of Munich; 2 patients at Hannover Medical School), were included in our study. Patients who received additional tumor therapies as bridging such as resection of individual lesions, RFA, or MWA were not included into our study since these therapies are established as a curative treatment option. Additionally, data showing excellent response by radiology and histopathology to these therapies is already available[12,32].

Observation period started with initial diagnosis through December 2019. To compare the different dose and fractionation regimens used for SBRT, the biological equivalent dose (BED) of the surrounding isodose was calculated according to the formula BED = nd (1+ d/$alphabeta$) (with n: Number of fractions, d: Single dose and $alpha/beta$ set to 10).

Number and size of HCCs were documented by magnetic resonance imaging or computed tomography scan at the time of diagnosis. Number of treatment cycles, time of treatment, and radiation dose were assessed when applicable. Additionally, age, sex, cause of liver cirrhosis (alcohol, chronic viral hepatitis, other) and serum AFP levels were analyzed. After transplantation, the presence of vital tumor tissue in explant livers was analyzed. Specifically, size and number of any remaining tumor nodules were determined macroscopically and by histopathology in order to identify differences in tumor response. The absence of vital tumor tissue was considered as complete response.

Statistical analysis

The study was designed as a retrospective multicenter longitudinal survey. All data were analyzed using Microsoft Excel (version 16) and SPSS (version 25). Statistics were performed using Fisher-Freeman-Halton test. Due to the limited sample size, no multivariate analysis was performed. Kruskal-Wallis tests as well as Mann-Whitney-*U* tests were used for comparisons of variables between groups, when appropriate. All statistical tests were performed two-sided using a significance level of $\alpha = 5\%$.

RESULTS

The study cohort comprised 27 subjects with HCC of whom 14 received TACE only, four SBRT only, and nine a combination of TACE and SBRT. Within the study cohort, 20 (74%) patients were male, 7 (26%) female. Mean patient age was 60 (SD ± 6) years ranging from 48 to 71 years. All patients suffered from cirrhosis, mostly due to alcohol (11/27; 40%) or hepatitis C (10/27; 37%).

Most patients had a single tumor lesion (20/27; 74%). Of the seven patients with two lesions, one patient received a combination of TACE and SBRT and one patient SBRT only, the others were in the TACE only group. At the time of diagnosis, mean tumor size was 29.3 mm (SD ± 9.5 mm). Median AFP was 8.0 ng/mL, with 1st quartile 5.0 ng/mL and 3rd quartile 58.0 ng/mL (range 1.2 to 2515 ng/mL).

Treatment plans were tailored to each individual patient and varied in number of TACE cycles (median = 2, range 1 to 5) and SBRT radiation dose (range 18.9 to 54 Gy, in 3 to 9 fractions, prescribed to the surrounding isodose) (Table 1). The most common schemes were 3 × 12.5 Gy prescribed to the 65% -isodose and 3 × 15 Gy prescribed to the 60%-isodose delivered every other day. There were no statistically significant differences in age, gender, origin of cirrhosis, tumor size or number of tumor lesions between groups (Table 1, Figure 1). LT was performed after a median interval of 114 d (range 1 to 786 d) from SBRT treatment (Supplementary Figure 1).

Analysis of explant livers by histopathology showed different treatment responses. In 9/27 patients (33%), no vital tumor was detected microscopically, which was considered as complete response (Figure 2A-D, Supplementary Figure 2). Strikingly, for the majority of patients in the TACE and SBRT combination therapy group a complete response was observed (8/9, 89%), compared to none in the TACE only group (0/14, 0%) and only one in the SBRT only (1/4, 25%) group ($P < 0.001$) (Table 2, Figure 2E). When tumor size at the time of initial diagnosis was compared to tumor size in liver explants, treatment with TACE alone led to a stabilization or a decrease in tumor size in the majority of patients, but could not stop tumor growth in all cases. In the combination group, the only sample with vital tumor showed disease stabilization (increase in size < 20%) with most lesions being completely necrotic by histopathology as described above. In the SBRT group, one completely necrotic tumor was observed, but no conclusions on treatment response could be made due to the small sample size (Figure 3).

Of note, the only patient with vital tumor in the TACE and SBRT group had by far the highest AFP level (2515 ng/mL, Figure 4) and the shortest time interval between SBRT and LT (29 d). The only patient with a complete response in the SBRT only group had the smallest tumor in this group (12 mm, BCLC 0), the longest time between SBRT and LT (256 d) and was transplanted due to deterioration of liver function. While there was a weak correlation between tumor size and treatment response in the overall patient cohort, the difference was not statistically significant (Supplementary Figure 3).

On follow-up, two patients suffered from extrahepatic recurrence after LT, of whom one was in the TACE only group (with vital tumor tissue by explant histology) and one patient was in the TACE and SBRT group (no vital tumor detected in explanted liver).

Table 1 Patients characteristics of all patients and separated into treatment groups, *n* (%)

Characteristics	Total number of patients (n^1 = 27)	TACE only (*n* = 14)	Combination of TACE and SBRT (*n* = 9)	SBRT only (*n* = 4)	*P* value
Male/female	20 (74)/7 (26)	12 (86)/2 (14)	5 (56)/4 (44)	3 (75)/1 (25)	
age < 60/≥ 60 yr	13 (48)/14 (52)	6 (43)/8 (57)	5 (56)/4 (44)	2 (50)/2 (50)	
mean age yr ± SD	60 ± 6	59.5 ± 8	61 ± 4	60 ± 2	0.963
Genesis of cirrhosis					
1 alcohol	11 (41)	6 (44)	3 (33)	2 (50)	
2 viral[2]	12 (44)	8 (57)	3 (33)	1 (25)	
3 others[3]	4 (15)	0 (0)	3 (33)	1 (25)	
Numbers of TACE treatment cycle[4]					
1	12 (44)	5 (36)	7 (78)	NA	
2	6 (22)	4 (29)	2 (22)		
3 or more	5 (19)	5 (36)	0 (0)		
Mean radiation dose in Gy		NA	40.00 ± 3.75	36.80 ± 17.56	0.586

[1]*n* = 27 is the number of patients included into our study.
[2]Ten patients suffered from hepatitis C virus (HCV), two from hepatitis B virus.
[3]One patient with combination of alcohol and HCV, three patients with autoimmune hepatitis.
[4]No statistical testing due to different therapeutic approaches. Dichotomous variables are presented in number and percentage, continuous variables in mean ± SD. TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy.

Table 2 Tumor response by treatment (including tumor characteristics), *n* (%)

	Total number of patients (n^1 = 27)	TACE only (*n* = 14)	Combination of TACE and SBRT (*n* = 9)	SBRT only (*n* = 4)	*P* value
Complete response	9 (33.3)	0 (0)	8 (88.89)	1 (25)	< 0.001
Number of tumor lesions					0.517
1	20 (74)	9 (64)	8 (89)	3 (75)	
2	7 (26)	5 (36)	1 (11)	1 (25)	
Mean tumor size[2]	29.3 ± 9.46	29.50 ± 7.63	27.67 ± 9.54	26.67 ± 14.50	0.389
BCLC[4]					
0	1 (4)	0 (0)	0 (0)	1 (25)	
A	26 (96)	14 (100)	9 (100)	3 (75)	
Median AFP[3,5]	8.0, 5.0/58.0	8.05, 5.2/84.2	8.0, 5.0/17.7	9.85, 8.0/11.85	

[1]*n* = 27 is the maximum number of patients included into our study.
[2]mean size of largest tumor in mm ± SD at time of diagnosis.
[3]Median AFP in ng/ml with 1st/3rd quartile at time of diagnosis.
[4]No statistical testing due to small sample size.
[5]No statistical testing due to high variation and SD. Patients characteristics of all patients and separated into treatment groups. Dichotomous variables are presented in number and percentage, continuous variables in mean ± SD. AFP: Alpha-fetoprotein; TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy; BCLC: Barcelona clinic liver cancer.

DISCUSSION

In this study, patients with HCC who received a combination therapy of TACE and SBRT before LT had a significantly higher rate of complete histopathological response than patients who received TACE or SBRT alone.

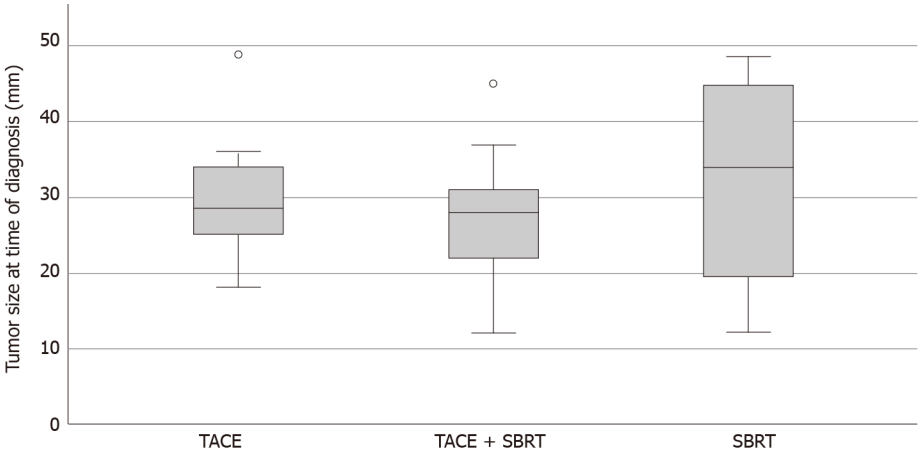

Figure 1 Box plot showing tumor size in each treatment group. Median is represented by bars, 25%-75% percentiles by boxes and outliers by markers. There were no statistically significant differences between groups. TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy.

Current bridging strategies to LT aim to stabilize the disease but are not sufficient to delay tumor growth in all patients[7]. Thermal ablation and TACE are the most commonly used bridging therapies before LT. However, RFA or MWA are not technically feasible in all patients mostly due to tumor location, and a complete pathologic response to TACE alone is found in less than 35% of patients receiving LT for HCC[21,33]. Disease progression poses a risk in these patients – especially in countries with long waiting times such as Germany, with an average of two to three patients per center removed due to tumor progression each year, revoking any curative treatment option. Based on the outcomes of previous studies indicating improved response after TACE and SBRT combination *vs* TACE alone in HCC[34,35], we used TACE and SBRT combination therapy as an individualized treatment approach in patients at risk for tumor progression beyond MC to achieve long term disease stabilization.

While a better outcome of the combination of TACE and SBRT was expected[28,30], the rate of complete tumor response by histopathology was surprisingly high in our patient cohort. In almost all patients who received combined TACE and SBRT, no residual vital tumor was detected in explant livers (TACE and SBRT 89% *vs* TACE alone 0%; $P < 0.001$). The only patient in the TACE and SBRT group with vital tumor tissue by histopathology had a very high AFP (2515 ng/mL) and was transplanted less than one month after SBRT treatment. On the other hand, one patient with complete response in the SBRT-only group had a lesion < 2 cm, an interval of more than 6 mo between SBRT and LT, and was transplanted for deterioration of liver function.

In the small group of patients treated with SBRT alone (four patients in which chemoembolization was not feasible for anatomical reasons or where treatment decision was made at an external hospital), only one of four patients had no vital tumor by histomorphology. Importantly, we had no indications for differences regarding SBRT schemes between groups in our study cohort (Table 1, Supplementary Figure 4 and 5). However, from a sample size this small and above all a very short time interval between SBRT and LT in three out of four patients in the SBRT only group (Supplementary Figure 1) it cannot be ruled out that SBRT alone might be equally efficient to TACE and SBRT combination therapy. However, recently published data from a cohort of 14 patients showing complete response by histopathology in 23.1% of tumor nodules in liver explants is in line with our data[36] – indicating that complete tumor necrosis is not commonly achieved after SBRT alone.

Importantly, none of our patients showed any higher grade treatment-related toxicities, which is in line with previous analyses[37,38]. While treatment side effects were not prospectively evaluated, there were no reports of deterioration of liver function in the TACE and SBRT group, or other higher-grade side effects observed at our centers. However, our study comprises a relatively small group of patients and almost all patients of our cohort had well-preserved liver function. Therefore, outcomes might have been different in patients with impaired liver function[39,40]. Clearly, long-term hepatic toxicity, which is mostly negligible in a pre-transplant setting, might be limiting in palliative treatment strategies[38].

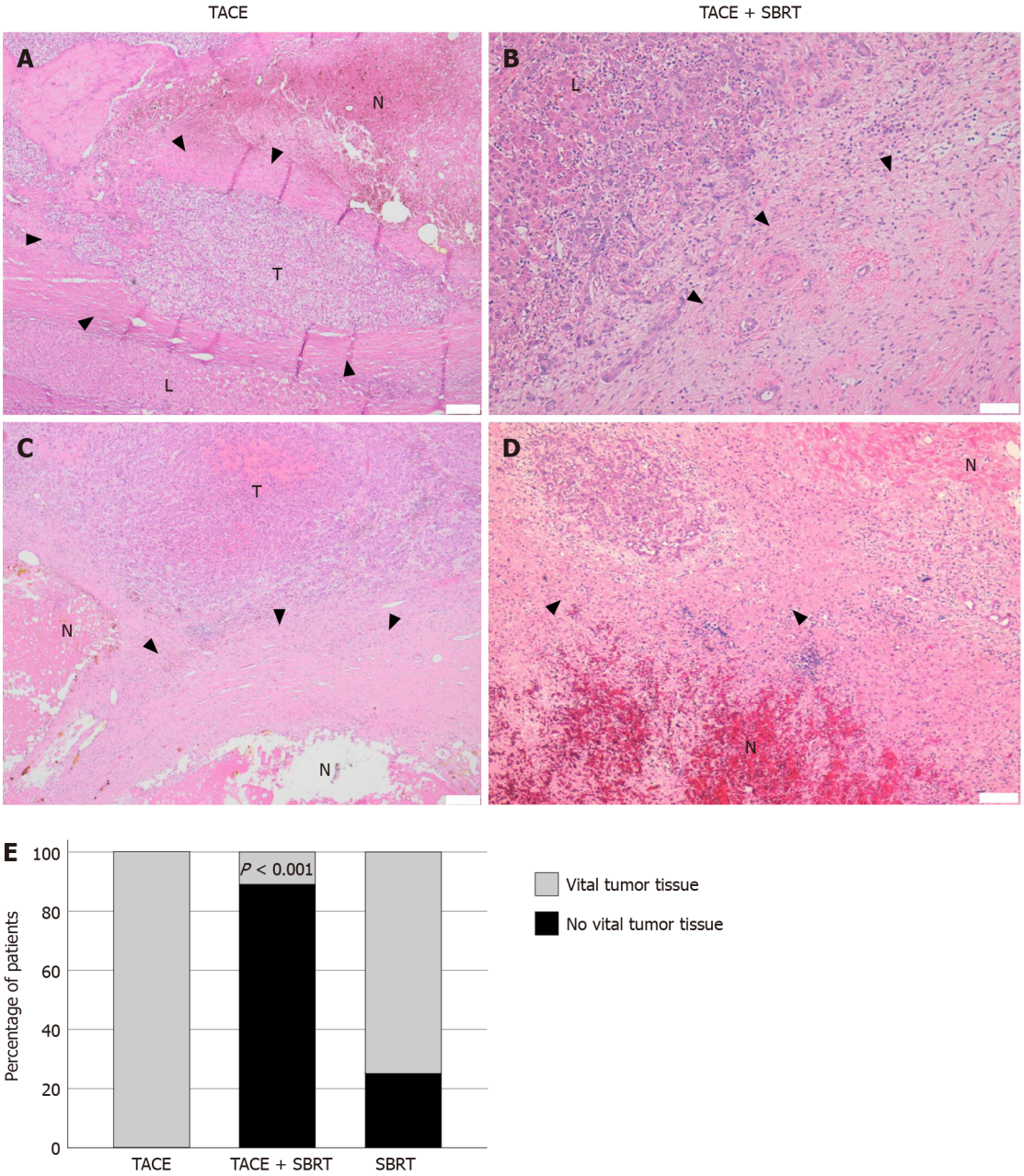

Figure 2 Tumor response by histopathology for each treatment group. A-D: Representative histopathology (Hematoxylin and eosin stain) of tumor lesions in explant livers after transarterial chemoembolization (TACE) (A, C; scale bar 200 μm) or TACE + stereotactic body radiation therapy (SBRT) (B, D; scale bar 100 μm). Samples show necrosis with granulation tissue and organization by connective tissue at the border area (arrowheads) to normal liver. Residual tumor tissue was observed in TACE only samples, while no vital tumor cells could be detected in most patients in the TACE + SBRT group (B, D); E: Bar graph displaying the proportion of vital tumor tissue in each treatment group. Combination therapy with TACE and SBRT leads to a statistically significantly lower number of residual tumor tissue in explant livers ($P < 0.001$). TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy; N: Necrosis; L: Normal liver; T: Tumor tissue.

Together, data from our study strongly indicate that TACE and SBRT combination therapy might lead to higher rates of complete histopathological tumor response than TACE alone. Nevertheless, this study has some limitations. A sample bias due to the multicenter, retrospective design, large duration of study recruitment and little opportunity to adjust for possible confounders due to small sample size cannot be ruled out. Furthermore, most of the patients with two tumor lesions were in the TACE group, which might have biased the results towards a lower percentage of complete response in this cohort. Additionally, the limited number of patients in this study does not allow to draw any conclusions on tumor recurrence or even overall survival. Our cohort accounts for less than 25% of all patients that were transplanted with HCC in Munich transplant centers as most HCC patients received additional bridging therapies such as thermal ablation or even resection whenever feasible. Therefore, whether these histopathological findings will translate into a survival benefit remains to be investigated prospectively in a larger patient cohort. For example, one patient

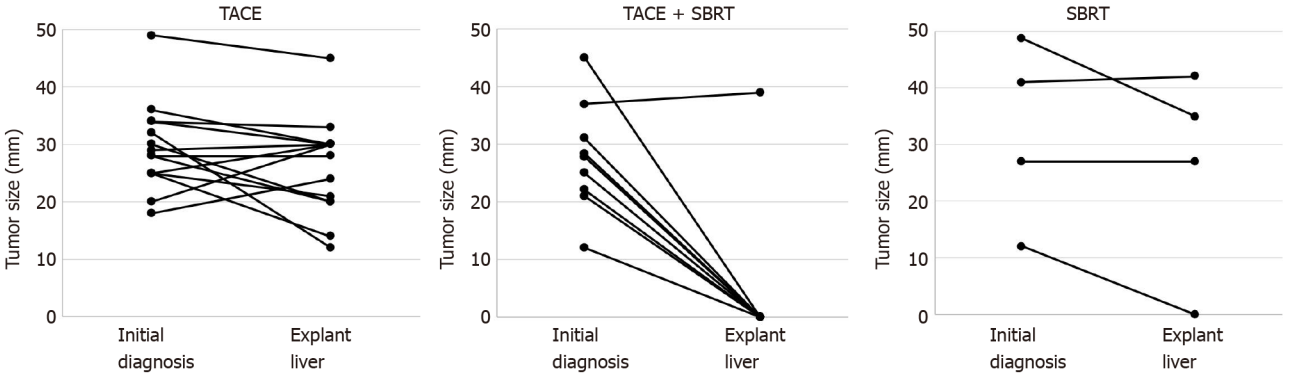

Figure 3 Tumor size at time of diagnosis and in explant histology for each treatment group. Tumor size at initial diagnosis was determined by radiology. When more than one tumor was present, the size of the largest tumor was graphed. In cases where no vital tumor tissue was detected, a size of 0 mm was graphed. TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy.

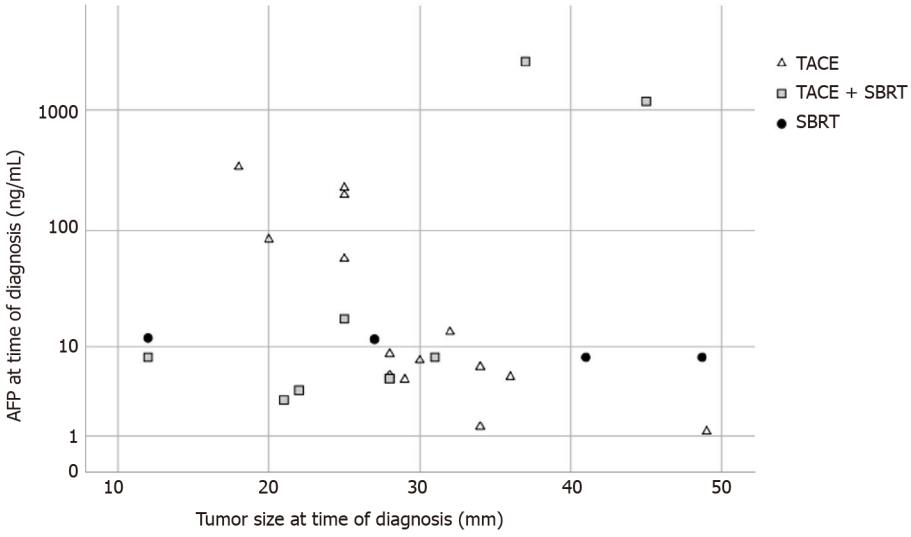

Figure 4 Correlation of alpha-fetoprotein and tumor size for each group. Scatter chart showing the correlation of alpha-fetoprotein in ng / mL and tumor size in mm for each group. AFP: Alpha-fetoprotein; TACE: Transarterial chemoembolization; SBRT: Stereotactic body radiation therapy.

who received TACE and SBRT combination therapy and showed a complete response in the explant liver developed metachronous metastatic disease less than 6 mo after liver transplantation. In this patient, metastases first occurred in the skull that was not routinely screened by standard tumor staging procedures while the patient was on the waiting list. If bone metastases to the skull were already present before completing SBRT bridging therapy before LT cannot be determined retrospectively but is certainly a possibility. The development of extrahepatic metastases therefore remains an eminent risk even with excellent local tumor control, yet it occurs very rarely at this stage. On the other hand, a patient with high AFP (2515 ng/mL) indicating limited prognosis was successfully transplanted after TACE and SBRT combination therapy. Despite only partial response with 20% of vital tumor tissue by histopathology, he shows no signs of tumor recurrence more than 4 years after LT.

More recently, down-staging to MC has been implemented in organ allocation criteria in several countries. In our cohort, a complete response with decrease of tumor size and loss of arterial hyperperfusion was routinely observed in the combination cohort (Figure 5).

