

# World Journal of *Gastroenterology*

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## Pain management in chronic pancreatitis incorporating safe opioid practices: Challenge accepted

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### Abstract

Patients with chronic pancreatitis often experience severe, unrelenting abdominal pain, which can significantly impact their quality of life. Pain control, therefore, remains central to the overall management of chronic pancreatitis. Most of the strategies aimed at treating the pain of chronic pancreatitis are based on expert opinion and vary from one institution to another, as there are no uniform guidelines to direct a stepwise approach towards achieving this goal. In this editorial, we comment on best practice strategies targeted towards pain control in chronic pancreatitis, specifically highlighting the use of opioid medications in this patient population. We discuss various safe and efficacious prescription monitoring practices in this article.

**Key Words:** Chronic pancreatitis; Chronic pain; Pain management; Opioid use disorder; Prescription opioid misuse; Prescription opioid abuse

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**Core Tip:** Pain management in chronic pancreatitis is complex; collaboration with local pain specialists maybe necessary to provide optimal care.

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## INTRODUCTION

Chronic pancreatitis (CP) is a fibro-inflammatory disorder characterized by irreversible parenchymal and ductal injury of the pancreas caused by a variety of genetic, metabolic and environmental factors. Globally, the prevalence of CP ranges between 36 and 125 per 100000 persons[1]. Patients with CP almost universally present with abdominal pain. Although the pathophysiology of the pain is poorly understood, it seems to be related to various factors such as pancreatic inflammation, duct obstruction and nerve damage[2-6].

With abdominal pain being the predominant symptom, pain management is central to the treatment of CP. Lifestyle modifications such as alcohol and tobacco cessation along with frequent consumption of small meals remain the first-line treatment for CP [7,8]. Efficacy of pancreatic enzymes has been debated based on results from multiple randomized controlled clinical trials, and the current consensus is that uncoated preparations containing large amounts of pancreatic enzymes can be used for pain control in selected patients, in addition to treating symptoms of pancreatic insufficiency[2,9]. Similarly, some studies have shown that antioxidants with or without other analgesics could be beneficial in treating pain *via* suppression of oxidative stress which would reduce pancreatic inflammation[10,11].

## PAIN MANAGEMENT STRATEGIES

Several experts have recommended following the World Health Organization (WHO) pain relief ladder while choosing the appropriate analgesic in CP[2,3,5,12]. Acetaminophen continues to remain the analgesic of choice. This is followed by neuromodulators such as gabapentin, pregabalin and tricyclic antidepressants. Antispasmodics and muscle relaxants such as baclofen and hyoscyamine have also been used as adjunctive therapies. Oftentimes, endoscopic intervention with sphincterotomy and stent placement is employed in patients with distal pancreatic duct obstruction. Surgical techniques aimed at ductal decompression or parenchymal resection or both, albeit limited by their invasive nature, are often employed in patients who have pain that is refractory to other treatment measures. In the United States, total pancreatectomy with islet auto transplantation has shown promising results, although there have been mixed results on long term diabetes control and insulin dependence[13-16]. Denervation techniques such as celiac plexus blocks are also used when other treatments fail[17]. Use of neurostimulation techniques such as spinal cord stimulation and transcranial magnetic stimulation have shown promising results, although rarely employed in standard practice owing to paucity of literature [18,19]. When these strategies fail, providers are often left with opioids for pain management although there is limited published literature outlining their use in patients with CP. Knowing about the devastating nature of the current opioid epidemic that has been prevalent for more than 25 years, it is important to have a mindful and cautious approach while effectively using opioids to treat pain in such patients[20,21].

Opioid use disorder (OUD) has evolved into a major health emergency over the last few years, contributing to more than 600000 deaths just in the United States, mandating judicious opioid use and close prescription monitoring in an attempt to prevent opioid misuse[22]. Although potentially effective in pain management, opioid use has serious consequences including narcotic bowel syndrome and opioid-induced hyperalgesia[23]. It has been shown that about 13%-50% patients with inflammatory bowel disease and irritable bowel syndrome use chronic opioids, and similarly, up to 66% patients of CP are known to use opioids[24-27]. Over the last two decades, opioid use among several gastrointestinal conditions with chronic pain has steadily continued to increase with an 88% increase in inflammatory bowel disease and a 57.6% increase in CP[27].

With the increased use of opioids, there remains a high level of concern among gastroenterologists and other providers for the risk of opioid dependence and abuse along with increased healthcare utilization. In CP, opioid use has limited data and uniform guidelines are lacking to direct prescription practices, which can lead to further apprehension and confusion among providers when deciding upon an ideal approach. Although the WHO pain relief ladder has been recommended by some experts while choosing an appropriate analgesic in patients with CP, it should be noted that this stepwise approach was originally designed for achieving pain control primarily in cancer patients, and the feasibility of its use has not been studied in CP[2,

12]. Absence of a uniform stepwise approach towards analgesic use in CP, may further result in irregular and scattered methods of opioid prescription among various providers, further adding to opioid overuse and associated adverse outcomes.

Curbing the overuse of opioids and regulating a streamlined opioid-prescription system, is a critical task for providers caring for patients with chronic pancreatitis. There is a need for treatment approaches that are successful in minimizing pain while minimizing the risk of developing OUD. Several pain societies have developed prescription guidelines for patients with non-cancer pain and thus it is important to partner with experienced pain clinics who can provide appropriate attention to patients requiring narcotic therapy[28-31].

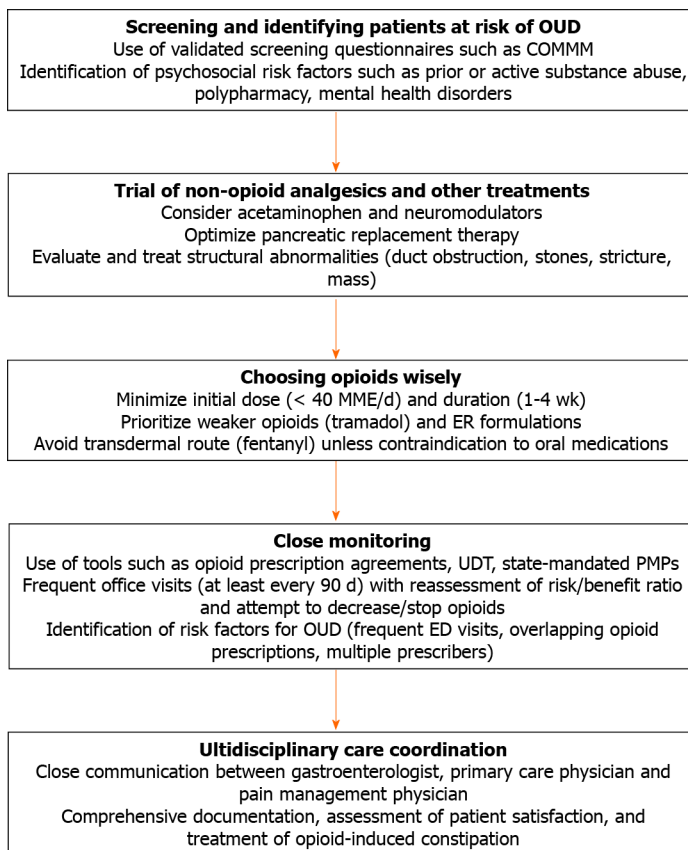
Selection of patients who would benefit from opioid therapy and predicting their risk of misuse potential should be carefully executed. Several validated screening tools for OUD can be used for this purpose including the Current Opioid Misuse Measure, which has been studied in nonalcoholic CP[32-35]. Clinicians should be vigilant for commonly encountered psychosocial risk factors that may be associated with a higher risk of abuse potential such as active or prior concurrent substance abuse, alcohol use, polypharmacy with sedating medications such as benzodiazepines, mental health disorders, *etc.*[36]. Since CP patients could often have comorbid substance abuse disorder, close attention should be paid to either completely avoiding or at least minimizing opioid prescriptions in patients with these risk factors[35,36].

While there is limited data on the opioid prescription guidelines in patients with CP, most experts recommend a one-to-four-week course of low dose opioids (less than 40 morphine milliequivalents) before committing to a long-term opioid therapy for patients with non-cancer pain. This is because higher doses of more than 60 to 90 morphine milliequivalents have been associated with increased risk of dependence and adverse outcomes[30,31,37]. Furthermore, there is a dearth of evidence to suggest better efficacy and safety profile of any one opioid over another[30]. Priority should be given to weaker opioids such as tramadol and extended-release formulations should be avoided due to their higher risk of overdose[30,37]. Transdermal opioids such as fentanyl should only considered when patients are unable to tolerate oral medications [30].

Prior to initiating long term opioid therapy, it is important to extensively educate the patient about its abuse potential and its associated consequences. Many states have mandated opioid treatment agreements that should be signed by both the prescriber and the patient and renewed on a regular basis[38-40]. Once long-term opioid therapy has been initiated, it is important to closely monitor for opioid misuse *via* various physician-based and state-mandated tools. Frequent office visits should be scheduled, no less than once in every 90 d[41]. During each visit, *via* means of a comprehensive history taking and physical exam, an assessment should carefully made to determine need for continuing opioid therapy. If continuing opioid therapy is deemed necessary, an attempt should be made to assess if the opioid dose can be progressively reduced to the minimum effective dose. Additionally, many experts recommend urine drug testing (UDT) prior to starting opioids and eventually for regular monitoring in an effort to curb opioid misuse[28,30]. The United States has mandated prescription monitoring programs (PMP) to track controlled prescriptions which is an efficient tool that can be regularly used by providers to reduce misuse of opioids and other controlled substances[28,30,31,37]. Close attention can thus be paid to patients with frequent emergency room visits who may be receiving multiple overlapping prescriptions of short course opioids. At any point, if lack of response, adverse events or opioid misuse are encountered, opioids may need to be discontinued[37]. Our approach is summarized in Figure 1.

## CONCLUSION

Abdominal pain is the central symptom for most patients with CP resulting in high healthcare utilization. While several non-opioid therapies exist, unfortunately patients remain symptomatic and thus opioids may be necessary to treat ongoing abdominal pain. With the ongoing opioid epidemic and elevated risk of OUD, several strategies should be incorporated while using opioids for the treatment of pain in patients with CP. These include robust screening measures to predetermine the risk of OUD, safe prescription practices using tools such as UDT and PMP, close clinical follow up and frequent reassessment to assess the need for continuing opioids. Finally, a multidisciplinary approach and coordination with pain management physicians is recommended to adequately cater to each patient's individual needs. Further studies and guidelines



**Figure 1 Stepwise approach to management of pain in chronic pancreatitis.** OUD: Opioid use disorder; COMMM: Current opioid misuse measure; ER: Extended release; UDT: Urine drug testing; PMP: Prescription monitoring program; ED: Emergency Department.

are needed to address opioid use in CP.

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## Pancreatitis and pancreatic cancer: A case of the chicken or the egg

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### Abstract

Acute pancreatitis (AP), chronic pancreatitis (CP) and pancreatic cancer are three distinct pancreatic diseases with different prognoses and treatment options. However, it may be difficult to differentiate between benign and malignant disease. AP may be a first symptom of pancreatic cancer, particularly in patients between the ages of 56 and 75 with presumed idiopathic AP who had a concomitant diagnosis of new-onset diabetes mellitus or patients who present with CP at diagnosis of AP. In these patients, additional imaging is warranted, preferably by endoscopic ultrasonography. CP may lead to pancreatic cancer through oncogenic mutations, mostly in patients with hereditary CP, and in patients in whom risk factors for pancreatic cancer (e.g., nicotine and alcohol abuse) are also present. Patients with *PRSS1*-mediated CP and patients with a history of autosomal dominant hereditary CP without known genetic mutations may be considered for surveillance for pancreatic cancer. Pancreatic inflammation may mimic pancreatic cancer by appearing as a focal mass-forming lesion on imaging. Differentiation between the above mentioned benign and malignant disease may be facilitated by specific features like the duct-penetrating sign and the duct-to-parenchyma ratio. Research efforts are aimed towards developing a superior discriminant between pancreatitis and pancreatic cancer in the form of imaging modalities or biomarkers. This may aid clinicians in timely diagnosing pancreatic cancer in a potentially curable stage.



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**Core Tip:** It is essential to distinguish acute pancreatitis and chronic pancreatitis (CP) from pancreatic ductal adenocarcinoma (PDAC), as these diseases have different treatment options and prognoses. Idiopathic acute pancreatitis may be a first symptom of PDAC, and endoscopic ultrasonography should therefore be considered. CP may obscure PDAC, and long-standing inflammation may cause PDAC, especially in hereditary CP and patients with a history of nicotine and alcohol abuse. Patients should be counselled in cessation of tobacco and alcohol use. Pancreatitis may mimic PDAC by presenting as a mass on imaging. Specific imaging features can aid in the differentiation between benign and malignant disease.

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## INTRODUCTION

Acute pancreatitis (AP) and chronic pancreatitis (CP) are inflammatory diseases of the pancreas. While AP is sudden and transient, CP is a longstanding inflammation of the pancreas characterized by pancreatic calcifications and exocrine pancreatic insufficiency. In CP, bouts of so-called acute-on-chronic episodes of pancreatitis may also occur.

Both AP and CP are benign diseases that are, in different ways, associated with pancreatic ductal adenocarcinoma (PDAC). Although these are three separate disease entities, inflammation and cancer can co-exist, and inflammation may cause cancer while cancer may also cause inflammation. This entails they are not always easy to distinguish from each other. As the treatment and prognosis of AP, CP and PDAC are completely different, it is important to understand the nature of the association between these diseases and be able to take the proper diagnostic steps to gather the correct diagnosis and subsequent treatment options.

In this review, we will focus on the relationship between AP, CP and PDAC and how to distinguish between these three disease entities.

## CLINICAL CASE: A 64-YEAR-OLD MAN WITH ABDOMINAL PAIN

A 64-year-old man visited the emergency department with acute abdominal pain in the epigastric region. Laboratory tests revealed an amylase level of 2100 U/L (normal < 107 U/L), a lipase level of 5548 U/L (normal 13-60 U/L) and normal liver enzymes, calcium and triglycerides. Computed tomography (CT) showed inflammation of the head of the pancreas (Figure 1). The patient was admitted with AP, received fluid resuscitation therapy and analgesics. Transabdominal ultrasound showed no cause for the AP episode. After 3 d, the patient was discharged with a diagnosis of idiopathic AP.

Approximately 1.5 years later, this patient was readmitted with a recurrent AP episode. CT of the abdomen revealed inflammation of the pancreas with a splenorenal fluid collection and slight dilatation of the pancreatic duct in the tail of the pancreas (Figure 2). Additional magnetic resonance imaging (MRI) showed a hypo-intense lesion in the pancreatic tail, causing a stenosis of the pancreatic duct (Figure 3). Endoscopic ultrasonography (EUS) with biopsy confirmed the diagnosis of PDAC (Figure 4). The patient underwent a pancreatic tail and spleen resection, and pathology revealed a radically resected T3N0 PDAC.

## AP AND PANCREATIC CANCER

The incidence of AP is rising, with a median increase of 3.4% per year[1]. Reported incidence rates ranges from 9.8 to 100 per 100000 in Europe and rise with age[1-3]. AP is often caused by alcohol or gallstone disease, although the incidence of these etiologies differ geographically[1].

A strong association between AP and PDAC has been established through multiple studies. A recent systematic review and meta-analysis by Liu *et al*[4] included 11 studies reporting on the risk of PDAC after an episode of AP and found an effect estimate of 2.07 [95% confidence interval (CI): 1.36-2.78] during a follow-up time exceeding 10 years. In a subgroup analysis of 103961 AP patients and 1442158 control subjects from five prospective studies in this systematic review, a relative risk of 7.81 (95%CI: 5.00-12.19) for PDAC was observed in patients with AP as compared to healthy control subjects.

Multiple hypotheses have been raised to explain this apparent association between AP and PDAC. Firstly, it has been suggested that risk factors for AP are similar to risk factors for PDAC. For instance, alcohol abuse is a prevalent etiology of AP and also a known risk factor for pancreatic cancer. Although the etiological value of nicotine abuse in AP is still a topic of discussion, it is an established risk factor for recurrent AP, while also being a strong risk factor for PDAC[5-7]. Secondly, in recurrent AP, the accumulating inflammatory states of the pancreas are thought to increase the chance of oncogenic mutations in pancreatic cells, leading to carcinogenesis in a similar fashion as CP, as elaborated below in the section on CP[8].

However, the most prevailing hypothesis has been that a single episode of AP cannot cause PDAC but that PDAC may cause AP. Although this is rare, it has been acknowledged that PDAC can cause an episode of AP by ductal obstruction before the PDAC has even been diagnosed[9]. Temporal data on the association of PDAC and AP endorse this hypothesis. In the previously mentioned meta-analysis by Liu *et al*[4], the strongest association between AP and PDAC was found in the first year after AP [effect estimate 23.47 (95%CI: 3.26-43.68)], while this association diminished over a course of 2 years after AP [effect estimate 9.82 (95%CI: 3.01-16.64)], 5 years [effect estimate 2.47 (95%CI: 1.93-3.02)], 10 years [effect estimate 1.69 (95%CI: 1.26; 2.11)] and over 10 years after AP [effect estimate 1.17 (95%CI: 0.78-1.57)]. A Danish matched-cohort study, which was included in this meta-analysis, also adjusted for alcohol- and smoking-related conditions and the Charlson Comorbidity Index score and excluded patients who developed CP or other exocrine pancreatic disease during follow-up[10]. This well-designed study found that a presumed idiopathic etiology appeared to be associated with the highest risk of PDAC [adjusted hazard ratio of 2.52 (95%CI: 1.83-3.47)]. This finding was supported by a recent cohort study, which found that biliary AP and a history of alcohol-related disease were predictors for no underlying PDAC [11]. In this study of 28231 patients with AP, 283 received the diagnosis PDAC during follow-up (overall risk 1.0%). They found several predictors for PDAC in patients with AP, including age between 56 and 75 at diagnosis of AP (risk approximately 1.6%), new-onset diabetes mellitus (DM) and new-onset CP [risk 2.0% (95%CI: 1.1-3.3%)] at diagnosis of AP. Interestingly, severe AP, smoking-related diseases, venous thromboembolism and previous malignancies were not associated with a higher risk for PDAC. A Swedish matched-cohort study of 49749 AP patients and 138750 matched subjects also identified recurrent pancreatitis as a risk factor for PDAC [hazard ratio 4.44 (95%CI: 1.181-10.89) after censoring for diagnosis of CP][12].

### Management

It has become more and more apparent that PDAC is a noteworthy etiology of AP, particularly in patients with presumed idiopathic etiology after standard diagnostic work-up. In these patients with idiopathic AP, primarily in patients aged 56-75, it is imperative to exclude the presence of an occult pancreatic tumor by providing additional imaging. Commonly, standard diagnostic work-up includes a personal history, laboratory tests (including liver enzymes, calcium and triglycerides) and transabdominal ultrasonography. EUS has been proven to be more sensitive for the detection of PDAC than CT (98 *vs* 74%) and transabdominal ultrasonography (94% *vs* 67%), especially for small lesions[13]. A meta-analysis comparing the diagnostic yield of EUS *vs* magnetic resonance cholangiopancreatography (MRCP) showed that EUS is superior to MRCP in detecting all etiologies in idiopathic AP[14]. Thus, mainly in patients aged 56-75 with idiopathic AP, recurring AP or new-onset DM or CP concomitant with their AP diagnosis, additional imaging is warranted, preferably using EUS.

## CP AND PANCREATIC CANCER

After a first episode of AP, 17% of patients have at least one recurrent attack, and 8% develop CP[5]. CP is a progressive inflammatory disease that often leads to pain, exocrine pancreatic insufficiency and DM. It is characterized by a pathologic response to pancreatic injury leading to irreversible parenchymal damage[15]. Estimates for global CP incidence range from 5.0 to 10.0 cases per 100000 person-years[16]. In the majority of cases, CP develops after a history of recurrent AP in patients with multiple environmental and genetic risk factors[17]. Alcohol and nicotine are the most important environmental risk factors for CP[5]. Genetic risk factors include mutations in the protease serine 1 (*PRSS1*) gene, which cause hereditary CP, and in the serine protease inhibitor Kazal-type 1 (*SPINK1*) and cystic fibrosis chymotrypsinogen C (*CFTR*) genes[18].

Lowenfels *et al*[19] were the first to describe the link between CP and progression to PDAC. In this study including 2015 CP patients, a cumulative risk for PDAC was observed in 1.8% (95%CI: 1.0-2.6) and 4.0% (95%CI: 2.0-5.9) 10 and 20 years after diagnosis, respectively[19]. To date, the increased risk of PDAC in CP patients has been confirmed by several meta-analyses[20-22]. However, the exact risk remains debated, mainly because different factors contribute to PDAC progression.

The risk of PDAC is much higher for patients with hereditary CP from *PRSS1* mutations [relative risk 69.0 (95%CI: 56.4-84.4)] and tropical CP [relative risk 100 (95%CI: 37.0-218)], when compared to patients with sporadic CP [relative risk 5.1 (95%CI: 3.5-7.3)][21,23-25]. Additionally, alcohol consumption and smoking, both well-known risk factors for PDAC, form important confounding factors as patients with CP often have a history of alcohol- and nicotine abuse[26]. Other risk factors are older age at disease onset, obesity, concurrent DM and pancreatic duct dilatation[27,28].

Even though many contributing factors play a role in the association between CP and PDAC, evidence suggests that a causal relationship is plausible. The pathway from CP to PDAC is not yet entirely understood. The prevailing hypothesis is that the long-standing inflammatory state of the pancreas associated with CP leads to oncogenic mutations. Several studies demonstrated that the pancreas of a notable proportion of CP patients harbor downregulating mutations of the tumor suppressing *p16*, *p53* and *SMAD4* genes and upregulating mutations of the oncogenic K-ras, tumor necrosis factor- $\alpha$  and nuclear factor  $\kappa$  B genes, which are also present in the majority of PDAC[8,29-31]. These mutations could subsequently facilitate carcinogenesis in patients who may already have a genetic disposition or environmental risk factors (e.g., nicotine abuse) for developing PDAC.

Additional to the risk of progression to PDAC in CP, there is a risk of PDAC being misdiagnosed as CP because discrimination between these diseases can be difficult. A retrospective study found that 5% of PDAC patients were misdiagnosed with CP, while actually having PDAC[32]. This finding has been supported by the temporal data of a recent meta-analysis of 13 studies[20]. This meta-analysis found a decrease in PDAC risk between a 2-year lag-period [effect estimate 16.16 (95%CI: 12.59-20.73)] and a 5- and 9-year lag-period [effect estimate 7.90 (95%CI: 4.26-14.66) and 3.53 (95%CI: 1.69-7.38), respectively][20]. The peak of diagnosis of PDAC in the first years after diagnosis of CP suggests that these were either patients with PDAC who were initially wrongfully diagnosed with CP or patients with CP and concomitant PDAC in whom the PDAC was not recognized at diagnosis of CP.

### Management

Special attention should be paid to the presence of PDAC in newly diagnosed CP patients, given the high risk of PDAC in the first years after CP diagnosis. Although the discussion is ongoing, international guidelines currently only consider surveillance of PDAC justified in patients with *PRSS1*-mediated CP and patients with a history of autosomal dominant hereditary CP without known genetic mutations, from the age of 40 until the patient is no longer suitable for (surgical) intervention. As small tumor lesions may be obscured by inflammation, fibrosis and calcification on EUS, it is advised to screen using CT imaging or MRI, although high quality evidence on the accuracy of different imaging modalities in detection of DPAC in CP patients is sparse [33]. Other contributing factors for PDAC progression that justify screening in patients with CP should be further clarified.

Additionally, time and effort should be invested in reducing environmental risk factors for PDAC, such as nicotine and alcohol abuse. These risk factors are prevalent among patients with CP and counselling and support should be provided to patients willing to quit smoking or drinking.



## DIAGNOSTICS: PANCREATITIS OR PANCREATIC CANCER?

Inflammatory diseases of the pancreas can sometimes mimic PDAC by the appearance of a focal, mass-forming pancreatitis. Although diffuse forms of pancreatitis are more common, some patients present with a focal pancreatitis, in particular autoimmune pancreatitis, groove pancreatitis and CP. Clinical symptoms of mass-forming pancreatitis often overlap with symptoms related to PDAC, including abdominal pain, weight loss and jaundice. In patients undergoing surgery for suspected (periampullary) cancer, up to 23% turn out to be chronic inflammatory lesions[34].

### Imaging

Similar to PDAC, focal CP can lead to focal parenchymal atrophy or enlargement and appears as a hypovascular mass on imaging, accompanied by upstream ductal dilation and atrophy[35]. Imaging features that point towards an inflammatory cause include an unobstructed or smooth tapering pancreatic duct through the hypovascular mass (duct-penetrating sign), while PDAC usually demonstrates an abrupt or irregular obstruction (Figure 2)[36-38]. The duct-penetrating sign is highly specific for an inflammatory mass (96%), with a sensitivity of 85%, and can be best visualized during MRCP[39]. In addition, collateral duct dilation or side-branch dilation in the upstream pancreas favors CP, due to traction effect of parenchymal fibrosis on the upstream healthy parenchyma[39,40]. In contrast, side-branches adjacent to malignancy are usually obliterated owing to mass-effect[39,41]. The presence of (pseudo)cysts and parenchymal or intraductal calcifications may suggest an inflammatory cause, although these findings can also sometimes be found in intraductal papillary mucinous neoplasms, especially with main duct involvement[39,42]. Peripheral displacement of calcifications can be seen in patients with a malignant mass in the background of CP[43]. Another imaging feature that can be used to differentiate between malignancy and inflammation is the extent of ductal dilation and parenchymal atrophy, expressed by the pancreatic duct-to-parenchyma ratio. A duct-to-parenchyma ratio  $\geq 0.34$  on EUS, indicating pronounced ductal dilation and parenchymal atrophy, is highly suggestive for PDAC instead of CP, with a diagnostic accuracy of 97%[44]. Signs that are more often observed in advanced PDAC compared to pancreatitis are vascular encasement by attenuating soft-tissue, caliber narrowing and occlusion[39]. Interestingly, a superior mesenteric artery-to-superior mesenteric vein ratio  $\geq 1.0$  favors the diagnosis of an malignancy[45]. Although not well-understood, enlargement of the superior mesenteric artery is probably caused by an increased resistance to blood flow in the pancreas of patients with PDAC, while in inflammatory conditions an increase in the diameter of the superior mesenteric vein can be observed secondary to the release of pancreatitis-induced vasoactive agents[39]. Imaging findings are summarized in Table 1.