Even though our study did not include any patients beyond MC, TACE and SBRT combination therapy might be efficient for down-staging patients to MC to reach requirements for LT. As a sample bias cannot be excluded due to the limited number of patients, retrospective design, and long recruitment time, further studies in a larger patient cohort are needed to confirm high treatment response to TACE and SBRT combination therapy and to clarify if these findings translate into a decreased number of waitlist removals due to tumor progression or into reduced rates of tumor

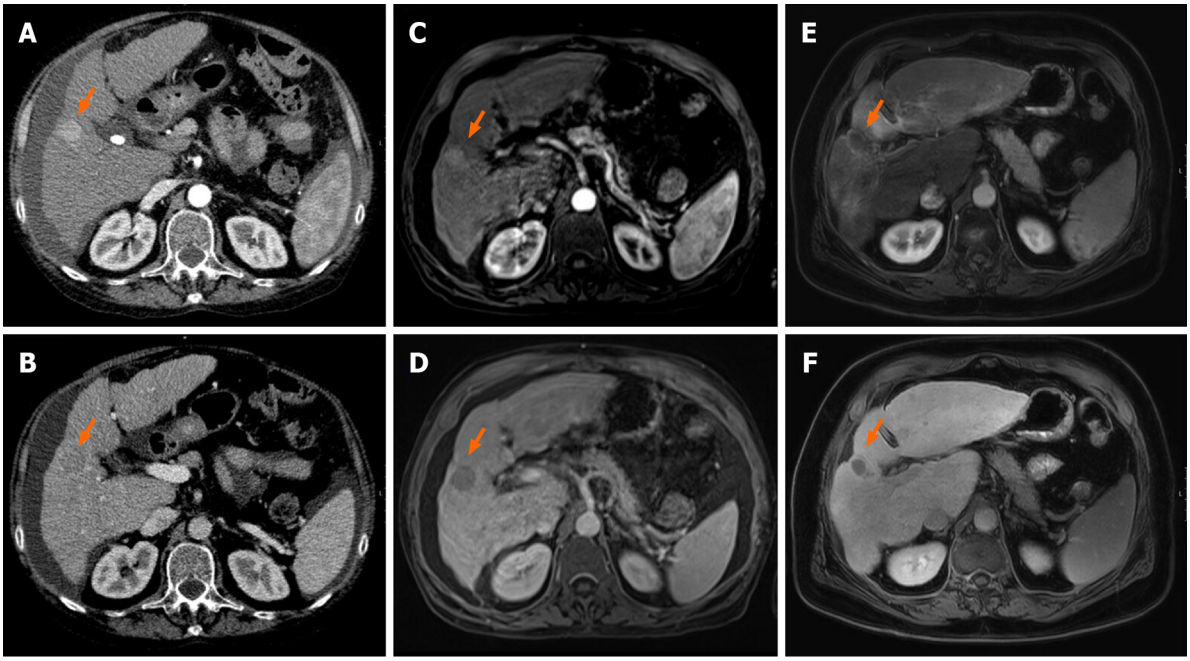

Figure 5 Imaging from before and after combination therapy in one patient in the transarterial chemoembolization + stereotactic body radiation therapy cohort. A, B: Contrast enhanced computed tomography (CT); C-F: Magnetic resonance imaging (MRI). Contrast enhanced CT and MRI, arterial phase (top row) and portal venous phase (bottom row) cross sectional imaging from before (A–D) and after (E, F) treatment. At baseline CT and MRI, a well-defined nodular lesion with typical contrast agent dynamics is noted in the right liver lobe (Arrows). After treatment, typical radiation induced peri-lesional hyperenhancement and no hepatocellular carcinoma-specific contrast agent uptake is noted.

recurrence after liver transplantation.

CONCLUSION

In summary, data from our study shows that patients not eligible for ablation or resection who received TACE and SBRT combination therapy were significantly more likely to have complete histopathological tumor response in explanted livers compared to patients treated with TACE or SBRT only. Whether TACE and SBRT combination therapy results in decreased number of waiting list removals and/or a reduced rate of tumor recurrence after LT needs to be evaluated prospectively in a larger patient cohort. Additionally, future studies will need to show if patients within MC who are not eligible for LT because of old age or relevant co-morbidities could benefit from TACE and SBRT combination therapy if curative resection or ablation is not possible due to tumor location.

ARTICLE HIGHLIGHTS

Research background

In patients with hepatocellular carcinoma (HCC) who are not eligible for resection or ablation therapy, liver transplantation presents a curative treatment option. Due to organ shortage there are long waiting times with the risk of tumor progression. Therefore, efficient bridging therapies are needed.

Research motivation

This study evaluated the treatment response to a combination therapy of transarterial chemoembolization (TACE) and stereotactic body radiation therapy (SBRT) as bridging to liver transplantation.

Research objectives

This study aimed to establish a pathologic response in explant livers after TACE and SBRT.

Research methods

Retrospective multicenter analysis of 27 patients that underwent liver transplantation and received either TACE or SBRT alone or a combination therapy of TACE and SBRT as bridging to liver transplantation.

Research results

About 89% of the patients in the TACE and SBRT combination group had no residual tumor tissue by histopathology, whereas 0% in the TACE only and 25% in the SBRT only group had a complete histopathological response.

Research conclusions

A combination of TACE and SBRT shows superior pathologic response in comparison to TACE or SBRT alone for bridging to liver transplantation in patients with HCC.

Research perspectives

If complete histopathological response in the TACE and SBRT combination group translates into a better progression free and overall survival needs to be evaluated in larger studies.

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ORIGINAL ARTICLE

Retrospective Study

Stem cell injection for complex anal fistula in Crohn's disease: A single-center experience

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Author contributions: Schwandner O performed the research and wrote the paper.

Institutional review board statement: According to the Ethics Committee of the Bavarian State Chamber of Physicians retrospective reviews of anonymized patient data do not require ethical approval, No. 2021-1022.

Informed consent statement: Patients were not required to give informed consent to the study because the retrospective analysis used anonymized clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: The author had honoraria from Takeda and Medtronic for invited presentations during advisory board meetings and satellite symposia. Neither funding nor financial support was administered for the current study.

Data sharing statement: No additional data are available.

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Abstract

BACKGROUND

Despite tremendous progress in medical therapy and optimization of surgical strategies, considerable failure rates after surgery for complex anal fistula in Crohn's disease have been reported. Therefore, stem cell therapy for the treatment of complex perianal fistula can be an innovative option with potential long-term healing.

AIM

To evaluate the results of local administration of allogenic, adipose-derived mesenchymal stem cells (darvadstrocel) for complex anal Crohn's fistula.

METHODS

All patients with complex anal fistulas associated with Crohn's disease who were amenable for definite fistula closure within a defined observation period were potential candidates for stem cell injection (darvadstrocel) if at least one conventional or surgical attempt to close the fistula had failed. Darvadstrocel was only indicated in patients without active Crohn's disease and without presence of anorectal abscess. Local injection of darvadstrocel was performed as a standardized procedure under general anesthesia including single-shot antibiotic prophylaxis, removal of seton drainage, fistula curettage, closure of the internal openings and local stem cell injection. Data collection focusing on healing rates, occurrence of abscess and follow-up was performed on a regular basis of quality control and patient care. Data were retrospectively analyzed.

RESULTS

Between July 2018 and January 2021, 12 patients (6 females, 6 males) with a mean age of 42.5 (range: 26-61) years underwent stem cell therapy. All patients had a minimum of one complex fistula, including patients with two complex fistulas in 58.3% (7/12). Two of the 12 patients had horse-shoe fistula and 3 had one complex fistula. According to Parks classification, the majority of fistulas were trans-

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sphincteric (76%) or suprasphincteric (14%). All patients underwent removal of seton, fistula curettage, transanal closure of internal opening by suture (11/12) or mucosal flap (1/12) and stem cell injection. At a mean follow-up of 14.3 (range: 3-30) mo, a healing rate was documented in 66.7% (8/12); mean duration to achieve healing was 12 (range: 6-30) wk. Within follow-up, 4 patients required reoperation due to perianal abscess (33.3%). Focusing on patients with a minimum follow-up of 12 mo (6/12) or 24 mo (4/12), long-term healing rates were 66.7% (4/6) and 50.0% (2/4), respectively.

CONCLUSION

Data of this single-center experience are promising but limited due to the small number of patients and the retrospective analysis.

Key Words: Complex anal fistula; Crohn's disease; Stem cell therapy; Mesenchymal stem cells; Darvadstrocel; Treatment; Surgery; Outcomes

Core Tip: This is a retrospective single-center study on the application of allogenic, adipose-derived mesenchymal stem cells (darvadstrocel) for complex perianal fistula associated with Crohn's disease, providing structured inclusion and exclusion criteria for 12 patients. The current data demonstrate that the management by local stem cell therapy is safe and effective. However, based on the small patient sample size and the retrospective analysis of routine clinical data, a general conclusion remains limited. Therefore, further prospective controlled studies are mandatory to assess the definite role of adipose-derived mesenchymal stem cells for complex anal fistula in Crohn's disease.

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INTRODUCTION

Both medical and surgical options for complex perianal fistulas associated with Crohn's disease remain difficult and challenging[1-5]. Despite recent progress in medical treatment including biologicals and actual trends in sphincter-preserving surgical techniques, considerable recurrence rates after definite surgery for Crohn's anal fistula have been documented[2,3,5]. As an interdisciplinary treatment regimen is the prerequisite for disease control and potential long-term remission of perianal fistulizing Crohn's disease, therapeutic goals include symptom improvement (*e.g.*, reduction of secretion, absence of pain), prevention of recurrent perianal abscess requiring further surgery, preservation of continence, improvement of quality of life and, finally, healing of the fistula.

Focusing on surgical procedures for complex anal fistulas, conventional procedures such as advancement flap repair have shown considerable failure rates for Crohn's fistulas, whereas innovative surgical options such as ligation of the intersphincteric fistula tract (LIFT), biomaterials (*e.g.*, plug) or video-assisted anal fistula treatment seem to have a 50% healing rate after 12 mo without significant impairment of continence[6]. Recently, several reports and randomized studies have demonstrated that stem cell therapy for Crohn's complex anal fistula has raised the healing rates after 12 mo[2,7,8].

Impressed by the encouraging healing rates reported from the ADMIRE trial[7,8], it was the aim of this retrospective single-center study to evaluate the current results of stem cell therapy for complex anal fistulas in Crohn's disease with special focus on indication, patient selection and long-term outcome.

MATERIALS AND METHODS

Clinical setting

Stem cell injection was performed by administration of human, allogenic, expanded adipose-derived mesenchymal stem cells (darvadstrocel) for complex anal fistulas associated with Crohn's disease in adult patients. For the current study, Alofisel® (5 million cells/mL) was used (Takeda GmbH, Konstanz, Germany). Upon authorization of the European Medicines Agency (initial authorization on December 14, 2017), Alofisel® is indicated in patients with non-active or mildly active Crohn's disease when fistulas show an inadequate response (failure either due to persistence or recurrence) to at least one conventional, biological or surgical therapy. Darvadstrocel was only administered if the fistulas had conditioning by curettage or seton drainage. All potential candidates for stem cell injection had fully informed consent of the innovative method and underwent a selection process by multidisciplinary evaluation and discussion (gastroenterology and proctology); finally, the center was trained by an educational and hands-on workshop for the use of darvadstrocel in complex fistulas in Crohn's disease to ensure that only specialist physicians perform the procedure. Moreover, intrainstitutional education was conducted for darvadstrocel administration (*e.g.*, pharmacy, surgical team).

Study population

All patients with complex anorectal fistulas associated with Crohn's disease who were amenable for definite fistula closure were potential candidates for darvadstrocel administration if at least one conventional or surgical attempt to close the fistula had failed. All patients suffered from complex fistula according to American Gastroenterological Association and Parks classification[9,10]. All patients had a minimum of one complex fistula, including patients with two complex fistulas in 58.3% (7/12). According to Parks classification, 76% of the fistulas were transsphincteric and 14% were suprasphincteric. Darvadstrocel was only indicated in patients without active Crohn's disease, ruled out by ileocolonoscopy, and without the presence of anorectal abscess assessed by clinical examination and/or pelvic magnetic resonance imaging (MRI). Additionally, all patients had interdisciplinary discussion prior to surgical treatment. Specific inclusion and exclusion criteria are outlined in Table 1.

Study design and outcome evaluation

To evaluate the outcome of patients who underwent local stem cell injection by the application of darvadstrocel, a retrospective analysis of existing routine clinical data was performed. In detail, regular clinical data of patient care and quality control (including patients' characteristics, type of therapy, outcome evaluation during routine clinical follow-up) were retrospectively analyzed in an anonymized fashion. Patients underwent darvadstrocel administration not for primary study purpose but for quality assurance and patient care. To assess outcome, criteria of healing included that both internal and external openings were closed, no abscess or fluid collection was present and if the patient was free of symptoms (*e.g.*, pain, secretion). Healing was assessed by clinical examination and proctoscopy. Strict criteria and definitions of persistence and recurrence are outlined in Table 2.

Special consideration for coronavirus disease 2019 pandemic

Due to the coronavirus disease pandemic in the beginning of 2020, the coronavirus disease 2019 (COVID-19) outbreak had direct implications for patients with perianal fistulizing Crohn's disease regarding their schedule for definite surgery. As a result of the primary outbreak in March 2020, no patients had elective surgery for Crohn's associated complex anal fistulas at our institution between March 2020 and July 2020 according to governmental restrictions (German Ministry of Health). Moreover, according to a second restriction period for elective procedures at our institution starting in November 2020, potential procedures were postponed to 2021. Finally, a relevant number of patients with fistulizing perianal Crohn's disease under immunosuppression and/or maintenance therapy (*e.g.*, biologicals) postponed their surgery to 2021.

Preoperative assessment

All patients amenable for stem cell injection were seen in the proctological office 2-4 wk prior to darvadstrocel treatment. Informed consent was given, including for efficacy from the ADMIRE study, innovation of the current technique, and monitoring of side effects or adverse events, surgical technique and follow-up observation. Procto-

Table 1 Inclusion and exclusion criteria for administration of darvadstrocel

Inclusion	Exclusion
Complex anal fistulas associated with Crohn's disease (including horse-shoe fistula)	Simple fistulas associated with Crohn's disease, rectovaginal fistula
At least one conventional, biological or surgical therapy prior to stem cell injection	No medical, biological or surgical therapy prior to stem cell injection
No active Crohn's disease confirmed by ileocolonoscopy	Active Crohn's disease confirmed by ileocolonoscopy
No perianal abscess or sepsis confirmed by clinical exam, endoanal ultrasound and/or pelvic MRI	Perianal abscess or significant inflammation in the pelvis or anorectal region confirmed by MRI
Interdisciplinary recommendation	No recommendation by multidisciplinary team
Patient compliant for medical consultation, follow-up examination and interdisciplinary monitoring of disease	Non-compliance of patient
Pretreatment of fistulas by seton drainage ("conditioning")	Fistulas without seton drainage or conditioning by curettage
Maximum of two fistulas with two internal and two external openings (each)	More than two complex fistulas
No anorectal stenosis	Presence of anorectal stenosis
Fully informed consent possible	No informed consent possible

MRI: Magnetic resonance imaging.

Table 2 Definitions of outcome

Parameter	Definition	Supplementary definition
Healing	Complete healing of internal and external opening; no abscess, no symptoms	Assessment by clinical examination including proctoscopy
Persistence	No complete healing with persistence of fistula (external opening) and/or symptoms	Defined postoperative observation period
Recurrence	Recurrence of fistula after period with complete healing and interval without symptoms	With or without occurrence of abscess

logical examination included clinical examination and proctorectoscopy to rule out abscess or proctitis in the immediate preoperative phase. No antibiotic treatment was administered; setons were controlled to be correct in place. No routine curettage of fistulas drained by setons was performed in the preoperative period, and no specific change for medical treatment was performed.

Surgical technique

All surgical procedures were performed under general anesthesia. The patient was placed in lithotomy position. A single-shot antibiotic prophylaxis was routinely administered (cefuroxime/metronidazole). Initially, a careful examination under anesthesia was performed to ensure that there was no presence of abscess or proctitis; moreover, assessment of fistula length, anatomy and topography according to Parks classification[10] was performed. After removal of setons, a vigorous curettage of the fistula tracts was performed by using a metallic curette. Additionally, injection of a sodium chloride solution (0.9%) was administered after curettage. No excision of the fistula tracts were conducted; however, the external openings were sparingly excised. Afterwards, the internal openings were closed by either direct suturing (absorbable suture, Vicryl 3/0; Ethicon EndoSurgery, Norderstedt, Germany) or by mucosal flap (absorbable suture, Vicryl 3/0) if necessary.

After preparing and resuspension of the stem cells and gentle aspiration by using a syringe and injection needles (22-G), darvastrocel was injected according to the manufacturer`s recommendations: Two vials were injected around the internal openings (transanal approach), and the other two vials were injected along the fistula tracts creating small deposits of the cell suspension along the fistulas from external openings (perianal approach). After injection, a soft massage along the fistula region was performed; finally, a sterile bandage was placed around the anal region.

Postoperatively, no specific restrictions related to feeding and mobility were present; after wound control on the first postoperative day, the patient was discharged from hospital. No specific wound management was proposed (only clear water twice a day). Maintenance therapy was given as planned prior to surgery. Neither intravenous nor oral antibiotic therapy was given in the postoperative period.

Follow-up

Regular follow-up examination was performed 2, 4 and 6 wk after surgery in the proctological office, to obtain clinical data related to quality assurance and patient care. Moreover, follow-up examination was advised at 6, 12 and 24 mo after stem cell injection. Additionally, gastroenterological monitoring was advised to provide regular monitoring of Crohn's disease. As clinical follow-up was primarily indicated for routine quality control and was not indicated for study purpose, regular follow-up did not include postoperative MRI as routine examination.

RESULTS

Study population and patient characteristics

Between July 2018 and January 2021, a total of 12 patients (6 females, 6 males) with a mean age of 42.5 (range: 26-61) years underwent stem cell therapy for complex anal fistula associated with Crohn's disease. All patients had medical therapy (infliximab, adalimumab, vedolizumab or azathioprine), and complex fistulas did not respond to either medical or surgical treatment prior to stem cell therapy. Mean duration of Crohn's disease was 19.3 years (range: 7-36 years), and median duration of perianal fistulizing Crohn's disease was 8.7 years (range: 1-16 years). All fistulas had at least one conventional surgical approach to close the fistula (except seton drainage) prior to stem cell therapy; mean number of surgical attempts to close the fistula was 2.0 (range: 1-4). Details on study population and patients' characteristics are detailed in Table 3.

Fistula characterization

All patients underwent fistula conditioning by seton drainage for a mean duration of 31.5 (range: 6-72) wk, and 4 patients had undergone fecal diversion due to severe perianal sepsis. All patients had a minimum of one complex fistula, including patients with two complex fistulas in 58.3% (7/12). Two of 12 patients had horse-shoe fistula, and 3 of 12 had one complex fistula. In total, 21 fistula tracts were documented in 12 patients. According to Parks classification, the majority of fistulas were trans-sphincteric (76%) or suprasphincteric (14%).

Surgery

All patients underwent removal of seton, fistula curettage, transanal closure of internal opening by suture (11/12) or mucosal flap (1/12) and stem cell injection. No intraoperative complications or side effects were noted. Postoperative morbidity included 1 patient with fewer (without signs of perianal sepsis) on the second postoperative day. No serious adverse events were documented.

Outcome

Details on efficacy evaluation and outcome are outlined in Table 4. At a mean follow-up of 14.3 mo (range: 3-30 mo), a healing rate was documented in 66.7% (8/12); mean duration to achieve healing was 12 wk (range: 6-30 wk). Four patients had either fistula persistence (n = 2, 16.7%) or fistula recurrence (n = 2; 16.7%). Within follow-up, 4 patients developed perianal abscess (33.3%) and required reoperation. From 4 patients with ileostomy, one stoma reversal was performed 2 years after fistula healing.

Focusing on patients with a minimum follow-up of 12 mo (6/12) or 24 mo (4/12), long-term healing rates were 66.7% (4/6) and 50.0% (2/4), respectively.

DISCUSSION

Despite significant progress in medical treatment, including biologicals and cell-based therapies, development of sphincter-saving surgical techniques and interdisciplinary co-working, failure rates after definite surgery for perianal fistulizing Crohn's disease are still high[3,11-13]. Moreover, in patients with severe and refractory perianal

Table 3 Patient population

	1	2	3	4	5	6	7	8	9	10	11	12
Age	26 yr	31 yr	52 yr	49 yr	39 yr	35 yr	61 yr	26 yr	54 yr	44 yr	45 yr	48 yr
Sex	Male	Female	Female	Female	Male	Female	Male	Female	Male	Male	Male	Female
CPF Parks classification (number of fistulas)	Transsphincteric (2)	Transsphincteric (2)	Suprasphincteric (1)	Transsphincteric (2)	Transsphincteric (1) Intersphincteric (1)	Transsphincteric (2)	Transsphincteric (2)	Transsphincteric (1)	Suprasphincteric (1) Transsphincteric (1)	Transsphincteric horseshoe-fistula (2)	Transsphincteric horseshoe-fistula (2)	Suprasphincteric (1)
Number of internal/external openings	2/2	2/2	1/1	2/2	2/2	1/2	2/2	1/1	2/2	1/2	1/2	1/1
Length of fistula tract	3 cm each	3 cm each	4 cm	2 cm; 5 cm	10 cm; 1.5 cm	12 cm; 1.5 cm	3 cm; 7 cm	3 cm	6.5 cm; 2.5 cm	2.5 cm; 2.5 cm	3.0 cm; 3.0 cm	3.0 cm
Previous medical treatment	Azathioprin	Azathioprin Infliximab	Azathioprin	Azathioprin	Azathioprin	Azathioprin	Azathioprin Infliximab	Azathioprin	Adalimumab	Azathioprin Adalimumab	Infliximab	Azathiopron Antibiotics
Actual medical treatment	Adalimumab	Ustekinumab	Azathioprin	None	Ustekinumab	Vedolizumab	Ustekinumab	Infliximab	Infliximab	Infliximab	Adalimumab	None
Previous surgical treatment for abscess and fistula	Multiple abscess excisions, previous fistula surgery (1)	Multiple abscess excisions, previous fistula surgery (1)	Multiple abscess excisions,previous fistula surgery (3)	Multiple abscess excisions, previous fistula surgery (2)	Multiple abscess excisions, previous fistula surgery (3)	Multiple abscess excisions, previous fistula surgery (2)	Multiple abscess and fistula surgeries (11)	Multiple abscess and fistula surgery (4)	Multiple abscess and fistula surgery (4)	Multiple abscess and fistula surgery (4)	Multiple abscess and fistula surgery (3)	Multiple abscess and fistula surgery (9)
Previous treatment (stoma)	No	Yes	No	Yes	No	No	No	No	No	Yes	Yes	No
Seton drainage prior to Darvadstrocel (duration)	Yes (6 wk)	Yes (4 mo)	Yes (10 mo)	Yes (18 mo)	Yes (8 wk)	Yes (12 wk)	Yes (9 mo)	Yes (60 wk)	Yes (36 wk)	Yes (48 wk)	Yes (24 wk)	Yes (12 wk)
Method of closure of internal opening	Suture (1), mucosal flap (1)	Suture (2)	Suture (1)	Suture (2)	Suture (2)	Suture (1)	Suture (2)	Suture (1)	Suture (2)	Suture (1)	Suture (1)	Suture (1)
Intraoperative adverse events	No	No	No	No	No	No	No	No	No	No	No	No
Postoperative adverse events	No	No	No	No	No	No	No	No	No	No	Fewer (day 2)	No

CD: Crohn's disease; CPF: Crohn's perianal fistula.