Even though secondary imaging features can provide valuable information for the differentiation between pancreatitis and PDAC, none of them are 100% specific or sensitive. The two conditions may not only have overlapping clinical and imaging features but can also coexist, and the diagnostic performance of imaging modalities remains operator-dependent. Recent advances in machine learning-based diagnostic models may overcome part of these limitations. Preliminary studies using machine-learning methods reported promising results in differentiating inflammatory from malignant lesions on EUS but need to be further evolved before implementation in clinical practice[46,47].

### Biomarkers

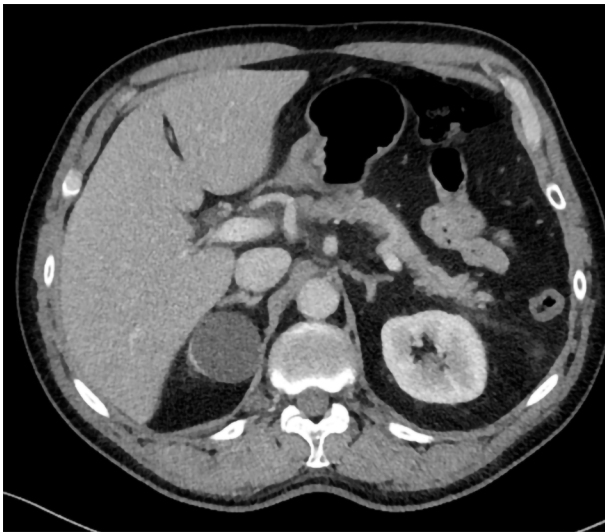
Serum carbohydrate antigen 19-9 (CA 19-9) is a widely used diagnostic tool for PDAC diagnosis. However, patients with CP often have elevation of serum CA 19-9 that is not indicative of malignancy but a result of pancreatic inflammation[48]. Therefore, the international consensus guideline do not recommend CA 19-9 as a diagnostic tool for PDAC in the CP population[33]. Over the last decades, several studies have investigated the use of other early predictive biomarkers (*i.e.* K-ras, interleukins, micro-RNAs and proteomics) in CP patients, however, no accurate biomarker is yet available for clinical use[49].

Many questions regarding the exact relative risk estimates of PDAC in CP patients remain, but most importantly, we need an adequate strategy for differentiation between CP and PDAC. Future research should devise the most optimal imaging methods and early detection biomarker to prevent misdiagnosis of PDAC as CP and, hence, delay of cancer diagnosis. As this delay compromises diagnosis of early-stage tumors and consequently resectable treatment options, optimizing diagnostic methods

**Table 1 Imaging findings favoring an inflammatory or malignant cause[39]**

Inflammatory cause	Malignant cause
Penetrating duct sign	Abrupt cutoff of the pancreatic duct
Collateral or side branch dilation	Upstream obliteration of side branches
Duct-to-parenchyma ratio $< 0.34$	Duct-to-parenchyma ratio $\geq 0.34$
AIP: occasionally perivascular involvement	Vessel encasement and caliber changes
SMA-to-SMV ratio $< 1.0$	SMA-to-SMV ratio $\geq 1.0$

AIP: Autoimmune pancreatitis; SMA: Superior mesenteric artery; SMV: Superior mesenteric vein.



**Figure 1** Abdominal computed tomography imaging of acute pancreatitis. Inflammation is present around the head of the pancreas.

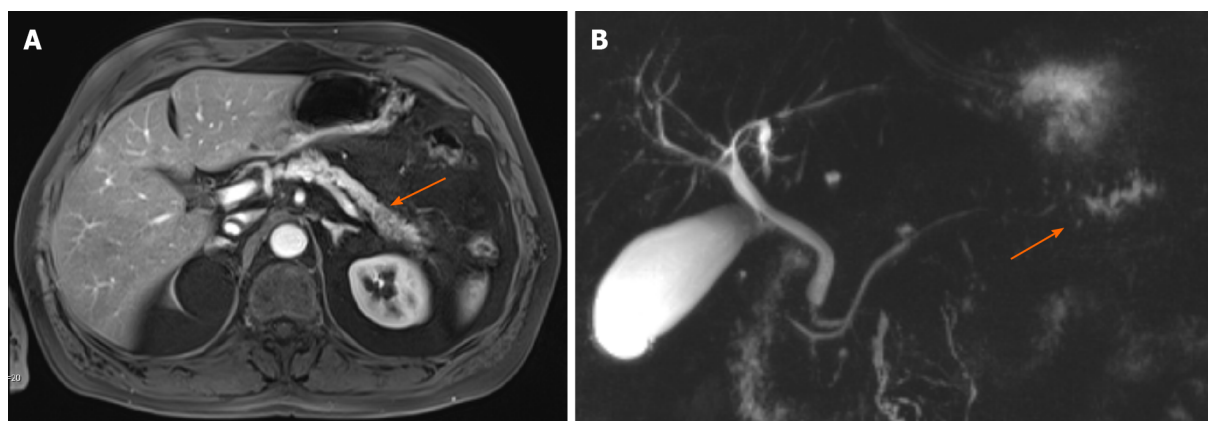


**Figure 2** Abdominal computed tomography imaging of acute pancreatitis. In the pancreatic tail, a dilatation of the pancreatic duct can be observed.

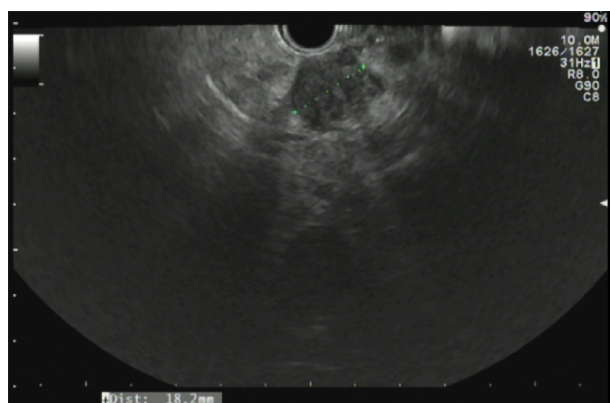
is clinically relevant.

## CONCLUSION

AP can be a first symptom of underlying PDAC, especially in patients with presumed idiopathic AP, between the ages of 56 and 75, and those who had a diagnosis of new-onset DM or CP. Additional imaging to exclude PDAC in these patients should at least



**Figure 3 Abdominal magnetic resonance imaging of the pancreas and magnetic resonance cholangiopancreatography.** A: Abdominal magnetic resonance imaging of the pancreas; B: Magnetic resonance cholangiopancreatography. A hypo-intense lesion (A, arrow) is causing a pancreatic duct stenosis with upstream dilatation of the pancreatic duct (B, arrow).



**Figure 4 Endoscopic ultrasonography of the pancreas.** A hypo-echoic lesion measuring 18.2 mm is present. Biopsy of this lesion revealed a pancreatic ductal adenocarcinoma.

be considered. EUS seems to be the preferred imaging modality.

CP, particularly hereditary CP, may lead to PDAC through oncogenic mutations caused by long-standing pancreatic inflammation, and CP patients may be exposed to overlapping risk factors for CP and PDAC. In patients with *PRSS1*-mediated CP or a history of autosomal dominant hereditary CP without known mutations, surveillance for PDAC can be considered, although the efficacy and modalities of surveillance are still up for debate.

Chronic pancreatic inflammation may present as a focal mass on imaging. Specific findings, such as the duct-penetrating sign (MRCP) and the duct-to-parenchyma ratio (EUS), may aid in the differentiation between pancreatitis and PDAC.

Currently, considerable effort is focused on finding a biomarker or machine-learning methods as a superior discriminant between CP and PDAC. Unfortunately, no clinically useful technique has yet emerged. Improving the possibility to differentiate between CP and PDAC, as well as identifying patients at risk of underlying or future PDAC, may give clinicians the opportunity to enhance the diagnostic process.

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## Pancreatic adenocarcinoma: A review of recent paradigms and advances in epidemiology, clinical diagnosis and management

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### Abstract

Pancreatic cancer is one of the dreaded malignancies for both the patient and the clinician. The five-year survival rate of pancreatic adenocarcinoma (PDA) is as low as 2% despite multimodality treatment even in the best hands. As per the Global Cancer Observatory of the International Agency for Research in Cancer estimates of pancreatic cancer, by 2040, a 61.7% increase is expected in the total number of cases globally. With the widespread availability of next-generation sequencing, the entire genome of the tumors is being sequenced regularly, providing insight into their pathogenesis. As invasive PDA arises from pancreatic intraepithelial neoplasia and mucinous neoplasm and intraductal papillary neoplasm, screening for them can be beneficial as the disease is curable with resection at an early stage. Routine preoperative biliary drainage has no role in patients suffering from PDA with obstructive jaundice. If performed, metallic stents are preferred over plastic ones. Minimally invasive procedures are preferred to open procedures as they have less morbidity. The duct-to-mucosa technique for pancreatojejunostomy is presently widely practiced. The role of intraperitoneal drains after surgery for PDA is controversial. Neoadjuvant chemoradiotherapy has been proven to have a significant role both in locally advanced as well as in resectable PDA. Many new regimens and drugs have been added in the arsenal of chemoradiotherapy for metastatic disease. The roles of immunotherapy and gene therapy in PDA are being investigated. This review article is intended to improve the understanding of the readers with respect to the latest updates of PDA, which may help to trigger new research ideas and make better management decisions.

**Key Words:** Pancreatic adenocarcinoma; Pancreatic cancer; Chemotherapy; Chemoradiotherapy; Pancreaticoduodenectomy; Distal pancreatectomy

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**Core Tip:** Pancreatic cancer is one of the dreaded malignancies for both patients and clinicians. This narrative review highlights the newer trends and achievements in the epidemiology, etiopathogenesis, screening, diagnosis and management of pancreatic adenocarcinoma (PDA). It is intended to improve the readers' understanding of the latest updates of PDA, which may help to trigger new research ideas and make better management decisions. Newer screening and diagnostic techniques will help in diagnosing the patients in early stages and prognosticate them better. The newer discoveries in drugs and management protocols will help increase the survival and quality of life of the patients.

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## INTRODUCTION

Pancreatic cancer is one of the dreaded malignancies for both patients and clinicians. For patients, it is associated with a poor survival rate and decreased quality of life due to local invasion and complications, and for the clinician, it is challenging to diagnose at an early stage and treat.

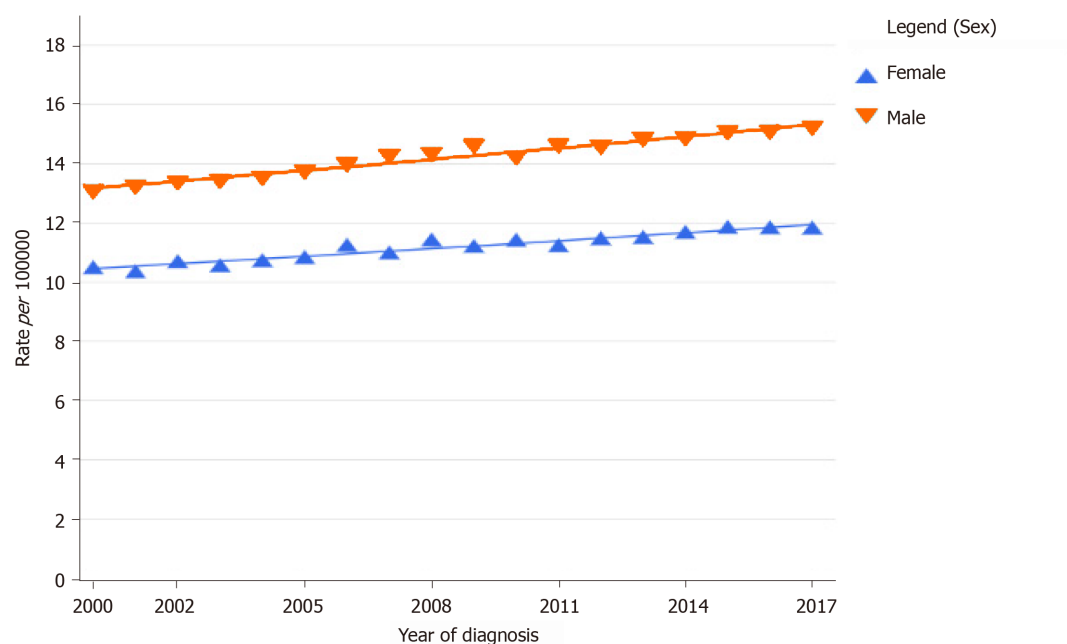
Pancreatic cancer is the third leading cause of cancer deaths in the United States of America and the seventh leading cause worldwide as per the 2018 GLOBACON data[1, 2]. It may arise from either the exocrine or the endocrine pancreas with the former being far more common than the latter. Pancreatic adenocarcinoma (PDA) and its subtypes constitute more than 90% of pancreatic tumors[3]. Most patients with PDA present at an advanced stage, which makes curative treatment virtually impossible[4]. The five-year survival rate of PDA is as low as 2% despite multimodality treatment even in the best hands[5]. The high mortality and morbidity associated with PDA have stimulated researchers all over the world to intensify the search for better diagnostic and treatment protocols.

Clinically, patients with PDA present to the healthcare facility with symptoms only in the advanced stage[6]. Early lesions have a good prognosis but are clinically silent. Diagnosis of an early-stage PDA is rare as there are no effective and reliable screening tools or investigations at present[7]. Surgery is the mainstay of treatment if therapy is planned with curative intention in PDA patients[8]. Patients with PDA from the surgical point of view are classified into resectable, borderline resectable, unresectable and metastatic disease categories at diagnosis. Chemotherapy and radiotherapy remain the backbone of the treatment for PDA with almost all patients requiring some form of chemoradiotherapy for curative or palliative purposes[9-11].

In view of the above facts, in the present narrative review, the authors have reviewed the latest trends in the epidemiology, diagnosis and management of PDA based on the published English literature so far. We have searched the literature using the keyword "PDA". This review article is intended to improve the understanding of the readers regarding the latest updates of PDA, which may help to trigger new research ideas and promote better management decisions.

## PARADIGMS IN EPIDEMIOLOGY

In the last decade, there has been a steady increase in the incidence and mortality of PDA across the globe for both males and females (Figure 1)[12,13]. Its incidence was higher among males when compared to females and continues to be so[1,12-14]. PDA constitutes about 2.5% of the total cancers diagnosed worldwide. The 2020 estimated crude incidence rate and age-standardized rate (ASR) of pancreatic cancer are 6.4% and 4.9%, respectively[1]. The ASR of pancreatic cancer is the highest in North America (8%) and Europe (7.8%) and lowest in Africa (2.3%)[1]. The incidence rate is also very high in countries with a high human development index (HDI) when



**Figure 1** Pancreas cancer-recent trends in surveillance, epidemiology, and end results age-adjusted incidence rates, 2000-2017[12]. Used with permission from National Cancer Institute.

compared to countries with low HDI (Figure 2)[1]. This difference can be attributed to the variation in the tobacco smoking, alcohol consumption and obesity rates among different countries[15-18].

The Surveillance, Epidemiology, and End Results Program of the National Cancer Institute reveals an age-specific trend in the increase of the incidence rate of pancreatic cancer in the age groups of 20-29 years and > 80 years in the United States[19]. The increase also varied with the stage of the disease at diagnosis. The last decade has shown a higher increase in the age-adjusted incidence rates of early and localized pancreatic cancer when compared to advanced disease. The age-adjusted incidence rate of metastatic disease has declined[12] (Figure 3). When the American Joint Committee on Cancer Tumor-Node-Metastasis staging was compared with the age-adjusted incidence rate, there was an increase in the stage I and II patients and a simultaneous decline in the stage III and IV patients at diagnosis, indicating that the evolving techniques of screening and diagnosis of pancreatic cancer are showing some result[14].

As per the Global Cancer Observatory of the International Agency for Research in Cancer estimates of pancreatic cancer, by 2040, a 61.7% increase is expected in the total number of cases globally. The most notable trend expected is the rapid increase in the number of cases from Africa (an increase of 100.1%) followed by Asia (81.5%). The expected increase from Europe and North America is less (Table 1)[1]. The mortality rates also follow the incidence rates with the expected increase in the mortality due to pancreatic cancer highest in Africa (Figure 4). There is an expected regional variation in the incidence among male and female sex in various continents[1,13]. The temporal trends of incidence and mortality of pancreatic cancer in various continents could be attributed to the temporal trends in tobacco smoking[15,20,21]. Anti-tobacco measures, which are being strongly advocated in developed countries, could be one of the main reasons for declining incidence rates[22]. On the other hand, the rise in the incidence and mortality rates in the developing and under-developed countries is a cause of concern and may be attributed to the lifestyle changes adopted as well as the impact of socioeconomic conditions.

Based on the present and expected trends of pancreatic cancer, there must be a widespread endorsement of healthy lifestyle practices and strong anti-tobacco laws. If not, the developing countries will have to bear the major brunt of the disease in the near future as the diagnostic and treatment services are still under-developed in these countries.

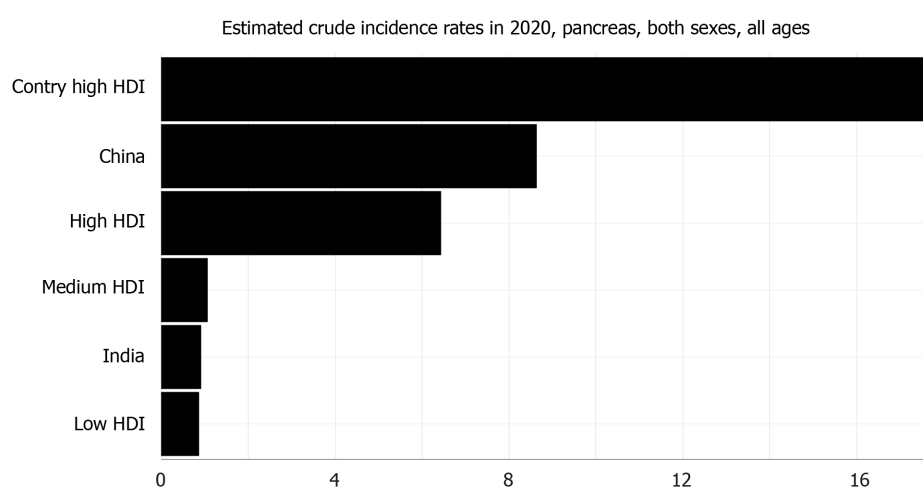
## NEWER CONCEPTS IN ETIOPATHOGENESIS

PDA is recently being called a genetic disease. With the widespread availability of next-generation sequencing, the entire genome of the tumors is being sequenced



**Table 1** The expected increase in the number of new cases among various continents by 2040[1]

Population	Number of new cases		Change in number of cases	Change in number of cases due to population
	2020	2040		
Africa	17070	34165	+ 100.1%	+ 100.1%
Asia	233701	424138	+ 81.5%	+ 81.5%
Europe	140116	178438	+ 27.4%	+ 27.4%
Latin America and Caribbean	37352	67836	+ 81.6%	+ 81.6%
Northern America	62643	89124	+ 42.3%	+ 42.3%
Oceania	4891	7933	+ 62.2%	+ 62.2%
Totals	495773	801634	+ 61.7%	+ 61.7%

**Figure 2** Estimated crude incidence rates in 2020, pancreas, both sexes, all ages as per human development index[1]. Used with permission from International Agency for Research on Cancer. HDI: Human development index.

regularly, providing insight into its pathogenesis. PDA has about 60 genetic alterations *per* tumor. The most important finding differentiating PDA from other cancers is the heterogeneity of the genome of each patient, meaning that each patient has a tumor with a specific genomic signature[23,24]. Four predominant genes have been identified in PDA. They are *K-ras*, *CDKN2A*, *TP53* and *SMAD4*. There are many other genes identified, but they are mutated at a lower frequency (less than 10%)[23].

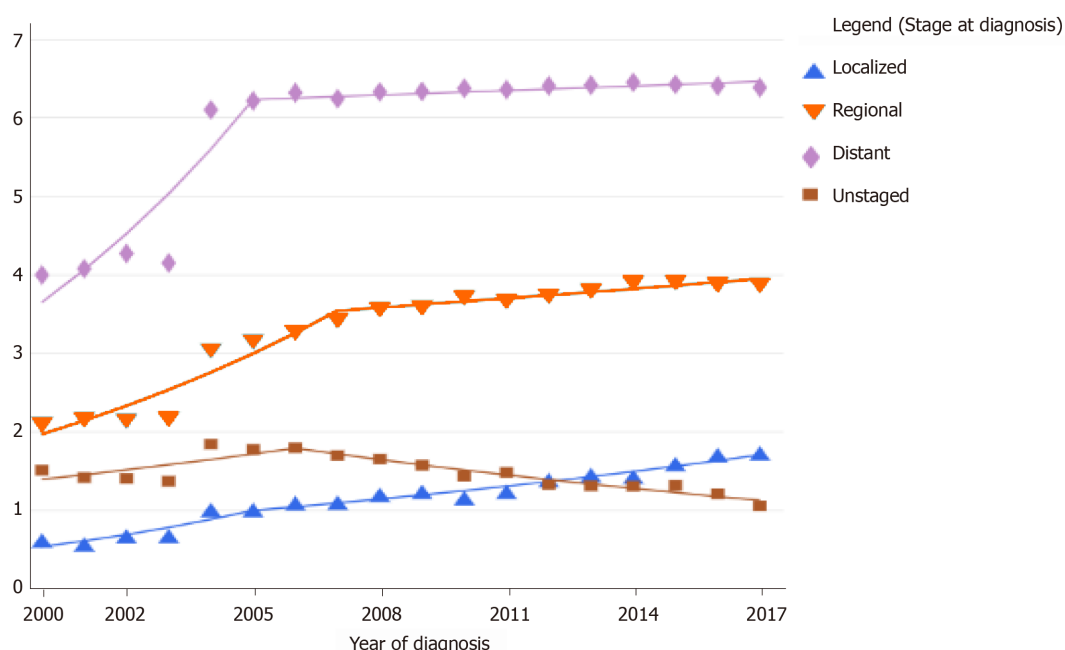
The *K-ras* oncogene is the most common gene mutation in PDA with an incidence of more than 90%[25]. Due to its high frequency of mutation, it is believed that the tumor pathogenesis revolves predominantly around the molecular pathways regulated by this gene and forms the basis for research on *K-ras* inhibitors. However, *K-ras* inhibitors were associated with high in-vivo toxicity[26]. *CDKN2A* gene is involved in the regulation of *RB1* and plays an important role in the G1/S checkpoint inhibition of the cell cycle[27]. *TP53* is the predominant DNA repair pathway gene and induces cell cycle arrest at G1 or G2 checkpoint[28]. *SMAD4* gene is a part of the transforming growth factor  $\beta$  pathway and regulates the G1/S checkpoint of cell cycle[28]. The group of genes involved in the genome maintenance DNA repair pathway (*BRCA2*, *PALB2*, *FANCC*, *FANCG*) constitute less than 10% of the mutated genes in PDA but are important as tumors deficient in these genes can be targeted with DNA damaging agents and poly ADP-ribose pathway inhibitor therapy[23,24,29]. Other gene mutations are involved in the regulation of pathways such as *Ras*, cell cycle regulators, WNT pathway and NOTCH pathway[23,24].

Invasive PDA arises from pancreatic intraepithelial neoplasia (PanIN). The lesions of PanIN progress from PanIN1 to PanIN3 by acquiring progressive mutations as shown in Table 2[30]. PanINs are also associated with lobulocentric acinar atrophy and local pancreatic inflammation secondary to obstruction of small duct secretions[31]. The time required from the onset of PanIN till progression to invasive carcinoma is about 10 years suggesting a significant lead time if PanIN are screened and detected

**Table 2 The histological features and genetic alterations in pancreatic intraepithelial neoplasia**

	PanIN-1	PanIN-2	PanIN-3
Histology	Columnar epithelial cells with basally oriented uniform and round nuclei	More nuclear changes such as loss of nuclear polarity, pleomorphism, hyperchromasia and nuclear pseudostratification	Cribriform pattern, budding cells into lumen and nuclear changes
Genetic changes	<i>K-Ras</i> mutations Telomere shortening	<i>CDKN2A</i> mutations	<i>TP53</i> loss <i>SMAD4</i> loss <i>BRCA2</i> loss

PanIN: Pancreatic intraepithelial neoplasia.

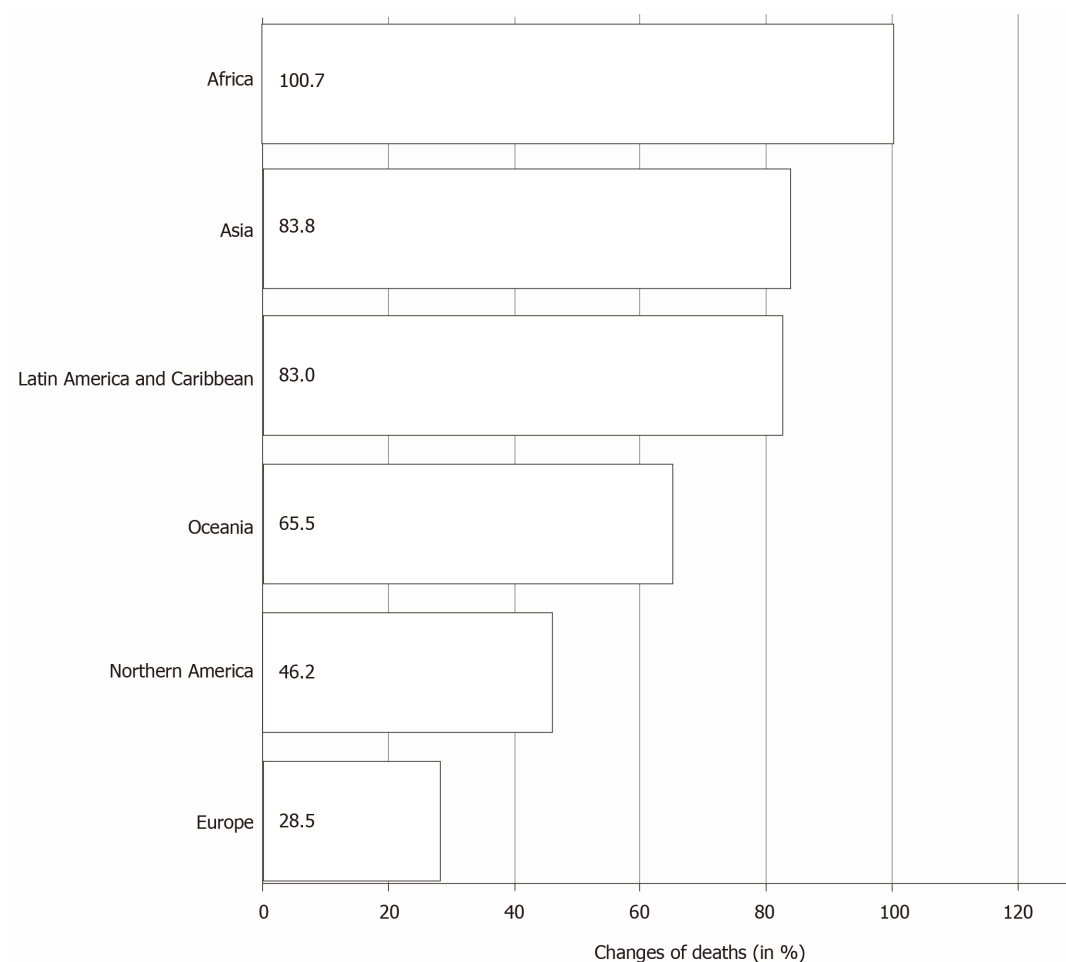


**Figure 3** Pancreas cancer-recent trends in surveillance, epidemiology, and end results age-adjusted incidence rates as *per stage at diagnosis*, 2000-2017[12]. Used with permission from National Cancer Institute.

early. The proteins expressed by PanIN and invasive carcinoma are similar, which can be used for screening these lesions[32-35]. The high-grade PanINs express mucins such as MUC1, MUC4, MUC5AC, and MUC6[36-38]. These can be used for screening and in the treatment of PanIN[39,40].

Based on the transcriptome analysis, PDA is divided into molecular subtypes[41]. The three subtypes named initially were classical, quasimesenchymal and exocrine-like, which have been modified as progenitor, squamous and aberrantly differentiated endocrine exocrine types, respectively, by the International Cancer Genome Consortium study[41]. An immunogenic subtype was also identified. The squamous subtype was associated with a poor prognosis similar to the basal subtype carcinomas of other organs and has a poor response to chemoradiotherapy and also exhibits *TP53* mutations more frequently[41]. Recently, RNA-based sequencing of PDA revealed the heterogeneity of the tumor at the molecular level[42]. RNA-based subgrouping may add further insights into the pathogenesis and tumor progression. The desmoplastic stroma is the predominant component of the PDA. Recent transcriptome analysis of the stromal cells revealed two subtypes similar to PDA cells—normal subtype and activated subtype[43]. The normal subtype resembles pancreatic stellate cells, and the inflammatory subtype has immunogenic signatures. The gene expression is also different for the two subtypes leading to the inference of the role of stromal and neoplastic cell interaction in determining the tumor heterogeneity[44].

The neoplastic cells of PDA are acclimatized to survive in a microenvironment of depleted oxygen and nutrients due to the poor vascularity and intense desmoplastic stroma of the tumor. The *K-ras* mutation up-regulates several metabolic pathways such



**Figure 4 Projected Changes of deaths due to pancreatic cancer from 2020 to 2040, both sexes, age (0-85+)**[1]. Used with permission from International Agency for Research on Cancer.

as glucose uptake and glycolysis[23]. Apart from the metabolic adaptations, cells survive by autophagy, mitophagy and macropinocytosis stimulated by the *K-ras* gene [24]. Genetic or pharmacological inhibition of autophagy leads to decreased tumor growth as seen in mouse models. Co-targeting with mitogen-activated protein kinase kinase/extracellular signal-regulated kinase inhibitors is an area of intense research in the treatment of pancreatic cancer[45,46]. Furthermore, the deregulation of these metabolic pathways is one of the reasons for resistance to chemotherapy in PDA[47-49].

## RISK FACTORS

Risk factors for PDA can be divided into modifiable and non-modifiable. Smoking has been proven beyond doubt to be the main modifiable risk factor. The risk is approximately twice in smokers, and they are at risk even after smoking cessation for about 20 years[50,51]. Alcohol is an additional risk factor in smokers but not in non-smokers [52]. Obesity is clearly associated with an increased risk of PDA as well as mortality [53]. Dietary factors such as consumption of red meat and processed foods are associated with an increased risk of PDA while consumption of fresh fruits and folate is protective[54,55]. Occupational exposure to nickel, cadmium and chlorinated biphenyls is associated with an increased risk[56].

Among the nonmodifiable risk factors, male sex, increasing age and African-American ethnicity are associated with an increased risk[13]. Genetic factors play an important role as 10% of the PDAs have a family history[57,58]. Mutations in the genes such as *BRCA2*, *PALB2*, *STK11*, *CDKN2A*, *APC*, Lynch syndrome genes, *ATM*, *FANCC* and *FANCG* are responsible for the familial causes of PDA[59]. Chronic pancreatitis is a risk factor for PDA, particularly chronic pancreatitis due to hereditary pancreatitis (*PRSS1/SPINK1* gene mutation)[60]. Diabetes type 1 and 2, particularly recent-onset

diabetes, are associated with an increased risk of PDA. However, the causal association was not proved and is a matter of debate as diabetes may be a manifestation of PDA[61]. The risk of PDA decreases with an increased duration of diabetes [62]. The association of *Helicobacter pylori* infection and PDA was proved in a meta-analysis[63]. There is also growing evidence of association of PDA with chronic diseases such as hepatitis B and C[64]. Recent studies have also proved the association between non-O blood group and PDA[64,65].

## RECENT ADVANCES IN SCREENING AND DIAGNOSIS

As invasive PDA arises from PanIN and from mucinous neoplasm and intraductal papillary neoplasm (IPMN) screening for these lesions can be beneficial as the disease can be cured with resection at an early stage. However, till now there is no approved and reliable screening test for PDA[66,67]. PDA is a cancer of comparatively low prevalence but with high mortality. Due to the non-availability of any standard, economical and reliable screening test, screening the entire general population for PDA is not possible. However, patients with a family history of pancreatic cancer have an increased risk of PDA and benefit from screening[66]. Screening is particularly recommended for those with at least two first-degree relatives with PDA or in patients with known familial syndromes. Even though IPMNs are visible on conventional imaging, PanIN are very small lesions of size less than 5 mm and are not identified on routine imaging studies[66]. Hence, we need to rely on biomarkers alone or in combination with imaging studies for screening.

The most commonly used biomarker is carbohydrate antigen (CA) 19-9, which is sialylated Lewis blood group antigen on MUC1 expressed by neoplastic cells of PDA and also by the normal cells of the pancreaticobiliary system, stomach, colon, endometrium and salivary glands[68]. It is elevated in only 65% of resectable pancreatic cancers and hence of low sensitivity[69]. It is also elevated in benign diseases of the biliary tract, biliary obstruction and also in malignancies arising from other organs, and hence it is also less specific[69,70]. CA19-9 cannot be used as a tumor marker in populations who do not express Lewis antigen (4%-15%)[66,69,70]. Therefore, it is primarily used to assess the response to treatment and in the follow-up of patients diagnosed with PDA[69,70]. Carcinoembryonic antigen (CEA) in the pancreatic juice can also be used to screen pancreatic cancer with reasonable accuracy [71,72]. However, the main limiting factor of CEA is the low sensitivity although specificity is high[71,72].

PAM4 is an anti MUC1 antibody, which is directed specifically against an epitope of MUC1 secreted by the pancreatic cancer cells absent in normal pancreas and other tissues and therefore found to be more specific and sensitive than CA19-9 in differentiating pancreatitis and pancreatic cancer[73]. Patients with advanced disease had higher values of PAM4 compared to early stages[74]. PAM4 can also be used to screen early lesions of PDA such as PanINs and IPMNs as the expression begins at an early stage of the disease and continues throughout[75]. The role of several new biomarkers such as CA494, CA50, CA242, CEA -related cell adhesion molecule 1, CAM 17.1-Ab, parathyroid hormone-related oncoprotein and serum beta-human chorionic gonadotropin in diagnosing early pancreatic lesions is growingly evident[70,76-80]. In a meta-analysis of seven studies evaluating the role of tumor M2-pyruvate kinase in screening pancreatic cancer, the conclusion was that the efficacy of tumor M2-pyruvate kinase was similar to CA19-9[81]. SPan-1 is also one of the markers studied for the diagnosis of exocrine pancreatic cancer, but it did not improve the rates when combined with CA19-9[82].

Various genetic and epigenetic mutations can be detected in the pancreatic juice obtained by endoscopic ultrasound (EUS) and endoscopic retrograde cholangiopancreatography[83]. *K-ras* mutations and *TP53* mutations identified in the pancreatic juice are associated with low specificity and sensitivity even though they are mutated in most of the PDAs[84,85]. DNA methylation abnormalities of a panel of genes were associated with a sensitivity of 82% and a specificity of 100% in identifying pancreatic cancer in a study[86]. Identification of mitochondrial mutations in the pancreatic juice is also being studied in diagnosing pancreatic cancer[87]. The main drawback of the above-mentioned tests is the requirement of invasive intervention to obtain the sample. Micro-RNAs are being intensely investigated in diagnosing various human cancers including pancreatic cancer[88]. Circulating tumor cells are also present in 47% of patients with PDA and are also associated with early lesions.



Multi-detector computerized tomography (MDCT) is presently the gold standard for the diagnosis of pancreatic lesions[89,90]. It is the virtual eye of a surgeon to assess the resectability of the tumor and also to accurately stage the disease[91,92]. The major drawback is the low sensitivity of MDCT in identifying lesions less than 2 cm and negligible sensitivity for identifying the pre-invasive lesions. Also, the routine use of MDCT for screening purposes in high-risk patients may increase the risk of radiation-induced secondary tumors. Hence, MDCT is preferably not used for the purpose of screening high-risk individuals and is comparatively inferior to EUS for the same[93]. Magnetic resonance imaging (MRI) is a non-ionizing investigation, which can image the entire abdomen as opposed to EUS. MRCP can provide very accurate details of the biliary and pancreatic ductal system and can identify small cystic lesions such as IPMNs. In fact, MRCP is proven to be superior to MDCT in detecting these lesions[93, 94]. In a prospective study evaluating the role of MRI in the screening of patients with P16 mutations, MRI was able to identify early lesions[95].

EUS plays an important role in the screening and diagnosis of PDA. EUS was shown to be superior to MDCT, MRI and positron emission tomography (PET) in detecting small lesions and lymph node involvement[96]. It was able to detect twice the number of lesions compared to MDCT and MRI when used for screening[94]. It can also be used for biopsying a suspected lesion. The major drawback is the invasiveness of the procedure and its operator dependence. It may also be associated with rare but severe complications such as iatrogenic gastrointestinal perforation. EUS is usually performed in sequence with biomarker tests (in those with elevated CA19-9) or after a basic imaging test rather than as a first option[66,97].

PET scan is one of the valuable investigations in PDA with good sensitivity but an average specificity. In two meta-analyses from Tang *et al*[98] and Wu *et al*[99], the pooled sensitivities were 90.1% and 87%, respectively, and pooled specificities were 80.1% and 83%, respectively[98,99]. The low specificity is because of the inability of the PET scan to differentiate between inflammatory lesions and neoplastic lesions[100]. The role of the PET scan in determining the T stage is limited and is surpassed by MDCT[101]. Even though the PET scan was able to pick up positive lymph nodes in other malignancies, the sensitivity of PET in accurately determining the N stage of PDA is limited[100,102]. However, a PET scan is invaluable in diagnosing metastatic PDA, which can alter the management of the patient[103].

## ADVANCES IN SURGICAL MANAGEMENT OF PDA

### *Preoperative preparation*

Surgical resection of PDA either pancreaticoduodenectomy (PD) or distal pancreatectomy (DP) with splenectomy is associated with high morbidity and mortality. Hence, thorough preoperative preparation is essential to avoid complications. The preoperative preparation is similar to any other major surgery except for two unique complications encountered in PDA: Obstructive jaundice and nutritional deficiencies. Obstructive jaundice is common in patients with carcinoma involving the head of the pancreas.

There were many studies evaluating the role of preoperative biliary drainage (PBD) *vs* surgery alone. Preoperative drainage is associated with improvement in the general condition of the patient and liver function. However, it is associated with significant side effects such as the introduction of infection into the biliary tree[104]. Furthermore, preoperative drainage will make the surgery challenging due to inflammation and fibrosis induced by the common bile duct (CBD) stents and decreased diameter of the CBD making anastomosis difficult. In a meta-analysis by Sewnath *et al*[105], the lack of benefit of PBD over direct surgery in patients of periampullary carcinoma with obstructive jaundice was proven[105], further confirmed by a Cochrane review and in a meta-analysis by Wang *et al*[106] and Fang *et al*[107]. It was proven in various studies that PBD and delayed surgery to improve the general condition were not associated with any improvement in survival[108]. The only indications accepted for PBD before surgery at present include patients with cholangitis, poor general condition and poor performance status precluding surgery and in whom neoadjuvant treatment is planned. PBD can be undertaken with the help of CBD stents or percutaneous transhepatic biliary drainage. Among the CBD stents, metallic or plastic ones can be selected. In a meta-analysis of five studies by Crippa *et al*[109], the rate of re-intervention and post-operative biliary fistula was shown to be lower in the case of metallic stents compared to plastic stents[109]. Hence, at present, the available evidence supports the use of metallic stents over plastic stents, in both unresectable

PDA and for PBD.

### Surgery

With the mortality and morbidity rates of pancreatic surgery improving over several decades, focus has been shifted to performing the surgeries using minimally invasive techniques. In a meta-analysis by Venkat *et al*[110] evaluating laparoscopic DP with open technique, they inferred that the laparoscopic technique was associated with lower blood loss, shorter hospital stay and lower overall postoperative complications compared to the open technique with no difference in the margin status and operating time[110]. Similar results were obtained in the meta-analysis by Jin *et al*[111].

The results from studies comparing laparoscopic PD (LPD) and open PD (OPD) are also encouraging. A summary of the outcomes of few recent studies in this regard is presented in Table 3. LPD was associated with lower intra-operative blood loss, faster recovery and shorter hospital stay with similar rate of post-operative complications and oncological outcomes[112-118]. However, in most of the studies, LPD was associated with greater operating times compared to OPD[112-118]. Robotic surgery has the added advantage of increased degrees of freedom and better images without motion artifacts compared to conventional laparoscopic surgery. In a retrospective study by Nassour *et al*[119], 428 minimally invasive PD surgeries were analyzed, and the 30-d complication rate was found to be the same between robotic and laparoscopic groups[119]. In a meta-analysis of 44 studies by Kamarajah *et al*[120] comparing robotic and conventional laparoscopic PD, the conclusion was that the robotic group was associated with lower conversion rates compared to the laparoscopic group[120]. No significant difference was noted in the operating times and blood loss between the two groups. The robotic surgery group had a shorter hospital stay compared to the laparoscopic group.

With the expertise of vascular reconstructions, venous involvement is no longer an absolute contraindication for resection of PDA with the criteria for resectability being updated regularly[121]. Venous reconstructions are widely practiced, and the present limiting factor seems to be arterial involvement. Hence, the approach has been shifted to the artery-first approach to determine the resectability of the tumor at the initial phase of the surgery itself. These include the posterior approach, medial uncinate approach, inferior infracolic approach, left posterior approach, inferior supracolic approach and superior approaches, which have become popular[122]. In a meta-analysis of 22 studies by Yu *et al*[123], PD combined with portal-superior mesenteric vein synchronous resection (PSMVR) was found to be associated with similar post-operative morbidity and mortality compared to the group without resection[123]. However, only the group with R0 resection had a significant improvement in survival [123]. In a similar meta-analysis by Bell *et al*[124], it was concluded that PSMVR was associated with a higher R1 rate, poor 5-year survival rate and was not cost effective [124]. In a meta-analysis of 26 studies by Mollberg *et al*[125] comparing pancreatectomy with arterial resection (AR) and without AR, pancreatectomy with AR was associated with an increased peri-operative mortality and poor 1-year and 2-year survival[125].

Post-operative pancreatic fistula (POPF) is one of the most dreaded complications of PD[126]. Many methods have been advocated to reduce its incidence, beginning with the type of pancreaticoenteric anastomosis. Several randomized control trial (RCTs) and meta-analysis have shown that pancreaticogastrostomy was associated with less incidence of POPF[127-129]. However, pancreatojejunostomy (PJ) is the mostly widely practiced technique because it is more physiological and is associated with lower long-term complications than pancreaticogastrostomy[130]. Several techniques of PJ have been compared in trials for the incidence of POPF. Among the duct-to-mucosa anastomoses, the Blumgart technique was found to be better compared to the Cattell-Warren technique[131]. Both the Blumgart and Kakita techniques were associated with similar results in many studies[132,133].

The continuous suturing technique was associated with a lower incidence of POPF compared to the interrupted suturing technique in several studies[134,135]. It is hypothesized that continuous sutures lead to a uniform distribution of tension along the suture line compared to intermittent sutures. A brief interest was sparked in the invagination techniques after the introduction of the jejunal eversion with binding PJ technique by Peng *et al*[136] and the end-to-side invagination technique by Berger *et al* [137], which showed better results with respect to POPF compared to the duct-to-mucosa technique[136,137]. However, the results were not reproduced in other trials [138]. In a study by Kojima *et al*[139], it was concluded that the Blumgart technique of PJ when combined with the tight dressings of the wound and drain sites (complete packing method) was associated with less incidence of POPF[139].

**Table 3 The outcomes of various latest studies comparing laparoscopic pancreaticoduodenectomy with open procedure**

Ref.	Study	Comparison	Outcome
Nickel <i>et al</i> [112], 2020	Meta-analysis of 3 RCTs	LPD and OPD	90-d mortality, post-operative complications and oncological outcomes were similar in both groups  Blood loss was less for LPD  Operating time was more for LPD
Yoo <i>et al</i> [113], 2020	Retrospective cohort study 359 patients	LPD and OPD	Post-operative complications and hospital stay were shorter for LPD  Operative time was longer for LPD  Recurrence free outcomes and overall survival rates were similar
Chen <i>et al</i> [114], 2020	Meta-analysis of 6 cohort studies	LPD and OPD for PDA	Number of lymph nodes harvested, number of positive lymph nodes, rate of adjuvant therapy, time to adjuvant therapy, 1 yr survival and 2 yr survival are same for both the groups
Zhou <i>et al</i> [115], 2019	Retrospective cohort study	LPD and OPD	Overall complications and survival were similar between the two groups
Chen <i>et al</i> [116], 2018	Retrospective cohort study of 102 patients	LPD and OPD	Intra-operative blood loss, post-operative recovery and hospital stay were shorter for LPD  Operative time was longer for LPD  Post operative complications were similar in both the groups
Dang <i>et al</i> [117], 2020	Retrospective cohort study	LPD and OPD	Intra-operative blood loss, operating time and hospital stay for shorter for LPD  30 d and 90 d mortality rates were better for LPD  Long term survival rates were similar
Palanivelu <i>et al</i> [118], 2017	RCT of 68 patients with periampullary carcinoma	LPD and OPD	Intra-operative blood loss and hospital stay for shorter for LPD  Operative time was longer for LPD  Post-operative complications were similar in both the groups

RCT: Randomized control trial; LPD: Laparoscopic pancreaticoduodenectomy; OPD: Open pancreaticoduodenectomy.

In a move to reduce the incidence of POPF, stenting of the PJ was evaluated in various trials. Stenting is hypothesized to prevent the pancreatic enzymes from coming in contact with the anastomosis, thereby promoting healing of the anastomosis. Studies comparing internal stents and no stents revealed no significant difference in the rate of POPF[140-142]. In trials comparing external stenting and no stenting, few trials have demonstrated the benefit of external stenting[142-145]. However, these stents are associated with complications such as tube-related complications, digestive enzyme loss and possible peritonitis during tube removal[141]. Moreover, no difference was observed between the internal and external stents in preventing the POPF[146]. Most of the high-volume centers do not stent the PJ at present.

In the case of DP, stump closure using hand-sewn or stapler techniques was evaluated with respect to POPF. There was no significant difference in the POPF rates between the two techniques, and the stapler technique is commonly used by most surgeons[147,148]. In a meta-analysis evaluating bare metallic staplers and reinforced staplers using bioabsorbable materials, the superiority of the reinforced staplers was not proven even though the rate of POPF was less in reinforced staplers[149]. Pancreaticoenteric anastomosis of the distal stump has been shown to reduce the rate of POPF but increased the rate of post-operative hemorrhage[150,151].

Topical application of fibrin sealants over pancreatic anastomosis has no effect on POPF incidence in various studies[152,153]. Similarly, omental wrapping around the pancreatic anastomosis has no effect on the POPF or post-operative hemorrhage[154]. Covering the distal pancreatic stump with a teres ligament patch has shown to reduce the rate of reoperations and readmissions compared to simple closure even though the rate of POPF was not significantly different between the two groups in a randomized control trial[155].

The role of prophylactic intraperitoneal drains following pancreatectomy is controversial. In case of DP, there is no role of prophylactic intraperitoneal drains as concluded in various studies[156,157]. Moreover, prophylactic drain placement

increases hospital stay. The PANDRA trial randomized 395 patients with PD with or without drains and concluded that there was no need for routine prophylactic intraperitoneal drainage[158]. However, other studies have reported increased mortality in patients who underwent PD without drains[159,160]. However, in low-risk patients, drains can be safely avoided[160]. If drains are placed, they must be removed as early as possible once their purpose is served as their prolonged placement may lead to intra-abdominal infections and increase the risk of POPF[161].

The role of somatostatin analogues in preventing POPF is unclear. Earlier RCTs revealed the efficacy of octreotide in preventing POPF. However, newer RCTs proved that there is no role of octreotide in preventing POPF[162-164]. Pasireotide was found to reduce the rate of POPF in one RCT by Allen *et al*[165], but not in other RCTs[165-167]. At present, the use of somatostatin analogues cannot be recommended due to inconsistent results in clinical trials.

## ADVANCES IN SYSTEMIC THERAPY

### Neoadjuvant therapy

Borderline and locally advanced lesions are started on chemotherapy  $\pm$  chemoradiation as *per* the present guidelines[168,169]. The current preferred regimen is 5-Fluorouracil, irinotecan and oxaliplatin (FOLFIRINOX)  $\pm$  chemoradiation, if the patient has good performance status[169-172]. Alternatively, gemcitabine and nab-paclitaxel  $\pm$  chemoradiation can also be used[170-172]. In patients with known mutations in the *BRCA* gene, substituting paclitaxel with cisplatin may provide added benefit[170]. Patients with poor performance status can be started on single-agent chemotherapy or provided with palliative care. The likelihood of resection depends upon the response to neoadjuvant therapy. As *per* the latest studies, the response should be measured by falling CA19-9 Levels and absence of disease progression while on neoadjuvant therapy rather than by assessing the radiological regression[173-176].

Most of the patients with PDA in the long term, even after complete resection, develop distant metastasis. This points to the notion of micrometastasis even in a resectable localized cancer at the time of presentation. Also, due to the morbidity associated with surgery, a significant proportion of patients are unable to receive adjuvant therapy or have a delay. The encouraging results of neoadjuvant and perioperative chemoradiation therapies in esophageal, gastric and rectal carcinoma have stimulated research on the role of neoadjuvant therapy in resectable lesions of PDA. It also improves the rate of R0 resections. Multiple trials have been conducted to assess the role of neoadjuvant chemotherapy and chemoradiation in resectable PDA (Table 4). The results of many of the latest trials support neoadjuvant chemotherapy therapy in resectable PDA[177,178]. The results of ongoing trials such as NEPAFOX, NorPACT-1, NEOPAC and NCT02562716 are eagerly awaited[179-183]. The role of neoadjuvant chemoradiotherapy in resectable PDA is proved in several trials[184-186].

### Adjuvant therapy

The trials of GITSG and EORTC 40891 have clearly proven the role of adjuvant chemoradiotherapy in PDA[187,188]. The ESPAC-1 trial has shown the beneficial effects of chemotherapy over chemoradiotherapy[189]. Even though the opinion on the role of adjuvant chemoradiotherapy is different among practitioners in Europe and the United States of America, most of them have a common opinion on the role of adjuvant chemotherapy. The CONKO-1 trial after showcasing the efficacy of gemcitabine in adjuvant chemotherapy has shifted the adjuvant chemotherapy regimens from 5-fluorouracil (5-FU) to gemcitabine-based regimens[190]. The ESPAC-3 trial has shown no significant difference between the 5-FU and gemcitabine regimen[191]. The ESPAC-4 trial has proved the increased efficacy of the gemcitabine and capecitabine combination over gemcitabine alone and is now the recommended regimen for adjuvant therapy[192]. The success of the FOLFIRINOX regimen in the metastatic setting has led to the evaluation of its role in the adjuvant setting. The PRODIGE 24 trial has proven the efficacy of FOLFIRINOX over gemcitabine, but its use is limited to patients with a good performance status[193]. In the Japanese trial JASPAC-01, S1 was proven superior to gemcitabine, but it is still not widely used outside Japan[194]. The CONKO-05 and CONKO-06 trials did not prove the efficacy of gemcitabine + erlotinib and gemcitabine + sorafenib, respectively, over gemcitabine alone[195,196].