Table 4 Efficacy evaluation and outcomes

	1	2	3	4	5	6	7	8	9	10	11	12
Length of follow-up (mo)	27	30	8	24	26	16	12	6	8	6	6	3
Efficacy evaluation (clinical)	Complete healing	Complete healing	Complete healing	Recurrence	Persistence	Complete healing	Complete healing	Complete healing	Persistence	Complete healing	Recurrence	Complete healing
Efficacy evaluation (MRI)	Not performed	Complete healing	Not performed	Recurrence	Persistence	Not performed	Not performed	Not performed	Not performed	Persistence	Recurrence	Not performed
Time to fistula closure	6 wk	12 wk	9 wk	---	---	12 wk	30 wk	9 wk	--	9 wk	---	10 wk
Incidence of abscess during follow-up	Yes	No	No	No	Yes	No	No	No	Yes	No	Yes	No
Further surgery during follow-up	Yes; Abscess excision (twice)	No	No	Seton drainage	Yes; Abscess excision	No	No	No	Yes; Abscess excision	No	Yes; Abscess excision	No
Maintenance therapy	Adalimumab	Ustekinumab	Azathioprin	Infliximab	Ustekinumab	Vedolizumab	Ustekinumab	Ceased	Infliximab	Infliximab	Infliximab	None
Stoma reversal (if present)	No stoma	Stoma reversal	No stoma	No	No stoma	No stoma	No stoma	No stoma	No stoma	No	No	No stoma

MRI: Magnetic resonance imaging.

disease, the decision to perform fecal diversion or even proctectomy has a tremendous impact on quality of life, particularly in younger patients. Impressed by the preliminary results of limited single-center studies and the encouraging data of the ADMIRE study[7,8], this was a retrospective single-center study analyzing routine clinical data on the application of allogenic, adipose-derived mesenchymal stem cells (darvadstrocel) for complex perianal fistula associated with Crohn's disease, providing structured inclusion and exclusion criteria in 12 patients.

In general, the optimal surgical treatment of complex anal fistulas in patients with Crohn's disease remains challenging. As the majority of patients need surgery for perianal fistulizing Crohn's disease and a high proportion of patients need further surgery due to abscess and recurrent fistula, the integrity of the anal sphincter is essential for preservation of continence[3]. Therefore, the risk of deterioration of continence status increases with the number of surgical procedures and the invasiveness of procedures performed. For complex anal fistulas according to

American Gastroenterological Association and Parks classification[9,10], transrectal flap procedures (advancement or mucosal flap repair) and the LIFT procedure can be considered as effective surgical options in stable disease and absence of proctitis and/or anorectal stenosis with acceptable healing rates[11-18]; however, fecal incontinence and soiling is reported in approximately 10% of patients after endorectal flap procedures[6,11,18]. Focusing on the LIFT procedure, the risk of incontinence is low, but about half of the patients need additional surgery due to recurrence[14-16]. The main "disadvantage" or limitation of both procedures are high complex fistulas with suprasphincteric course or multi-tract fistulas with two internal and external openings ("multiple branching tracts"). In these patients, long-term seton drainage is the favored option; alternatively, proctectomy can be considered. Based on this "surgical dilemma", the novel therapeutic approach of local stem cell therapy seems to be an alternative for highly selected patients with multi-tract, complex fistulas.

In the meantime, a variety of limited studies demonstrated that local injection of mesenchymal stem cells can induce long-term fistula healing without the risk of incontinence and without serious adverse advents related to the mesenchymal stem cells themselves[19-22]. Following the results of the multicenter, phase III randomized control trial (ADMIRE), the application of allogenic, adipose-derived, mesenchymal stem cells combined with transanal closure of the internal opening and fistula curettage, a 50% healing rate of stem cell therapy compared with a 34% success in the placebo group was documented after 24 wk, and healing rates were sustained to 52 wk [7,8].

Impressed by these results and personally searching for the "best option" in patients with multi-branching or two-tract complex fistula, it was the aim of the current single-center experience to evaluate the outcome of local injection of darvadstrocel in a highly selected group of patients. Following the current single-center experience with stem cell therapy, strict inclusion and exclusion criteria were defined. In accordance with the inclusion criteria of the ADMIRE study[7,8], only patients with complex fistulas associated with Crohn's disease–in the majority two complex fistula, suprasphincteric fistula and horse-shoe-fistula, without active luminal Crohn's disease (stable disease under medical treatment) and no evidence of perianal sepsis were included. In contrast, simple fistulas or rectovaginal fistulas were excluded for stem cell therapy. Moreover, in the current study, patients with single-tract intersphincteric or transsphincteric fistulas as well as relatively short single-tract fistulas (fistula length less than 3 cm) were not candidates for stem cell therapy; in these patients, advancement flap repair, mucosal flap repair or LIFT procedure was the preferred option. Within the observation period, a total of 28 patients underwent flap or LIFT procedure for complex fistula associated with Crohn's disease (data not shown).

All patients had drainage of fistulas by seton for a minimum of 6 wk. In contrast to the ADMIRE study[7,8], 4 patients who underwent fecal diversion due to perianal sepsis related to complex fistulas years before admission to our center were included in the current study after interdisciplinary discussion, as the only alternative surgical treatment would have been a proctectomy in these patients, and patients refused this option. Finally, all 12 patients had at least one attempt to surgically close the fistula with no success; thus, all fistulas treated with stem cell therapy were recurrent complex fistula.

Focusing on surgical technique and administration of darvadstrocel, no difficulties or intraoperative morbidity were documented. One patient had fewer on the second postoperative day without any signs of perianal sepsis. No serious adverse events in the immediate postoperative course were noted.

After a mean follow-up of 14.3 mo (range: 3-30 mo), healing rate was 66.7% (8 of 12 patients). Healing was based on strict criteria and was assessed by clinical examination and proctoscopy in the follow-up period. In terms of postoperative MRI (5/12 patients), 1 female patient with clinical evidence of fistula healing also had radiological healing as documented by MRI, whereas 3 patients had radiological recurrence (2) or persistence (1). One patient with clinical healing had radiologic persistence of fistula without signs of contrast enhancement. Focusing on patients with a minimum follow-up of 12 mo (6/12) or 24 mo (4/12), long-term healing rates were 66.7% (4/6) and 50.0% (2/4), respectively.

The occurrence of perianal abscess during follow-up was relatively frequent in the current collective. Four patients (33.3%) developed perianal abscess in the postoperative course, and surgery (abscess excision) was required (1 patient had abscess formation twice). Interestingly, the occurrence of perianal abscess was late (3, 4, 7, 9 and 22 mo after darvadstrocel administration), and abscess localization was near to one former external opening. The incidence of abscess was related to recurrence in 1 patient (supralevatoric abscess with proctosigmoiditis), occurred with exacerbation of

systemic disease (subcutaneous abscess with active inflammation of ileocolic region and proctitis), and was associated with fistula persistence in 2 patients. As a consequence of the relatively high incidence of perianal abscess following stem cell therapy in the current series, a more wide or radical excision of the external opening should be recommended in future procedures to prevent perianal abscess. Moreover, patients with the occurrence of abscess could not be defined "in remission"; however, there was no change in medical treatment. Therefore, the differentiation of abscess as a local problem following fistula surgery or as a problem of systemic disease with direct implications for medical treatment should be more in focus between gastroenterologists and surgeons.

Analyzing the further course of patients with long-term healing in terms of medical therapy, 1 patient had ceased maintenance therapy after recommendation of the gastroenterologist. Focusing on patients who had fecal diversion in the past, one female patient underwent stoma reversal 2 years after stem cell injection. In the other 3 patients, follow-up is still too short, as stoma reversal should be advised not prior to a minimum period of 6 mo after definite fistula surgery (1 patient), and the other two patients had recurrence.

Specifically addressing the observation period related to the COVID-19 pandemic, stem cell therapy was not performed within March 2020 and July 2020 and within a second period starting in November 2020 due to general restrictions concerning elective operations in Germany. Based on governmental restrictions and in-hospital limitations (*e.g.*, reduced capacity in the surgical theater) as well as logistic reasons (*e.g.*, transportation of stem cells under specific conditions) patients with stable disease and without signs of active perianal disease were postponed. This was in accordance with other European and United States experiences related to the management of patients with inflammatory bowel disease[23,24].

The current results clearly demonstrate that an innovative sphincter-sparing surgical approach including fistula curettage, transanal closure of internal openings and local injection of darvadstrocel leads to promising long-term healing rates in patients with no "effective" alternative, such as flap repair or LIFT procedure. However, fundamental limitations of this single-center experience are the small number of patients, the retrospective design, and the absence of a control group. Therefore, the definite role of local application of mesenchymal stem cells has to be discussed within multidisciplinary round tables or consensus conferences to have clear position statements[25-28]. Actually, we are still in the episode of a "learning curve" in terms of patient selection, ideal surgical technique, interdisciplinary treatment discussion, role of maintenance therapy and evaluation of outcomes, among others. Therefore, generally accepted indications and pathways of stem cell therapy for complex fistulas in Crohn's disease cannot be derived; however, the current results should be a plea for further standardization and interdisciplinary consensus.

CONCLUSION

These single-center data demonstrate that local injection of adipose-derived mesenchymal stem cells (darvadstrocel) is safe and effective in patients suffering from perianal fistulizing Crohn's disease. Providing long-term healing in 66.7% of patients, stem cell therapy seems to be an innovative and promising surgical therapy for complex anal fistula associated with Crohn's disease. However, this single-center experience is limited due to the small number of patients and the retrospective assessment of routine clinical data related to quality control. As a technical consequence of a high incidence of perianal abscess during follow-up, a wide excision of the external opening should be recommended for future procedures. Finally, further interdisciplinary efforts including controlled studies are necessary to evaluate the definite role of stem cell therapy for complex anal fistula in Crohn's disease.

ARTICLE HIGHLIGHTS

Research background

Despite significant progress in medical therapy and surgical options, definite surgery for complex anal fistula in Crohn's disease remains challenging.

Research motivation

At present, failure rates after surgery for complex anal fistula associated with Crohn's disease are still high, and surgical options in patients with recurrent and/or multi-tract fistula are limited.

Research objectives

The primary objective was to assess whether local stem cell injection is associated with acceptable healing rates in a routine clinical setting.

Research methods

Providing strict inclusion and exclusion criteria, 12 patients with complex anal fistulas associated with Crohn's disease underwent local application of allogenic, adipose-derived mesenchymal stem cells (darvadstrocel). Darvadstrocel was only indicated in patients without active Crohn's disease confirmed by ileocolonoscopy and without presence of anorectal abscess. Study design was retrospective and routine clinical data were analyzed.

Research results

Twelve patients (6 females, 6 males) with complex anal fistula associated with Crohn's disease underwent fistula curettage, transanal closure of internal openings and local darvadstrocel administration. Fifty-eight percent of patients had two complex fistulas, and seventy-six percent of the fistulas were transsphincteric. After a mean follow-up of 14.3 mo, a healing rate of 66.7% (8/12) was documented. Perianal abscess occurred in 33.3% of patients during follow-up.

Research conclusions

This single-center experience demonstrates that local stem cell injection for complex perianal fistulizing disease is safe and provides acceptable healing rates. However, conclusions are limited due to the small number of patients and the retrospective study design.

Research perspectives

Based on the current results, local stem cell injection could be a new "puzzle piece" for effective treatment of complex anal fistulas associated with Crohn's disease.

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ORIGINAL ARTICLE

Observational Study

Laparoscopic lateral lymph node dissection in two fascial spaces for locally advanced lower rectal cancer

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Abstract

BACKGROUND

The procedure for lateral lymph node (LLN) dissection (LLND) is complicated and can result in complications. We developed a technique for laparoscopic LLND based on two fascial spaces to simplify the procedure.

AIM

To clarify the anatomical basis of laparoscopic LLND in two fascial spaces and to evaluate its efficacy and safety in treating locally advanced low rectal cancer (LALRC).

METHODS

Cadaveric dissection was performed on 24 pelvises, and the fascial composition related to LLND was observed and described. Three dimensional-laparoscopic total mesorectal excision with LLND was performed in 20 patients with LALRC, and their clinical data were analyzed.

RESULTS

The cadaver study showed that the fascia propria of the rectum, urogenital fascia, vesicohypogastric fascia and parietal fascia lie side by side in a medial-lateral direction constituting the dissection plane for curative rectal cancer surgery, and the last three fasciae formed two spaces (Latzko's pararectal space and paravesical space) which were the surgical area for LLND. Laparoscopic LLND in two fascial spaces was performed successfully in all 20 patients. The median operating time, blood loss and postoperative hospitalization were 178 (152-243) min, 55 (25-150) mL and 10 (7-20) d, respectively. The median number of harvested LLNs was 8.6

written consent prior to study enrollment.

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(6-12), and pathologically positive LLN metastasis was confirmed in 7 (35.0%) cases. Postoperative complications included lower limb pain in 1 case and lymph leakage in 1 case.

CONCLUSION
Our preliminary surgical experience suggests that laparoscopic LLND based on fascial spaces is a feasible, effective and safe procedure for treating LALRC.

Key Words: Locally advanced low rectal cancer; Lateral lymph node dissection; Fascial anatomy; Visceral fascia; Vesicohypogastric fascia; Cardinal ligament

Core Tip: The procedure for lateral lymph node dissection (LLND) is complicated, with a high incidence of complications. We developed a technique of laparoscopic LLND based on two fascial spaces to simplify the procedure. By cadaveric dissection, we found that urogenital fascia, vesicohypogastric fascia and parietal fascia lie side by side and formed two spaces (Latzko's pararectal space and paravesical space) which were the surgical area for LLND. 3D-Laparoscopic LLND in two fascial spaces was performed successfully in 20 patients with locally advanced low rectal cancer, and the results showed that it was a feasible, effective and safe procedure.

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INTRODUCTION

For rectal cancers below the peritoneal reflection, the incidence of lateral lymph node (LLN) metastasis is reported to be 15%-20%[1-3]. However, routine LLN dissection (LLND) for locally advanced low rectal cancer (LALRC) is still a controversial issue. In addition to the controversy on the indication for LLND, another argument against LLND is that the procedure is complicated, and consequently results in a high incidence of complications[4]. Earlier studies showed that LLND was associated with 30%-70% of urinary disorders and 80%-100% of sexual dysfunction at the initial stage [5]. Despite the introduction of pelvic autonomic nerve preservation, a Japanese clinical trial showed that urinary dysfunction and postoperative complications occurred in 59% and 5% of patients, respectively[6]. In this study, we do not discuss the indications for LLND but focus on how to improve the accuracy and safety of the procedure for laparoscopic LLND based on the fascial space approach.

Total mesorectal excision (TME) is the gold standard surgery for rectal cancer and has stimulated a tremendous upsurge of interest in "fascia anatomy" in colorectal surgery[7-10]. The theory of "fascia anatomy" has changed the traditional view of surgical anatomy focusing on organs and blood vessels, and holds that surgical dissection should be performed along a pre-existing embryological plane (space) formed by fasciae. However, the detailed anatomy of fasciae and spaces related to LLND is not clear, and therefore standard surgical procedures have not been established. In the last few years, there have been numerous anatomical research studies on pelvic fascia[11,12]. Based on the new understanding of fascial composition in the pelvis, we have developed a laparoscopic LLND in two fascial spaces formed by three layers of fasciae to treat LALRC. We describe the anatomical basis, surgical procedure and summarize our initial clinical results.

P-Editor: Ma YJ

MATERIALS AND METHODS

Cadavers

Twenty-three formalin-preserved and 1 fresh cadavers (12 males and 12 females) were dissected at the Anatomy Department of Shanghai Jiaotong University School of Medicine. Eighteen specimens (9 males and 9 females) were separated into two hemi-pelvises through the mid-sagittal plane. Detailed dissections were performed macroscopically with the assistance of binocular loupes (Heine, Germany, HR 2.5 mm × 340 mm). Cadavers with malformations, abdominal or pelvic adhesions, or a history of abdominal or pelvic surgery were excluded.

Patients

From July 2018 to October 2020, a total of 20 patients with LALRC underwent 3D-laparoscopic TME with LLND at Yangpu Hospital affiliated to Tongji University School of Medicine. There were 16 males and 4 females, with a median age of 58.2 years (range: 48-68 years). All patients were pathologically diagnosed by endoscopic biopsy and evaluated by computed tomography (CT) and magnetic resonance imaging (MRI) before surgery. All tumors were located below the peritoneal reflection, and the median distance between the inferior margin of the tumor and anal verge was 4.7 cm (range: 2.5-6.8 cm). The preoperative T stage was T3 in 13 patients and T4 in 7 patients. Preoperative MRI showed uni-LLN enlargement (the largest short diameter > 7 mm) in all cases.

Methods

This was a descriptive study, and was approved by the local Ethics Committee (LL-2020-KXJS-004) and performed in accordance with the Declaration of Helsinki[13].

Cadaver dissection: The details of the dissection procedure were described in our previous studies[11,12]. The main observation points were as follows: (1) The distribution of visceral fascia in the pelvic cavity and its association with the urogenital and vesicohypogastric fasciae; (2) The association of the hypogastric nerve and ureter with the urogenital fascia; (3) The association of the urogenital and vesicohypogastric fasciae with the cardinal ligament; (4) The association of Denonvilliers' fascia with the pelvic plexus; (5) The association of internal iliac blood vessels and their branches with the vesicohypogastric fascia; and (6) The distribution and variation of the branches of the internal iliac artery.

Surgical technique: TME: All operations were performed by the same experienced surgical team using the Karl Storz Image1 S™ 3D system. The patient was placed in a modified lithotomy position. Laparoscopic TME was performed using a five-trocar method through the traditional medial-to-lateral approach, and a detailed description of this surgical procedure was given previously[14].

LLND: (1) Separation of the urogenital fascia to expose the medial boundary of Latzko's pararectal space (Figure 1). The peritoneum was incised lateral to the ureter, and the dissection continued to the vas deferens (male) or the round ligament of the uterus (female). The ureter was pulled medially to expose the urogenital fascia containing the hypogastric nerve. There is an embryonic plane between the urogenital fascia and internal iliac lymph nodes. Dissection was continued along the surface of the urogenital fascia, and was stopped at the level of the pelvic plexus to avoid injuring the neurovascular bundle; (2) Separation of the obturator fascia (parietal fascia) to expose the lateral boundary of the paravesical space (Figure 2). The external iliac lymph nodes were removed along the external iliac artery, and the dissection was continued along the medial surface of the iliopsoas muscle deep into the obturator fascia. An avascular plane exists between the obturator lymph nodes and the obturator fascia. The obturator nerve and vessels were identified at the obturator. A further dissection along the obturator fascia was performed to expose the parietal fascia covering the levator ani muscle (superior fascia of the pelvic diaphragm), and then the dissection plane was connected to the TME surgical plane; (3) Separation of the vesicohypogastric fascia to expose the medial boundary of the paravesical space (Figure 3). The umbilical artery was identified, and the dissection was continued along the lateral border of the umbilical artery to expose the vesicohypogastric fascia. The vesicohypogastric fascia covers the lateral side of the branches of the internal iliac artery. An avascular plane also exists between the obturator lymph nodes and the vesicohypogastric fascia. The superior border of the distal part of the vesicohypogastric fascia fuses with the obturator fascia where the obturator was easily identified, and the obturator nerve and vessels were exposed. The inferior border of the

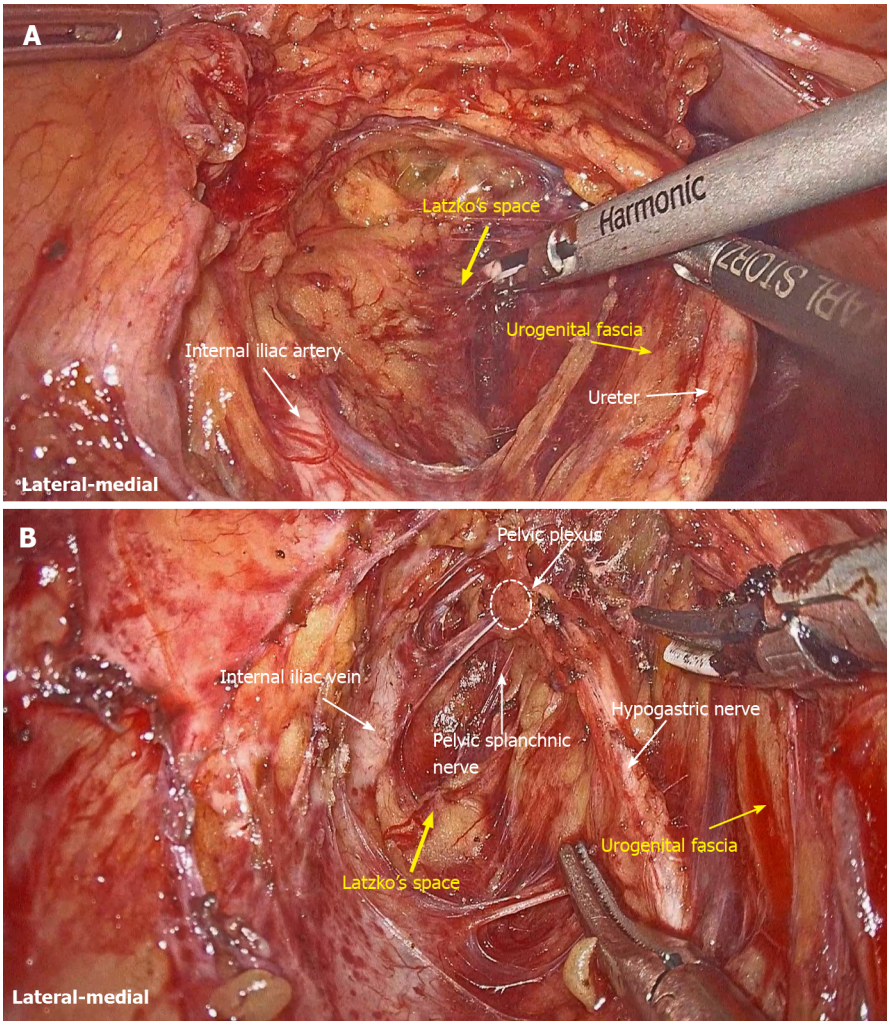

Figure 1 Urogenital fascia is separated to expose the medial boundary of the Latzko's pararectal space, and the dissection continues to the level of the pelvic plexus. A: Medial boundary of the Latzko's pararectal space; B: Dissection continues to the level of the pelvic plexus.