**Table 4 Studies showing the role of neoadjuvant chemotherapy and chemoradiotherapy in resectable pancreatic adenocarcinoma**

Ref.	Type of study	Type of neoadjuvant therapy	Drugs	Results
Tajima <i>et al</i> [177], 2012	Retrospective pilot study	Chemotherapy	Gemcitabine and S1	The 3 yr survival rates of NACT group (55.6%) was higher than control group (29.6%)
O'Reilly <i>et al</i> [178], 2014	Phase II trial non randomized	Chemotherapy	Gemcitabine and oxalipatin	Resectability was 71% Overall survival was 21.7 mo
Motoi <i>et al</i> [179], 2013	RCT- NACT <i>vs</i> direct surgery	Chemotherapy	Gemcitabine and S1	Results awaited
Scott <i>et al</i> [180], 2017	RCT- NACT <i>vs</i> direct surgery	Chemotherapy	FOLFIRINOX	Results awaited
Labori <i>et al</i> [181], 2017	RCT- NACT <i>vs</i> direct surgery	Chemotherapy	FOLFIRINOX	Results awaited
Heinrich <i>et al</i> [182], 2011	RCT- NACT <i>vs</i> direct surgery	Chemotherapy	Gemcitabine and oxalipatin	Results awaited
Sohal <i>et al</i> [183], 2017	RCT-FOLFIRINOX <i>vs</i> GnP	Chemotherapy	FOLFIRINOX <i>vs</i> Gemcitabine and nab paclitaxel	Results awaited
Turrini <i>et al</i> [184], 2009	Prospective study	Chemoradiotherapy	5-Fluorouracil and cisplatin with radiotherapy	Resectability rate is 82.6% Median overall survival for resected patients is 23 mo
Golcher <i>et al</i> [185], 2015	RCT- NACRT <i>vs</i> direct surgery	Chemoradiotherapy	Gemcitabine and Cisplatin with radiotherapy	R0 resection rate (52%) and median overall survival after tumor resection (27 mo) was greater NACRT arm
Okano <i>et al</i> [186], 2017	Prospective study	Chemoradiotherapy	S-1 with radiotherapy	1-yr and 2-yr survival rates are 91% and 83% in resectable group

RCT: Randomized control trial; NACT: Neoadjuvant chemotherapy; NACRT: Neoadjuvant chemoradiotherapy; FOLFIRINOX: 5-Fluorouracil, irinotecan and oxalipatin.

Combination chemotherapy regimens are now being recommended over single agent gemcitabine for patients with metastatic disease with good performance status. The ACCORD trial has laid the ground for the FOLFIRINOX regimen as the preferred regimen over gemcitabine[197]. Several new second-line regimens were added to the arsenal after the success of the MPACT and NAPOLI-1 trials[198,199]. The trial comparing extracellular matrix degrader pegvorhialuronidase alfa (PEGPH20) was stopped as its primary end-point was not met[200]. The results of the clinical trial AVENGER 50 comparing modified FOLFIRINOX with or without CPI-613 are awaited [201].

Studies on the role of immunotherapy in cancers are encouraging, but that is not the case with PDA[202]. The highly desmoplastic stroma and absence of any effector cells in the tumor microenvironment seem to be the predominant reason for the failure of immunotherapy[203]. Even though pembrolizumab is approved in patients with microsatellite instability, the latest results from the KEYNOTE-158 study are disappointing[204]. Two new targeted therapy drugs have been approved. Larotrectinib was approved in patients with tropomyosin receptor kinase (TRK) fusion-positive tumors[205]. Entrectinib, a multiple tyrosine kinase inhibitor, was also approved in patients with *NTRK1/2/3*, *ROS1*, or *ALK* gene fusions[206]. Cisplatin-based regimens are recommended in patients with germline mutations in *BRAC2* [207]. Based on the results of the POLO trial, olaparib is approved as maintenance therapy in patients with metastatic PDA and *BRCA2* mutation and with a good performance status[208].

Gene therapy for pancreatic cancer is being widely studied. A number of clinical trials and ongoing studies have been conducted in this regard[209]. Even though the results of gene therapy in phase 1 trials are encouraging, the same is not being replicated in the phase 2 studies in comparison with standardized treatment. The role of miRNAs such as *miR-4516* is being studied, which may be the future target for therapies[210]. The role of intra-operative chemotherapy in PDA is being studied in the combiCaRe trial[211].

## CONCLUSION

This narrative review highlights the newer trends and achievements in the epidemiology, etiopathogenesis, screening, diagnosis and management of PDA. The newer trends in epidemiology will help us to predict the population and countries at risk in the near future. The newer concepts in the field of etiopathogenesis will help us to understand this stubborn disease better. Newer screening and diagnostic techniques will help in diagnosing the patients at an early stage and prognosticate better. The newer discoveries in drugs and management protocols will help the physicians and surgeons increase the survival and quality of life of the patients.

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## Silencing hepatitis B virus covalently closed circular DNA: The potential of an epigenetic therapy approach

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### Abstract

Global prophylactic vaccination programmes have helped to curb new hepatitis B virus (HBV) infections. However, it is estimated that nearly 300 million people are chronically infected and have a high risk of developing hepatocellular carcinoma. As such, HBV remains a serious health priority and the development of novel curative therapeutics is urgently needed. Chronic HBV infection has been attributed to the persistence of the covalently closed circular DNA (cccDNA) which establishes itself as a minichromosome in the nucleus of hepatocytes. As the viral transcription intermediate, the cccDNA is responsible for producing new virions and perpetuating infection. HBV is dependent on various host factors for cccDNA formation and the minichromosome is amenable to epigenetic modifications. Two HBV proteins, X (HBx) and core (HBc) promote viral replication by modulating the cccDNA epigenome and regulating host cell responses. This includes viral and host gene expression, chromatin remodeling, DNA methylation, the antiviral immune response, apoptosis, and ubiquitination. Elimination of the cccDNA minichromosome would result in a sterilizing cure; however, this may be difficult to achieve. Epigenetic therapies could permanently silence the cccDNA minichromosome and promote a functional cure. This review explores the cccDNA epigenome, how host and viral factors influence transcription, and the recent epigenetic therapies and epigenome engineering approaches that have been described.

**Key Words:** Chronic hepatitis B virus; Epigenetic gene silencing; Functional cure; Hepatocellular carcinoma; Hepatitis B surface antigen

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**Core Tip:** Epigenetic regulation of the hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) minichromosome is important for establishing and maintaining infection. To do this HBV manipulates several cellular pathways, resulting in an intricate and complex interplay between the virus and the host. Epigenetic silencing of the cccDNA could permanently inhibit viral transcription. Therapies such as immune modulators, small molecules, and epigenome engineering tools could silence HBV DNA to promote a functional cure.

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## INTRODUCTION

Chronic HBV infection (CHB) remains largely incurable and is a major risk factor for the development of hepatocellular carcinoma (HCC)[1]. Regrettably, only a small percentage of chronically infected individuals are currently afforded therapy[2] and the annual rate of hepatitis-related deaths remains high. This underpins the importance of developing new therapeutics to treat the estimated 257-291 million people afflicted by this life-threatening disease[3]. Licensed direct-acting antivirals such as nucleoside/nucleotide analogues help mitigate infection by preventing viral replication but have little effect on established covalently closed circular DNA (cccDNA)[4]. Immunotherapy approaches using interferon alpha (IFN- $\alpha$ ) have demonstrated antiviral efficacy, particularly when using pegylated formulations, and have been paired with various nucleoside/nucleotide analogues for combination therapy[5]. However cost, serious side effects, and mixed results have limited the broad clinical feasibility of IFN-based therapies in their current form. Persistence of the cccDNA as a minichromosome-like structure in the nucleus of long-lived hepatocytes may account for the inability of these antivirals to achieve cure, even after long-term treatment[6]. A sterilizing cure can only be realized following complete elimination of intrahepatic cccDNA, while permanent loss of serum hepatitis B surface antigen (HBsAg) would achieve a functional cure. The cccDNA is thought to endure for years [7] and as the template for viral mRNA synthesis[8], it drives viral replication and remains the major obstacle in the development of curative antiviral therapies.

In recent years, treatments designed to directly silence or eliminate the cccDNA minichromosome have gained interest as they may provide a means for achieving cure [9]. Gene editing tools such as designer nucleases and nickases disrupt the viral DNA *via* site-directed cleavage. This stimulates either targeted mutagenesis or degradation of the cccDNA[10,11]. Zinc finger (ZF) nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided nucleases have all demonstrated antiviral potential in preclinical models of infection. The simplicity of the CRISPR/Cas system has led to a surge in the field of anti-HBV gene editing, particularly as multiple studies have reported degradation of cccDNA following cleavage[12-15]. The CRISPR/Cas constructs developed by Seeger and Sohn[16] were shown to edit HBV DNA 15000 times more efficiently than IFN- $\alpha$ -induced APOBEC deamination. However, concerns regarding off-target cleavage, liver-specific delivery and expression, and Cas9 immunity will need to be addressed to ensure safety without compromising efficacy [17-20]. Viral vectors, including adeno-associated viruses (AAVs) and high-capacity adenoviral vectors (HCAV) have recently been explored as hepatotropic delivery vehicles[13,21,22]. To overcome the packaging limitations of AAVs, the smaller *Staphylococcus aureus* endonucleases have been combined with HBV-specific guide RNAs to demonstrate cccDNA-targeting and antiviral efficacy in hNTCP-HepG2 cells [22] and transgenic mice[21]. On the other hand, the large packaging capacity of HCAVs have been exploited to accommodate multiple HBV guide RNAs along with the larger *Streptococcus pyogenes* Cas9[13], which may improve the efficacy of this gene editing approach. Importantly, iterative modifications to the effector domains of traditional gene editing platforms have given rise to several new tools. CRISPR/Cas

base editors comprising an APOBEC1 deaminase, Cas9 nickase, and uracil glycosylase inhibitor introduced point mutations in cccDNA as well as integrated viral DNA[23]. A number of new epigenome engineering platforms have also been developed, which could be used to generate targeted epigenetic changes to control gene expression[24]. Such an approach may be well suited to treating CHB, as epigenetic modifications regulate cccDNA minichromosome organization and can either promote or repress viral transcription[25-27]. Host and/or virus-specific epigenetic therapies could promote silencing of HBV cccDNA and achieve a functional cure. This review will explore the formation and epigenetic regulation of the cccDNA minichromosome, how host and viral factors influence transcription, and whether epigenome editing could be used to silence HBV cccDNA permanently.

## CCC DNA FORMATION AND ORGANIZATION OF THE MINICHROMOSOME

cccDNA biogenesis is a multi-step process that relies on a number of host-cell DNA synthesis and repair factors[28,29]. Although the exact process is not fully understood it involves: (1) Removal of the covalently attached viral polymerase from the 5' end of the negative DNA strand; (2) Removal of the short RNA oligomer attached to the 5' end of the positive strand which is derived from the priming of positive-strand DNA synthesis; (3) Removal of one copy of the short terminal redundancy from the negative-strand; (4) Positive-strand elongation; and (5) Covalent ligation of the two strands[30-32]. Evidence from animal models has shown that between 1 and 50 copies of cccDNA may accumulate per infected cell[33-36]; however, more recent reports estimate up to 15 copies per cell[37,38]. Multiple rounds of infection are not necessary to establish the cccDNA pool as recycling of newly synthesized relaxed circular DNA (rcDNA) has been shown to occur, and contributes to the maintenance of cccDNA copy numbers[39-41]. Increasing evidence also indicates that the size of the pool is controlled by different host and viral mechanisms[42], but that initial *de novo* cccDNA formation is sufficient to maintain viral replication in the absence of rcDNA recycling [43].

Many different host factors have been implicated in cccDNA biogenesis, including host DNA polymerases[32,44], ligases[45], flap endonuclease I (FEN1)[46], topoisomerase I and II[47], SAMHD1[48], and tyrosyl-DNA phosphodiesterase 2[31]. Five essential components for cccDNA formation have recently been described by Wei and Ploss[49], who developed a cell-free biochemical system to mimic cccDNA biogenesis. The components are proliferating cell nuclear antigen (PCNA), replication factor C complex, DNA polymerase  $\delta$ , FEN1, and DNA ligase 1. Confirmation of these requirements in hepatocytes will be needed to validate the cell-free biochemical system[50]. Aphidicolin and CD437 were shown to block host DNA polymerases  $\alpha$ , which in turn inhibited intracellular amplification of cccDNA[44]. Also disubstituted sulfonamide compounds, CCC-0975 and CCC-0346, influenced conversion of rcDNA to cccDNA, most likely as a result of inhibiting deproteination[51]. Blocking the conversion of rcDNA to cccDNA with small molecule inhibitors may prevent accumulation of new replication-competent viral genomes in the nucleus of infected hepatocytes, but will not prevent *de novo* cccDNA biogenesis.

Once formed, cccDNA is organized into a nucleosome-decorated minichromosome [8,52]. It associates with histone and non-histone proteins, the latter of which are derived from both the host and the virus[29,33]. As a hepatotropic virus, the viral genome contains binding sites for ubiquitous and liver-specific transcription factors [53]. Thus, the recruitment, activity, and dynamic interplay of several host and viral factors are essential for efficient HBV gene expression[25-27]. Several histone proteins, transcription factors, chromatin modifying enzymes, as well as additional host enzymes and coactivators have been reported to bind to cccDNA (Table 1). Recruitment of these factors plays an essential role in regulating viral transcription which is amenable to epigenetic influences. Apart from host factors, viral elements such as HBx (HBV protein X) and HBc (HBV protein core) have been shown to influence gene transcription as well as reduce nucleosomal spacing, indicating a role in promoting viral replication and structural maintenance of the cccDNA minichromosome[8]. Thus, it is conceivable that post-translational modifications (PTMs) such as DNA methylation and histone modifications could be used to regulate cccDNA.

## HOST FACTORS AND THE EPIGENETIC REGULATION OF HBV

Epigenetic modifications are reversible heritable phenotypic changes that alter chemical signatures on chromosomes without affecting the DNA sequence. This highly

**Table 1 Host cell factors involved in the epigenetic maintenance of the covalently closed circular DNA minichromosome**

	Category	Epigenetic effect	Effect on HBV	Ref.
<b>Chromatin remodeling enzymes</b>				
Histone acetyltransferase p300/CBP	Writer	Increases H3K122a	Activation	Ananthanarayanan <i>et al</i> [99] and Tropberger <i>et al</i> [221]
P300/CBP-associated factor (PCAF)	Writer		Activation	Levrero <i>et al</i> [82] and Rivière <i>et al</i> [93]
Histone deacetylase 1 (HDAC1)	Eraser	Reduces acetylation of H3K9 and H3K27 with IFN	Inhibition	Pollicino <i>et al</i> [57] and Liu <i>et al</i> [98]
Sirtuin 1 and 3 (SIRT1/3)	Eraser	Reduces H3K9me3	Inhibition	Belloni <i>et al</i> [105] and Ren <i>et al</i> [114]
Enhancer of zeste homolog 2 (EZH2)	Writer	Increases H3K27ac and H3K27me3	Inhibition	Zhang <i>et al</i> [89]
Histone acetyltransferase 1 (HAT1)	Writer	Overexpression of HAT1 promotes acetylation of H3K27, H4K5 and H4K12	Activation	Yang <i>et al</i> [195]
Mixed lineage leukemia protein 3 (MLL3)	Writer	Increases H3K4me3	Activation	Tropberger <i>et al</i> [86] and Ananthanarayanan <i>et al</i> [99]
Protein arginine methyltransferase (PRMT)	Writer	PRMT5 interacts with HBc to increase H4R3me2s	Inhibition	Zhang <i>et al</i> [89]
Demethylases (KDMs)	Eraser	Increases H3K79me and function to transcriptional repression <i>via</i> SIRT1-mediated chromatin silencing	Inhibition	Kang <i>et al</i> [222]
Histone methyltransferase suppressor of variegation 3-9 homolog 1 (SUV39H1)	Writer	Increases H3K9me3	Inhibition	Peng and Karpen[223,224]
DNA methyltransferase (DNMTs)	Writer		Inhibition	Vivekanandan <i>et al</i> [81]
Methyl-CpG binding protein (MBPs)	Reader	Recruit chromatin remodeling and histone-modifying complexes to methylated sites resulting in histone methylation	Inhibition	Lopez-Serra and Esteller[94]
<b>Cellular transcription factors</b>				
Activating transcription factor 2 (ATF 2)		Inhibition viral transcription	Inhibition	Choi <i>et al</i> [225]
cAMP response element binding protein (CREB)		Enhances transcription	Activation	Kim <i>et al</i> [226] and Song <i>et al</i> [227]
Nuclear factor 1 (NF1)	Reader		Activation	Ori <i>et al</i> [228] and Shaul <i>et al</i> [229]
Transcription factor Yin Yang 1 (YY1)			Inhibition	Belloni <i>et al</i> [199] and Nakanishi-Matsui <i>et al</i> [230]
Specificity protein 1 (SP1)	Reader		Activation	Raney and McLachlan[231], Raney <i>et al</i> [232] and Li and Ou[233]
Nuclear Transcription Factor Y (NF-Y)		Recruit enzymes that both methylate and acetylate histone proteins	Activation	Lu and Yen[234], Maity and de Crombrughe[235] and Nardini <i>et al</i> [236]
Activator protein 1(AP-1)			Activation	Ren <i>et al</i> [111] and Choi <i>et al</i> [237]
TATA binding protein (TBP)	Reader		Activation	Bogomolski-Yahalom <i>et al</i> [238] and Chen <i>et al</i> [239]
Prospero-related homeobox protein (Prox1)			Inhibition	Qin <i>et al</i> [240]
Nuclear factor kappa-B (NF-κB)			Inhibition	Lin <i>et al</i> [241]
Histone-lysine N-methyltransferase SETDB1	Writer	In the absence of HBx, SETDB1 increases H3K9me2 and H3K9me3	Inhibition	Rivière <i>et al</i> [93]
<b>Hepatocyte factors</b>				
Retinoid X receptors (RXRα)		Increases acetylation of histones H4 and H3 by recruiting p300 to cccDNA minichromosome	Activation	Zhang <i>et al</i> [89] and Nkongolo <i>et al</i> [242]
Small heterodimer partner (SHP)			Inhibition	Oropeza <i>et al</i> [243]
CAAT enhancer-binding protein		C/EBP in low concentrations; C/EBP in high	Activation;	Raney and McLachlan[231] and

$\alpha$ and $\zeta$ (C/EBP)	concentrations	Inhibition	López-Cabrera <i>et al</i> [244]
Hepatocyte nuclear factor 1 $\alpha$ and $\beta$ (HNF1)	HNF1 and Oct 1 are essential co-activators of transcription. High HNF1 levels increase NF- $\kappa$ B expression and resulting in transcription inhibition	Activation and inhibition	Zhou and Yen[245], Zheng <i>et al</i> [246] and Lin <i>et al</i> [247]
Hepatocyte nuclear factor 3 $\alpha$ , $\beta$ , and $\gamma$ (HNF3)	Functions as a chromatin remodeler	Activation	Chen <i>et al</i> [248] and Li <i>et al</i> [249]
Hepatocyte nuclear factor 4 (HNF4)		Activation	Zheng <i>et al</i> [246], Cho <i>et al</i> [250] and Long <i>et al</i> [251]
Testicular orphan receptor 4 (TR4)		Inhibition	Lin <i>et al</i> [252]
Type I interferon (IFN- $\alpha$ )	Reduced acetylation of H3K9 and H3K27	Inhibition	Pollicino <i>et al</i> [57], Liu <i>et al</i> [98] and Yuan <i>et al</i> [253]

cccDNA: Covalently closed circular DNA; NF- $\kappa$ B: nuclear factor-kappa B.

regulated process has a critical impact on gene expression, cell function as well as cell behavior[54]. Epigenetic marks can be added directly to DNA or placed on histone tails. Methylation of cytosines within CpG dinucleotides is the most common DNA epigenetic mark, and is critical for controlling transcription, genomic imprinting and cell type identity maintenance[55]. The wrapping of DNA around histones creates highly condensed chromatin structures with protruding N-terminal histone tails amenable to PTMs[56]. Histone acetylation and DNA methylation are required for cccDNA formation and viral replication[57], highlighting the importance of the epigenome in HBV infection.

Numerous proteins are involved in the epigenetic regulation of genes and are generally categorized as writers, readers and erasers (Table 1)[54]. Writers are responsible for establishing epigenetic marks while readers recognize modified residues and recruit other protein complexes to regulate gene expression. Erasers are enzymes that remove epigenetic marks. Epigenetic therapies generally target one or more of these proteins, and studies using this approach for other chronic viral infections[58,59] suggest that this may be a promising treatment strategy for CHB.

### **Methylation for transcriptional gene silencing**

In mammalian cells, methylation is a common cellular defense mechanism known to silence invading foreign DNA[60]. It involves the addition of a methyl group from the methyl donor, S-adenosyl methionine, to the fifth carbon of cytosine in DNA (5mC). DNA methylation is most commonly detected within CpG islands and is associated with transcriptional gene silencing[61]. These inheritable 5mC marks can be 'written' (added) *de novo* in unmethylated regions which are either maintained or actively 'erased' (removed)[62]. In mammals, methyl-CpG binding proteins (MBPs) are responsible for the identification of methylation patterns[63] and a family of DNA methyltransferases (DNMTs) is responsible for establishment and maintenance of these patterns[64]. The DNMT family consists of DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (DNMT3-like). DNMT1 is primarily responsible for the maintenance of the methylation patterns during cell division and shows a preference for hemimethylated DNA[65,66]. *De novo* methylation patterns are established by the two active DNMT3A and DNMT3B enzymes and their activity is enhanced by interaction with DNMT3L which lacks catalytic activity[67,68]. In addition, DNMT2 plays an important role in methylation of structural RNA[69]. More than 20 years ago, it was discovered that integrated HBV DNA could be methylated and this was frequently observed in CHB patients with HCC[70]. Since then methylation of non-integrated HBV DNA as well as cccDNA has been observed in patients' liver samples [71,72]. Several studies have reported that HBV cccDNA can be methylated to various extents, resulting in repression of transcription, decreased viremia and loss of hepatitis B e antigen (HBeAg)[71,73,74]. Thus, the methylation pattern of cccDNA is a crucial component of viral replication and may affect pathogenesis.

HBV cccDNA has three predicted CpG islands which are strategically located in the regulatory elements of the viral genome[75]. The first CpG island (CpG I) overlaps the ATG start site of the sequence encoding the small HBsAg, the second (CpG II) overlaps the enhancer I and II, the core promoter, and HBx promoter sequence, and the third (CpG III) spans the SpI promoter and ATG start codon of the polymerase gene[72,76]. Among the 10 distinct HBV genotypes (A-J), CpG I is present in five genotypes (A, B, D, E, and I) whereas CpG II and III have been shown to exist in all genotypes[76].



Varying degrees of methylation have been reported to occur at the three CpG islands. In patients with CHB, methylation frequencies of 14, 0.6 and 3.7% within CpG I, II and III, respectively, were observed[77]. However, a separate computational study revealed that 50% of HBV sequences lacked CpG I, whereas CpG II and II were conserved across genotypes[76]. This suggests that methylation patterns are likely to differ between the genotypes. Vivekanandan *et al*[72] demonstrated that increased methylation of CpG I and II correlated with reduced viral protein production when analyzing HBV DNA samples isolated from CHB liver biopsies. In addition, the authors showed that patients with occult HBV infection had increased methylation at CpG II when compared to non-occult CHB individuals. A subsequent study compared the methylation status of CpG II in liver tissues from HBeAg+ and HBeAg- individuals [71]. Methylation was higher in HBeAg- samples (48%) compared to HBeAg+ samples (14%), indicating that an increase in methylation may reduce viral protein production. Similarly, a link between methylation of CpG III and lower HBsAg levels has been reported[74]. A cohort of cirrhosis patients failed to show an association between cccDNA methylation and HBsAg expression, but did indicate that higher methylation density was associated with lower viral load and virion productivity[73]. These results show the impact methylation can have on viral gene expression in CHB[78]. A recent comprehensive meta-analysis revealed significant hypermethylation of certain host genes depending on the geographical location of the population[79]. This included six genes in HBV-positive carcinoma tissues (*p16*, *RASSF1A*, *GSTP1*, *APC*, *p15* and *SFRP1*), two genes in HBV-positive carcinoma sera (*p16* and *APC*) and one gene in HBV-positive adjacent tissues (*GSTP1*). The study also indicated that DNA methylation could lead to the development of HBV-related HCC, an important consideration for epigenetic therapy.

The gene silencing effects of methylation have also been validated in different cell culture models of HBV replication. Transfection of HepG2 cells with methylated HBV DNA led to a reduction in viral mRNA levels, decreased HBV core antigen (HBcAg) and HBsAg expression, and reduced secretion of viral proteins[80]. *In vitro* infection experiments showed an increase in DNMT expression in response to HBV, which led to hypermethylation of the viral DNA, a reduction in viral mRNAs and proteins, and decreased HBV replication[81]. Furthermore, co-transfection of HBV DNA and DNMT3a was associated with decreased production of HBeAg and HBsAg[81].

### **Histone modifications and the cccDNA epigenome**

HBV cccDNA is associated with host histones, whose “bead-on-a-string” organization serves to compact the viral genome and provide a means for regulating gene expression[52]. As such transcription can be controlled by epigenetic modifications to the cccDNA-bound histones, thereby regulating HBV replication[82]. Nucleosome-associated histones undergo numerous types of PTMs that are generally reversible and mainly localized at the amino-terminal histone tails. These direct modifications of the N-terminal tails most commonly include acetylation, methylation, ubiquitination, phosphorylation, SUMOylation, ADP-ribosylation, and deamination[83]. Histone modifications can also indirectly regulate chromatin structure by serving as binding sites for the recruitment of other regulatory proteins[84]. Current studies have shown that PTMs significantly impact HBV replication, maturation and infection[85,86]. Several enzymes such as histone acetyltransferases (HATs) and deacetylases (HDACs), lysine and protein arginine methyltransferases (KMTs and PRMTs), demethylases (HDMTs), kinases, phosphatases, ubiquitin ligase, and deubiquitinases, modify cccDNA-associated histones[83,87]. The epigenome is further influenced by HBx and HBc, adding to the intricate and complex interplay between host and viral proteins on the cccDNA minichromosome (discussed later). Regulation of histone modifications has been proposed as a likely method to reduce cccDNA[8,52,57]. HATs facilitate the acetylation of lysine residues, making the histone-associated DNA more accessible to transcription factors, effectors and RNA polymerase II, to promote gene expression[88, 89]. Conversely, HDACs catalyze the removal of acetyl groups from lysine residues, leading to heterochromatin formation and gene repression[90,91]. Although less characterized, MBPs are reported to recruit HMTs and HDACs to promote histone methylation, which in turn recruits heterochromatin protein 1 factors (HP1) to promote DNA methylation[92-94].

HBV transcription is regulated by the acetylation status of cccDNA bound histones 3 and 4 (H3 and H4) both in cell culture models of viral replication[57,95] and in CHB patients[57]. Recruitment of HDAC1 and hypoacetylation of H3 and H4 is associated with low HBV replication and viremia *in vitro* and *in vivo*[57]. In this study, Pollicino *et al*[57] demonstrated that acetylation of cccDNA-bound H4 correlated with high levels of replication, an effect that was maintained when using HDAC inhibitors. Further-



more, the recruitment of the HATs, CREB-binding protein (CBP) and p300 as well as HDAC1 was associated with low HBV replication in an HBx-dependent manner. Nurf, an E3 ubiquitination ligase, has been shown to reduce acetylation of HBc[96]. Gong and colleagues previously reported similar results for acetylation of cccDNA-bound H3, and identified roles for histone methylation and phosphorylation in controlling viral replication[85]. Surprisingly, HDAC inhibitors suppressed duck hepatitis B virus (DHBV) cccDNA transcription and reduced viral replication in a dose-dependent manner[97,98], suggesting cell and virus-specific epigenetic factors differ between the avian and human models[98].

Genome wide studies of PTMs on the cccDNA minichromosome revealed high levels of trimethylation at lysine 4 of histone H3 (H3K4me3) and acetylation of lysine residues of histone H3 (H3K27ac and H3K122ac) at specific loci, which lead to gene activation or repression[86]. H3K4me3 modifications are crucial for active transcription of the sodium-taurocholate cotransporting polypeptide (NTCP) gene in HepG2 cells[99]. MLL3, a component of the ASCOM complex, is responsible for this modification and may indirectly facilitate HBV infection, given that NTCP is the hepatotropic receptor[97,99]. Knockdown of another methyltransferase, the enhancer of zeste homolog 2 (EZH2), resulted in upregulation of HBsAg and HBeAg production indicating that EZH2 represses HBV gene expression[89]. A recent study by Zhang *et al*[89] showed that PRMT5 induced symmetric dimethylation of arginine 3 on histone H4 (H4R3me2s) on cccDNA when associated with the hSWI/SNF chromatin remodeling complex and HBc. This epigenetic modification repressed viral transcription by inhibiting binding of RNA polymerase II. Interestingly, encapsidation of pregenomic RNA was also restricted in an epigenetically-independent manner, suggesting multiple antiviral roles for PRMT5[89].

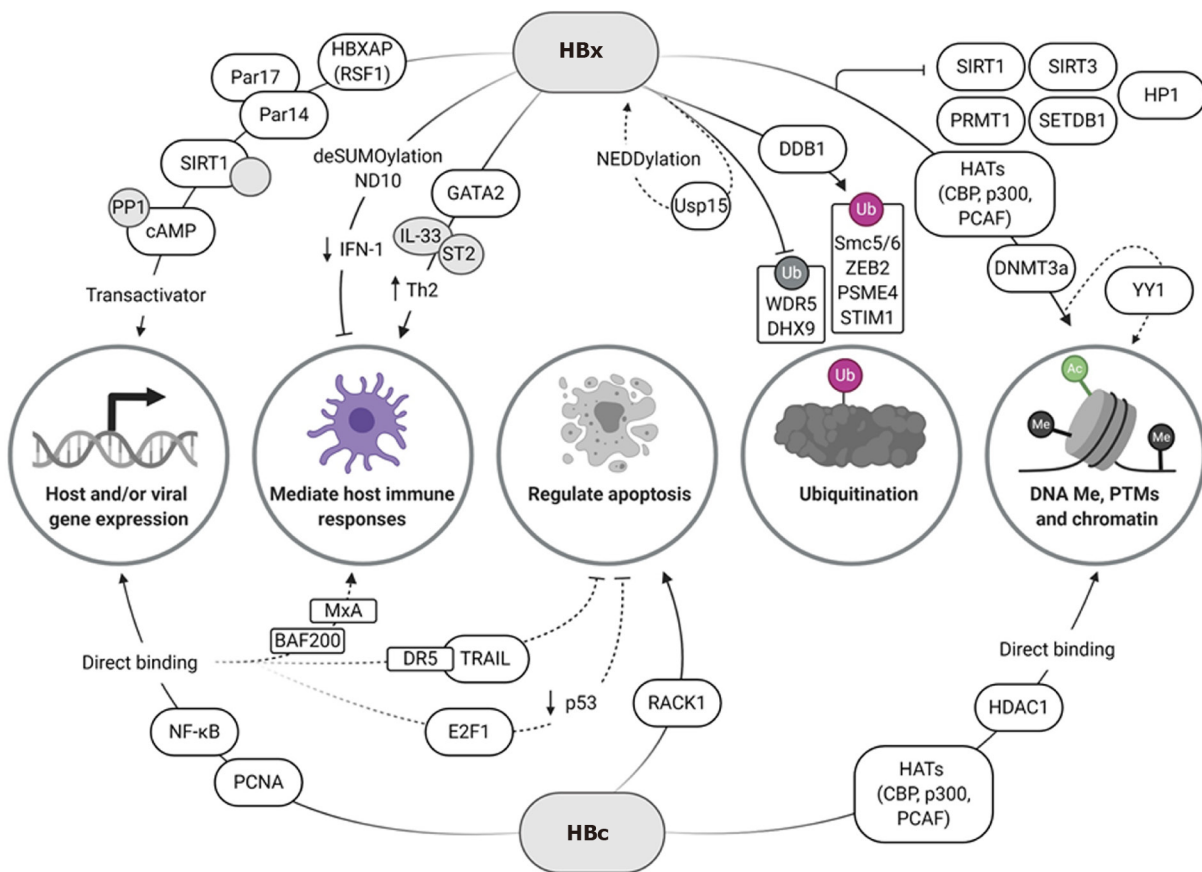
## VIRAL FACTORS AS EPIGENETIC REGULATORS

Two viral proteins, HBx and HBc, have been identified as important modulators of HBV transcription which influence the cccDNA epigenome (Figure 1). HBx is a small 17 kDa oncogenic protein that has been shown to interact with multiple host factors to manipulate both host and viral gene expression, mediate host immune responses, and control apoptosis[100]. Although HBx lacks DNA-binding motifs and does not directly bind to the cccDNA minichromosome, it is able to act as a trans-activator of viral replication by recruiting other factors to the minichromosome[101]. HBc is a 21 kDa structural polypeptide that forms the viral nucleocapsid and is essential for reverse transcription of the pgRNA[102]. As such, HBc is important for the packaging and secretion of new virions during HBV replication. Interestingly, it also forms part of the cccDNA minichromosome and is involved in its epigenetic regulation[8,57]. Unlike HBx, the HBc C-terminal domain (CTD) is capable of binding directly to cccDNA[103] as well as host gene promoter regions[104]. As such, HBc binding affects minichromosome organization as it can adjust the number and spacing of cccDNA-associated nucleosomes[57,105,106].

### ***HBx acts as a ‘master regulator’***

HBx has been shown to recruit a variety of host transcription factors and co-activators to regulate cccDNA transcription (Figure 1). These include ATF/CREB, ATF3, c/EBP, NF-κB, IL-6, Ets, Egr, SMAD4, Oct1, RXR receptor, and p53[82]. HBx-associated protein HBxAP (RSF1) has been shown to bind to HBx *via* its plant homology domain, and acts as a co-activator of viral transcription[107]. Recently, Saeed *et al*[108] demonstrated that HBx can directly bind to parvulin 14 (Par14) and 17 (Par17), which increased the stability of HBx and mediated its translocation to the nucleus and mitochondria. In addition, binding of Par14 and Par17 to the cccDNA at enhancer and promoter regions resulted in upregulation of viral transcription, an effect that was abrogated in the absence of HBx[108]. HBx has also been shown to recruit, block and promote degradation of host antiviral proteins that would normally prevent HBV replication and cccDNA persistence, as well as activate host genes to enhance viral gene expression.

Initiation of viral replication and stabilization of the cccDNA minichromosome is thought to occur through HBx-mediated PTMs of histones associated with hypomethylated CpG islands (mainly GpG I and III)[74,109]. Recruitment of HATs such as CBP, p300, and the p300/CBP-associated factor (PCAF) to the cccDNA minichromosome allows for upregulation of viral transcription[105]. In the presence of



**Figure 1** Hepatitis B virus proteins X and core act on multiple host cell pathways to promote viral persistence. An intricate epigenetic regulatory network is established during Hepatitis B virus infection. Hepatitis B virus proteins X and core contribute to this by either directly or indirectly controlling gene expression, modifying chromatin structure or location, mediating host immune responses, regulating apoptosis, and promoting or preventing ubiquitination. Pathways discussed in 'Viral factors as epigenetic regulators' are depicted here. Created with BioRender.com. HBx: Hepatitis B virus X protein; HBc: Hepatitis B virus core protein; IFN: Interferon; IL: Interleukin; NF-κB: nuclear factor-kappa B; PTM: Post-translational modifications; SIRT1: Sirtuin 1; YY1: Yin-Yang 1; HATs: Histone acetyltransferases; HDAC: Histone deacetylase; PCNA: Proliferating cell nuclear antigen; HBXAP: HBx-associated protein; RSF1: Remodeling and spacing factor 1; Par14: Parvulin 14; Par17: Parvulin 17; PP1: Protein phosphatase 1; cAMP: Cyclic adenosine monophosphate; ND10: Nuclear domain 10; GATA2: GATA binding protein 2; ST2: Interleukin 1 receptor-like 1; Th2: T helper cell type 2; Usp15: Ubiquitin-specific peptidase 15; DDB1: Damaged DNA-binding protein 1; Smc5/6: Structural maintenance of chromosomes 5/6; ZEB2: Zinc finger E-box-binding homeobox 2; PSME4: Proteasome activator subunit 4; STIM1: Stromal interaction molecule 1; WDR5: tryptophan-aspartic acid (WD) repeat domain 5 protein; DHX9: DEXH-box RNA helicase 9; ub: Ubiquitination; SIRT3: Sirtuin 3; PRMT1: Protein arginine methyltransferase 1; SETDB1: SET domain bifurcated histone lysine methyltransferase 1; HP1: Heterochromatin protein 1 factors; CBP: CREB-binding protein; PCAF: p300/CBP-associated factor; DNMT3a: DNA methyltransferase 3a; MxA: Myxovirus resistance gene A; DR5: Death receptor 5; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; E2F1: E2F transcription factor 1; RACK1: Receptor for activated protein kinase C 1.

an HBx mutant, Belloni *et al*[105] showed increased recruitment of HDAC1 and sirtuin 1 (SIRT1) to the cccDNA, which correlated with a reduction in acetylation of cccDNA-bound H4 and viral transcription. These results suggest a role for HBx in promoting cccDNA histone acetylation to enhance viral transcription. However, the involvement of SIRT1 in the promotion or suppression of HBV replication appears to vary. Other studies found that SIRT1 positively regulated HBV replication by deacetylating PGC-1α and FXRα[110], and promoting binding of activator protein 1 to the core promoter [111]. However, these studies did not investigate the role of SIRT1 in cccDNA-dependent models of viral replication. More recently Deng *et al*[112] showed that in HepG2-NTCP cells, SIRT1 complexes with and stabilizes HBx, and promoted the recruitment of HBx and other co-activating factors to cccDNA. This increased viral transcription in a deacetylase-independent manner. These results suggest a dual role for SIRT1 in the regulation of HBV replication, which may be dependent on the presence or absence of HBx.

Binding of HBx to PRMT1 inhibited the methyltransferase activity of this enzyme, ultimately enhancing viral replication[113]. Recently, HBx was found to inhibit SIRT3 expression by downregulating its expression and possibly inhibiting its recruitment to the cccDNA[114]. SIRT3 promotes cccDNA-histone methylation (H3K9me3) by directly removing H3K9ac marks and recruiting histone methyltransferase SUV39H1 and SETD1A to the cccDNA[114]. However, in the presence of HBx, H3K9me3 marks

were reduced and H3K4me3 and H3K9ac modifications increased to promote HBV transcription. This effect has also been observed for other transcriptional repressors. HBx reduced the chromatin structure alteration-mediated repression of histone-lysine N-methyltransferase SET domain, bifurcate 1 (SETDB1) and HP1[93]; however, the exact molecular mechanisms of this rescue remain to be elucidated. Upregulation of DNMTs by HBx and direct interaction of HBx with DNMT3A has been shown to either upregulate transcription of host genes, or block activity by facilitating host promoter methylation[115]. For example, SOCS-1, SUFU and TIRAP are downregulated, and this could be important in mediating an immune response in CHB, albeit the exact mechanisms still need to be elucidated[81,116]. HBx-mediated downregulation of tumor suppressors may be a factor leading to the development of HCC[115,117-119]. HBx-mediated deSUMOylation removes transcription factors from nuclear domain 10 (ND10), which in turn downregulates IFN-I-response pathway elements and host epigenetic modifications by p300/HDAC1, resulting in viral persistence[120]. Host gene promoters and enhancers can also be stimulated by HBx to regulate HBV replication[121-123]. Upregulation of host ST2 expression by HBx-GATA2 binding to its promoter and subsequent induction of the IL-33/ST2 axis stimulated a Th2 immune response, instead of the desired antiviral Th1 response[124], which is likely to play a role in the chronicity of infection[125].

As stated before, CREB is recruited by HBx to the cccDNA minichromosome; however, HBx also increases this activator's longevity[126]. By binding to protein phosphatase 1, the activating phosphorylation induced by cAMP has a longer half-life, resulting in increased viral transcription[126]. Interestingly, not only does HBx mediate recruitment of factors directly to the cccDNA, but also promotes spatial localization of the minichromosome. Shen *et al*[127] showed that HBx and the transcription factor Yin-Yang 1 (YY1) aligned the cccDNA minichromosome with a region of the human genome rich in a highly active enhancer element, resulting in increased HBV replication.

Negative regulation by HBx has also been shown through an interaction with peroxiredoxin 1 (Prdx1), a cellular hydrogen peroxide scavenger, which recruits exosome component 5 (Exosc5) to degrade HBV RNA. Although the ability of Prdx1 to bind to HBV RNA was independent of HBx, HBx was required for degradation of the RNA[128].

### **HBx-mediated ubiquitination**

Indirect epigenetic regulation can also occur through HBx-mediated proteasomal degradation of host factors, or by preventing degradation or deactivation of host factors. Damaged DNA-binding protein 1 (DDB1), an adapter protein for the cullin-RING ligase 4 (CRL4) E3 Ligase complex, is responsible for recruitment of DDB1-Cul4-associated factors (DCAFs), which allow for ubiquitination and subsequent degradation of proteins[129]. Interactions between HBx and DDB1 were implicated in early studies[130]. The interaction is mediated by a motif in HBx that mimics the DCAF proteins' binding motif, allowing HBV to hijack the host protein ubiquitination system[131]. Years later, Decorsière *et al*[132] and Murphy *et al*[133] identified the structural maintenance of chromosomes 5/6 (Smc5/6) complex as a target for HBx-mediated proteasomal degradation. The Smc5/6 complex is a host antiviral restriction factor that enables transcriptional repression of the cccDNA minichromosome, by localizing to the ND10 without inducing an innate immune response[134]. However, the epigenetic effect is transient as it does not silence HBx mRNA expression, ultimately leading to Smc5/6 degradation and initiation of viral transcription[135]. Like Smc5/6, other HBV restriction factors are degraded by HBx-mediated polyubiquitination, to increase viral transcription and HBV replication[136]. This includes ZEB2, which inhibits HBV replication by interaction with the core promoter[137], proteasome activator subunit 4 (PSME4), which induces degradation of acetylated histones involved in upregulation of HBV[138,139], and stromal interaction molecule 1 (STIM1), known to regulate cytoplasmic calcium levels[140].

On the other hand, HBx is also able to inhibit proteasomal degradation[141]. This has recently been shown for the WD repeat domain 5 protein (WDR5), a core subunit of H3K4 methyltransferase complexes[142]. HBx stabilized WDR5 by preventing DDB1/cullin-4-induced degradation and in turn, promoted viral gene expression[142]. Shen *et al*[143] demonstrated how HBx could also inhibit MDM2-mediated degradation of DExH-box RNA helicase 9 (DHX9) to enhance viral DNA synthesis.

The epigenetic regulation of viral transcription is further enhanced as a result of HBx-mediated *trans*-activation of its own promoter[144]. Along with this, post-translational modifications of HBx through interactions with the deubiquitinating ubiquitin-specific peptidase 15 (Usp15) enzyme, increase the half-life of the viral protein[145].

This may also be a result of the close interaction of HBx with components of the proteasome machinery, such as the DDB1-CRL4 complex, which may inadvertently result in ubiquitination of HBx itself. HDM2-mediated NEDDylation of HBx was also found to occur, probably because of its close proximity to Cul4, resulting in increased stability and chromatin localization[101,146]. NEDDylation-dependent reduction of ubiquitination and subsequent degradation of HBx by E3 Ligases, such as Siah-1, may account for the increased stability of this viral protein. MLN4924 (pevonedistat), a NEDD8-activating enzyme inhibitor, has been shown to impede HBV replication by reducing cullin[147] and HBx NEDDylation[146]. In addition, MLN4924 promotes upregulation of phosphorylated extracellular signal-regulated kinases (ERKs) resulting in reduced HNF1 $\alpha$ , HNF4 $\alpha$  and C/EBP $\alpha$  transcription factor levels[147], which are known activators of HBV transcription. Overall, the maintenance of persistent HBV infection by HBx contributes to HCC, by inducing host epigenetic modifications implicated in cancer.

### **HBc associates with cccDNA and host genes**

Direct interaction of the HBc CTD[103] with CpG islands across the cccDNA, especially CpG II, has been shown to induce hypomethylation and subsequent upregulation of viral transcription and regulation[74,148]. This hypomethylation causes increased CBP binding and resulting histone acetylation, further increasing viral gene expression[57,148] albeit at lower levels than with HBx[149,150]. The association of HBc with HDAC1 was also shown[148], as well as P300 and PCAF/CBP [151]. Increased NF- $\kappa$ B binding upstream of *ENII* induced by HBc is thought to increase pre-C promoter activity[152]. Host DNA polymerase coordinator PCNA, which is known to maintain genetic and epigenetic integrity[153], is recruited to cccDNA by HBc which upregulates viral gene expression and plays a role in the development of HCC. However, the exact mechanism of this process is still unclear [154].

HBc can additionally mediate host gene expression through the binding of HBc to endogenous promoter regions. Guo *et al*[104] identified nearly 3100 host promoter regions with potential HBc binding sites. Examples of these include binding to the MxA promoter to evade a host IFN-induced immune response[155] and competitive binding to BAF200, resulting in the downregulation of interferon-induced transmembrane protein 1 (IFITM1) mRNA[156]. Binding of HBc to the death receptor 5 (DR5) promoter reduced DR5 expression, thereby reducing cellular apoptosis through TRAIL which is thought to support CHB infection[157].

Transcription factor binding to mediate host expression has also been shown. The binding of HBc to E2F1 prevented its natural association with the p53 promoter, and hence decreased p53 levels[158]. This could reduce p53-related apoptosis[159] and along with DR5 downregulation further support the development of chronic infection. However, competitive binding of HBc to the receptor for activated protein kinase C 1 (RACK1) prevented phosphorylation of mitogen-activated protein kinase 7 (MKK7) which sensitized cells to apoptosis[160]. This suggests a dual role for HBc in the control of apoptosis and requires further study to determine the impact of these pathways on HBV infection. The subcellular location of HBc is dependent on cell cycle phase, with *in vitro* studies showing that predominantly nuclear localization occurs during G1 phase[161]. Since E2F transcription factors and p53 both have effects on cell cycling[162-164] and are differentially regulated during HBV infection, further investigation of this phenomenon and the possibility of epigenetically controlling the cell cycle is needed. HBc has also been shown to recruit APOBEC3A/B to cccDNA to promote deamination[165]. Since so many HBc binding sites are predicted to occur in endogenous promoter regions, there is the possibility that APOBEC3A/B is recruited to host genes to influence their expression, and may play a role in the development of HCC[166].

## **REGULATORY NONCODING RNAS**

Noncoding RNAs (ncRNAs) consist of small ncRNAs (< 200 nt) and long ncRNA (lncRNA > 200 nt)[167]. Small ncRNAs, including miRNAs, are involved in post-transcriptional gene silencing[167], while lncRNA are implicated in a plethora of host functions including post-transcriptional and chromatin modifications[168]. By altering the host's epigenetic signature, HBV dysregulates the ncRNA landscape to control viral replication and influence hepatocarcinogenesis.



### Epigenetic influences of miRNAs

miRNAs have been widely studied in HBV infection, and have been shown to facilitate or inhibit viral replication[169]. An increase in replication can occur by targeting host factors that normally restrict viral replication, for example miR-15b downregulates HNF-1 $\alpha$ [170], or by indirectly upregulating factors to promote replication, such as miR-1 upregulation of farnesoid X receptor  $\alpha$  (FRX $\alpha$ ) expression[171]. Interestingly, miR-1-mediated downregulation of E2F5 and HDAC4 was implicated in G1 cell cycle arrest and upregulation of hepatocyte differentiation factors, to increase HBV replication[171]. This may be related to other viral epigenetic factors such as HBc, which showed similar associations. Indirect FRX $\alpha$  upregulation by miR-449a targeting of CREB5[172], HDAC4 downregulation by miR-548ah[173] and G1 arrest by miR-125b-5p-mediated inhibition of retinoblastoma protein phosphorylation[174] implicate redundant epigenetic pathways in HBV regulation.

A recent study by Moon *et al*[175] has suggested that miR-20a can act in an epigenetic manner and promote methylation of cccDNA. They found that AGO2-miR-20a binding to the cccDNA may recruit DNMT3 to silence gene expression; however, further studies are needed to confirm the exact mechanism. Interestingly miR-146a could play a role in cccDNA formation, through a positive feedback loop with FEN1[176], and in silencing ZEB2 expression[177].

Induction of autophagy has been associated with many miRNAs including miR-146a-5p[178], the miR-99 family[179] and miR-192-3p, which is downregulated by HBx[180]. miR-155 has also been implicated in autophagy; however, this miRNA is downregulated following HBV infection[181]. HBV upregulates expression of miR-192-5p and miR-215-5p, which results in downregulation of apoptosis[182]. Contrasting effects were observed during HBV-mediated upregulation of miR-194-5p, which resulted in the downregulation of anti-apoptotic proteins SODD and cFLIP and sensitization of liver cells to apoptosis[182]. Once again, the role of HBV in promoting and preventing apoptosis needs further clarification.

Certain cancer-related miRNAs, namely miR-15a/miR-16-1, the miR-17-92 cluster and miR-224, are also associated with decreased viral replication through modification of cccDNA promoters and histones[183]. Interestingly, miR-122, which targets the HBV polymerase ORF and *core* 3'UTR[184], has also been shown to inhibit HBV by targeting cyclin G1 to increase p53-mediated inhibition of replication[185]. However, HBx abrogates these antiviral effects by downregulating miR-122[183]. Host miRNAs can also indirectly inhibit viral replication by regulating various host factors[169] HBx-mediated downregulation of miR-122 alters regulation of its natural host targets, including heme oxygenase-1[186], CCNG1 and NDRG3[187]. Similarly, miR-141 was implicated in targeting both PPARA[188] and SIRT1[189]. Reduced SIRT1 inhibited autophagy, and miR-130a targets liver pyruvate kinase which is thought to reduce energy supply and hence HBV replication[190]. Induction of immune suppression may occur through the increase of miR-199a-5p, miR-221-3p and Let-7a-3p, as shown in immune tolerant HBV-infected patients[191]. HBV-miR-3, a miRNA produced by the virus, reduces replication by directly targeting viral transcripts[192] or upregulating the anti-HBV IFN immune response[193].

### Newly discovered lncRNAs implicated in the control of HBV

lncRNAs have been identified as important polyfunctional epigenetic regulators and are associated with disease progression, particularly carcinogenesis[194]. Novel lncRNAs related to development of HBV-associated HCC are continuously being identified; however, a few have also been implicated in epigenetic regulation of viral transcription. lncRNAs highly up-regulated in liver cancer (HULC), DLEU2, and lncRNA 32 have been identified as epigenetic regulators of HBV. HAT1, which is co-activated by HBx-Sp1 binding, is transported to the cccDNA by HULC in an HBc-dependent manner[195]. HULC also mediates upregulation of HBx and the subsequent HBx-STAT upregulation of miR-539, which targets APOBEC3B. This results in reduced cccDNA degradation, with resultant increased transcription of viral genes, viral persistence, and HCC progression[196]. Interestingly, the promotion of HBV transcription by the lncRNA HULC is dependent on both HBx and HBc, which forms a positive feedback loop[195]. In the case of lncRNA DLEU, HBx both upregulates its expression and is co-recruited with it to the cccDNA, where the complex may bind to the histone methyltransferase EZH2[197]. Computational analysis of this interaction suggests that binding alleviates EZH2 transcriptional repression[197]. lncRNAs have also been implicated in the host antiviral response. To increase IFN-stimulated gene (ISG) expression, lncRNA 32 binds to activating transcription factor 2 (ATF 2) and promotes gene expression. However, during HBV



infection, the expression of this lncRNA is reduced[198], ultimately dampening the immune response to the virus.