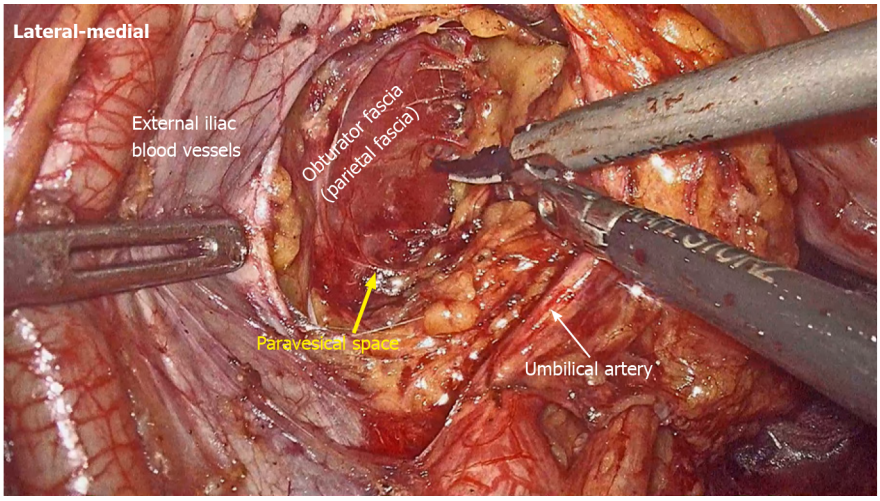

Figure 2 Obturator fascia is separated to expose the lateral boundary of the paravesical space.

vesicohypogastric fascia reaches the superior fascia of the pelvic diaphragm; (4) Dissection of the obturator lymph nodes in the paravesical space between the vesicohypogastric and parietal fasciae (Figure 4). Dissection of the paravesical space included three steps. At the lateral side, the obturator nerve was skeletonized, and the distal obturator vessels were cut and ligated at the obturator canal. At the medial side,

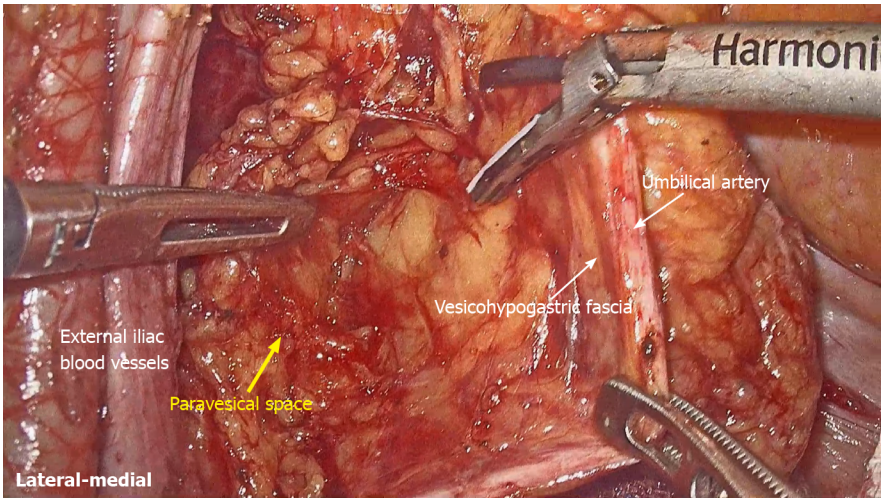

Figure 3 Vesicohypogastric fascia is separated to expose the medial boundary of the paravesical space.

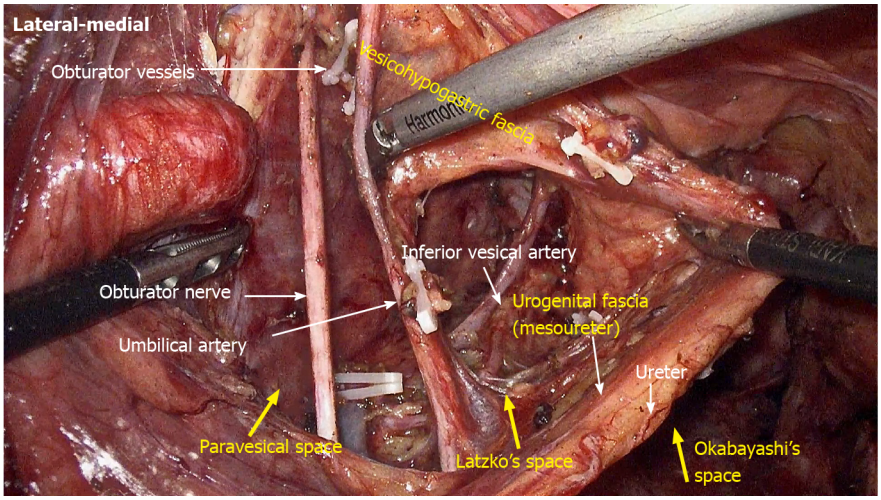

Figure 4 Obturator lymph nodes are dissected in the paravesical space.

the dissection began with removal of the common iliac lymph nodes, followed by dissection along the bifurcation of the internal and external blood vessels and the proximal vesicohypogastric fascia. The proximal obturator vessels were ligated and cut at their origins. At the dorsal side, after exposing the lumbosacral trunk, the dissection started from the bottom of the obturator fossa, and then extended ventrally, to expose Alcock's canal (pudendal canal) and the superior fascia of the pelvic diaphragm. The dissection also allowed the paravesical space to connect to the surgical plane of TME; and (5) Dissection of the internal iliac lymph nodes in Latzko's pararectal space between the urogenital and vesicohypogastric fasciae (Figure 5). The medial side of the vesicohypogastric fascia was dissected to expose the lateral boundary of Latzko's pararectal space. After identifying the trunks of the internal iliac vessels, the dissection was performed along the lateral surface of the vesicohypogastric fascia, in order to sever the branches of the internal iliac vessels at their origins. It should be noted that there was no need to identify each branch. Dissection around the umbilical artery, superior vesical artery, internal pudendal artery, and inferior vesical artery (male) or uterine artery (female) was required for internal iliac lymph nodes cleaning.

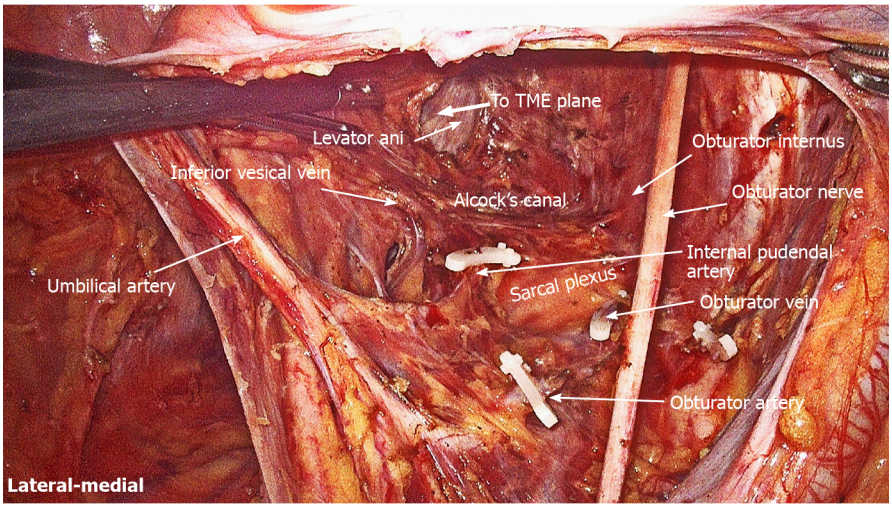

Figure 5 Internal iliac lymph nodes are dissected in the Latzko's pararectal space.

RESULTS

Anatomical observations

Fasciae related to LLND: (1) Fascia propria of the rectum (Figure 6). The fascia propria of the rectum is a thin fascia layer surrounding the mesorectum. It extends downward and shows tight contact with the visceral fascia below the level of Waldeyer's fascia; (2) Urogenital fascia (Figure 7). The urogenital fascia extends posterolateral to the rectum, providing a hammock-like support to the rectum. It can be recognized as the dense connective tissue which contains the hypogastric nerve and ureter. The urogenital fascia connects with Denonvilliers' fascia anterior to the rectum. The urogenital fascia is actually the visceral fascia described by Heald. The mesoureter is defined as a sheet of connective tissue surrounding the ureter. Actually, it is part of the urogenital fascia suspended below the ureter (Figure 4); and (3) Vesicohypogastric fascia (Figure 8). The vesicohypogastric fascia is a triangle-shaped structure, and its boundaries are formed by the umbilical artery, the tendinous arch of the pelvic fascia and the lateral surface of the bladder. The urogenital and vesicohypogastric fasciae blend with each other in the tendinous arch of the pelvic fascia, and present in a V-shape relationship.

Spaces related to LLND: (1) Pararectal space (Figure 9). The pararectal space is a potential space between the rectum and internal iliac vessels, and is divided into Latzko's and Okabayashi's pararectal spaces by the mesoureter. In the view of "fascia anatomy", Latzko's pararectal space is the space between the urogenital fascia and vesicohypogastric fascia, and Okabayashi's pararectal space is the space between the fascia propria of the rectum and the urogenital fascia; and (2) Paravesical space (Figure 9). The paravesical space is a potential space between the umbilical artery and the external iliac vein. From the perspective of "fascia anatomy", the paravesical space is the space between the vesicohypogastric fascia and parietal fascia.

Vascular anatomy related to LLND: As shown in Figure 10, the anatomical relationship between the branches of the internal iliac blood vessel is complicated, but there are still some anatomical characteristics to discriminate them. The first branch of the internal iliac artery is the umbilical artery. The superior vesical artery always arises from a common trunk with the umbilical artery. The vessels crossing above and below the ureter are the uterine artery and deep uterine vein, respectively. The vesical veins drain into the superficial and deep uterine veins or the internal iliac vein and are distributed along the bladder and uterus/vagina in a sagittal plane. The inferior gluteal artery and internal pudendal artery are two major branches arising from the internal iliac artery and enter into the lesser sciatic foramen. However, the inferior gluteal artery passes between the S2 and S3 nerves, and then enters into the lesser sciatic foramen behind the S3 nerves; while the internal pudendal artery goes directly into the lesser sciatic foramen. As the course of the internal pudendal artery in the pelvic cavity is longer, it is regarded as the terminal branch of the internal iliac artery.

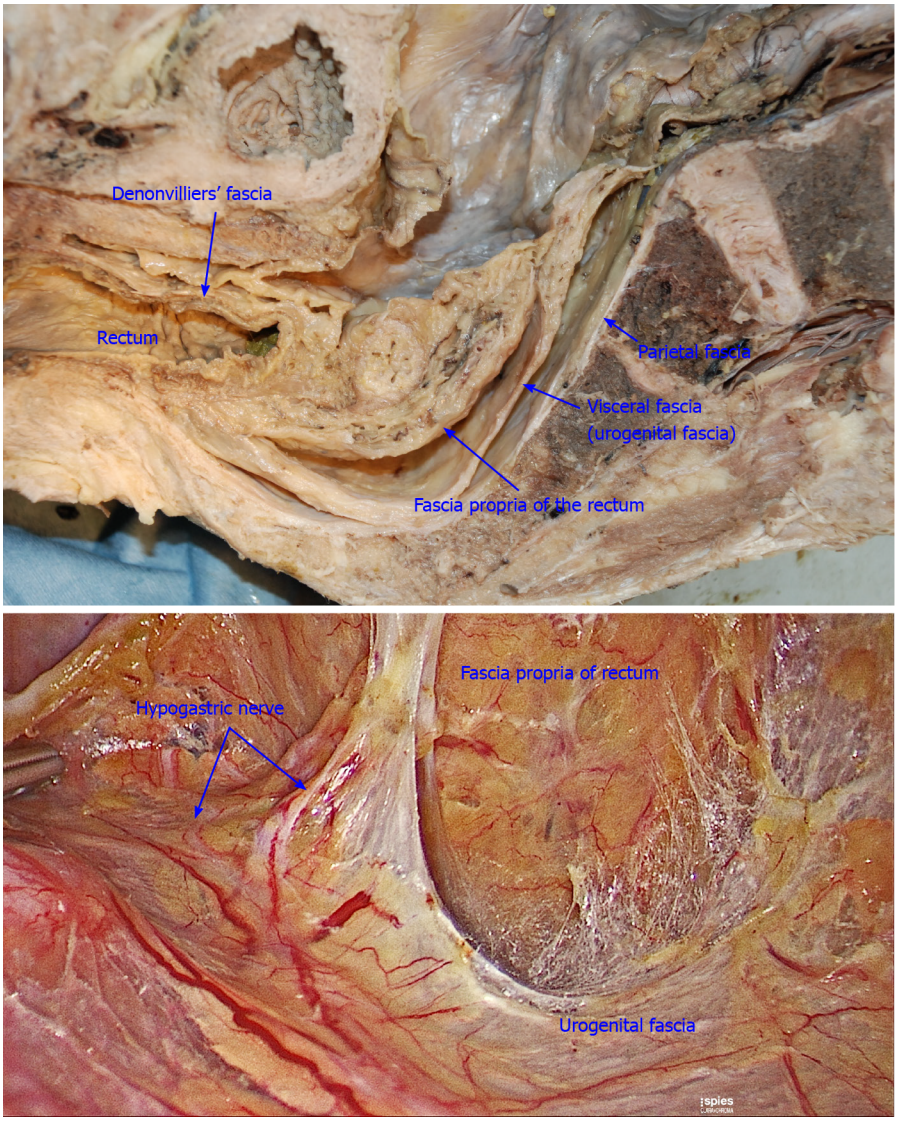

Figure 6 Fascia propria is the innermost fascia around the rectum, and appears as a thin and transparent fascia.

Nerve anatomy related to LLND: As shown in Figure 10, the hypogastric nerve is found within the urogenital fascia, which passes from the posterior to the anterior-lateral aspect of the rectum and fuses with Denonvilliers' fascia in a fan shape. The pelvic plexus is located exactly external to the junction of the urogenital and Denonvilliers' fascia. The pelvic plexus is made up of the hypogastric nerve and the pelvic splanchnic nerve, and give off branches to the bladder and the uterus. All these nerves are located in the same connective tissue plane and presents as a T-shape "pelvic nerve plate".

LLND based on fascial anatomy

3D-Laparoscopic LLND was performed successfully in all 20 patients without conversion to laparotomy. Of these patients, 12 underwent low anterior resection, 7 cases underwent intersphincteric resection, and 1 case underwent abdominoperineal resection. The median operating time for LLND was 178 (range: 152-243) min, with a median blood loss of 55 (range: 25-150) mL. The median length of postoperative hospitalization was 10 (range: 7-20) d.

All patients received uni-LLND, and the postoperative pathology showed that LLN metastasis was present in 7 (35.0%) cases. Of these, 6 cases had both mesenteric and internal iliac lymph node metastases, and 1 case had only internal iliac lymph node metastasis. The median number of harvested LLNs was 8.6 (range: 6-12).

Postoperative complications included lymph leakage in 1 case and lower limb pain in 1 case (grade I based on Clavien-Dindo classification). No other postoperative complication was observed. The former was cured after fasting for 4 d, and the latter

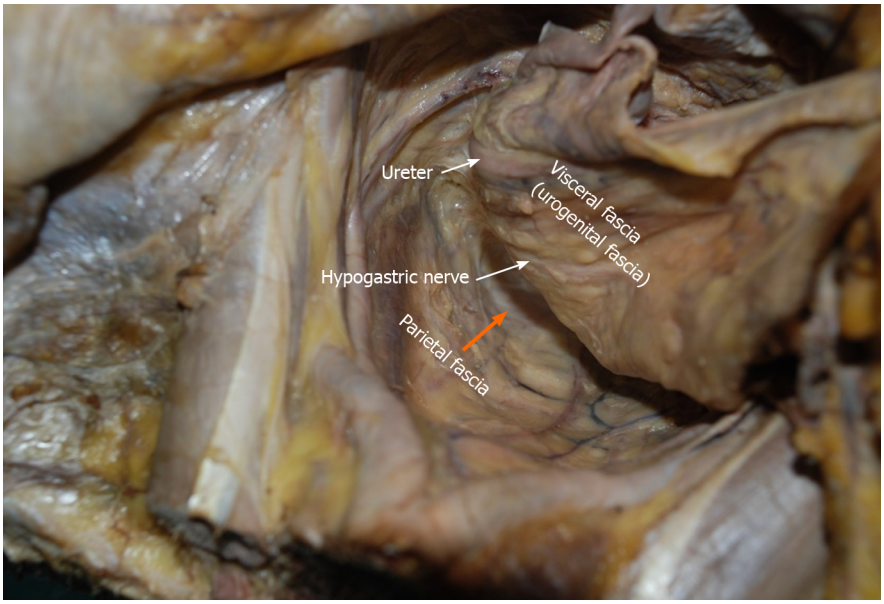

Figure 7 Urogenital fascia extends posterolaterally to the rectum, with the hypogastric nerve and ureter running in it. The "Holy plane" for total mesorectal excision is between the urogenital fascia (visceral fascia) and the parietal fascia (orange arrowhead).

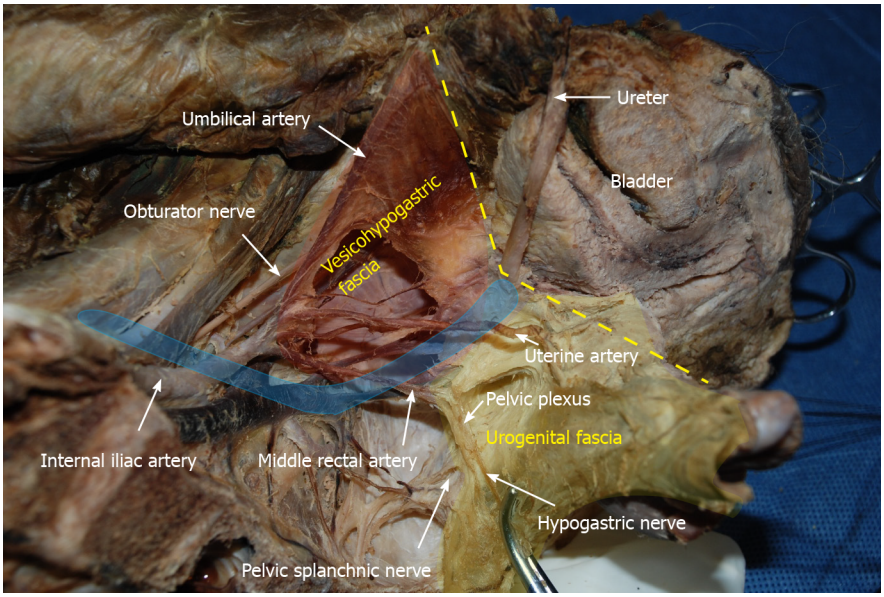

Figure 8 Vesicohypogastric fascia and urogenital fascia. Vesicohypogastric fascia is a triangular fascia composed of umbilical artery, tendinous arch of pelvic fascia and bladder (red shadow). The urogenital fascia (yellow shadow) and the vesicohypogastric fascia blend with each other and appear as a V-shape (yellow dotted line). The ureter is pulled up and the actual position is shown in blue shadow.

resolved spontaneously 1 mo after hospital discharge.

DISCUSSION

With the application of TME in clinical practice, the concept of performing surgery along the embryological space formed by fasciae has been established in colorectal surgery[15]. However, there is still a lack of data to illustrate fascial composition related to LLND; therefore, it is impossible to establish standard LLND procedures based on fascial anatomy. This is the first study to outline the terminology of fasciae and spaces used in colorectal surgery and gynecology to clarify the surgical plane of LLND. We have put forward the concept of two-space dissection for LLND, and located important blood vessels and nerves using landmark fasciae in each space. By

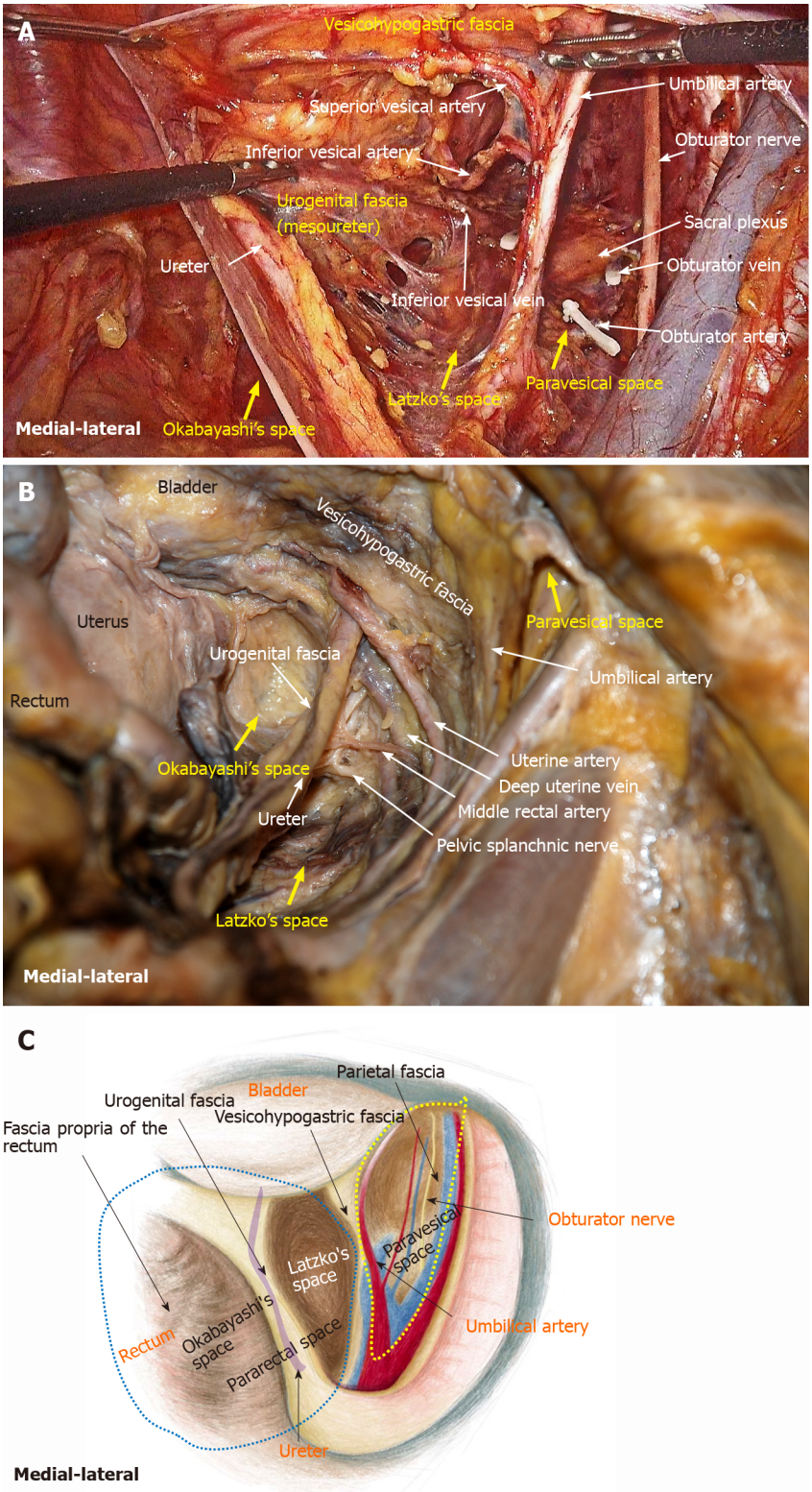

Figure 9 Fascial spaces related to rectal cancer surgery are showed in photograph of operation, photograph of cadaver dissection and diagram. A: Photograph of operation; B: Photograph of cadaver dissection; C: Photograph of diagram. The pararectal space (blue dotted line) can be divided into Okabayashi's space and Latzko's space. Fascia propria of the rectum, urogenital fascia, vesicohypogastric fascia and parietal fascia form Okabayashi's, Latzko's space and paravesical space (yellow dotted line). The last two spaces are the surgical area for lateral lymph node dissection.

applying these anatomical findings in practice, the initial clinical results showed that the fascial space approach can significantly reduce intraoperative blood loss and maintain a clear surgical field. Moreover, this approach can easily expose and protect the ureter, the hypogastric nerve, pelvic plexus, lumbosacral trunk and sacral plexus, which significantly decreased the incidence of postoperative urogenital dysfunction.

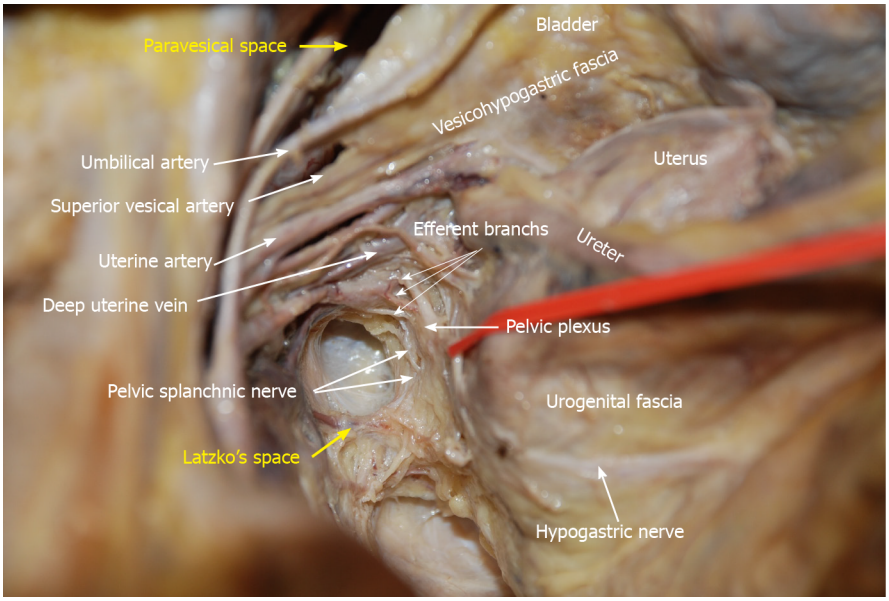

Figure 10 Anatomy of the branches of the internal iliac blood vessel and nerves related to lateral lymph node dissection. The hypogastric nerve, pelvic splanchnic nerves and pelvic plexus are located in the same connective tissue plane and presents as a T-shape "pelvic nerve plate".

There are many diverse descriptions on the fascia composition related to rectal cancer surgery[15]. As described in the literature, the fascia propria of the rectum is part of the visceral fascia, and the visceral fascia together with Denonvilliers' fascia constitute the morphology of the "mesorectum"[16]. This has become the knowledge on classical pelvic anatomy and has been widely accepted by colorectal surgeons. However, we found that the fascia propria of the rectum and visceral fascia are two independent layers of fascia. The fascia propria of the rectum presents as a barely visible translucent layer which is the innermost layer of fascia surrounding the rectum and perirectal fat (Figure 6). The visceral fascia was the densest portion of pelvic fascia, which stretched "like a hammock" posterolateral to the rectum and fused anteriorly with Denonvilliers' fascia (Figure 7). In fact, "visceral fascia" is a general term used to describe the fascia ensheathing organs[17], and therefore, it is inappropriately used to describe specific fascia. Actually, the visceral fascia described by Heald *et al*[7] in TME is just the urogenital fascia. The urogenital fascia originates from the fusion of the anterior renal fascia and posterior renal fascia below the inferior pole of the kidney. Muntean *et al*[18] described the hypogastric nerve and the ureter embedded in this fascia, hence the name urogenital fascia. However, Kinugasa demonstrated a protective fascia overlying the hypogastric nerves and proposed that "prehypogastric nerve fascia" seemed more appropriate[19]. In some patients with an enlarged hypogastric nerve, the hypogastric nerve was identified posterior to the urogenital fascia in this study (Figure 1B). The urogenital fascia carries the ureter and the hypogastric nerve to the bladder and continues with the vesicohypogastric fascia, and the two fasciae blend with each other anterior-laterally in the tendinous arch of the pelvic fascia (Figure 8). The obturator fascia is one of three parts of the parietal fascia [20]. In summary, the fascia propria of the rectum, urogenital fascia, vesicohypogastric fascia and parietal fascia lie side by side in a medial-lateral direction, and constitute the dissection plane for curative rectal cancer surgery (Figure 9).

In gynecological anatomy, the paravesical space is a potential space between the umbilical artery and the external iliac vein[21]. Given that the umbilical artery is the landmark of the vesicohypogastric fascia and the obturator fascia (parietal fascia) follow the same plane as the external iliac vein, the paravesical space can be considered to be located between the vesicohypogastric fascia and the parietal fascia (Figure 9). Gynecological anatomy also defines the pararectal space as a loose connective tissue space between the rectum and the internal iliac vessels, and is further divided into the medial Okabayashi's space and lateral Latzko's space by the ureter [21]. According to our findings, the fascia propria of the rectum is the innermost layer covering the rectum, the mesoureter is a part of the urogenital fascia, and the umbilical artery constituting the superior border of the vesicohypogastric fascia is the first branch of the internal iliac artery. Therefore, in the view of fascia anatomy, Okabayashi's space can be described as the space between the fascia propria of the

rectum and the urogenital fascia, and Latzko's space lies between the urogenital fascia and the vesicohypogastric fascia (Figure 9). Okabayashi's space corresponds to the resection area of TME, while Latzko's pararectal space and the paravesical space constitute the surgical field for LLND. Moreover, the vesicohypogastric fascia divides the LLNs into obturator lymph nodes and internal iliac lymph nodes.

The advantage of the fascial space approach lies in its ability to accurately locate important anatomical structures, for example, the hypogastric nerve is located in Latzko's space, while the lumbosacral trunk and sacral plexus are located in the paravesical space (Figures 1B and 5). Dissection of Latzko's space mainly involves the hypogastric nerve and inferior vesical artery. As the hypogastric nerve runs in the urogenital fascia, the urogenital fascia is recommended to be separated first and pulled medially to protect the hypogastric nerve. Separation of the urogenital fascia is always stopped at the level of the pelvic plexus to avoid damage to the neurovascular bundle. In fact, the hypogastric nerve and the pelvic plexus are easily exposed and protected using the fascial anatomy approach. Therefore, we consider that possible nerve damage depends more on the surgical procedures for TME rather than those for LLND. In TME, if we follow the traditional view to dissect in the "Holy plane" between the visceral fascia (urogenital fascia) and parietal fascia, the hypogastric nerve will undoubtedly be impaired (Figure 7). According to our viewpoint, the appropriate plane for TME is between the fascia propria of the rectum and the visceral fascia (urogenital fascia) (Figure 6), which is consistent with the opinions of Kinugasa *et al* [19]. We also showed that the mesoureter is a part of the urogenital fascia (Figure 9B); thus, care should be taken to preserve the integrity of the mesoureter to avoid damage to the hypogastric nerve, and the traditional procedure such as isolating the ureter and picking it up by a rubber band should be abandoned.

The inferior vesical artery is a common site for internal iliac lymph node metastasis [22]. It is the last branch of the internal iliac artery, and located just above the infrapiriformis foramen. Therefore, the inferior vesical artery can be considered the distal end of the vascular dissection in LLND. It should also be noted that the branches of the internal iliac artery are varied in their number, origin, location and course. For instance, our previous study showed that the middle rectal artery was only observed in 29.2% (7/24) specimens, and its diameter was mostly less than 1.5 mm[12]. As a result, we suggest that it is unnecessary to expose each branch of the internal iliac vessels in LLND. The blood vessels requiring dissection include the umbilical artery, superior vesical artery, internal pudendal artery, and inferior vesical artery (male) or uterine artery (female). Many classic anatomical works believe that the inferior vesical artery does not exist in females. In "Gray's anatomy", the inferior vesical artery is described as being replaced by the ovarian artery or uterine artery[23]. However, a cadaver study by de Treigny *et al*[24] indicated that the inferior vesical artery does exist in females, and originates mostly from the common trunk with the umbilical or uterine arteries. Whichever is the case, it is clear that it is difficult to identify the inferior vesical artery in females during LLND.

Dissection of the paravesical space can be completed from three directions. The lateral dissection begins at the external iliac lymph nodes and extends downward to the obturator fascia (parietal fascia). The medial dissection is performed along the vesicohypogastric fascia. The vesical veins change direction sagittally and are distributed in the distal part of the vesicohypogastric fascia. Therefore, special attention should be paid to avoid damage to the vesical veins when the distal end of the obturator vessels (at the obturator) is isolated and ligated. The proximal dissection begins at the bifurcation of the internal and external iliac vessels, followed by exposing the lumbosacral trunk. After dissecting the bottom of the obturator fossa, Alcock's canal and the superior fascia of the pelvic diaphragm are exposed, forming a connection between the paravesical space and the TME surgical plane. Although the sacral plexus is formed by the lumbosacral trunk and other rami of S1-S4, they have different anatomical locations. The sacral plexus is located between the obturator vein and internal pudendal artery (Figure 11). The sacral plexus is usually covered by the parietal fascia, which should be preserved to avoid postoperative limb pain. This might be the reason why one patient in this study developed limb pain after surgery. In addition, the lymphatic chain connects with the inguinal lymph nodes and the obturator nodes at the distal external iliac vein; therefore, the distal end should be ligated to avoid lymph leakage. In this study, one patient suffered postoperative lymph leakage, possibly because the lymphatic vessels were not securely ligated when dissecting the superior inguinal lymph node (stated by Fujii *et al*[25]) located in the angle between the external iliac vein and pubis bone.

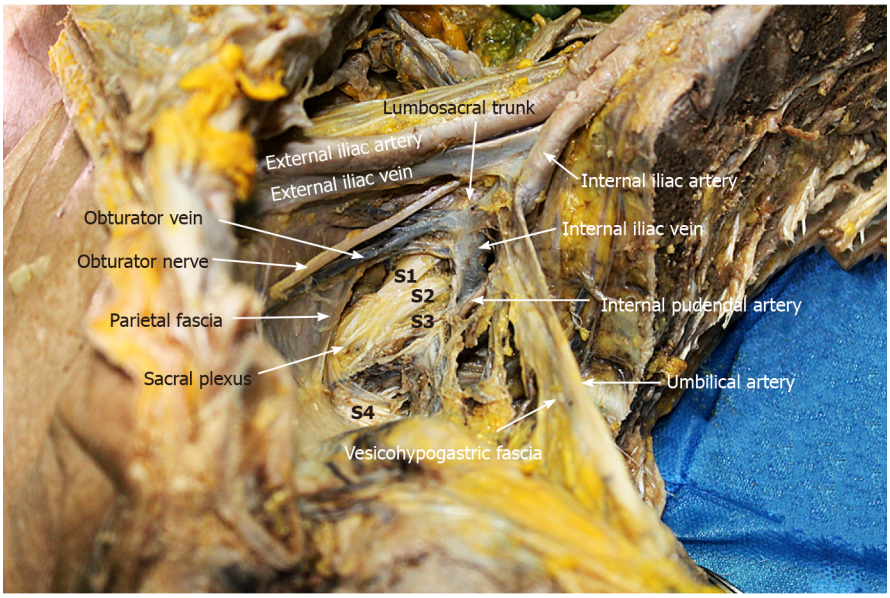

Figure 11 Lumbosacral trunk is located at the bifurcation of internal and external iliac vessels, while sacral plexus is located between obturator vein and internal pudendal artery. Sacral plexus was covered by parietal fascia.

CONCLUSION

In conclusion, laparoscopic LLND in two fascial spaces has several significant advantages, including easy identification of the surgical plane and clear location of nerves and blood vessels. Our preliminary surgical experience has shown that it is a feasible surgical approach for treating LALRC, which not only improves surgical safety but also ensures radical resection of the tumor. However, further studies are warranted to validate these results and optimize the surgical procedure.

ARTICLE HIGHLIGHTS

Research background

Lateral lymph node (LLN) metastasis is a major cause for local recurrence in rectal cancer. In Japan, LLN dissection (LLND) is the standard treatment for locally advanced low rectal cancer (LALRC). However, the procedure is complicated with significant morbidity. In recent years, with the rise of "fascia anatomy", more and more surgeons began to explore LLND based on the fascial space approach.

Research motivation-

LLND is a challenging procedure due to its technical difficulty and higher incidence of complications. The development of "fascia anatomy" provides a new sight for improving the accuracy and safety of laparoscopic LLND. However, the detailed anatomy is not clear and a standard surgical procedure has not yet been established.

Research objectives

We developed a technique of laparoscopic LLND in two fascial spaces formed by three layers of fasciae. This study aimed to describe the surgical procedure on an anatomical basis and to summarizes our preliminary surgical experiences in the treatment of LALRC.

Research methods

Detailed pelvic dissections were performed in 24 cadavers, and the fasciae and spaces related to LLND were observed and described. 20 patients with LALRC received 3D-laparoscopic total mesorectal excision with LLND at our hospital from July 2018 to October 2020, and their surgical videos and clinical data were analyzed.

Research results

The urogenital fascia lies posterolateral to the rectum, and the hypogastric nerve and ureter are observed to be enveloped in it; vesicohypogastric fascia shows a triangle shape formed by the umbilical artery, the tendinous arch of the pelvic fascia and the lateral border of the bladder. In all 24 cadavers, urogenital fascia, vesicohypogastric fascia and obturator fascia (parietal fascia) were located lateral to the rectum in a medial-to-lateral direction and form the Okabayashi's pararectal space and paravesical space, respectively, which were the surgical area for LLND. Laparoscopic LLND was performed successfully in all 20 LALRC patients with a median postoperative hospitalization of 10 (7-20) d. The median operating time was 178 (152-243) min, with a median blood loss of 55 (25-150) mL. The median number of harvested LLNs was 8.6 (6-12), and 7 patients (35.0 %) had LLN metastasis. Postoperative complications included lymph leakage and lower limb pain in 1 case, respectively.

Research conclusions

This study indicated that urogenital fascia, vesicohypogastric fascia and parietal fascia lie side by side in the pelvis and formed two spaces (Latzko's pararectal space and paravesical space), which were the surgical area for LLND. Performing LLND in two fascial spaces is a feasible surgical approach, which improves surgical safety while ensuring radical tumor resection.

Research perspectives

The present study preliminarily explored the clinical significance of laparoscopic LLND in two fascial spaces for treating LALRC. However, large studies with long-term follow-up and more detailed clinical data are needed to confirm these findings.

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SYSTEMATIC REVIEWS

Disorders of the brain-gut interaction and eating disorders

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Author contributions: Stanculete MF, Chiarioni G, Popa SL, Dumitrascu DI searched the literature, analyzed the results and made substantial contribution to the writing of the manuscript; Dumitrascu DL gave the idea of this review, suggested the methodology, analyzed the results, and revised the manuscript; all authors approved the final version of the manuscript.

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Abstract

BACKGROUND

Eating disorders (ED) involve both the nervous system and the gastrointestinal tract. A similar double involvement is also found in disorders of the brain-gut interaction (DGBI) and symptoms are sometimes similar.

AIM

To find out where there is an association and a cause-effect relationship, we looked for the comorbidity of DGBI and ED.

METHODS

A systematic review was undertaken. A literature search was performed. Inclusion criteria for the articles retained for analysis were: Observational cohort population-based or hospital-based and case-control studies, examining the relationship between DGBI and ED. Exclusion criteria were: Studies written in other languages than English, abstracts, conference presentations, letters to the Editor and editorials. Selected papers by two independent investigators were critically evaluated and included in this review.

RESULTS

We found 29 articles analyzing the relation between DGBI and ED comprising 13 articles on gastroparesis, 5 articles on functional dyspepsia, 7 articles about

Manuscript source: Invited manuscript

Specialty type: Gastroenterology and hepatology

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Peer-review report's scientific quality classification
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Grade B (Very good): 0
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Grade D (Fair): 0
Grade E (Poor): 0

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functional constipation and 4 articles on irritable bowel syndrome.

CONCLUSION
There is no evidence for a cause-effect relationship between DGBI and ED. Their common symptomatology requires correct identification and a tailored therapy of each disorder.

Key Words: Eating disorders; Dyspepsia; Constipation; Irritable bowel syndrome, Anorexia; Gastroparesis

Core Tip: Functional gastrointestinal disorders actually defined disorders of the brain-gut interaction (DGBI) by the new Rome IV criteria share similar symptoms with eating disorders (ED). Etiology is ill defined for both disorders thou a negative impact on quality of life and a high socio-economic burden is reported. We looked for the comorbidity of DGBI and ED, in order to find out where there is an association and a cause-effect relationship.

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INTRODUCTION

Eating disorders (ED) are psychiatric disorders defined by abnormal eating habits that negatively affect a person's physical or mental health and frequently begin in late childhood or early adulthood[1]. The pathogenesis of ED is still unclear, although it has been demonstrated that both genetic and environmental factors are involved. The most frequent ED include binge ED (eating a large amount of food in a short time), anorexia nervosa (eating an extremely small quantity of food due to a fear of gaining weight in contrast with low body weight in reality), bulimia nervosa (characterized by binge eating followed by purging, an attempt to get rid of the food consumed by vomiting or taking laxatives), pica (eating non-food items like hair, paper, sharp objects, metal objects, soil or glass), night ED (delayed circadian pattern of food intake) and avoidant/restrictive food intake disorder (eating only within an extremely narrow repertoire of foods)[1,2].

Epidemiological studies show that in the developed world, binge eating disorder affects about 1.6% of women and 0.8% of men, anorexia nervosa about 0.4%, and bulimia about 1.3% of young women[3,4]. At some point in their lifetime, more than 4% of women have anorexia, 2% have bulimia, and 2% have a binge eating disorder. In less developed countries, the rates of ED are lower[3,4]. Females are nine times more frequent than males to suffer from ED. ED do not include obesity. Notwithstanding visible progress in the current therapeutic methods[4], ED result in more than 7000 deaths a year, covering the highest mortality rate in all mental illnesses.

Disorders of the brain-gut interaction (DGBI) previously known as functional gastrointestinal disorders, involve visceral hypersensitivity and motility disturbances of different parts of the gastrointestinal tract[5]. DBGI are defined by several variable combinations of chronic or recurrent gastrointestinal symptoms that do not have an identified underlying pathophysiology. In the absence of any biological marker or endoscopic modifications, the identification and classification of DGBIs is based on symptoms[6,7]. Patients suffering from DGBIs typically present with various symptoms as early satiety, postprandial fullness, bloating, nausea, emesis, and epigastric pain[6-8]. Gastrointestinal motility disorders are the result of the dysfunction of the extrinsic nervous system, enteric nervous system, interstitial cells of Cajal (or intestinal pacemakers), or smooth muscle[6-8]. The type of disorder in transit (delay or acceleration) and the region of the gastrointestinal tract affected are the main criteria that are used for the classification. The neural control of the gastrointestinal

tract is a subject that is still generating interest and debates[9]. The close connections between the central nervous system and the enteric system are realized by ascending and descending fibers[10]. The ascending tract sends information from the digestive tube to the spine, hypothalamus, diencephalon and the cerebral cortex[9,10]. The descending tracts are responsible for the motility of the muscular layers of the digestive tract as well as glandular secretion[9]. A large part of the descending neurons has its origin in the prefrontal cerebral cortex[10]. This area is responsible for emotions and personality. Therefore, gastrointestinal motility is influenced by psychological factors[11-14].

Therefore, we looked for the comorbidity of DGBI and ED, in order to find out where there is an association and where there is a cause-effect relationship.

MATERIALS AND METHODS

A thorough literature search was undertaken. PubMed, Cochrane Library, EMBASE, and WILEY databases were screened for relevant publications regarding DGBI in ED. The search terms included: ("Eating disorders" OR "disorders of gut-brain interaction" OR "functional gastrointestinal disorders" OR "neurogastroenterological disorders" OR "gastrointestinal motility disorders") AND ("anorexia nervosa" OR "bulimia nervosa" OR "binge eating disorders") AND ("gastroparesis" OR "functional dyspepsia" OR "irritable bowel syndrome" OR "functional bowel disorders"). Inclusion criteria of original articles in this systematic review were as follows: Observational cohort population-based or hospital-based and case-control studies, examining the relationship between gastrointestinal disorders and ED. Exclusion criteria were: Studies written in languages other than English, abstracts, conference presentations, letters to the editor and editorials (Figure 1). The articles included in this review refer only to DGBI present in ED. Searches were carried out by two independent investigators.

RESULTS

We found 29 articles analyzing the relation between DGBI and ED comprising 13 articles regarding gastroparesis (Table 1), 5 articles on functional dyspepsia (FD) (Table 2), 7 articles regarding functional constipation (FC) (Table 3) and 4 articles on irritable bowel syndrome (IBS).

Gastroparesis

Gastroparesis is a neuromuscular disorder of the upper gastrointestinal tract characterized by delayed gastric emptying in the absence of mechanical obstruction of the stomach[15,16]. The clinical outcome of the delayed gastric emptying is a combination of symptoms, including early satiety, postprandial fullness, nausea, vomiting, belching, and bloating. The complicated overlap between upper gastrointestinal symptoms leads to a difficult diagnosis between gastroparesis and other DGBIs, such as FD, gastroparesis like syndrome and other organic gastrointestinal disorders[17]. A precise diagnosis requires measurement of gastric emptying using gastric scintigraphy, which is considered a gold standard diagnosis technique or using breath testing[15-17].