## ANTI-HBV EPIGENETIC THERAPY

A number of epigenetic strategies has been identified as having therapeutic potential for CHB[27]. Some promote the host's natural antiviral defense mechanisms while others aim to target specific pathways involved in the epigenetic control of cccDNA.

### *IFN- $\alpha$ therapy*

The antiviral and epigenetic properties of IFN- $\alpha$  therapy are well established[199], and have been adopted as a strategy to treat CHB[5]. Type 1 IFNs interact with the IFN- $\alpha$ / $\beta$  receptor complex to modulate transcription of ISGs, ultimately to evoke strong innate immune responses against viral infections[200]. Administration of IFN- $\alpha$  to HBV-infected cells and HBV-infected chimeric uPA/SCID mice resulted in the inhibition of viral replication through epigenetic regulation of cccDNA[105]. Hypoacetylation of cccDNA-bound histones was achieved following the active recruitment of histone deacetylases hSIRT1 and HDAC1, methyltransferase EZH2, and the transcriptional repressor YY1[199]. Binding of transcription factors STAT1 and STAT2 to the IFN- $\alpha$  sensitive response element on active cccDNA was shown to be reduced after IFN- $\alpha$  treatment[199]. More recently, the structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1) and promyelocytic leukemia protein have, along with STAT1, been implicated in the stimulation of cccDNA-associated histone PTMs in response to IFN- $\alpha$ [201]. Furthermore, IFN- $\alpha$ -based treatment may regulate the SMC5/6 complex to promote transcriptional repression of cccDNA[202].

When comparing IFN- $\alpha$  treatment to the effects of C646 (a small molecule epigenetic modifying agent that inhibits p300/CBP), Tropberger *et al*[86] demonstrated that C646 reduces HBV transcription in primary human hepatocytes in a dose-dependent manner. Like IFN- $\alpha$ , C646 treatment reduced the levels of active PTMs but without activating the innate immune response. However, neither treatment reduced cccDNA levels[86]. When combined with lymphotoxin- $\beta$  receptor activation, IFN- $\alpha$  treatment did eliminate cccDNA in an APOBEC-dependent manner[165]. Yet the clinical utility of currently licensed IFN- $\alpha$ -based treatments has proved tricky because some patients respond to therapy, but others show no benefit. High costs and adverse side effects have also limited the use of IFN- $\alpha$ . Alternatives are being investigated and an iterative approach has led to the identification of new IFNs with increased potency, such as the recently described IFN- $\alpha$  14[203].

### *Small molecule inhibitors and epigenetic drugs*

Epigenetic therapies and immunomodulators have shown promise as anti-HCC agents [204], of which HDAC inhibitors such as 5-Azacytidine (5-Aza) and the EZH2 inhibitor 3-Deazaneplanocin A (DZNep) have recently been explored as combination therapies [205]. Such therapies have also been proposed for the treatment of CHB to silence the cccDNA minichromosome. Two small molecule inhibitors, AGK2 and GS-5801, are currently being investigated as anti-HBV epigenetic drugs[206,207]. AGK2 is an inhibitor of the SIRT2 deacetylase, while GS-5801 prevents erasing of epigenetic marks by lysine demethylase 5 (KDM5). Both have been shown to repress viral replication; however, preliminary results from a single dose of GS-5801 suggest that H3K4me3 may not be permanently associated with cccDNA[207]. Recently Dicoumarol, an inhibitor of NAD(P)H:quinone oxidoreductase 1 (NQO1), has been shown to promote the silencing of cccDNA[208] indirectly. The epigenetic effect of Dicoumarol is thought to arise from the destabilization of the HBx/NQO1 interaction, as the upregulation of NQO1 was shown to increase the half-life of HBx[208]. Although promising, the feasibility of using epigenetic modulators to silence HBV is complex, especially as the virus employs many normal host factors to regulate gene expression. The influence on development of HBV-related HCC and viral integration are additional important considerations for advancing epigenetic therapy. For instance, treating HCC with HDAC inhibitors is likely to promote viral transcription[57], while epigenetic silencing cccDNA gene expression by increasing DNA methylation may promote hepatocarcinogenesis[209].

### Targeted epigenetic gene silencing

Epigenome engineering has become a popular targeted gene therapy approach to promote locus-specific epigenetic changes to treat genetic diseases[24]. Effector domains based on a variety of transcriptional activators, repressors, and chromatin remodeling enzymes including the typical writers, readers and erasers (Table 1) have been fused to DNA binding domains (DBD) to promote site-specific modifications. The targeting capabilities of epigenome engineering tools could overcome the non-specific effects epigenetic drugs may have on host gene expression. As such, cccDNA epigenome editors are currently being developed as a novel antiviral gene therapy for CHB (Figure 2).

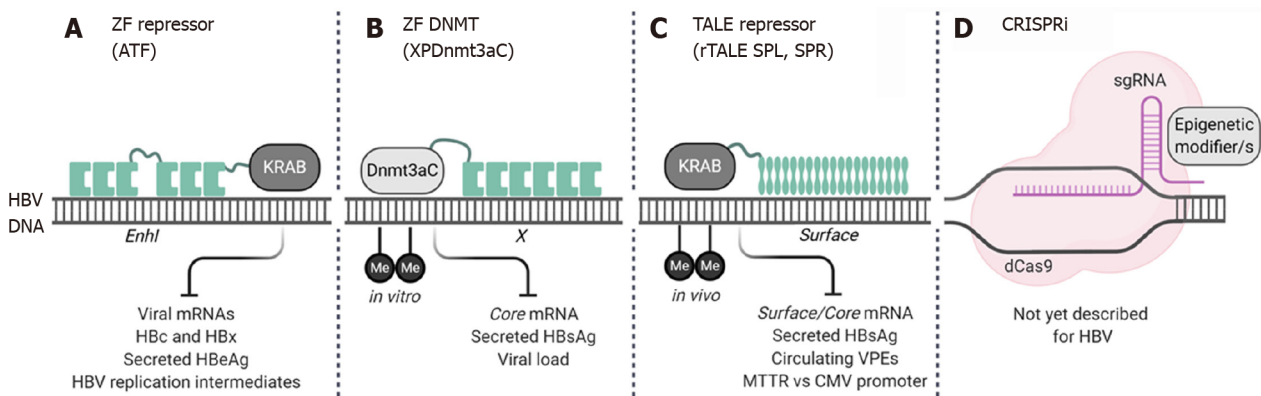
ZF and transcription activator-like effector (TALE) DBDs have been used to direct different epigenetic effectors, primarily transcriptional repressors, to the HBV genome [210-213]. In 2013, Zhao *et al* [210] described the first ATF which was designed to target an 18 bp region of the HBV X or enhancer I (EnhI) sequence. The ATF was generated by joining six ZF DBDs to the Krüppel-associated box (KRAB) repression domain. Although not tested in a typical HBV replication model, the ATF achieved repression of the HBx promoter in a Hep3B reporter cell line, although accompanying cellular growth arrest was also observed[210]. Building on this study, Luo and colleagues demonstrated the antiviral activity of their EnhI-specific ATF in HepG2.2.15 cells and transgenic mice[211]. Viral replication was inhibited, in this case without affecting cellular growth, despite both models harboring integrated HBV sequences. Interestingly, the EnhI ZF-array alone achieved transcriptional repression, an effect that has previously been reported when using anti-HBV TALENs[214]. The antiviral effect of the ATF was further confirmed in the HBV transgenic mouse model where reductions in viral DNA, HBcAg and HBeAg were observed[211]. By replacing the repressor domain with a methyltransferase, ZF arrays have also been used to achieve targeted methylation of viral DNA. Xirong *et al* [213] fused the C-terminal region of the DNMT3A to a six-finger ZF (XPDnmt3aC) and tested the epigenome editor in HepG2 cells and HBV transgenic mice. The XPDnmt3aC was designed to bind to the HBx promoter region and achieved *de novo* methylation of the HBV DNA in HepG2 cells which was accompanied by a reduction in markers of viral replication. However, in transgenic mice the antiviral effect lasted for about two weeks[213], suggesting that if epigenetic modification was achieved it was not permanent.

Anti-HBV repressor TALEs (rTALEs) are engineered proteins with DBDs derived from the *Xanthomonas* bacteria which have been fused to the KRAB effector domain [212]. Two rTALEs, SPL and SPR, which were designed to bind to the surface ORF were tested in both *in vitro* and *in vivo* models of viral replication. Both the SPL and SPR rTALEs inhibited HBV replication, and significant reductions in HBsAg, viral mRNAs, and circulating viral particles were observed[212]. Importantly, a comparison between the liver specific mouse transthyretin (MTTR) or ubiquitously active CMV promoters was performed. The MTTR conferred liver-specific expression which may improve the safety of this epigenome engineering approach. Quantitative DNA methylation analysis using EpiTYPER™ Technology[215] was used to show that increased targeted methylation of intrahepatic HBV DNA was achieved at CpG II [212]. This confirmed the principle of this epigenome engineering approach; however, further validation of these rTALEs on cccDNA is warranted.

Interestingly, despite widespread studies using CRISPR/Cas gene editing technologies to disrupt cccDNA, the alternative epigenome editing platforms have yet to be investigated. CRISPRi and CRISPRa are RNA-guided epigenome modifiers that can repress or promote gene expression, and are generated by fusing single or multiple effector domains to a dead (nuclease deficient) Cas[216]. This means that site-directed epigenetic modifications can be achieved by designing guide RNAs to bind to the cccDNA. Although not yet reported, the field of CRISPR epigenome engineering is constantly expanding and may present a novel way of silencing cccDNA.

## CONCLUSION

The epigenetic regulation of the cccDNA minichromosome to either promote or repress viral transcription involves an intricate association between host cell factors and viral proteins that is not yet fully understood. While there is currently debate around whether HBV should be considered a stealth virus[217], it is clear that it can manipulate the host cells replication, transcription and translation machinery for its own benefit. HBx appears to have a key role in viral persistence, not only as a regulator of cccDNA but also as a modulator of RNAs, ubiquitination, apoptosis, and



**Figure 2 Novel epigenome engineering tools that target hepatitis B virus DNA.** Three different types of epigenetic editors have been designed to silence viral gene expression. The Zinc finger (ZF) repressor-based artificial transcription factors (ATF) comprise two three-finger ZF DNA binding domains (DBD) which are fused, with a C-terminal linker peptide, to the Krüppel-associated box (KRAB) repressor domain. ATFs were designed to target the *EnhI* region (A). A ZF-based methyltransferase (DNMT) designed to target the *X* region (XPDnmt3aC). The XPDnmt3aC comprises a six-finger ZF DBD fused to the catalytic domain of Dnmt3a (Dnmt3aC). Methylation of the viral DNA was achieved *in vitro* (B). To generate transcription activator-like effector (TALE) repressors, TALE DBDs targeting two regions of *surface* were fused to the KRAB repressor domain (N-terminal). *In vivo*, methylation of intrahepatic viral DNA was shown (C). Although not yet described for HBV, the CRISPRi epigenome editing tools provide a novel means of silencing covalently closed circular DNA. RNA guides (sgRNA) targeting the viral genome could be used to recruit a dead Cas (dCas9) with one or more epigenetic modifying domains (D). Created with BioRender.com. HBV: Hepatitis B virus; ZF: Zinc finger; ATF: Artificial transcription factor; HBx: Hepatitis B virus X protein; HBc: Hepatitis B virus core protein; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; TALE: Transcription activator-like effector; MTTR: Mouse transthyretin; CMV: Cytomegalovirus; VPE: Viral particle equivalent.

antiviral responses. Although these intricate host-viral pathways require further clarification, the research to date has helped to identify possible feedback loops which could act as new targets for anti-HBV therapy.

There are conflicting views regarding the involvement of certain proteins, for example the epigenetic and deacetylase-independent roles of SIRT1. While some factors may have dual roles, the model systems used to evaluate epigenetic regulation of HBV need to be considered carefully. Although CHB is associated with HCC, epigenetic therapies designed to achieve a functional cure are likely to be given prior to the development of cancer. Using different liver cancer cell lines may skew the HBV epigenetic landscape as regulation of the host cells' epigenome and gene expression is irregular. Establishing an ideal model system is however, easier said than done. Infectious primary human hepatocyte-based models require specialized facilities, as for example is required for the generation of humanized mouse models[218]. Similarly, studies on primary human hepatocytes and three-dimensional liver cultures are costly. Viruses infecting small animals, like the DHBV and woodchuck hepatitis virus, which are commonly used to test novel therapies, may also pose problems for analysis of the cccDNA epigenome. The different effects that HDAC inhibitors had on HBV and DHBV infection suggest differences between the hosts' endogenous epigenetic pathways. Standardizing cccDNA quantification using droplet digital polymerase chain reaction and the liver biopsy-adapted chromatin immunoprecipitation (ChIP)-quantitative polymerase chain reaction technique (micro-ChIP) may help detect low levels of cccDNA as well as measure epigenetic marks[219].

Epigenetic drugs, improved interferon therapies, and epigenome editing tools are at an interesting stage of development, as new ways to silence cccDNA transcription. Accomplishing targeted epigenetic modifications and long-term viral suppression will be important, and consideration of the HCC epigenetic profile will need careful evaluation. Integrated viral sequences may also be amenable to cccDNA-specific epigenome editing, and undesirable off-target effects on the host genome need to be carefully assessed. Next-generation sequencing platforms like RNA-Seq would help to establish transcriptome profiles and identify potential off-target sites. To date only four targeted epigenome editors have been investigated, and although action on HBV DNA has been shown, a direct effect on the cccDNA minichromosome is yet to be established. Furthermore, as with designer nucleases, liver-specific delivery[19] and immune responses to the foreign TALE and Cas proteins will need to be addressed [220]. Delivering epigenome editors as *in vitro* transcribed mRNA transcripts would avoid the packaging constraints of viral vectors[10]. Despite the current challenges associated with epigenetic therapies, there is potential for such an approach to achieve cccDNA gene silencing and perhaps cure. As this relatively new field of research continues to grow, overcoming the hurdles could lead to a promising class of new

antivirals.

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## COVID-19-associated diarrhea

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### Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) recently emerged as a highly virulent respiratory pathogen that is known as the causative agent of coronavirus disease 2019 (COVID-19). Diarrhea is a common early symptom in a significant proportion of patients with SARS-CoV-2 infection. SARS-CoV-2 can infect and replicate in esophageal cells and enterocytes, leading to direct damage to the intestinal epithelium. The infection decreases the level of angiotensin-converting enzyme 2 receptors, thereby altering the composition of the gut microbiota. SARS-CoV-2 elicits a cytokine storm, which contributes to gastrointestinal inflammation. The direct cytopathic effects of SARS-CoV-2, gut dysbiosis, and aberrant immune response result in increased intestinal permeability, which may exacerbate existing symptoms and worsen the prognosis. By exploring the elements of pathogenesis, several therapeutic options have emerged for the treatment of COVID-19 patients, such as biologics and biotherapeutic agents. However, the presence of SARS-CoV-2 in the feces may facilitate the spread of COVID-19 through fecal-oral transmission and contaminate the environment. Thus gastrointestinal SARS-CoV-2 infection has important epidemiological significance. The development of new therapeutic and preventive options is necessary to treat and restrict the spread of this severe and widespread infection more effectively. Therefore, we summarize the key elements involved in the pathogenesis and the epidemiology of COVID-19-associated diarrhea.

**Key Words:** COVID-19; Diarrhea; Ionic imbalance; Viroporin; Angiotensin-converting enzyme type 2; Leaky gut

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**Core Tip:** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) replicates in enterocytes, triggers ionic imbalances, activates the NLRP3 inflammasome pathway, induces apoptosis, and exerts a dual effect on the autophagic process. These effects of SARS-CoV-2 lead to the development of leaky gut. Increased permeability triggers the absorption of lipopolysaccharide into the circulation, further exacerbating inflammation induced by viral infection. In addition to drugs that affect the inflammatory response and viral replication, agents targeting autophagy and apoptosis appear to be potentially suitable for the treatment of coronavirus disease 2019 (COVID-19). The fecal-oral route of SARS-CoV-2 transmission calls for strict and more consistent adherence to hygiene rules to prevent the spread of COVID-19.

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## INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) recently emerged as a highly virulent respiratory pathogen that is known as the causative agent of coronavirus disease 2019 (COVID-19)[1]. SARS-CoV-2 enters the human body through the airways and multiplies in the lungs. This novel coronavirus causes mild, severe, and critical respiratory disease in 81%, 14%, and 5% of cases, respectively[2]. It may also enter the bloodstream, which results in viremia and systemic spread throughout the body.

In addition to the airways, the virus can multiply in the gastrointestinal (GI) tract (GIT), urinary tract, and central nervous system. The infection elicits an intemperate immune response characterized by a life-threatening cytokine storm and a corrupted interferon (IFN) system, which is unable to eliminate the pathogen effectively. As a result, a systemic inflammatory response syndrome occurs[3,4]. In the severe and critical clinical manifestations of COVID-19, atypical pneumonia leading to progressive respiratory failure develops[2].

As of the 13<sup>th</sup> of January 2021, about 90 million people have been infected, and nearly 2 million people have died during the COVID-19 pandemic[5]. Although the leading COVID-19 symptoms are due to involvement of the respiratory system, it often causes GI symptoms as well. Thus, we examined the current state of knowledge on the pathogenesis, occurrence rate, clinical significance, and epidemiological consequences of COVID-19-associated diarrhea.

## TAXONOMIC CLASSIFICATION, STRUCTURE, AND REPLICATION OF SARS-COV-2

SARS-CoV-2 belongs to the genus *Betacoronavirus* of the family *Coronaviridae*, which comprises enveloped viruses with positive-sense single-stranded RNA genomes[1,6,7]. The spherical or elliptical virions are pleomorphic with diameters of 80-160 nm[8,9]. The capsid has helical symmetry, which is built up by the nucleocapsid (N) protein. The spike (S), membrane (M), and envelope (E) proteins are located in the virion envelope[10]. The S protein forms protrusions of 20 nm in length that provide a characteristic crown-like appearance, which is reflected in the name of the viral family. The S-protein is responsible for binding to the cell surface receptor[10].

Besides the S, E, M, and N structural protein genes, the genome of SARS-CoV-2 contains open reading frames (Orfs) that encode nine accessory proteins (3a, 3b, 6, 7a, 7b, 8, 9b, 9c, and 10) and two polyproteins (pp1a and pp1ab)[10-15]. Polyproteins pp1a and pp1ab are cleaved by viral proteases to form unique non-structural proteins (Nsp), which play an important role in viral replication[10]. Although the accessory proteins of SARS-CoV-2 are not essential for viral multiplication, they are implicated in the pathogenesis[10].

As the first step in infection, the S protein of SARS-CoV-2 binds to its corresponding cell-surface receptor, angiotensin-converting enzyme type 2 (ACE2)[16,17]. The S protein has two subunits: S1 and S2. The S1 subunit has a receptor-binding domain and is responsible for receptor engagement, whereas the S2 subunit is involved in the fusion process[17-19]. Following ACE2 binding, cellular proteases such as transmembrane protease/serine subfamily member 2 (TMPRSS2), TMPRSS4, and cathepsin L cleave S protein into S1 and S2 subunits, and the virus enters the host cell by receptor-mediated endocytosis[17,19-23]. Other proteases have also been shown to be able to cleave S protein, including furin, trypsin-like proteases, elastase, plasmin, and factor Xa. This suggests that these enzymes may also facilitate entry or expand the tissue tropism of SARS-CoV-2[23-27].

Within the endosome, cathepsin-mediated activation of the S protein continues, eventually causing the S2 subunit to gain a fusogenic effect that triggers the fusion of the viral envelope and the endosomal membrane[25]. The nucleocapsid is then released into the cytoplasm, where the translation of Orf1a and Orf1b results in the formation of pp1a and pp1ab, from which Nsp1-16 are generated by proteolysis. Nsp12 functions as an RNA-dependent RNA polymerase and associates with Nsp7 and Nsp8 to form the core of the replication and transcription complex (RTC) of SARS-CoV-2[28,29]. The cofactors Nsp7 and Nsp8 form a hexadecameric ring structure that has a primase function and generates RNA primers for the synthesis of the negative-sense RNA[28,29].

RTC synthesizes the genomic RNA and a set of SARS-CoV-2 mRNAs through full-length and subgenomic negative-sense RNA intermediates[28,29]. The replication of the viral genome and transcription of viral genes takes place in double-membraned vesicles[30,31]. The SARS-CoV-2 replication compartment provides a protected environment which inhibits the antiviral effects of IFN and other cellular antiviral defense mechanisms by hiding the viral genome, transcripts, and replicative intermediates from cellular nucleic acid sensors.

The viral mRNAs are translated in the rough endoplasmic reticulum, leading to the formation of accessory proteins and structural proteins (N, M, E, and S). The M, E, and S proteins then become embedded in the endoplasmic reticulum, whereas the N proteins assemble with the newly synthesized full-length positive-sense RNA to form the nucleocapsid[30,31]. After being transported to the ERGIC (endoplasmic reticulum-Golgi intermediate compartment), the nucleocapsids bud through the ERGIC membrane into its lumen[30,31]. The mature virions reach the cytoplasm membrane *via* vesicular transport and are released from the cell[30,31].

## THE MAIN CELLULAR EFFECTS OF SARS-COV-2

During multiplication, SARS-CoV-2 modulates several cellular aspects, including signaling, transcription, translation, cell division, the IFN system, autophagy, and apoptosis, as well as the biogenesis, function, and morphology of mitochondria and intracellular vesicles. Phosphoproteomic profiling has revealed that SARS-CoV-2 infection affects the activity of 97 kinases. The activities of several members of the p38 pathway and the guanosine monophosphate-dependent protein kinases are upregulated, while cell cycle kinases (CDK1/2/5), cell growth-related signaling pathway kinases (AKT1/2), and regulators of the cytoskeleton are down-regulated[32]. The functional changes in the signal transduction pathways have been shown to play an important role in SARS-CoV-2-induced cytoskeletal damage, cytokine production, and slow-down in cell proliferation at the S/G2 transition phase[32].

Transcriptomic profiles of SARS-CoV-2-infected primary human bronchial epithelial cells, lung biopsy, and bronchoalveolar lavage fluid samples of COVID-19 patients have demonstrated upregulated expression of genes implicated in metabolism, immunity, and the stress responses of the endoplasmic reticulum and mitochondria[33-35]. It has been shown that the M protein, Nsp7, and ORF9c stimulate lipogenesis, while Nsp7, Nsp12, and ORF8 trigger endoplasmic stress response, and Nsp7 induces mitochondrial dysfunction[34]. Moreover, the M and E proteins, along with Nsp3a, Nsp6, Nsp8, Nsp10, and Nsp13, were shown to be able to modify the structure and function of the endomembrane system and vesicle trafficking, thereby facilitating several steps of viral multiplication[36].

Interestingly, the expression of genes involved in the humoral immune response and innate immune response-activating signal transduction are increased, whereas genes implicated in cytokine-mediated signaling pathways are down-regulated[33]. A multiplex gene expression analysis showed that the genes involved in type I IFN

signaling were highly up-regulated, whereas the expression of IFN-stimulated genes (*ISGs*) was decreased in severe COVID-19 patients[37]. The levels of pro-inflammatory cytokines measured in sera of COVID-19 patients were highly increased in a pattern corresponding to a cytokine storm[38-40].

Consistent with this observation, transcriptional activation of pro-inflammatory cytokine genes was also detected in peripheral blood mononuclear cells and broncho-alveolar lavage fluid[41]. The sera and lung tissue samples of patients have shown interleukin (IL)-1 $\beta$ , IL-6, IL-10, IL-18, IL-33, transforming growth factor- $\beta$ , IFN- $\gamma$ , CSF2/GM-CSF (colony-stimulating factor 2/granulocyte-macrophage colony-stimulating factor), CSF3/G-CSF, CC chemokines [CCL2/MCP-1, CCL3/MIP-1A, CCL4/MIP-1B, CCL5/RANTES, CCL8, CCL3L1] and CXC chemokines [CXCL1, CXCL2 and CXCL10/IP10][38,39,41,42]. However, during SARS-CoV-2 infection, the production of type I and III IFNs is decreased[37,43]. Thus, these data clearly demonstrate that SARS-CoV-2 infection alters both the transcriptional and translational patterns in cells profoundly[32-43].

Other observations indicate that SARS-CoV-2 could trigger several cell-death processes, including apoptosis, necrosis, pyroptosis, and anoikis, depending on the type of cell[44-47]. The death of infected cells may contribute to tissue damage and induce an inflammatory reaction[44-47]. It has also been revealed that SARS-CoV-2 Orf 3a stimulates the formation of the autophagic Beclin-1-Vps34-Atg14 complex while simultaneously inhibiting the Beclin-1 complex containing the UVRAG adaptor protein[48]. Orf 3a thereby exerts a dual effect on the autophagic process manifesting in the induction of the initial steps and a block in the fusion of the autophagosomes with lysosomes[48].

## DIARRHEA IN COVID-19

GIT involvement is frequent in COVID-19 patients and includes anorexia, nausea, vomiting, diarrhea, and abdominal pain[49-62]. Among the specific GI symptoms, diarrhea is the most common. Based on different studies, the prevalence of diarrhea might range from 2% to 49.5%[50,61,63]. COVID-19-associated diarrhea is characterized by loose or watery stools and is usually mild, self-limiting, and can even be the only symptom of the infection[49,52,58,59,63]. The average frequency of bowel movements is in the range of 3.3-4.3 times per day[53,58], and the average duration of diarrhea is 3-5.4 d[52,53,58,59,63]. In some cases, however, diarrhea is more severe, with patients experiencing more frequent bowel movements of up to 18-30 times per day[58,63].