The etiology of gastroparesis is multifactorial: Diabetes mellitus, infectious, connective tissue diseases, prior gastric surgery, visceral ischemia, myopathic diseases, neurological diseases (most frequent Parkinson disease), coma or artificial ventilation and medications (especially opiate narcotic analgesics and anticholinergic agents)[17]. Nevertheless, the etiology cannot be established in more than 50% cases, representing idiopathic gastroparesis. The pathogenesis of gastroparesis is also complex, and abnormalities in fundic tone, gastroduodenal dyscoordination, a weak antral pump, gastric dysrhythmias, abnormal duodenal feedback justify the reason why gastroparesis is considered a part of a broader spectrum of gastric neuromuscular dysfunction that includes impaired gastric accommodation[15-17]. Epidemiological evidence is still lacking. The number of gastroparesis patients in the United States is estimated to be more than 4 million[17]. Gastroparesis is associated with ED. A study performed by Szmukler *et al*[18] investigated 20 patients presenting ED using gastric scintigraphy [18]. From a total of 20 patients, 8 had anorexia nervosa, 10 had both anorexia nervosa and bulimia nervosa, and 2 with bulimia nervosa alone. In this group, gastric half-

Table 1 Studies investigating the association between gastroparesis and eating disorders

Ref.	Aims	Study population	Assessment instruments	Results and conclusions
Szmukler *et al*[18], 1990	To determine the natural history of delayed gastric emptying of solid foods in AN	20 consecutive female inpatients. 8 restrictive AN. 10 AN and BN. 2BN. Mean age: 22.8 ± 5.2 yr. Duration of illness: 49.0 ± 37.4 mo	Scintigraphy; HET; BMI	HET > 110 min. HET significant negative correlation with BMI; delayed gastric emptying in AN improves quite rapidly as feeding recommences
Hutson and Wald[19], 1990	To measure: Gastric emptying of a mixed liquid and solid meal in patients with AN, BN, and HC; the relationship of body weight and gastrointestinal symptoms to gastric emptying	11 BN. 10 AN. A sex-matched HC	A dual radioisotope technique	Gastric emptying of solids in patients with BN was similar to that in HC (gastric T1/2 131 ± 15 min *vs* 119 ± 7 min; mean ± SEM). AN patients had overall delayed emptying (182 ± 31 min; $P < 0.05$); gastric emptying of liquids was similar in the BN and HC (gastric T1/2 48 ± 5 min and 49 ± 4 min, respectively), AN tended to have prolonged gastric emptying (65 ± 11 min, P = NS). There was no correlation between body weight, gastrointestinal symptoms, and gastric emptying
Benini *et al* [20], 2004	To compare dyspeptic symptoms and gastric emptying times. To examine the relationship between dyspeptic symptoms, gastric motility, behavioral and psychological features of eating disorders and general psychopathology. To study the effect of simple reefeding and of long-term rehabilitation on gastric symptoms and on parameters of psychopathological distress	23 AN. 12 binge/purging subtype. Mean age 19.9 ± 0.7 yr; mean BMI 13.2 ± 0.6, 11 restricting subtype; mean age 25.4 ± 1.1 yr; mean BMI 15.5 ± 0.7. 24 HC age and sex matched	Ultrasonographic gastric-emptying test, psychopathological questionnaires (SCL-90, EDI, EDE-Q). The bowel symptom questionnaires. VAS for hunger and epigastric fullness	Gastric symptom scores: Markedly higher in AN than in HC; improved significantly with treatment; no correlation between entry values of gastric emptying symptoms and questionnaire score was found; long-term rehabilitation improves gastrointestinal symptoms, gastric emptying and psychopathological distress in an independent manner, but not short-term refeeding
Inui *et al* [21], 1995	Analyzing gastrointestinal motility abnormalities in ED patients	26 female patients. 9 AN (mean age 22.5 ± 2.0 yr). 10 AN and BN (mean age 22.2 ± 1.6 yr). 7 BN (mean age 19.2 ± 1.2 yr). 9 HC	Gastric emptying: Radionuclide technique SDS; CAS	ED patients had delayed gastric emptying after ingestion of a solid meal. The patients has high depression and anxiety scores
Dubois *et al* [22], 1979	Measure of gastric emptying and gastric output concurrently in a group of patients with AN before and after weight gain	15 female AN age 14-32 yr; weight 34 ± 1 kg; 11 HC (8 male and 3 female) age 20-31 years old weight 68 ± 3 kg	Dye dilution technique; Barium meal x-ray examination	Fractional gastric emptying rate was significantly less in AN patients than in controls during basal conditions and following a water load, but not during maximal doses of pentagastrin. Emptying is inversely correlated with body weight in healthy controls. Gastric emptying is abnormally low in AN patients, even after weight gain
Kamal *et al* [23], 1991	To determine whether small bowel transit time or colonic transit time is delayed in AN and BN. To determine whether delays in gastrointestinal transit are correlated with symptoms of constipation or bloating	10 AN (9 female, 1 male). 18 BN (15 female, 3 male). 10 female HC	Whole-gut transit was tested by the radiopaque marker technique, mouth-to-cecum transit time was assessed by the lactulose breath test	Whole-gut transit time was significantly delayed in both AN (66.6 ± 29.6 h) and BN (70.2 ± 32.4 h) compared with HC (38.0 ± 19.6 h). Mouth-to-cecum transit time longer in AN (109.0 ± 33.5 min) and BN (106.2 ± 24.5 min) than in HC (84.0 ± 27.7 min), but these differences were not statistically significant
Robinson *et al*[24], 1988	Determinants of delayed gastric emptying in AN and BN patients	22 AN patients (21 female and 1 male). 10 BN female. 10 HC (8 female and 2 male)	Gamma camera technetium ^{99}m-sulphur colloid	Only gastric emptying rates of the solid meal and glucose solution were significantly delayed. The gastric disturbance was confined to patients with AN patients selecting their own diet. Patients receiving adequate nutrition on the ward had normal gastric emptying and weight gain in this group had no significant effect on emptying. Slow emptying was observed in patients who maintained a low weight solely by food restriction as well as in patients whose AN was complicated by episodes of bulimia. Gastric emptying in BN was normal
Bluemel *et al*[26], 2017	Relationship of postprandial gastrointestinal motor and sensory function with body weight	24 AN [BMI 14.4 (11.9–16.0) kg/m^2]. 16 OB [34.9 (29.6-41.5) kg/m^2]. 20 HC [21.9 (18.9-24.9) kg/m^2]	MRI and 13C-lactose-ureide breath test	Gastric half-emptying time (t_{50}) was slower in AN than HC ($P = 0.016$) and OB ($P = 0.007$). A negative association between t_{50} and BMI was observed between BMI 12 and 25 kg/m^2 ($P = 0.0$). Antral contractions and oro-cecal transit were not different. Self-reported postprandial fullness was greater in AN than in HC or OB ($P < 0.001$). After weight rehabilitation, t_{50} in AN tended to become shorter ($P = 0.09$) and postprandial fullness was less marked ($P < 0.01$)

Holt *et al* [27], 1981	Gastric emptying of the solid and liquid components of a physiological test meal	10 AN female patients, age 17-32 yr, mean weight 42 kg. 12 HC (6 females, 6 males, age 32-65 yr; mean weight 67 kg	Scintiscanning method	Significantly slower gastric emptying was found for both the liquid and the solid components of the meal in AN patients compared with HC. Emptying during the early phase (0-40 mm after meal ingestion) was not significantly differently in the two groups
Abell *et al* [28], 1987	Gastrointestinal and neurohormonal function measuring gastric electrical activity, antral phasic pressure activity, gastric emptying of solids and liquids, and hormonal and autonomic function in AN patients	8 AN (2 male and 6 female), age: 16-31 yr. 8 HC (2 male and 6 female) age19-34 yr	Gastric electrogastrography and manometry (fasting and postprandially), radioscintigraphic gastric emptying test, cold pressor test	AN patients: Increased episodes of gastric dysrhythmia (mean percentage of dysrhythmic time: 9.75 patients *vs* 0.48 controls during fasting, $P < 0.02$; 7.21 patients *vs* 0.18 controls postcibally, $P < 0.001$); impaired antral contractility (mean motility index, 12.8 patients *vs* 14.2 controls, $P < 0.002$); delayed emptying of solids; decreased postcibal blood levels of norepinephrine and neurotensin; impaired autonomic function
Rigaud *et al* [29], 1988	Effects of renutrition on gastric emptying in AN patients	14 AN inpatients (13 female and 1 male); duration of illness: 9 mo-40 yr; mediane 5.9 yr); age 18-61 yr	Double-isotope technique (^{111}In) DTPA and ^{99m}Tc-ovalbumin	Gastric emptying can be improved by a renutrition program in AN
Waldholtz *et al*[30], 1990	To determine the type and frequency of gastrointestinal symptoms. To follow symptoms during refeeding prospectively. To develop guidelines for gastrointestinal testing and intervention in hospitalized AN patients	16 AN consecutive patients in their early 20 s, chronically ill (4.5 ± 1.2 yr); 71.6% ± 2.9% of matched population weight, 12 HC	AN patients rated on 12 gastrointestinal symptoms before and after nutritional rehabilitation. GISS (24 questions); blood tests physical examination	Belching did not improve during treatment. No patients required endoscopy, x-ray evaluation, or antipeptic regimens. Although severe gastrointestinal symptoms are common in AN, they improve significantly with refeeding
Murray *et al* [33], 2020	To identify the frequency of FED symptoms and evaluate the relations between FED symptoms, gastrointestinal symptoms, and gastric retention	288 patients (ages 17-78 yr; 77.5% female). Age 42.7 ± 16.3 yr; BMI 26.3 ± 6.5 (kg/m^2). AN 5 (2.0%) Other Specified FED 23 (9.4%) Unspecified FED-Restrictive 24 (8.3%)	GES, NIAS, EDDS, PAGI-SYM, GCSI	FED symptoms: Were common (55%), particularly ARFID symptoms (23%-40%); Were associated with greater GI symptom severity, but not gastric retention

GP: Gastroparesis; GI: Gastrointestinal; AN: Anorexia nervosa; BN: Bulimia nervosa; ED: Eating disorders; HC: Healthy controls; HET: Initial gastric half-emptying time; BMI: Body mass index; SCL-90: Symptom Check List-90; EDI: Eating disorders inventory; EDE-Q: Eating disorders examination-questionnaire; VAS: Visual analogue scale; SDS: Self-rating depression scale; CAS: Cattell anxiety scale; OB: Obesity; MRI: Magnetic resonance imaging; DTPA: Diethylenetriaminepentacetic acid; GCSI: Gastroparesis cardinal symptom index; PAGI-SYM: Patient assessment of upper GI symptoms; GCSI: Gastroparesis cardinal symptom inventory; EDDS: Eating disorder diagnostic scale; FED: Feeding or eating disorder; NIAS: Nine item avoidant/restrictive food intake disorder survey; GES: Gastric emptying scintigraphy; ARFID: Avoidant/restrictive food intake disorder.

emptying time (HET) showed a significant negative correlation with body mass and no correlation with age, gender, duration of illness or use of psychiatric medication. In the second part of the study, 12 patients with delayed gastric emptying were retested after one month, and HET had improved in 9 of 12 ($P = 0.0005$)[18]. Further, the normalization of the majority of scintigraphy parameters occurred while the body mass index was still subnormal (less than 20.3) and with amenorrhea still present. The conclusion of the study was that delayed gastric emptying is present in ED and improves quite rapidly after restarting normal feeding. Therefore, the gastrointestinal motility disorder is secondary to insufficient food intake and is not the primary disorder.

A study performed by Hutson *et al*[19] analyzed gastric emptying of a mixed liquid and solid meal in 11 patients with bulimia nervosa and was compared with ten patients with anorexia nervosa and a sex-matched control population[19]. The authors of the study decided to use a dual radioisotope technique in order to measure gastric

Table 2 Studies analyzing the association between functional dyspepsia and eating disorders

Ref.	Aims	Study population	Assessment instruments	Results and conclusions
Santonicola *et al*[37], 2012	Prevalence of FD	20 AN, 6 BN, 10 EDNOS, 9 CT, 32 OB, 22 HC	Rome III criteria (18 questions diagnosis of FD and its subgroups PDS and EPS)	90% AN, 83.3% BN, 90% EDNOS, 55.6% OB and 18.2% CT met PDS criteria. Emesis was present in 100% BN patients, 20% EDNOS, 15% AN, 22% of CT subjects, 5.6% HC. Postprandial fullness intensity-frequency score was significantly higher in AN, BN, EDNOS. Nausea and epigastric pressure were increased in BN and EDNOS
Porcelli *et al* [38], 1998	Presence of lifetime ED in patients referred for FGID	127 consecutive patients (42 FD, 28 IBS 20 FAP, 37 with FD and IBS; male and 83 females; 163 control subjects gallstone disease	GSRS; HADS (HADS-A and HADS-D)	Past ED were significantly more prevalent in FGID (15.7%) than in gallstone disease patients (3.1%) (chi-square = 14.6, $P < 0.001$). FGID patients with past ED were significantly younger, more educated, more psychologically distressed, more dyspeptic, and more were women than FGID patients without past ED
Cremonini *et al*[39], 2009	Severity of BE episodes would be associated with upper and lower GI symptoms	4096 subjects (population-based survey of community residents found through the medical record linkage system) > 18 yr	Questionnaire measuring GI symptoms, frequency of BE episodes and physical activity level	BE disorder: Was present in 6.1% subjects, was independently associated with upper. GI symptoms: Acid regurgitation heartburn, dysphagia, bloating and upper abdominal pain, was associated with lower GI symptoms: diarrhea, urgency, constipation and feeling of anal blockage. The associations independent of the level of obesity
Jáuregui *et al* [40], 2011	QoL in FD patients psychopathological features that underlie the FD	245 people (mean age 28.36 ± 11.26 yr; 189 female and 56 male) 78 patients with ED (70 female and 8 male, mean age 22.88 ± 8.28 yr), 90 university students with associated FD (76 female and 14 male, mean age 22.49 ± 4.27 yr); 77 psychiatric patients (non-ED) (43 female and 34 male, mean age 40.78 ± 9.40 yr)	NDI-SF, BDI, STAI, TSF-Q, VAS	Satiation and bloating were significantly higher in ED patients. Correlations between dyspepsia and TSF were initially positive and significant in all cases, but significance was only maintained in the group of ED patients. Predictors of quality of life in ED patients: dyspepsia, depressive symptomatology, TSF-conceptual, TSF-interpretative and total TSF
Santonicola *et al*[41], 2019	Relationship among anhedonia, BED and upper gastrointestinal symptoms in 2 group of morbidly OB with and without SG	81 OB without SG, 45 OB with SG, 55 HC	BDI, STAI, SHAPS, ROME IV criteria for FD and its subtypes	OB without SG showed a higher prevalence of PDS, mood disorders and anxiety when positive for BE behavior compared to those negative for BE behavior, no differences were found in SHAPS score. OB with SG showed a higher prevalence of PDS compared to OB without SG. BED and depression are less frequent in the OB with SG, while state and trait anxiety are significantly higher. The more an OB with SG is anhedonic, less surgical success was achieved

FD: Functional dyspepsia; ED: Eating disorders; HC: Healthy controls; AN: Anorexia nervosa; BN: Bulimia nervosa; EDNOS: Eating disorders not otherwise specified; CT: Constitutional thinners; OB: Obesity; PDS: Postprandial distress syndrome; EPS: Epigastric pain syndrome; FGID: Functional gastrointestinal disorders; IBS: Irritable bowel syndrome; FAP: Functional abdominal pain; GSRS: Gastrointestinal symptoms rating scale; HADS: Hospital Anxiety and Depression Scale; BE: Binge eating; GI: Gastrointestinal; QoL: Quality of life; NDI-SF: Nepean Dyspepsia Index-Short Form; BDI: Beck Depression Inventory; STAI: State-Trait Anxiety Inventory; TSF-Q: Thought Shape Fusion-Questionnaire; VAS: Visual Analogue Scales; SG: Sleeve gastrectomy; SHAPS: Snaith-Hamilton pleasure scale.

emptying. Similar to previous studies, the relationship between body mass index and gastrointestinal symptoms were also examined. Authors reported at gastric emptying of solids in patients with bulimia nervosa was similar to that in controls (gastric T1/2 131 ± 15 min *vs* 119 ± min; mean ± SEM) and anorexia nervosa patients had overall significantly delayed emptying (182 ± 31 min[19]. Gastric emptying of liquids was similar in bulimic patients and healthy controls[19]. The study did not find any correlation between body mass index, gastrointestinal symptoms, and gastric emptying, indicating that gastrointestinal symptoms are unreliable criteria of gastric emptying in patients with ED[19]. Although gastric scintigraphy is the gold standard technique for measuring gastric emptying, not all studies use it. A study performed by

Table 3 Studies analyzing the association between functional constipation and eating disorders

Ref.	Aims	Study population	Assessment instruments	Results and conclusions
Chun *et al*[47], 1997	Colorectal function measuring colonic transit and anorectal function in AN with constipation during treatment with a refeeding program	Prospective study 13 AN females; 20 age-matched, female HC	Radiopaque marker technique; anorectal manometry	Colonic transit is normal/returns to normal in the majority of AN patients once they are consuming a balanced weight gain or weight maintenance diet for at least 3 wk
Sileri *et al*[48], 2014	Prevalence and type of defecatory disorders in AN patients	85 patients (83 female and 2 male); mean age 28 ± 13 yr; BMI 16 ± 2 kg/m^2; 57 HC, BMI 22 ± 3 kg/m^2	WCS, OD score, FISI	All results influenced by the severity of the disease (BMI; duration). The percentage of defecatory disorders rises from 75 to 100% when BMI is < 18 kg/m^2 and from 60% to 75% when the duration of illness is ≥ 5 yr (P < 0.001 and P = 0.021)
Chiarioni *et al*[49], 2000	Anorectal and colonic function in AN patients complaining of chronic constipation	12 AN female (age 19-29 yr) chronic constipation. 12 female HC	Anorectal manometry; radiopaque technique; test of rectal sensation	AN patients: anorectal motor abnormalities (slow colonic tranzit time, pelvic floor dysfunction)
Boyd *et al*[50], 2005	Prevalence and type of FGIDs in AN, BN and EDNOS patients; relationships between psychological features, eating-disordered attitudes and behaviours, demographic characteristics and the type and number of FGIDs	101 consecutive female AN (n = 45, 44%), EDNOS (n = 34, 34%), BN (n = 22, 22%). Mean age 21 yr	Rome II modular questionnaire GI, ENS, BDI, STAI, BSI somatization subscale, EEE-C, version 4, EDI-2, EAT	52% IBS (constipation-predominant 22%, diarrhoea-predominant 6%, alternating 24%), FH (51%), FAB (31%), FC (24%), FDys (23%), FAno (22%). 52% of patients exhibited 3 or more coexistent FGID diagnoses. Psychological variables (somatization, neuroticism, state and trait anxiety), age and binge eating were significant predictors of specific, and > 3 coexistent FGIDs
Murray *et al*[51], 2020	Frequency of and relation between EDs and constipation in patients with chronic constipation referred for anorectal manometry	279 patients with chronic constipation (79.2% female). Average age (SD) 46.6 ± 17.2 yr	EAT, PAC-SYM, HADS, VSI, ARM, colonic transit testing (24 radiopaque markers)	19% had clinically significant ED pathology. ED pathology might contribute to constipation *via* gastrointestinal-specific anxiety
Dykes *et al*[52], 2001	Past and current psychological factors associated with slow and normal transit constipation.	28 consecutive constipated female patients, mean age 38.2 yr (SD 10.8 yr)	SCID, SF-36, EAT	1/5 current affective disorder, 2/3 previous affective disorder, 1/3 distorted attitudes to food
Waldholtz *et al*[30], 1990	Type and frequency of GI symptoms. To follow symptoms during refeeding prospectively. Guidelines for gastrointestinal testing and intervention in hospitalized AN patients	16 consecutive AN patients chronically ill (4.5 ± 1.2 yr); 71.6% ± 2.9% of matched population weight, 12 HC	AN patients rated on 12 gastrointestinal symptoms before and after nutritional rehabilitation GISS (24 questions); blood tests physical examination	Belching did not improve during treatment; no patients required endoscopy, x-ray evaluation, or antipeptic regimens; although severe gastrointestinal symptoms are common in AN, they improve significantly with refeeding

AN: Anorexia nervosa; BN: Bulimia nervosa; EDNOS: Eating disorders not otherwise specified; HC: Healthy controls; BMI: Body mass index; WCS: Wexner constipation score; OD score: Obstructed defecation score; FIOSI: Fecal incontinence severity index; FGIDs: Functional gastrointestinal disorders; ENS: Eysenck neuroticism scale; BDI: Beck Depression Inventory; STAI: The State-Trait Anxiety Inventory; BSI: Brief Symptom Inventory; EEE-C: Eating and Exercise Examination/computerized; EDI-2: Eating Disorder Inventory-2; EAT: Eating attitudes test; ED: Eating disorders; IBS: Irritable bowel syndrome; FH: Functional heartburn; FAB: Functional abdominal pain disorder; FC: Functional constipation; Fano: Functional anorectal pain disorder; PAC-SYM: Patient Assessment of Constipation Symptom Questionnaire; VSI: Visceral Sensitivity Index; ARM: High-Definition Anorectal Manometry; SCID: Structured Clinical Interview for DSM; SF-36: Short Form (36) Health Survey.

Benini *et al*[20] analyzed 23 anorexic patients using an ultrasonographic gastric-emptying test and psychopathological questionnaires, before and after 4 and 22 wk rehabilitation[20]. The result was that gastric symptom scores were markedly higher in patients than in controls and improved significantly with treatment. Further, no correlation between entry values of gastric emptying symptoms and questionnaire score was found[20]. The study summarized that long-term rehabilitation improves gastrointestinal symptoms, gastric emptying, and psychopathological distress, and short-term does not.

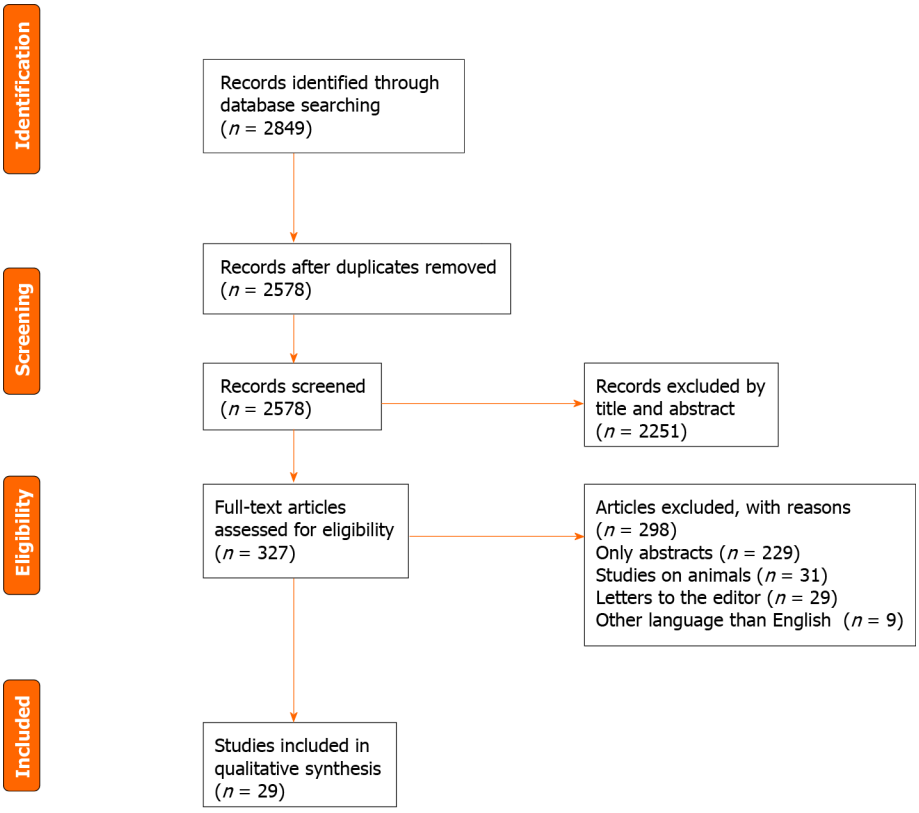

Figure 1 PRISMA flow diagram for study selection.

A study performed by Inui *et al*[21] analyzed gastrointestinal motility abnormalities in 26 female patients who met the DSM-III-R criteria for ED[21]. Gastric emptying was measured using a radionuclide technique, and all patients were additionally evaluated using a self-rating depression scale and the Cattell anxiety scale. Nine patients were diagnosed with anorexia nervosa, 10 with anorexia nervosa and bulimia nervosa, and 7 with bulimia nervosa. In addition, the time expressed in minutes at which half the meal was emptied from the stomach was measured. Patients with anorexia nervosa, anorexia nervosa with bulimia nervosa, and bulimia nervosa all had delayed gastric emptying as compared to nine normal healthy controls and had delayed gastric emptying after ingestion of a solid meal, regardless of DSM-III-R classification[21]. The authors concluded that impaired gastric motility might be caused not only by food restriction or emesis but also by other independent factors from the patient nutritional state[21].

The intricate effect of depression and anxiety shows that gastroparesis pathogenesis is more complex, and psychotherapy may have an essential role in the nutritional rehabilitation of patients with ED. Anorexia nervosa and bulimia nervosa were the most frequent ED associated with gastroparesis, a fact demonstrated by numerous studies over the last 6 decades[22-30]. Limited evidence regarding the association of gastroparesis and pica, night eating disorder and avoidant/restrictive food intake disorder was found[22-30].

FD

FD is one of the most frequent DGBIs and is defined using the Rome IV criteria as any combination of the following symptoms: Postprandial fullness, early satiety, epigastric pain, and epigastric burning that are severe enough to interfere with the usual activities and occur at least three days *per* week over the past three months with an onset of at least six months before presentation[31].

FD includes three syndromes: (1) Postprandial distress syndrome; (2) Epigastric pain syndrome; and (3) Overlapping postprandial distress syndrome and epigastric pain syndrome[31-33]. The pathophogensis of FD is multifactorial and incompletely understood. Dysfunctional gastrointestinal motility (antral hypomotility, delayed or rapid gastric emptying, impaired gastric accommodation), psychological stress, visceral hypersensitivity, psychiatric disorders (depressive disorder, anxiety disorder),

gastric or duodenal hypersensitivity to specific types of food and gastric distension, have been incriminated as pathogenesis mechanisms[34-44]. Smokers, women, patients with Helicobacter pylori infection, ED patients, and nonsteroidal anti-inflammatory drug users have an increased risk of developing FD[34-44].

The dyspeptic symptoms may be encountered in ED. Thus, a study performed by Santonicola *et al*[37] analyzed the prevalence of FD in ED patients, constitutional thinner subjects with no pathology, obese patients, and healthy volunteers[37]. The patients were recruited from a clinic specialized in treating ED, and the study groups included 20 anorexia nervosa patients, six bulimia nervosa patients, ten unspecified eating disorder patients, nine constitutional thinner subjects, 32 obese patients and 22 healthy controls[37]. The presence of epigastric pain syndrome and postprandial distress syndrome were diagnosed according to Rome III criteria. The intensity and frequency score of early satiety, epigastric fullness, epigastric pain, epigastric burning, epigastric pressure, belching, nausea, and vomiting were measured by a standardized questionnaire[37]. The result was that 90% of anorexia nervosa patients, 83.3% of bulimia nervosa patients, 90% of unspecified eating disorder patients, 55.6% of constitutionally thin subjects and 18.2% of the healthy volunteers met the postprandial distress syndrome criteria (χ^2, $P < 0.001$) and only one bulimia nervosa patient met the epigastric pain syndrome criteria[37]. Emesis was present in 100% of bulimia nervosa patients, in 20% of ED not otherwise specified patients, in 15% of anorexia nervosa patients, in 22% of constitutional thinner subjects, and, in 5.6% healthy volunteers (χ^2, $P < 0.001$)[37]. The pathologic eating behavior causes dysfunctional gastrointestinal sensitivity and motility, and after a variable period of time, the resulting DGBIs can persist independently of the eating disorder that originally caused the motility dysfunction[38].

A study performed by Cremonini *et al*[39] analyzed the gastrointestinal symptoms associated with binge ED[39]. A population-based survey of community residents through a mailed questionnaire was performed, and a total of 4096 subjects were included in the study. Binge eating disorder was present in 6.1% of subjects and was associated with the following gastrointestinal symptoms: Acid regurgitation, heartburn, dysphagia, bloating and epigastric pain, diarrhea, constipation and feeling the sensation of anal blockage[39]. The study demonstrated that both upper and lower gastrointestinal symptoms appear in binge ED.

Although the number of studies analyzing ED and FD is small, dyspeptic symptoms are more common in anorexia nervosa and bulimia nervosa patients.

A limitation of most studies regarding FD in ED is that a clear delimitation between meal-related gastrointestinal symptoms is lacking, and future studies analyzing the theoretical and clinical implications might help in developing a more efficient diagnosis and therapeutic scheme[40,41].

FC

FC is defined according to the Rome IV criteria as a change in bowel habit, or defecatory behavior that results in acute or chronic symptoms or diseases that would be resolved with the relief of constipation and a patient must have experienced at least two of the symptoms over the preceding three months[45,46]. FC is present also in ED. Thus, a study by Chun *et al*[47] analyzed the prevalence of FC in anorexic patients by measuring colonic transit and anorectal function[47]. The first study group consisted of 13 anorexic females, and the second study group consisted of 20 healthy female control subjects. Colonic transit was measured using a radiopaque marker technique, and anal sphincter function, rectal sensation, expulsion dynamics, and rectal compliance were measured with anorectal manometry[47]. The result showed that colonic transit returns to normal in the majority of patients after they start to finish a specialized refeeding program[47].

A study performed by Sileri *et al*[48] analyzed the prevalence of defecatory disorders in anorexic patients[48]. The Wexner constipation score (WCS), Altomare's obstructed defecation score (ODS), and the fecal incontinence severity index were used to evaluate constipation and incontinence of 83 female anorexic patients and 57 healthy volunteers. The result showed that constipation (defined as WCS ≥ 5) was present in 83% of anorexic patients and in 12% of healthy controls ($P = 0.001$), while obstructed defecation syndrome (defined as ODS ≥ 10) was present in 84% of anorexic patients and in 12% of healthy controls ($P < 0.001$)[48].

A study performed by Chiarioni *et al*[49] evaluated the prevalence and pathogenetic mechanisms of FC in 12 female anorexic patients and 12 healthy female controls[49]. Pelvic floor dysfunction was analyzed using an anorectal manometry, and colonic transit time was measured by a radiopaque marker technique.

A subgroup of 8 patients was retested after a specialized refeeding program[49]. The results showed that 66.7% of anorexic patients had slow colonic transit times, and 41.7% had pelvic floor dysfunction. The specialized refeeding program normalized the colonic transit time in the subgroup of 8 patients, but pelvic floor dysfunction did not normalize in these patients[49]. The study demonstrated that anorectal motility dysfunctions and delayed colonic transit are frequent in anorexic patients, a result also shown by other studies[30,50-52].

Although, the relation between FC and ET was analyzed in a limited number of studies, a significant association between the disorders was found.

IBS

IBS is one of the most frequent DGBIs characterized by abdominal pain and altered bowel habit in the absence of a specific organic pathology[53,54]. Epidemiologic studies show that the prevalence of irritable bowel syndrome is 10%-20% and the incidence of 1%-2% *per* year[53,54]. Perkins *et al*[55] performed a study about the prevalence of IBS in a large sample of ED patients analyzing the timing of onset of the ED symptomatology and assessing if they are predictors of IBS. The result of the study was that 64% of ED patients met the Manning criteria for IBS and 87% had developed their ED before the onset of IBS symptoms, with a mean period of time of 10 years between the onset of ED and IBS demonstrating that EDs increase the risk of IBS[55].

A study performed by Dejong *et al*[56] examined the prevalence of IBS in patients with bulimia nervosa and showed a high prevalence of IBS in the bulimia nervosa group (68.8%) [b]. IBS diagnosis was assessed using the Manning criteria and even if the study demonstrated a high incidence of IBS in patients with BN, the relationship between those disorders remain unclear[56]. A study performed by Sullivan *et al*[57] on 48 IBS patients, 32 ED patients, 31 inflammatory bowel disease (IBD) patients and 28 healthy controls analyzed the relationship between IBS, IBD and ED. The results showed that the eating Attitudes Test score for the IBS group was higher than IBD and control group (P = 0.05), demonstrating the correlation between IBS and ED[57].

The relation between clinical characteristics of ED and IBS was investigated in a study performed by Tang *et al*[58] on 60 IBS patients. The result was that the severity of IBS symptomatology was correlated with Perfectionism and Ineffectiveness and severe episodes of emesis were associated with self-reported binge-purge behaviors measured by the Bulimia subscale of the EDI[58]. Bulimia nervosa was more common in IBS patients, but no data about the effect of psychiatric therapy on IBS symptoms was found.

DISCUSSION

In this review we looked for current evidence of the association of neurogastroenterological disorders with ED. We identified 29 studies, focusing on four gastrointestinal disorders associated with ED (gastroparesis, FD, FC and irritable bowel syndrome). Despite progress in the field of neurogastroenterology, we were not able yet to identify a causative relation between neurogastroenterological disorders and ED.

One of the strengths of this study is that it highlights the overlap between ED and neurogastroenterological disorders, a fact of paramount importance for the management of those patients who often are affected with both type of disorders, but, just as often, are treated for only one disorder, depending on whether they visit the psychiatrist or the gastroenterologist.

No relation between gastroesophageal reflux disease and ED was found[11-14]. A limited number of studies investigated the rumination syndrome but we were not able to find supportive data about the association between rumination and ED.

The small number of patients included in the majority of studies represent the main limitation and future studies under the support of international collaboration might help in developing a more efficient therapy. Another limit of most studies regarding FD is that a clear delimitation between meal-related gastrointestinal symptoms is lacking, and future studies using an animal model might help in developing precise diagnosis tools[40-44]. Anorexia nervosa and bulimia nervosa were the most frequent ED associated with FD[37-39]. Most studies about FC showed that anorectal motility dysfunctions and delayed colonic transit are frequent in anorexic patients and anorexia nervosa was the most frequent eating disorder associated with FC[47-53]. IBS was associated with anorexia nervosa and bulimia nervosa[55-58].

Anorexia nervosa and bulimia nervosa were the most frequent ED associated with gastroparesis[18-21]. A major limit in studies concerning gastroparesis was the fact

that not all studies use gastric scintigraphy for diagnosis, although it is the gold standard technique for measuring gastric emptying. Thereby, a lack of a clear distinction between gastroparesis and FD was present[20,21,59].

Clear evidence for a cause effect relationship between ED and DGBI, still does not exist. More powerful studies are required. In respect to therapy of DGBI in ED, the absence of randomized controlled trials (RCTs) is the main reason why no guidelines for gastrointestinal symptomatology in ED exist. These limitations can be overcome by projecting larger RCTs with significant samples of ED and DGBI patients.

CONCLUSION

There is no evidence for a cause-effect relationship between DGBI and ED. Their common symptomatology required correct identification and a tailored therapy of each disorder.

ARTICLE HIGHLIGHTS

Research background

Eating disorders (ED) involve both the nervous system and the gastrointestinal tract. A similar double involvement is also found in disorders of the brain-gut interaction (DGBI) and symptoms are sometimes similar.

Research motivation

We aimed to understand the management of patients who often are affected with both DGBI and ED, but, just as often, are treated for only one disorder, depending on whether they visit the psychiatrist or the gastroenterologist.

Research objectives

This systematic review aimed to evaluate the comorbidity of DGBI and ED, in order to find out where there is an association and a cause-effect relationship.

Research methods

A thorough literature search was undertaken. PubMed, Cochrane Library, EMBASE, and WILEY databases were screened for relevant publications regarding DGBI in ED.

Research results

We found 29 articles analyzing the relation between DGBI and ED comprising 13 articles on gastroparesis, 85 articles on functional dyspepsia, 7 articles about functional constipation and 4 articles on irritable bowel syndrome.

Research conclusions

There is no evidence for a cause-effect relationship between DGBI and ED. Their common symptomatology requires correct identification and a tailored therapy of each disorder.

Research perspectives

The absence of randomized controlled trials (RCTs) is the main reason why no guidelines for gastrointestinal symptomatology in ED exist. These limitations can be overcome by projecting larger RCTs with significant samples of ED and DGBI patients.

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SYSTEMATIC REVIEWS

Weight loss interventions in living donor liver transplantation as a tool in expanding the donor pool: A systematic review and meta-analysis

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Abstract

BACKGROUND

With increasing rates of liver transplantation and a stagnant donor pool, the annual wait list removals have remained high. Living donor liver transplantation (LDLT) is an established modality in expanding the donor pool and is the primary method of liver donation in large parts of the world. Marginal living donors, including those with hepatic steatosis, have been used to expand the donor pool. However, due to negative effects of steatosis on graft and recipient outcomes, current practice excludes overweight or obese donors with more than 10% macro vesicular steatosis. This has limited a potentially important source to help expand the donor pool. Weight loss is known to improve or resolve steatosis and rapid weight loss with short-term interventions have been used to convert marginal donors to low-risk donors in a small series of studies. There is, however, a lack of a consensus driven standardized approach to such interventions.

AIM

To assess the available data on using weight loss interventions in potential living liver donors with steatotic livers and investigated the feasibility, efficacy, and safety of using such donors on the donor, graft and recipient outcomes. The principal objective was to assess if using such treated donor livers, could help expand the donor pool.

METHODS

We performed a comprehensive literature review and meta-analysis on studies

The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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examining the role of short-term weight loss interventions in potential living liver donors with hepatic steatosis with the aim of increasing liver donation rates and improving donor, graft, and recipient outcomes.

RESULTS
A total of 6 studies with 102 potential donors were included. Most subjects were males (71). All studies showed a significant reduction in body mass index post-intervention with a mean difference of -2.08 (-3.06, 1.10, I^2 = 78%). A significant reduction or resolution of hepatic steatosis was seen in 93 of the 102 (91.2%). Comparison of pre- and post-intervention liver biopsies showed a significant reduction in steatosis with a mean difference of -21.22 (-27.02, -15.43, I^2 = 56%). The liver donation rates post-intervention was 88.5 (74.5, 95.3, I^2 = 42%). All donors who did not undergo LDLT had either recipient reasons or had fibrosis/steato-hepatitis on post intervention biopsies. Post-operative biliary complications in the intervention group were not significantly different compared to controls with an odds ratio of 0.96 [(0.14, 6.69), I^2 = 0]. The overall post-operative donor, graft, and recipient outcomes in treated donors were not significantly different compared to donors with no steatosis.

CONCLUSION
Use of appropriate short term weight loss interventions in living liver donors is an effective tool in turning marginal donors to low-risk donors and therefore in expanding the donor pool. It is feasible and safe, with comparable donor, graft, and recipient outcomes, to non-obese donors. Larger future prospective studies are needed.

Key Words: Living donor liver transplant; Living liver donors; Liver steatosis; Weight loss interventions; Donor outcomes; Recipient outcomes

Core Tip: Living donor liver transplantation is an established modality in expanding the donor pool but is limited by donor safety concerns and recipient and graft outcomes due to high prevalence of hepatic steatosis in obese or overweight donors. Weight loss is known to improve or resolve steatosis and help convert marginal donors to low-risk donors in a small series of studies. Our meta-analysis demonstrates that short term weight loss intervention, is feasible and safe in significantly reducing hepatic steatosis in living liver donors undergoing donor evaluation and has the potential to safely expand the donor pool.

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INTRODUCTION

With the steady increase in liver transplantation (LT) over the last 2 decades, and the donor pool remaining largely stagnant, the shortage of organs for transplantation has become even more pressing. This has led to an increase in median time on the wait list for transplantation, especially in patients with a model for end-stage liver disease (MELD) greater than 15[1]. Consequently, as per United Network for Organ Sharing (UNOS) data, every year more than 1200 patients are being removed from the liver transplant wait list[1].

Living donor LT (LDLT) has the potential of increasing the donor pool and lowering the wait list mortality. LDLT offers recipients the advantage of a high-quality graft and the possibility of avoiding delisting, deconditioning over time, or death due to a change in clinical status. In addition, LDLT has the benefit of scheduling the

transplantation as an elective surgery[2]. In parts of the world including South Korea, Japan, India, and Taiwan, LDLT is the primary modality of offering organs to patients in need for LT[3].

Although the number of LDLTs has steadily increased in the United States in the last few years, the total number of LDLTs has lagged in comparison to high volume centers in Asia. The number of deceased donor liver transplants in the United States in 2019 was 8372. The number of LDLTs during the same year was 524, a mere 6% of total liver transplants performed[4].

Most patients listed for liver transplant struggle to find a suitable living donor[5,6]. One way to address the shortage of donors is to use marginal living donors, including those with hepatic steatosis. The negative effects of such steatotic grafts on liver donation and transplantation are well known, including higher incidence of severe ischemic damage resulting in primary dysfunction or primary non function of graft, biliary strictures, and decreased one-year graft survival[7-9]. In a study by Gabrielli *et al*[10], recipients who received non-heart beating liver grafts with macrovesicular steatosis had significantly lower 3-year overall survival.

Compounding the problem of organ shortage is the dramatically rising rate of obesity around the world[11]. In the United States in 2012, 69% of the population was overweight [body mass index (BMI) > 25] and 35% was obese (BMI > 30)[12]. Obesity is a strong risk factor for hepatic steatosis and steatosis is seen on liver biopsy in 76% of potential living liver donors with a BMI of more than 28[13].

The aim of our study was to summarize the current evidence on the role of short-term interventions for weight loss, such as diet and medications, in obese or overweight potential living liver donors with steatotic livers. The objectives were to assess the effectiveness of these interventions in reducing donor BMI and liver steatosis, turning marginal donors to low-risk donors, and examining the impact of steatosis reduction or resolution on short-term donor and recipient morbidity, mortality, and graft outcomes.

MATERIALS AND METHODS

We used PubMed as our primary electronic search database. Keywords used for search criteria included LDLT, living liver donors, diet therapy, fatty liver, steatosis, and short-term weight loss interventions. Studies were analyzed using the Population, Intervention, Comparison, and Outcomes methodology and all studies that met our eligibility criteria were included (Table 1).

Studies that investigated weight loss strategies for potential living liver donors were reviewed. Our eligibility criteria included adult (age > 18 years), overweight or obese potential living liver donors with biopsy proven and or radiologically assessed hepatic steatosis who underwent weight loss interventions and who had post intervention assessment of liver steatosis, with liver biopsy with or without radiologic modalities and post intervention assessment of weight loss. Studies should have also analyzed donor, recipient, and graft outcomes, including perioperative complications as graded by Clavien-Dindo classification[14], and donor and recipient morbidity and mortality. Six studies that met our criteria were finally included in our study.

Variables that were examined in each study included exclusion criteria, treatment modality, diagnostic modalities to assess for pre- and post-intervention hepatic steatosis (such as liver biopsies, computed tomography, or magnetic resonance imaging), pre- and post-intervention BMIs, total bilirubin, liver transaminases [aspartate transaminase, alanine amiotransferase (ALT)]. In addition, liver donation rates, donor and recipient perioperative complications (graded according to Clavien's scale), and donor, graft and recipient outcomes were examined with each study.

Statistical analysis

We used meta-analysis techniques to calculate the pooled estimates following the methods suggested by DerSimonian and Laird using the random-effects model. Mean difference and odds ratio were calculated using random-effects model for continuous and binary variables, respectively. When the incidence of an outcome was zero in a study, a continuity correction of 0.5 was added to the number of incident cases before statistical analysis. Heterogeneity was assessed between study-specific estimates by using Cochran Q statistical test for heterogeneity, and the I^2 statistics. I^2 values of < 30%, 30%-60%, 61%-75%, and > 75% were suggestive of low, moderate, substantial, and considerable heterogeneity, respectively. A P value of ≥ 0.05 was used 'a-priori' to define statistical significance. The analysis was done using RStudio and RevMan

Table 1 Overview of interventions used for body mass index and steatosis reduction, and donor, recipient and graft outcomes following liver transplantation

Study number	Ref.	*n*	Type of intervention	Treatment duration	BMI reduction	Steatosis reduction	Liver donation	Donor, graft, recipient outcomes
1	Fujii *et al*[15], 2020	8	< 1600 Kcal/d + exercise 20 min x 3/wk ± statins	Median of 58 d	Yes (P = 0.0009)	Yes (P = 0.0006)	8	No significant difference from controls
2	Doyle *et al* [16], 2016	16	Optifast VLCD: 1000 kcal/ d	Median of 7.3 wk	Yes (P < 0.001)	Yes (P < 0.001)	14 (1 inadequate volume, 1 fibrosis)	No significant difference from controls
3	Choudhary *et al*[17], 2015	16	1200 kcal/d + 200 to 400 kcal/d exercise ± statins	Mean 28 ± 10 d	Yes (P = 0.006)	Yes (P = 0.008)	14 (2 had NASH/fibrosis)	No reported complication in perioperative period
4	Oshita *et al* [18], 2012	42	800 to 1400 kcal/d diet + 100 to 400 kcal/d exercise	Median 2.9 mo	Yes (P < 0.0001)	Yes, to < 20 %	41 (1 had stage 2 fibrosis)	No different from control group
5	Nakamuta *et al* [19], 2005	11	1000 kcal/d diet + exercise (600 kcal/d) + Bezafibrate	Mean 37.8 ± 4.6 d	Yes(P = 0.0033)	Yes(P= 0.0028)	7 (2 recipient deaths, 1 inadequate GRWR)	No different from control group
6	Hwang *et al* [20], 2004	9	Diet (25-30 calories x ideal body weight) + exercise	Median of 3 mo	Yes (P = 0.0001)	Yes (P = 0.006)	9	No different from control group

BMI: Body mass index; GRWR: Graft weight/recipient weight ratios; NASH: Nonalcoholic steatohepatitis; VLCD: Very low calorie diet.

software.

RESULTS

Six studies were included following our literature search. Data and results from these studies are outlined in Table 1. The largest study had a sample size of 42 patients and the smallest study, had a sample size of 8.