Rare cases with more severe GI symptoms have also been reported, such as acute hemorrhagic colitis and GI bleeding[53,54,64]. Furthermore, the relationship between GI symptoms and the severity of the disease has been investigated. Statistically significant differences were not observed between COVID-19 patients with and without GI symptoms in clinical severity, length of hospital stay, and mortality rate[49, 59,60]. SARS-CoV-2 mRNA could be detected in the stool of COVID-19 patients in 22%-54.5% of cases, and occasionally, the virus is detectable in the stool even after the airway samples become negative[54,57,58,65-69]. Positive results have been obtained from real-time reverse transcriptase-polymerase chain reaction (Rt-PCR) tests of stool even in patients without GI symptoms[58]. In patients with GI symptoms, the total time between the onset of symptoms and viral clearance is significantly longer than in those with only respiratory manifestations[58,70].

The reason why GI symptoms occur in only a subset of COVID-19 patients is currently unknown. There are no significant differences between the two patient groups in terms of demographics and certain coexisting conditions, such as pregnancy, cancer, chronic renal disease, chronic obstructive pulmonary disease, or immunosuppression. A study conducted by Jin *et al*[52] revealed that the rate of chronic liver disease in COVID-19 patients with GI symptoms is much higher than among those without GI symptoms. Moreover, the incidence of COVID-19 with GI symptoms displays familial clustering[52]. Based on these interesting observations, it is reasonable to infer that genetic, immunological, and epidemiological factors are involved in the development of COVID-19-associated diarrhea.



## PATHOGENESIS OF COVID-19-ASSOCIATED DIARRHEA

ACE2, the cellular receptor of SARS-CoV-2, is widely expressed in many types of cells and tissues of the GIT, including the esophagus, stomach, small intestine, colon, rectum, pancreatic exocrine glands and islets, and gallbladder[71]. The expression level of ACE2 in the GIT is highest in the ileum epithelial cells, especially in the absorptive enterocytes[72]. It has also been demonstrated that ACE2 is co-expressed with TMPRSS2/4 proteases in the GIT, with the highest level in the ileum[73]. These observations indicate that several cell types in the GIT are potentially susceptible to SARS-CoV-2 infection[71-73].

Studies demonstrating that viral RNA can be detected in the stool samples of COVID-19 patients indicate that SARS-CoV-2 can indeed infect the GIT[54,57-59,66-68]. It is estimated that feces and GI tissues contain  $10^4$ - $10^8$  and  $10^0$ - $10^4$  RNAs per gram, respectively[74,75]. Further studies revealed that SARS-CoV-2 establishes a productive infection in intestinal epithelial cells and human small intestinal organoids, leading to the production of new infectious progeny virions[20,76]. Viral particles within intracytoplasmic vesicles and aggregates of SARS-CoV-2 virions attached to the surface of enterocytes have been detected in intestinal organoids and post-mortem GIT samples from COVID-19 patients by electron microscopy[76,77]. These observations indicate that the GIT can be an entry site and an extra-pulmonary target organ of SARS-CoV-2[20,71-73,76,77].

Further analyses were performed to determine whether infectious viruses are present in the GIT or feces. In most cases, efforts to cultivate infectious SARS-CoV-2 from feces have failed, although Xiao *et al*[78] recently reported the successful isolation of the virus from stool samples by using the Vero E6 cell line. Simulated large intestinal fluid was shown to reduce the infectivity of the virus significantly[20]. Thus, it is possible that most of the virus that multiplies in the enterocytes may be inactivated in the lumen of intestines within a short time after release.

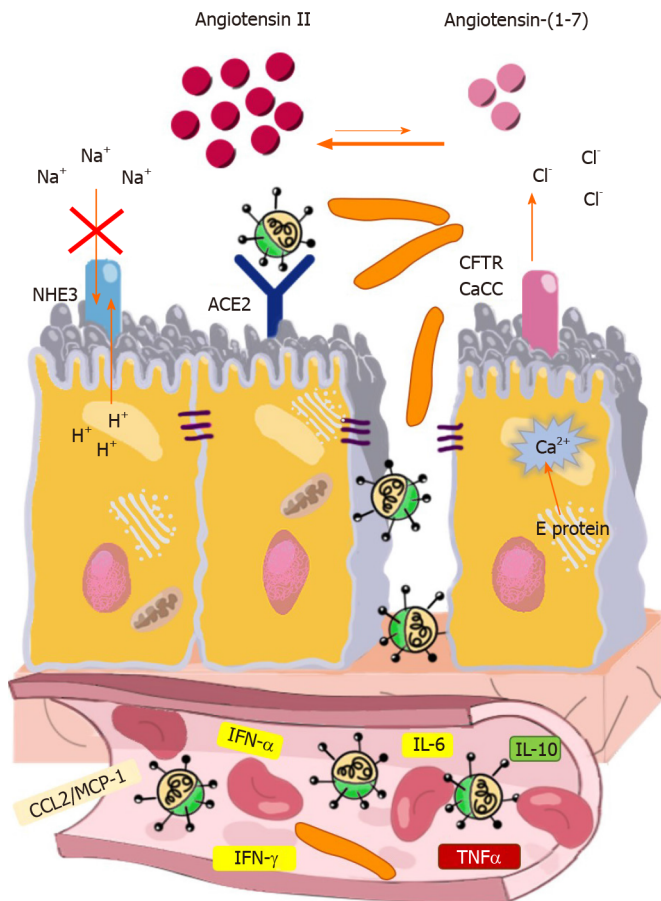
*In vitro* cultivation of SARS-CoV-2 has demonstrated that this virus elicits a cytopathic effect (CPE) on some cell lines, whereas in other cell types, no cytomorphological abnormalities could be observed despite efficient viral replication[79]. In human airway epithelial cells, SARS-CoV-2 causes CPE characterized by the formation of multinucleated syncytia and cilium shrinking, and cell death largely occurs by way of apoptosis[45]. In contrast, the colorectal adenocarcinoma Caco-2 cell line proved to be susceptible to infection, but the multiplication of SARS-CoV-2 was not accompanied by a visible CPE[79]. Likewise, intense tissue damage was not observed in the GIT of COVID-19 patients[80].

SARS-CoV-2 can establish a persistent infection in human C2BBel intestinal cells expressing a brush border[81]. Moreover, SARS-CoV-2 was shown to be more effective in inducing the production of IFN- $\alpha$ , IFN- $\beta$ , IFN- $\lambda$ 1, IFN- $\lambda$ 2, and IFN- $\lambda$ 3 in human intestinal tissues *ex vivo* than in lung tissue[80]. Therefore, it is also conceivable that a specific immuno-inflammatory environment develops in the lungs and GIT as a result of infection, which affects the rate of viral replication and cell demise in different ways.

Although SARS-CoV-2 causes no extensive tissue damage in the intestines, the infection seems to harm the enterocytes in a much more sophisticated way. E protein was shown to bind to the tight junction-associated PALS1 (Proteins Associated with Lin Seven 1)[82]. PALS1 interacts with PATJ (PALS1-Associated Tight Junction protein) and CRB3 (Crumbs 3), and the PALS1/PATJ/CRB3 complex that forms is essential for the maintenance of tight junctions connecting epithelial cells[83]. E protein causes functional impairment of PALS1 and interferes with the formation of tight junctions, leading to the disruption of intestinal barrier integrity[82]. By using a biomimetic gut-on-chip system, Guo *et. al.* elegantly demonstrated that SARS-CoV-2 infection destroys tight junctions and adherent junctions in both the endothelium and intestinal epithelium, which in turn may lead to leaky gut syndrome, local and systemic invasion of normal microbiota members, and immune activation[84] (Figure 1).

The E protein of SARS-CoV-2 is a single-spanning membrane protein that forms a homopentameric ion channel, which displays selective permeability for monovalent ions ( $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$ ) and  $\text{Ca}^{2+}$ [85]. E protein accumulates in the endoplasmic reticulum and ERGIC/Golgi membranes and transports  $\text{Ca}^{2+}$  from these compartments to the cytoplasm. Elevated cytoplasmic  $\text{Ca}^{2+}$  concentration can increase the rate of apical  $\text{Cl}^-$  exit across the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels and cyclic-nucleotide-activated cystic fibrosis transmembrane conductance regulator[86].

SARS-CoV-2 also has another ion-channel protein, Orf3a, which is a  $\text{K}^+$  ion channel viroporin that exhibits plasma membrane and endomembrane localization[46,87]. Orf3a in the cytoplasmic membrane may cause leakage of  $\text{K}^+$  ions from enterocytes.



**Figure 1 Mechanism involved in coronavirus disease 2019-associated diarrhea.** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) binds to its corresponding cell-surface receptor, angiotensin-converting enzyme type 2. In the intestines, SARS-CoV-2 viroporins, E protein, dysregulation of the renin-angiotensin-aldosterone system triggering ionic imbalance, disruption of barrier integrity and inflammation play important roles in the development of coronavirus disease 2019-associated secretory diarrhea and leaky gut. ACE: Angiotensin-converting enzyme; IFN: Interferon; IL: Interleukin; TNF $\alpha$ : tumor necrosis factor alpha; CFTR: CF transmembrane conductance regulator; CaCC: Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel; NHE3: Na<sup>+</sup>-H<sup>+</sup> exchanger 3.

Moreover, intracellular ionic imbalances triggered by SARS-CoV-2 viroporins (E protein and Orf3a) can lead to the activation of the NLRP3 inflammasome (NOD-, LRR-, and Pyrin domain-containing 3). This results in the secretion of IL-1 $\beta$  and cell death in a process called pyroptosis[44,46]. By activating innate immune cells, IL-1 $\beta$  contributes to the development of a local inflammatory environment and a systemic cytokine storm. The direct action of viroporins and the indirect effects of cytokines together can trigger an ionic imbalance of enterocytes, which may contribute to the development of diarrhea (Figure 1).

During its multiplication, SARS-CoV-2 disturbs the function of the renin-angiotensin-aldosterone system (RAAS). The main components of this system are ACE, angiotensin II, and AT1R. Angiotensin II is known to elicit vasoconstriction, oxidative stress, and inflammation following binding to AT1R[88]. The ACE2/angiotensin (1-7)/Mas pathway is an important physiological negative regulator of the ACE/angiotensin II/AT1R axis and exerts anti-inflammatory effects[88]. SARS-CoV-2 uses ACE2 for entry as a receptor, which becomes degraded in the endolysosomal compartment after being internalized along with the virion particles[17,19,20].

The viral infection has been shown to increase the expression of ADAM (A Disintegrin and Metalloprotease) metalloproteinase domain 17 enzyme, which is endowed with sheddase activity[89]. ADAM17 functions in the ectodomain shedding of tumor necrosis factor alpha (TNF- $\alpha$ ), EGFR ligands, and ACE2[90,91]. ADAM17-mediated cleavage decreases ACE2 Levels on the cytoplasm membrane and thereby shifts the delicate balance towards the ACE/angiotensin II/AT1R pathway. In turn, this can lead to pro-inflammatory predominance. It has also been demonstrated recently that ACE2 forms dimer-of-heterodimer complexes with the neutral amino acid transporter B<sup>0</sup>AT1 (Broad neutral Amino acid Transporter 1)[91]. B<sup>0</sup>AT1 is involved in the Na<sup>+</sup>-coupled transportation of tryptophan, phenylalanine, glutamine, and leucine[91].

The ADAM17-mediated cleavage of ACE2 ectodomain and attachment of SARS-CoV-2 to the ACE2:B<sup>0</sup>AT1 complex may potentially compromise the transport of Na<sup>+</sup> and neutral amino acids[88]. Impairment of the ACE2:B<sup>0</sup>AT1 complex and the consequential amino acid starvation can decrease Na<sup>+</sup> uptake and affect the activation state of the mechanistic target of rapamycin (mTOR) complex, which is an important regulator of autophagy, xenophagy, metabolism, and various immune processes[88,92-95]. Dysregulated RAAS may aggravate ionic imbalance and inflammation, which may affect the metabolic state of cells, the composition of the microbiota, and cell viability, leading to increasingly severe intestinal dysfunction[88,90,91,94,95] (Figure 1).

## VIRUS-INDEPENDENT CAUSES OF COVID-19-ASSOCIATED DIARRHEA

If diarrhea is not included in the presenting symptoms and develops after admission, it becomes challenging to ascertain the cause of diarrhea. Several confounding variables, such as the hyperinflammatory response, altered gut flora, secondary bacterial infections, antiviral agents, antibiotics, enteral feeding, and the use of proton pump inhibitors can potentially cause diarrhea in hospitalized COVID-19 patients.

In approximately 20% of COVID-19 patients, the infection progresses to severe and critical phases in which an extrapulmonary hyperinflammatory state develops due to cytokine release syndrome[96]. Several cytokines may affect the course and clinical manifestations of SARS-CoV-2 infection by increasing the intestinal and vascular permeability as well as triggering the formation of thrombi in the small blood vessels and the alteration of intestinal microbiota, leading to bacterial translocation towards the bloodstream and the mesenteric lymph node[3,4]. The cytokine-mediated GI damage may thereby further intensify the systemic immunological response and contribute to the deterioration of the patient's condition. A study conducted by Zhang *et al*[97] revealed that the pro-inflammatory cytokine patterns are different in COVID-19 patients with and without diarrhea. Moreover, diarrhea patients were more likely to develop cytokine release syndrome and multi-organ damage[97]. Interestingly, the levels of TNF- $\alpha$ , IL-6, and IL-10 were significantly higher in the sera of diarrhea patients than in the non-diarrhea group[97]. TNF- $\alpha$  is known to increase the expression of adhesion molecules on the surface of endothelial cells, platelets, and leukocytes, thereby facilitating the adhesion of thrombocytes to the vessels and initiating the formation of thrombi in the microcirculation of the GIT and other organs. These effects increase vascular permeability and can lead to inflammation and disseminated intravascular coagulation. Furthermore, TNF- $\alpha$  has the ability to disrupt the intestinal tight junction barrier, which in turn contributes to the development of leaky gut[98]. IL-6 exerts dual effects on the intestinal epithelium. It increases gut permeability to small molecules with a radius < 4Å (< 0.4 nm) *via* activating claudin-2 gene expression[99]. However, by stimulating epithelial proliferation and regeneration, IL-6 plays a beneficial role in the maintenance of intestinal epithelial integrity during acute injury[100]. IL-10 is an anti-inflammatory cytokine that restricts uncontrolled immune responses to the intestinal microbiota and defends gut barrier integrity[101,102]. In light of these data, it is reasonable to infer that TNF- $\alpha$  may be an important factor in COVID-19-associated diarrhea, whereas without IL-10, the cytokine storm and intestinal injury would be even more devastating. However, further studies are needed to identify the precise role of each cytokine in the development of COVID-19-associated diarrhea. Such studies could also contribute to a better understanding of the potential adverse effects of anti-cytokine therapies on the GIT.

Interesting observations revealed that the composition of intestinal microbiota is profoundly altered in COVID-19 patients: The diversity is highly reduced, the proportion of beneficial commensal bacteria is decreased and the opportunistic pathogens are enriched compared with that found in healthy controls[103,104]. It has also been demonstrated that some *Bacteroides spp.*, capable of decreasing ACE2 expression in mice, displayed inverse correlation with the fecal SARS-CoV-2 load [104]. The immune system and the intestinal microbiota are in a continuous dialog. The presence of commensal microorganisms shapes host immunity, and alterations in microbiota composition may lead to increased susceptibility to various pathological conditions, including infections, inflammation, and metabolic and autoimmune disorders. Thus, the altered microbiota observed in COVID-19 patients may be an additional factor contributing to the development of diarrhea by weakening colonization resistance, decreasing the production of beneficial bacterial metabolites, and triggering a local immune recalibration.

For clinical improvement and treatment of secondary bacterial infections, COVID-19 patients are treated with antiviral agents, antibiotics, and corticosteroids. Antiviral agents such as the RNA polymerase inhibitors favipiravir and remdesivir may cause diarrhea[105]. Diarrhea is also a common adverse drug reaction to antibiotics such as cephalosporins, macrolides and fluoroquinolones, largely due to destruction of the normal intestinal microbiota. Moreover, treatment of COVID-19 patients with broad-spectrum antibiotics has the potential to increase the risk of *Clostridioides difficile* (*C. difficile*) infection, including in survivors even long after recovery. In co-infections with SARS-CoV-2 and *C. difficile*, intestinal damage is more extensive and diarrhea symptoms are more severe[106]. To counteract the detrimental effects of various proinflammatory cytokines, biological therapy is used in selected patient groups. IL-6 and IL-6 receptor inhibitors, such as tocilizumab, sarilumab and siltuximab, represent another class of drugs that often cause diarrhea[107]. Although enteral feeding has well-established, clear advantages over parenteral nutrition[108], adverse events, like diarrhea, may develop. Tube feeding-related diarrhea can occur for several reasons, mostly related to the circumstances of feeding (adaptation time, perfusion speed, temperature) or the composition of the used enteral formula (osmolality, fat content, nutrient intolerance), and can be managed easily with careful observation of the patients[109]. Not only is the use of PPIs during the course of COVID-19 infection controversial[110], such drugs may induce diarrhea in general through the alteration of GI microbiota by different mechanisms, including the direct consequences of increased gastric pH itself. This safety issue was evaluated by a number of meta-analyses based on retrospective observational or case-control studies, which suggested an increased risk for enteral infections, especially *C. difficile* infections, in PPI-treated patients[111-113]. In contrast, a recent long-term prospective study (COMPASS) failed to show an increased risk of *C. difficile* infection in PPI users and only a slight increase of enteral infections in general[114].

## POTENTIAL CONSEQUENCES OF COVID-19-ASSOCIATED DIARRHEA

A great body of experimental and clinical evidence demonstrates that SARS-CoV-2 infects and replicates in the GIT, and the stool contains high copies of viral RNA, although the amount of infectious virus in the stool appears to be low. The presence of SARS-CoV-2 in the feces may potentially facilitate the spread of COVID-19 through fecal-oral transmission among humans and contaminate the environment[115-117]. Thus, SARS-CoV-2 infection of the GIT has important epidemiological significance.

The feces of COVID-19 patients pose a serious epidemiological risk, which justifies the use of all available methods of prevention, including protective equipment, disinfection procedures, and vaccination. However, further studies are needed to establish the efficiency of the fecal-oral spread of SARS-CoV-2 precisely. It would be very useful if the concentration of infectious virion particles in the stool were determined in asymptomatic individuals and different patient groups under standardized parameters when discharge frequencies and the grade on the Bristol stool scale are precisely recorded. It is possible that the rate of virus inactivation in the intestinal lumen may significantly differ in COVID-19 patients.

SARS-CoV-2 can extensively contaminate the environment, and viral RNA can be detected in sewage and solid waste[118,119]. Measurement of SARS-CoV-2 RNA in wastewater is used for local monitoring of the epidemic situation, which facilitates the implementation of preventive measures. Wastewater epidemiology involves using Rrt-PCR to determine SARS-CoV-2 RNA in sewage, but how long the virus survives in this environment has not been measured[118,119]. It would be essential to determine the concentration of infectious virion particles to elucidate the risk of SARS-CoV-2 transmission *via* wastewater contamination.

## CONCLUSION

Among the specific GI symptoms, diarrhea is the most common in COVID-19 patients. The ACE2 receptor and other elements required for the attachment of this virus to the various cell types are extensively expressed throughout the GIT. SARS-CoV-2 can establish a productive infection in the enterocytes, leading to mild cellular damage. The infection evokes an inflammatory response in the intestines, which is characterized by the production of various pro-inflammatory cytokines and chemokines, many of which are known to increase intestinal permeability. Direct effects of SARS-



CoV-2 viroporins and dysregulation of the intestinal RAAS triggering ionic imbalance and inflammation in the intestines seem to play important roles in the development of COVID-19-associated secretory diarrhea and leaky gut.

Infection in the lungs and GIT also seems to display some different tissue-specific features. The production of type I and III IFNs is more efficient in the GIT than in the lungs. The antiviral IFNs may restrict viral replication in the GIT to some extent, which may allow the development of a less cytopathogenic or persistent form of infection in this anatomical region. SARS-CoV-2-mediated dysregulation of the ACE2:B<sup>0</sup>AT1 complex may modify the biological response of cells to the infection, and in enterocytes, it may contribute to the development of diarrhea by inducing amino acid starvation, which can decrease Na<sup>+</sup> uptake. These effects are not seen in the lungs, however, as ACE2 does not form a complex with B<sup>0</sup>AT1 in this organ. SARS-CoV-2 infection of the GIT is of pivotal epidemiological significance, but further studies are needed to assess the extent of this risk.

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## Assessment of liver disease in patients with chronic hepatitis C and unhealthy alcohol use

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### Abstract

Hepatitis C virus (HCV) infection and unhealthy alcohol use are major drivers of the burden of liver disease worldwide and commonly co-occur. Assessment of underlying liver damage is a cornerstone of the clinical care of patients with chronic HCV infection and/or unhealthy alcohol use because many of them are diagnosed at advanced stages of disease. Early diagnosis of liver disease before decompensated liver cirrhosis becomes established is essential for treatment with direct acting antivirals and/or abstinence from alcohol consumption, which are the main therapeutic approaches for clinical management. In this review, we discuss current knowledge around the use of non-invasive methods to assess liver disease, such as abdominal ultrasound, controlled attenuation parameter, transient elastography, magnetic resonance imaging, and indices based on serum markers of liver injury.

**Key Words:** Hepatitis C virus; Alcohol; Liver fibrosis; Non-invasive methods; Ultrasound; Transient elastography

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**Core Tip:** In this review, we discuss current knowledge around the use of non-invasive methods to assess underlying liver disease in patients with hepatitis C virus infection and/or unhealthy alcohol use. A timely diagnosis of liver disease is of the outmost



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importance to avoid progression to decompensated liver disease and liver cancer. Antiviral treatment and abstinence from alcohol use are cornerstones of clinical care for these patients.

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## INTRODUCTION

Chronic hepatitis C virus (HCV) infection and unhealthy alcohol intake are common co-occurring conditions, in part because people with HCV often consume more alcohol than the general population[1]. Conversely, alcohol use is associated with a higher prevalence of HCV infection exposure and greater HCV infection persistence; patients with alcohol use have a lower likelihood of spontaneously clearing acute HCV infection[2]. For these reasons, the prevalence of HCV infection among patients with alcohol use is higher than in the general population[3], which is usually around 1% in Western countries[4]. We have found a prevalence > 20% in our cohort of patients with alcohol use disorder (AUD) and admitted for hospital detoxification[5], which is much higher than many values reported in a meta-analysis published in 2013[3]. This contrast underscores the relevance of a history of prior injection drug use and other risk factors for the acquisition of HCV in an urban population of patients with AUD[6].

In addition to higher infection exposure and persistence, alcohol use may affect HCV viral replication in certain subsets of patients[7] and is associated with progressive liver fibrosis and more extensive liver injury than in patients without alcohol use [8]. HCV is also associated with increased mortality and other poor health outcomes in different subsets of patients with alcohol-use problems[9,10].

Currently, alcohol use is a major contributor to decompensated cirrhosis in patients with HCV infection[11]. Moreover, with the current availability of efficacious treatments for viral hepatitis, alcohol-related liver disease is becoming a major driver of liver disease burden worldwide[12].

HCV infection needs to be detected as early as possible among patients with unhealthy alcohol intake, and accurate assessment of alcohol intake is necessary in patients with HCV infection[12]. Assessment of underlying liver disease is crucial, given that many patients are diagnosed only after their liver disease has reached an advanced stage[13].

## ASSESSMENT OF ALCOHOL USE IN PATIENTS WITH LIVER DISEASE

The American Association for the Study of Liver Diseases and European Association for the Study of the Liver guidelines for the treatment of alcohol-related liver disease recommend using the alcohol use disorders identification test (AUDIT) to identify patients with AUD[14,15]. AUDIT includes 10 questions and explores consumption, dependence and alcohol-related problems according to the number of positive answers[16]. For patients with an AUDIT > 7, Diagnostic and Statistical Manual of Mental Disorders 5<sup>th</sup> edition is an appropriate tool used for the assessment of clinical, personal and social alcohol-related problems[17]. The severity of AUD, that encompasses both alcohol dependence and alcohol abuse, is based on the number of criteria that the patient meets (mild AUD: 2-3 criteria, moderate AUD: 4-5 criteria and severe AUD > 6). AUD represents the more extreme form of unhealthy alcohol use, a term that will be used throughout the manuscript[17].

In terms of amount, unhealthy alcohol use means drinking more than the recommended amount of alcohol by the National Institute of Alcohol abuse and Alcoholism [2 United States standard drinks (12oz of beer or 5oz of wine) *per day* or 14 drinks *per week* for men and 1 drink *per day* or 7 United States standard drinks *per week* for women or men aged > 65], what includes the spectrum from risky alcohol use to AUD[18].

It is important to note that for those who already have HCV infection there is no safe threshold of alcohol consumption and abstinence from alcohol should be the main treatment goal[12].

## **PATHOLOGIC FEATURES OF HCV-RELATED LIVER DISEASE AND ALCOHOL-RELATED LIVER DISEASE**

The natural history of HCV-related liver disease is characterized by progressive liver fibrosis that eventually leads to liver cirrhosis and hepatocellular carcinoma[19]. Many cofactors are associated with a faster progression of liver fibrosis in patients with HCV infection, including age, duration of HCV infection, male sex, obesity, the presence of hepatitis B virus (HBV) and/or human immunodeficiency virus (HIV) co-infection, alcohol use, and immune suppression[20].