Some inferences can be made based on the data analyzed. Most subjects (71) were males. There were no reported dropouts from the treated group due to adverse events. All studies showed a significant reduction in BMI post-interventions (Figure 1) with a mean difference of -2.08 (-3.06, 1.10, I^2 = 78%). All six studies showed a significant reduction (P < 0.05) in steatosis (Table 1). A significant reduction or resolution of hepatic steatosis was seen in 93 of the 102 in the intervention group (91.2%). Three of the 6 included studies had both pre- and post-intervention liver biopsies, and these studies were included in the Forest plot (Figure 2) to allow for a standardized comparison of intervention outcomes; they showed a significant reduction in steatosis with a mean difference of -21.22 (-27.02, -15.43, I^2 = 56%).

Majority of donors who underwent weight loss interventions successfully underwent living liver donation (Figure 3) with the rate being at 88.5% (74.5-95.3%, I^2 = 42%). Prospective donors, who did not undergo donor hepatectomy, were either waiting to donate at study conclusion or had either steatohepatitis (2 in Choudhary group, 1 in Doyle group), inadequate Graft weight/recipient weight ratios (GRWR) (1 in Nakamuta group, 1 in Doyle group) or recipient causes for not donating (2 recipient deaths in the Nakamuta group).

Post-operative biliary complications (Figure 4) in the intervention group were not significantly different compared to control (non-intervention) donors with odds ratio of 0.96 [(0.14, 6.69), I^2 = 0]. The overall post-operative donor, graft, and recipient outcomes in the diet treated donors were also not significantly different when compared to non-diet treated donors. All studies were limited by their small sample size, and some by their retrospective study design.

DISCUSSION

With increasing rates of LT and a stagnant donor pool, the annual wait list removals

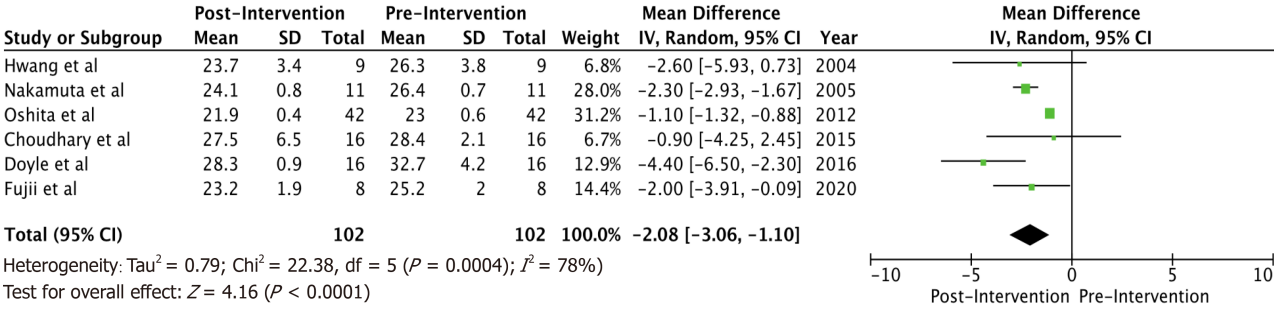

Figure 1 Forest plot showing mean difference in body mass index post-pre intervention in the intervention groups. Mean difference = -2.08 (-3.06, 1.10, I^2 = 78%), with a significantly lower post-intervention body mass index as compared to the pre-intervention body mass index. CI: Confidence interval; SD: Standard deviation.

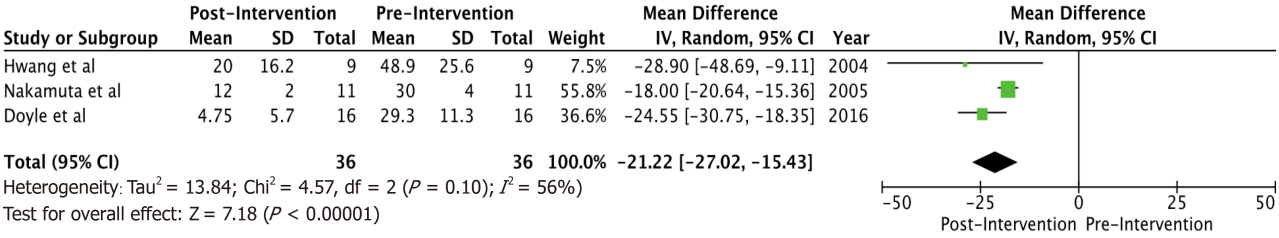

Figure 2 Forest plot showing mean difference in steatosis post-pre intervention. Mean difference = -21.22 (-27.02, -15.43, I^2 = 56%), with significantly lower post intervention steatosis as compared to pre intervention steatosis. CI: Confidence interval; SD: Standard deviation.

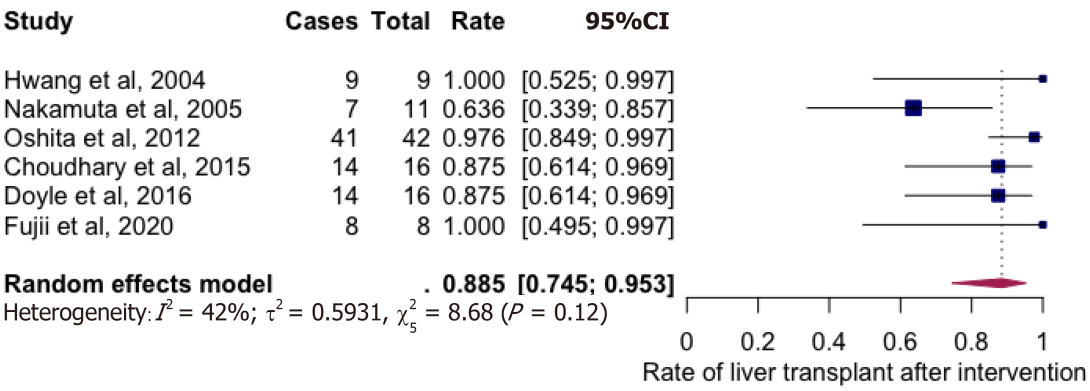

Figure 3 Living liver donation rates after weight loss interventions. Rate = 88.5% (74.5%-95.3%, I^2 = 42%). CI: Confidence interval.

have remained high. The use of extended criteria donors including those with steatotic livers to expand the donor pool is a viable option in expanding the donor pool. The data on donor, graft and recipient outcomes in grafts used for potential donors with steatotic livers who have undergone weight loss interventions, is however sparse. Compounding this issue is the increasing rates of obesity has made donor safety and successful recipient outcomes, an even greater challenge.

We, therefore, analyzed current literature on the role of short-term dietary interventions in preparing potential donors with hepatic steatosis for LDLT with the aim of safely and effectively expanding the donor pool and improving donor and recipient outcomes. Studies included, have been summarized in Table 1.

In study number 1 by Fujii *et al*[15], 8 potential donors were examined from October 2009 to August 2015. Exclusion criteria were age greater than 65 and steatohepatitis. Steatosis was diagnosed based on a liver to spleen (L/S) ratio of < 1.1 and/or hepatic attenuation of < 55 Hounsfield units (HU) on non-enhanced CT. Donors without fatty liver (n = 21) during the study period, were selected as a control group. Treatment efficacy was serially evaluated and when L/S was ≥ 1.1, and hepatic attenuation was ≥ 55 HU, a liver biopsy was performed. When macrovesicular steatosis of < 10% was confirmed, donors were taken up for partial hepatectomy. A significant reduction in

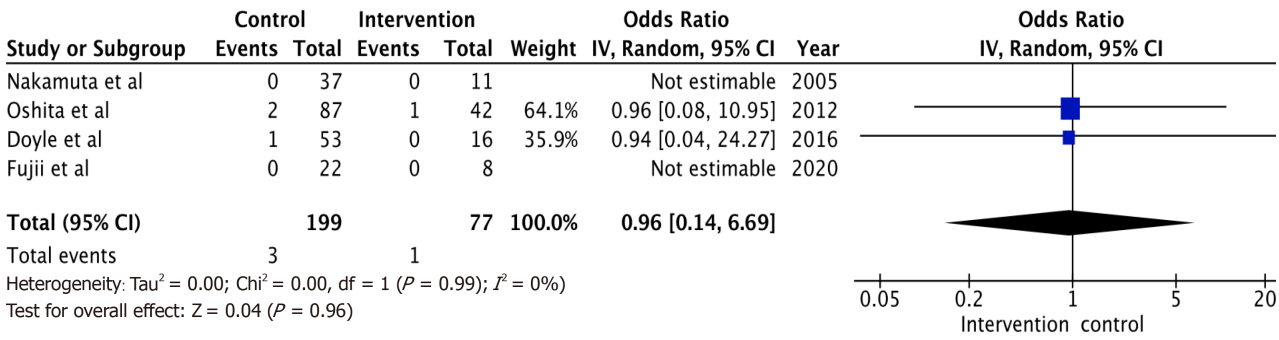

Figure 4 Odds ratio comparing rate of donor post-operative biliary complications in intervention group compared to control donors - odds ratio 0.96 (0.14, 6.69), I^2 = 0.

mean BMI (25 ± 2.0 to 23.2 ± 1.9, P = 0.0009) and L/S ratio [0.95 (0.62-1.06) to 1.2 (1.12-1.46), P = 0.003] were seen. All 8 in the study group showed < 10% steatosis on intra-operative biopsy and underwent partial donor hepatectomy. No major complications (Clavien grade IIIa or greater) were seen. No significant difference in graft function were observed between the 2 groups with 100% Graft and patient survival at 3 mo. They concluded that preoperative treatment for fatty liver was effective and treated potential donors can undergo LDLT without jeopardizing donor safety.

Study number 2 from University of Toronto by Doyle *et al*[16], retrospectively analyzed 16 potential donors from September 2011 to December 2014. Subjects were followed until September 2015. Potential donors with nonalcoholic steatohepatitis (NASH) were excluded and those with steatosis of > 10% who underwent treatment with Optifast, were included. Baseline pre-treatment liver biopsies were performed in the first 8 but after observing promising preliminary results, the authors proceeded directly to dietary intervention in the remaining 8, based on imaging. All underwent liver biopsies at treatment completion. A targeted BMI reduction of 10%, guided treatment duration. The control group (n = 53) included all non-Optifast donors had intraoperative liver biopsy showing < 10% macrovesicular steatosis, as part of the Adult-to-Adult Living Donor Liver Transplantation Cohort Study (A2ALL) consortium. The pre-intervention mean BMI of Optifast donors was 32.7 kg/m^2 [95% confidence interval (CI): 30.5-34.9 kg/m^2] and was higher than non-Optifast donors at 26.4 kg/m^2 (95%CI: 25.4-27.4 kg/m^2; P < 0.001).

Optifast was well tolerated and all 16 completed intervention. The mean BMI decreased to 28.3 kg/m^2 (95%CI: 26.3-30.2 kg/m^2; P < 0.001). All post-intervention biopsies demonstrated ≤ 10% macrovesicular steatosis with mean steatosis reduction from 29.3% to 4.75% (P < 0.001). Fourteen underwent partial hepatectomy with no reported donor mortality and no significant difference in surgical complications (P = 0.11), Clavien scores (P = 0.28), or mean length of stay (P = 0.82) between recipients of both groups. The authors concluded that Optifast can potentially eliminate or significantly reduce steatosis in donors being evaluated for LDLT, with donor and recipient outcomes equivalent to outcomes in non-steatotic donors.

Study number 3 by Choudhary *et al*[17], from July 2010 to January 2015, prospectively analyzed 16 potential liver donors. They had pre- and post-intervention liver biopsies and imaging and the study group was selected from a potential donor pool of 188 biopsy proven NAFLD. Exclusion criteria were metabolic syndrome, NASH, or steatosis of > 30%. Liver attenuation index (LAI, defined as liver attenuation minus splenic attenuation), was used for initial screen of steatosis. Prospective donors with LAI of 0 - 5 or liver attenuation < 53 HU (indicating steatosis), and presence of dyslipidemia or BMI > 28 kg/m^2, underwent biopsy. Donors with a remnant volume of < 30% also had biopsy and had repeat biopsy prior to hepatectomy.

The mean weight loss was 7 ± 4.3 kg with a significant post-intervention BMI reduction (P = 0.006) and improvement in LAI (P = 0.008). A median decrease in steatosis from 15% to 5% was seen in fifteen, including normalization in 7. Two donors had steatohepatitis, steatosis > 20% with borderline liver remnant and did not undergo liver donation. Fourteen underwent liver donation with all donors and their recipients having an uneventful post-operative course. The authors concluded that, in motivated younger liver donors with no comorbidities, steatosis is reversible in a short duration by aggressive lifestyle modifications.

Study number 4 by Oshita *et al*[18] compared outcomes of diet treated (n = 42) to non-diet treated donors (n = 87), from April 2003 to March 2010. Steatosis was assessed by pre-intervention L/S ratio and post-intervention biopsy. Pre-intervention exclusion criteria were diabetes mellitus and L/S ratio of ≥ 1.2. Post-intervention exclusion criteria were macrovesicular steatosis of > 20%.

BMI was reduced from 23.3 ± 0.6 to 21.9 ± 0.4 kg/m^2 (P < 0.0001). ALT, γ-GTP, and total cholesterol showed significant improvements (P = 0.0128, 0.0016, and 0.0004, respectively). Forty in the intervention group had stage 0/1 fibrosis with ≤ 20% steatosis and one had stage 2 fibrosis. One had inflammation and did not undergo liver donation. Forty-one treated donors underwent LDLT with no significant differences in perioperative lab data and complications (Clavien grading), including recipient biliary complications compared to controls. Overall, 1-, 3-, and 5-year recipient survival were not significantly different between the study and control groups (P = 0.455). The authors concluded that with appropriate selection criteria, use of diet-treated donors is feasible and safe with respect to donor and recipient outcomes.

Study number 5 by Nakamuta *et al*[19], tested short-term weight loss interventions on 11 potential donors with ≤ 30% combined microvesicular and macrovesicular steatosis. All had pre- and post-intervention liver biopsies. The control group included 37 donors without hepatic steatosis. The study was conducted from May 2003 to July 2004.

A significant reduction in steatosis (30% ± 4% to 12% ± 2%, P = 0.0028) and BMI (26.4 ± 0.7 kg/m^2 to 24.1 ± 0.8 kg/m^2, P = 0.0033) was seen. All had post-intervention normalization of liver enzymes, total cholesterol, and triglycerides. Seven underwent LDLT and one at study conclusion was waiting for donation. No adverse postoperative events were observed in study group donors or recipients with no difference in graft function. The authors concluded that short-term interventions are effective in reducing steatosis and can contribute to a safer LDLT.

In study number 6 by Hwang *et al*[20], from January 2001 to December 2002, 9 potential liver donors were examined. Exclusion criteria were a combined macro- and microvesicular steatosis of > 30% and or alcohol intake > 40 gm/wk. All underwent pre- and post-intervention liver biopsies and CT assessment of steatosis. In addition, all in the study group had intra-operative liver biopsies. All except one potential donor had pre-intervention elevation in LFTs. All nine in the intervention group showed a significant reduction in BMI (25.3 ± 3.8 to 23.7 ± 3.4, P = 0.0001) and in steatosis (48.9% ± 25.6% to 20.0% ± 16.2%, P = 0.006). All nine underwent donor hepatectomy with an uneventful post-operative course recovered and all recipients survived at 15 mo post-transplantation (study completion). They concluded that short-term weight loss in donors reduces steatosis and can contribute to expanding the donor pool.

Prior research has shown that hepatic steatosis adversely affects donor and recipient outcomes in LT and increases the likelihood of graft damage[21,22]. Marsman *et al*[23] reported that transplantation of livers with up to 30% steatosis resulted in a decreased 4-mo graft survival and 2-year patient survival rate. These findings, along with several other studies showing adverse outcomes with steatotic grafts[7-9], has led to the current practice of excluding potential overweight or obese donors with more than 10% macro vesicular steatosis[24]. In an analysis of the A2ALL database, only 15% of all living donors had a BMI of 30 or more[25]. As per UNOS database, in 2019, of the 874 donor livers discarded, 650 (74%) were in donor BMIs of 25 or more[1].

A few studies have used overweight or obese donors. Knaak *et al*[26], showed that donors with BMI of > 30 but < 35, had equivalent outcomes to non-obese donors. However, all potential donors with > 10% hepatic steatosis were excluded from their study. Also, certain donor characteristics separated them from other LDLT programs, including the use of Graft with higher GRWR in the obese donor group (mean of 1.42 ± 0.44%), a number much higher than the standard practice of using a GRWR cutoff of ≥ 0.8% and the greater use of male donors who tend to have larger liver volumes.

To avoid graft size mismatch, preoperative donor liver volumetry is done using the standardized GRWR. The donor graft weight is derived from CT volumetric assessment of the proposed graft to be harvested and the recipient's required standard liver volume (SLV) is calculated from the recipient's body weight[27]. GRWR is then expressed as the ratio of graft volume (expressed in kg) to the recipient's SLV calculated from the recipient's weight. Calculating GRWR is important in preventing overestimation of the donor's standard liver volume (that can result in excessive hepatic resection and consequent liver failure) and in preventing underestimation of the recipient's standard liver volume that could lead to small-for-size syndrome. The generally accepted GRWR threshold is 0.8%. Some authors have proposed the lowering of threshold to between 0.6 to 0.8% under specific circumstances including

donor age < 45 years, MELD score < 20, no graft steatosis and specific anatomic graft requirements. In such highly select cases, using a lower GRWR threshold in combination with grafts with no steatosis could lead to safe expansion of the donor pool with additional decrease in donor morbidity by preferentially selecting left lobe over right lobe grafts.

Calorie restriction, weight loss, and exercise are still recommended as the initial treatment for fatty liver. In a recent randomized control trial using paired biopsies of 261 patients with NASH who underwent dietary and lifestyle changes for a duration of 52 wk, 72 (25%) achieved resolution of steatohepatitis, 138 (47%) had reductions in NAFLD activity score (NAS) and 56 (19%) had regression of fibrosis[28]. The degree of weight loss correlated independently with all NASH histology. In those who achieved 10% or more weight loss, 90% had resolution of NASH and 45% had regression of fibrosis[28].

As our analysis has shown, there is promising data regarding short term interventions in decreasing or eliminating macro-vesicular steatosis, turning marginal steatotic donors to low-risk donors, and in positively impacting donor and recipient outcomes. All six studies included in this review (Table 1), however, are limited by their small sample size. All studies except one[15], used liver biopsy to quantify steatosis pre-donor hepatectomy and used non-invasive modalities for fat estimation, as an adjunct. Only one of the included studies is in Western population[16] making it difficult to extrapolate findings to our patient population. In addition, study inclusion and exclusion criteria were varied and so were the interventions. Despite these variabilities, most in the study groups tolerated the interventions well, and showed no increase in donor, graft or recipient morbidity or mortality as compared to non-diet treated donors.

Overall, the combination of short-term dietary intervention with low calorie diet (most studies had < 1200 kcal/d) for a duration ranging from 4 to 12 wk with exercise, and/or pharmacotherapy, was safe, well tolerated, and showed good treatment adherence. These interventions were effective in significantly reducing donor BMI with a pooled weighted difference of -1.6 (-4.4 to -1.1, CI of 0.95) and significantly reduced liver steatosis, leading to successful liver donation (88.5%) in the diet treated group. With respect to complications, diet-treated donors did extremely well, with only one donor in the Oshita group having Clavien grade III biliary stenosis. Outcomes of recipients who received grafts from diet-treated donor were not significantly different from recipients of grafts from non-diet treated donor. Grafts from diet treated donors functioned similarly to grafts from donors without obesity. The use of diet-treated donors is feasible with respect to safety of the donor and the outcome of the recipient in LDLT when strict selection criteria are used.

CONCLUSION

Short term dietary interventions, in conjunction with exercise and pharmacotherapy, is feasible and safe with good donor adherence. Our study has shown that such interventions significantly reduce and, in some help resolve hepatic steatosis in potential donors undergoing evaluation for LDLT. We conclude that, carefully selected steatotic diet treated living liver donors have equivalent donor, graft and recipient outcomes compared to those receiving grafts from non steatotic donors. It therefore has the potential to safely expand the donor pool and consequently, decrease the number of wait list removals.

ARTICLE HIGHLIGHTS

Research background

The rates of liver transplantation having increasing but the donor pool has largely remained stagnant leading to high removals from liver transplant waitlists. Living donor liver transplantation (LDLT) using fatty liver could potentially be used to expand the donor pool. However, due to negative effects of steatosis on Graft and recipient outcomes, current practice is to exclude overweight or obese donors with steatosis livers. Data on feasibility, efficacy, and safety of using weight loss interventions marginal donors to low-risk donors is lacking. The aim of the study was to evaluate the feasibility safety and efficacy of short-term weight loss interventions in converting marginal living liver donors to low-risk donors.

Research motivation

Data on safety, efficacy and donor, graft and recipient outcomes when using short term weight loss interventions to convert marginal steatotic liver grafts in LDLT, to low-risk grafts, is lacking. With continuing shortage of organs for transplantation, we looked into the safety and efficacy of using treated steatotic donors, for LDLT.

Research objectives

We did a meta-analysis on the feasibility, safety, and efficacy of weight loss interventions in converting marginal living liver donors to low-risk donors and analyzed the perioperative donor, graft and recipient outcomes.

Research methods

We performed a systematic review and meta-analysis on studies examining the role of short-term weight loss interventions in potential living liver donors with hepatic steatosis with the aim of increasing liver donation rates and improving donor, graft, and recipient outcomes.

Research results

A total of 6 studies with 102 potential donors were included. Most subjects were males (n = 71). All studies showed a significant reduction in body mass index post-intervention with a mean difference of -2.08 (-3.06, 1.10, I^2 = 78%). A significant reduction or resolution of hepatic steatosis was seen in 93 of the 102 (91.2%). Comparison of pre- and post-intervention liver biopsies showed a significant reduction in steatosis with a mean difference of -21.22 (-27.02, -15.43, I^2 = 56%). The liver donation rates post-intervention was 88.5 (74.5, 95.3, I^2 = 42%). All donors who did not undergo LDLT had either recipient reasons or had fibrosis/steatohepatitis on post intervention biopsies. Post-operative biliary complications in the intervention group were not significantly different compared to controls with an odds ratio of 0.96 [(0.14, 6.69), I^2 = 0]. The overall post-operative donor, graft, and recipient outcomes in treated donors were not significantly different compared to donors with no steatosis.

Research conclusions

Our study has shown that using liver grafts from potential living liver donors with hepatic steatosis undergoing short term weight loss interventions, have comparable donor, graft, and recipient outcomes, to donors with no hepatic steatosis.

Research perspectives

Use of appropriate short term weight loss interventions in living liver donors is a feasible, safe, and effective tool in turning marginal donors with liver steatosis to low-risk donors and therefore can help in expanding the donor pool.

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