Alcohol-related liver disease includes several histological abnormalities ranging from simple steatosis to cirrhosis and liver cancer[21,22]. These various histological abnormalities can be present at the same time in a liver biopsy[21,22]. Alcoholic hepatitis is a complication associated with acute liver failure, an increased risk of infection, and high mortality that can be seen at any point in the natural history of alcohol-related liver disease[12].

Liver fibrosis is the best predictor of progression to decompensated cirrhosis in both chronic HCV infection and alcohol-related liver damage[23]. Liver biopsy is the preferred method to evaluate liver fibrosis, but it is costly, invasive[24] and rarely performed in patients with current alcohol or other substance use disorders[25]. In the present review, we will not discuss the particularities of liver biopsy[26] and focus instead on currently available non-invasive methods to assess the extent of liver damage in patients with HCV and/or unhealthy alcohol use.

## **THE NEED FOR ASSESSMENT OF LIVER DAMAGE**

A timely diagnosis of liver disease in asymptomatic patients with HCV infection and/or AUD contributes to a better prognosis and facilitates treatment[27,28]. As noted, in patients with AUD, liver involvement is the main culprit of alcohol-related disease burden, and liver disease is usually detected in the advanced stages[28]. Almost 75% of patients with alcohol-related liver disease present with a non-elective hospital admission because of decompensated cirrhosis[29], so efforts should prioritize early identification of patients at risk for end-stage liver disease.

Despite the availability of efficacious treatments for viral hepatitis, the liver disease burden is expected to increase because of the growing prevalence of overweight and obesity and other unhealthy lifestyles in developed countries, which is predicted to drive an increase in the prevalence of non-alcoholic liver disease. In addition, many patients with metabolic syndrome also drink alcohol, and many patients with AUD have overweight or obesity and/or a sedentary lifestyle[30].

## **NON-INVASIVE METHODS FOR ASSESSMENT OF LIVER DAMAGE**

### ***Liver ultrasound***

Liver ultrasound is both inexpensive and easily available, but it is rarely performed in patients with AUD and no apparent liver disease. The latest American Association for the Study of Liver Diseases guidelines for the management of chronic HCV infection are focused on the test-and-treat paradigm but recommend abdominal ultrasound for hepatocellular carcinoma surveillance only if the patient has already developed cirrhosis[31]. Although the latest European guidelines recommend liver ultrasound to early detect non-alcoholic steatohepatitis[32], there are no guidelines for the early detection of liver abnormalities among patients with high alcohol intake. Screening approaches for patients at risk for alcohol-related liver disease thus are a matter of debate[33,34]. It is also important to note that, in general, liver cancer surveillance among individuals with alcohol-related liver disease is far from optimal, and hepatocellular carcinoma is usually diagnosed at an advanced stage[22].

On abdominal ultrasound, liver steatosis has a hyperechogenic appearance because of the increased parenchymal reflectivity of intracellular fat accumulation[35]. Fat

content affects the sensitivity of abdominal ultrasound for detecting liver steatosis, and sensitivity severely decreases at fat contents lower than 10%–20% [28].

The precision of abdominal ultrasound in differentiating between steatosis and fibrosis is higher when liver fibrosis is more advanced because of an increase in coarse echoes without posterior beam attenuation [35–37]. Steatosis is usually classified as “mild,” “moderate,” or “severe” [37,38].

Despite operator dependency for the assessment of steatosis, there is agreement that liver ultrasound is inexpensive and reliable for the detection of moderate or severe liver steatosis [39,40]. The accuracy of abdominal ultrasound is lower for mild steatosis, however, it can be increased with a computer-aided method [40]. Ultrasound findings contribute to the detection of alcohol-related liver disease features in addition to liver steatosis, including (among others): Liver size, edge bluntness, parenchyma coarseness, surface nodularity, inferior vena cava abnormalities, the presence of portal hypertension, and spleen size [41].

The authors of a Cochrane review published in 2016 commented on the need for studies of large sample size to assess the efficacy of abdominal ultrasound in patients with alcohol-related liver disease [42], in part because their review could only include two studies [42]. Of these two studies, the first was performed in France in 1985 and included 126 patients with alcoholism [43]. In addition to abdominal ultrasound performed in the entire cohort, 100 patients also underwent liver biopsy. In that group of 100 patients, cirrhosis of the liver was confirmed in 72, and abdominal ultrasound had a sensitivity of 81% and a specificity of 79% to detect cirrhosis [43].

The second study was published in 2013 and performed in Korea and included 230 patients (81% male) who underwent liver biopsy, elastography and abdominal ultrasound [44]. The mean age of participants was 50.4 years. Ultrasound suggested heterogeneous liver in 199 patients (86.5%) and liver cirrhosis in 170 (74%). Cirrhosis of the liver was found in 111 participants, for a sensitivity of 94% and specificity of 49% for ultrasound detection of cirrhosis of the liver [44]. Because of the small number of patients included and the differences in the selection criteria in each of these two studies, the authors of the review could not reach any conclusion about the use of abdominal ultrasound for detecting underlying advanced liver disease among patients with unhealthy alcohol use [42].

In 2018, we published a study describing ultrasound findings among 301 consecutive AUD patients without clinically relevant liver disease who were admitted for hospital treatment of the disorder at Hospital Universitari Germans Trias i Pujol [34].

We obtained clinical and laboratory parameters at admission. An ultrasound was performed so as to identify the presence of liver steatosis, hepatomegaly, heterogeneous liver, and portal hypertension.

For this work, we studied unadjusted associations of ultrasound abnormalities with three prevalent conditions: Alcohol-related liver injury (ALI), advanced liver fibrosis (ALF) and HCV infection. ALI was defined as the presence of at least two of the following: Aspartate aminotransferase (AST) levels  $\geq 74 < 300$  U/L, AST/alanine aminotransferase (ALT) ratio  $> 2$ , and total bilirubin  $> 1.2$  mg/dL. ALF was measured with fibrosis-4 (FIB-4) [45] and was defined as a FIB-4 score  $\geq 3.25$ . We performed logistic regressions to detect if ALI, ALF and/or HCV were associated with having two or more abnormalities in the liver ultrasound.

In summary, 80% of the patients were male, had a median age of 46 years, drank a median of 180 grams of alcohol *per day* upon admission and 21.2% had HCV infection. Median serum AST was 42 U/L, median serum ALT was 35 U/L, the prevalence of ALI and ALF was 16% and 24%, respectively. A total of 57.2% patients had steatosis, 49.5% had hepatomegaly, 17% had heterogeneous liver, while 16% had portal hypertension. Of interest, 77% of patients had one abnormality, and 45% had  $\geq 2$ .

In the multivariate models, both ALI and ALF were significantly associated with the presence of  $\geq 2$  abnormalities, with odds ratios (ORs) of 5.2 for ALI and 4.7 for ALF. HCV infection did not predict the presence of more than two abnormalities [34].

Most patients included in the study who had only one ultrasound abnormality had mild to moderate steatosis or hepatomegaly, which are both potentially reversible with alcohol cessation. In light of those findings, we believe that abdominal ultrasound could be implemented in the everyday clinical care of patients seeking treatment for AUD, as it could help in clinical decision-making and treatment selection.

Liver abnormalities were quite common in the study, even in patients without HCV, ALI, or ALF, and only 31% of patients without any of those conditions had a completely normal ultrasound. Accordingly, we think that sharing information about ultrasound abnormalities may promote alcohol cessation in patients with AUD who are unaware of their underlying liver disease [34]. Other researchers have previously

reported that sharing findings suggestive of poor health outcomes with patients leads to a decrease in unhealthy alcohol use[46].

Liver cancer has an incidence of 2.9% *per year* in individuals who have cirrhosis of the liver[22]. Hepatocellular carcinoma entails a dire prognosis if not detected early [47], which is why patients with alcohol-related end stage liver disease should undergo an abdominal ultrasound every 6 months[22]. However, periodic surveillance is challenging because many patients miss appointments[22], and less than 30% of liver cancers are detected by surveillance in Europe and the United States[22,48]. In our series, liver cancer was found in two participants[34].

Besides the early identification of steatosis or cirrhosis of the liver, abdominal ultrasound could be used for detecting other forms of liver damage. In fact, alcohol use is common among individuals with other forms of liver disease, and there is no safe level of alcohol consumption in these situations[8,30]. As previously mentioned, alcohol use is a cofactor in liver disease progression in individuals with HCV, HBV, non-alcoholic fatty liver disease, and hemochromatosis, among others. A threshold of a consumption of more than 30 grams of alcohol *per day* for men and 20 g for women is arbitrarily used to support the diagnosis of alcohol-related liver disease[12]. Nevertheless, as overweight and obesity are highly prevalent in the developed world, alcohol-related and non-alcoholic steatosis can co-occur[49]. In our case series of AUD patients, the median body mass index was 24.7, indicating that a sizable proportion of participants were overweight or obese[34].

In addition, liver ultrasound can also detect other forms of liver disease that are less prevalent, like parenchymatous or vascular diseases (non-cirrhotic portal hypertension, hepatic vein congestion, and Budd Chiari).

### Transient elastography

Transient elastography has been used for more than a decade to accurately assess liver fibrosis, measuring liver stiffness in patients with chronic HCV infection or HBV infection, with or without HIV co-infection, and in non-alcoholic liver disease[50-52]. In transient elastography, a piston vibrator that is placed in the intercostal space generates a shear wave, and velocity is measured below the skin surface. It measures liver stiffness in kilopascals (kPa), and elastography readings usually range from 2.5 to 75 kPa[53].

The method has also been used to a lesser extent for analyzing liver injury in patients with AUD. A study that included 199 consecutive patients at risk for alcohol-related liver disease found that transient elastography provided an assessment of fibrosis (either significant fibrosis, Ishak score  $\geq 3$ ) and cirrhosis (Ishak score  $\geq 5$ ) that was comparable to liver biopsy with high accuracy (area under the curve  $\geq 0.92$ )[33]. Some authors have pointed out that steatohepatitis may lead to overestimation of liver fibrosis[54]. The risk of overestimation especially applies in patients who are abstinent, as liver stiffness decreases with cessation of alcohol use, especially with baseline values higher than 7 kPa[55].

In 2016, a systematic review and meta-analysis found that transient elastography could reliably exclude ALF or cirrhosis of the liver. The authors suggested that the same cut-offs described for viral hepatitis-related liver disease should be applied with caution to other forms of liver damage, particularly alcohol-related liver disease[56]. Mueller and colleagues have suggested adapting elastography cut-offs in patients who present with elevated AST or bilirubin[54]. The studies in that meta-analysis, which included only patients with alcohol-related liver disease, had a wide range of participant numbers and an ALF prevalence ranging from 53% to 80%[57-61].

Results of an individual-level meta-analysis published in 2018 that included 1026 patients suggested that cut-offs for transient elastography should be higher[58]. In that study, higher concentrations of AST and bilirubin were significantly associated with higher liver stiffness values ( $P < 0.01$ )[58]. In addition, the presence of non-severe alcoholic hepatitis was associated with liver stiffness ( $P < 0.0001$ ). Also, higher AST ( $P < 0.01$ ) and higher bilirubin ( $P = 0.01$ ) levels and increased prothrombin activity ( $P = 0.01$ ) were significantly associated with non-severe alcoholic hepatitis. That meta-analysis described specific liver stiffness cut-offs based on concentrations of AST and bilirubin[58].

French researchers used transient elastography to assess ALF in a cohort study of patients with HCV/HIV co-infection. They found that ALF was more prevalent among patients with an alcohol-related diagnosis (OR 3.06; 95%CI: 1.42-6.60) compared to those with non-hazardous alcohol intake[62].

A recently published study describes the use of transient elastography for the measurement of liver and spleen stiffness (a surrogate marker of portal hypertension), as well as spleen length in a retrospective cohort of 499 patients with HCV or alcohol-



related liver disease[63]. Participants with HCV had higher mean spleen stiffness and spleen length but lower liver stiffness when compared to participants with alcohol-related liver disease. The authors concluded that the use of those measurements, as well as the spleen stiffness/liver stiffness and spleen length/spleen stiffness ratios, could be helpful to measure burden of disease and risk for disease-specific complications[63].

Other researchers have confirmed that elastography is a good predictor of clinical events[64]. Transient elastography has also been proven to be cost-effective in primary care[65], and other authors have used it to detect chronic alcohol-related liver disease after alcohol cessation[66] or to detect improvements in liver fibrosis after successful HCV antiviral therapy[67].

Transient elastography thus represents a promising tool for assessing underlying liver damage in patients with unhealthy alcohol use and/or HCV infection[28]. It is important to note that transient elastography results may be affected by the thickness of subcutaneous fat, width of intercostal spaces, by the presence of ascites, by the patients' breathing or by an uneven distribution of liver fibrosis. In addition, the presence of hepatic congestion or steatosis may also distort transient elastography results[28,56].

Table 1 includes a summary of the most relevant studies that have used transient elastography in patients with alcohol-related liver disease.

### **Controlled attenuation parameter**

The controlled attenuation parameter (CAP) is a non-invasive tool for detecting steatosis. It measures ultrasound attenuation during transmission through fatty liver tissue[68]. CAP software can be incorporated into transient elastography devices, thus facilitating the non-invasive measurement of steatosis and fibrosis[28].

Cut-offs for moderate and severe liver steatosis were defined in a recently published patient-level meta-analysis that included 2735 participants with liver biopsy and CAP. Their diagnostic accuracy ranged from 0.65 to 0.90[69]. The etiology of liver disease was HBV in 37% of patients, HCV in 36% and or non-alcoholic fatty liver disease in 20%, and participants with unhealthy alcohol use were under-represented[69]. As all patients included had liver diseases that are strongly associated with liver fibrosis, these cut-offs require further validation in other and healthier populations[70].

A study by Thiele and colleagues included 562 patients with alcohol-related liver steatosis and found that CAP above 290 dB/m ruled in any steatosis with a 88% specificity and a 92% positive predictive value, whereas CAP below 220 dB/m ruled out steatosis with a 90% sensitivity but with a 62% negative predictive value[71]. The authors concluded that it was useful for the diagnosis of severe alcohol-related steatosis and could also rule in any liver steatosis. In patients admitted for alcohol detoxification who did not have obesity, CAP rapidly declined[71]. This finding underscores the synergic effect of obesity and alcohol use in patients seen in clinical practice[72].

A study by Unalp-Arida and colleagues measured liver stiffness and CAP in 4870 participants in the National Health and Nutrition Examination Survey cohort, and found that liver stiffness in the highest quartile was associated, among others, with HCV infection, increased age and body mass index and CAP[73].

### **Virtual touch quantification**

Virtual touch quantification is a point shear wave elastography technique, using acoustic radiation force impulse technology also offers good diagnostic accuracy for liver fibrosis assessment[74]. It has been used in patients with chronic liver disease of different origins, mainly due to viral hepatitis[75,76].

### **Magnetic resonance imaging**

Magnetic resonance imaging and proton magnetic resonance spectroscopy are promising methods for quantifying hepatic fat content[35]. Magnetic resonance measurement of liver steatosis does not appear to be affected by the type of liver disease or the presence of liver inflammation or iron overload, features that may distort results obtained with other non-invasive methods. Despite these potential advantages, magnetic resonance imaging is costly and time-consuming[28]. Moreover, information is scarce about its applicability in alcohol-related liver disease.

### **Laboratory-driven indices**

There are several non-invasive indices that are derived from laboratory parameters aimed for the estimation of liver fibrosis. Some of those parameters are used in

**Table 1 Studies that have used transient elastography in patients with alcohol-related liver disease**

Ref.	Number of patients	Advanced liver fibrosis ( $\geq 3$ )	Sensitivity <sup>1</sup>	Specificity <sup>1</sup>
Nguyen-Khac <i>et al</i> [58], 2018	103	51%	87%	80%
Nahon <i>et al</i> [59], 2008	147	75%	87%	89%
Kim <i>et al</i> [107], 2009	45	80%	97%	78%
Mueller <i>et al</i> [57], 2010	101	45%	91%	75%
Janssens <i>et al</i> [60], 2010	49	65%	72%	76%
Fernandez <i>et al</i> [61], 2015	135	53%	91%	68%

<sup>1</sup>Sensitivity and specificity are calculated using liver biopsy as the gold standard.

everyday clinical practice, including AST, ALT, and platelets. As far back as 1991, Poynard and colleagues described the poly-gamma-l-glutamic acid index, which included prothrombin time, gamma-glutamyl transferase (GGT), and apolipoprotein A1[77]. In a group of 333 individuals with high alcohol consumption, the index correctly classified a heavy drinker as harboring liver cirrhosis most of the time (89%) at a value  $\geq 9$ [77]. The accuracy increased with the addition of alpha-2 macroglobulin (known as the PGAA index)[78].

Other tests, such as the enhanced liver fibrosis (ELF) test, FibroTest (FT), and HepaSCORE, are commercially available and combine different serum markers that are not used in everyday clinical practice. ELF includes type III procollagen peptide, hyaluronic acid, and tissue inhibitor of metalloproteinase-1[79]. FT, also known as FibroSure in the United States, combines age and sex, alpha-2 macroglobulin, haptoglobin, apolipoprotein A1, GGT, total bilirubin, and ALT[80]. HepaSCORE includes bilirubin, GGT, hyaluronic acid, alpha-2 macroglobulin, age, and sex[81]. The patented indices seem to perform better than non-patented versions against the gold standard of liver biopsy[82], but because of their cost, they are less frequently used in health systems with budget constraints.

Among the indices that include routinely measured parameters, the most commonly used are FIB-4[45] and the AST/platelet ratio index (APRI)[83]. Both indices have been validated against liver biopsy in patients with HCV and in patients with HCV and HIV co-infection[84-86]. Both FIB-4 and APRI are better suited for the detection of the absence of liver fibrosis or the presence of ALF[45,83]. Experience with their use in patients with unhealthy alcohol use is far more limited[87], and some authors have expressed concerns around the potential overestimation of liver fibrosis in patients with alcohol-related liver disease[87,88].

Lieber and colleagues, publishing in 2006, reported assessment results for 1308 patients from two United States veterans' administration cooperative studies of alcoholic liver disease for the accuracy of APRI in non-invasive detection of liver fibrosis[87]. APRI had low sensitivity (13.2%) and specificity (77.6%) for the non-invasive estimation of significant fibrosis in individuals with alcohol-related liver disease, including those who also had HCV.

In different series of patients with HCV with or without co-infection, results have been mixed regarding if non-invasive indices are able to detect the association between alcohol use and liver fibrosis, probably because of how alcohol consumption was assessed and because of unmeasured confounding in the different cohorts studied. A cross-sectional study in a cohort of patients with HIV/AIDS in Baltimore found that heavy alcohol use was associated with ALF measured with the APRI score[89]. However, when patients were stratified according to the presence of HCV infection, the association between APRI score and unhealthy alcohol use was only found among those with no HCV infection[89].

In a cohort that only included women, Blackard and colleagues did not find an association between alcohol use and FIB-4 values as a continuous variable among patients with HCV/HIV co-infection, whereas immune suppression was associated with higher FIB-4 values[90]. In a more recent study in the same cohort, Kelly and colleagues found that light or moderate alcohol consumption (1-7 drinks *per week*), which was reported in 35.7% of the 686 patients included, was not associated with fibrosis progression measured by several FIB-4 determinations over time[91]. Conversely, drinking 8-14 drinks *per week* was associated with minimal acceleration of fibrosis progression, while drinking more than 14 drinks *per week* was clearly

associated with increased fibrosis progression[91].

In a study performed in our cohort of patients with AUD who were admitted for hospital treatment in 2012, FIB-4 used as a continuous variable was significantly higher among those with HCV/HIV co-infection compared to those with HCV infection only[92]. When we stratified results by the presence of HIV infection, we found that alcohol use affected FIB-4 values only in those with HCV infection, whereas immune depression exerted a more negative role than alcohol consumption in those with co-infection[92].

In the HIV-LIVE cohort in Boston, involving a cohort of patients with HIV infection and alcohol problems, lifetime alcohol consumption measured with the Lifetime Drinking History questionnaire was not associated with FIB-4 values < 1.45 consistent with the absence of fibrosis. The adjusted (A)ORs were 1.12 (95%CI: 0.25–2.52) for a lifetime consumption of 150–600 kg of alcohol *vs* < 150 kg (reference category) and 1.11 [(95%CI: 0.52–2.36) for > 600 kg *vs* < 150 kg; global *P* = 0.95][93]. Similar results were found for the presence of ALF (FIB-4 > 3.25) and with the use of APRI instead of FIB-4. Results did not differ among patients with HCV co-infection[93].

An analysis from the veterans aging cohort study (VACS) included a large number of patients (701 with HIV/HCV coinfection, 1410 with HIV infection, with 296 HCV infection, and 1158 with neither HIV nor HCV infection) and a different measure of alcohol consumption (AUDIT-C questionnaire and/or presence of alcohol-related diagnoses). The authors reported greater risks for ALF (measured with FIB-4) among patients with co-infection who had nonhazardous drinking (OR = 14.2; 95%CI: 5.91–34.0) or hazardous/binge drinking (OR = 18.9; 95%CI: 7.98–44.8), or who exhibited alcohol-related diagnoses (OR = 25.2; 95%CI: 10.6–59.7) in comparison to uninfected Veterans who were nonhazardous drinkers[94].

In a more recent study by our group that included 1313 patients with AUD who were admitted for hospital detoxification, 30.6% had ALF, estimated with a FIB-4 value > 3.25. Patients with HCV infection, who represented 18% of the total study population, were two times more likely than those without HCV infection to present with ALF (OR = 2.1; 95%CI: 1.5–3.1, *P* < 0.01)[25].

Despite these different results in terms of accuracy or the ability to detect associations between the presence either of AUD or HCV and ALF, non-invasive indices are performed in patients with alcohol or other substance use disorders that will not undergo a biopsy[93,95]. In addition, non-invasive indices can predict mid-term mortality in epidemiological studies[95,96], which is why FIB-4 has been included in the VACS cohort index[97]. This index, initially intended to predict mortality, also predicts the occurrence of other health outcomes[98], such as incident heart failure in patients living with HIV[99].

The Forns index, which was described in 2002, has been less frequently used in patients with unhealthy alcohol use[100]. Originally developed for detection of the absence of liver fibrosis in a cohort of patients with HCV, it includes platelets, cholesterol, and GGT levels, as well as age[100]. In a study by Naveau and colleagues published in 2009, the accuracy of the Forns index for detecting biopsy-proven ALF was lower than that of other non-patented biomarkers (APRI and FIB-4)[82]. Furthermore, other researchers have expressed concerns regarding the use of the Forns index in alcohol-related liver disease, given that GGT levels differ between patients with HCV or alcohol-related liver disease[101]. Despite those concerns, in our group, we have used the Forns index to estimate liver fibrosis in drug users with HCV infection with or without HIV infection[96] and in patients with AUD[25]. The Forns index accurately predicted mid-term mortality and detected ALF in a fashion that was comparable to APRI and FIB-4[25].

Table 2 includes a summary of the more relevant papers describing assessment of alcohol use with non-patented indices to estimate liver fibrosis in patients with HCV infection.

### Combination of non-invasive methods

Because non-invasive indices are better suited for the detection of either the absence of liver fibrosis or the presence of ALF, several approaches of the sequential use of different methods have been proposed. A study published in 2017 by Spanish researchers showed that use of the ELF test with or without a confirmation elastography was cost-effective compared with a single liver biopsy for the detection of liver fibrosis in patients with HCV and alcohol-related liver disease[102].

Another new approach is the use of both FIB-4 and transient elastography to detect ALF in patients with FIB-4 values within the intermediate range (1.45–3.25)[103]. This approach has been used in a Russian cohort of individuals with high alcohol intake, HIV infection, and a high prevalence of chronic HCV infection[103].

**Future directions for the non-invasive assessment of liver damage**

The measurement of small non-coding microRNAs (miRNAs) is a promising field [104]. In fact, miR-155 and miR-122 have been proposed as potential markers of different forms of liver diseases, including those related to HCV and alcohol [105].

Other markers that could be used in the future are extracellular vesicles, the small membrane vesicles released by cells that transport mRNA, miRNA, proteins, and lipids, and circulating nucleic acids, including cell-free DNA and cell-free non-coding RNA [106].

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**CONCLUSION**

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Underlying liver disease can be accurately estimated with the use of several non-invasive methods that spare the necessity of performing a liver biopsy. The widespread use of these methods can help to accurately identify patients at risk for the development of end-stage liver disease. In addition to treatment with direct-acting antivirals for those with HCV infection, abstinence from alcohol consumption should be strongly recommended, given the overlap between alcohol-related and metabolic liver diseases.



**Table 2 Studies that used non-patented indices to estimate liver fibrosis in patients with hepatitis C virus infection and unhealthy alcohol use**

Ref.	Setting	Non-invasive method	Method for detecting alcohol consumption	Finding
Lieber <i>et al</i> [87], 2006	Veterans' affairs studies (2) of alcoholic liver disease	APRI <sup>1</sup>	Average alcohol intake	Low sensitivity and specificity of APRI in comparison to liver biopsy, especially in patients with HCV
Chaudhry <i>et al</i> [89], 2009	HIV Hopkins clinical cohort	APRI <sup>1</sup>	Past 6 mo of hazardous drinking	No effect of alcohol on APRI values in HCV/HIV co-infection
Blackard <i>et al</i> [90], 2011	WIHS cohort	FIB-4 <sup>2</sup>	Recent drinking	No association between alcohol intake and FIB-4 values in HCV/HIV co-infection
Muga <i>et al</i> [92], 2012	Patients with AUD admitted for detoxification	FIB-4 <sup>2</sup>	Past 6 mo of unhealthy drinking	No association between FIB-4 and alcohol use in HCV/HIV co-infection
Fuster <i>et al</i> [93], 2013	HIV-LIVE cohort	FIB-4 <sup>2</sup> and APRI <sup>1</sup>	LDH	No association between LDH and liver fibrosis measured with FIB-4 or APRI
Lim <i>et al</i> [94], 2014	VACS cohort	FIB-4 <sup>2</sup>	AUDIT-C <sup>3</sup>	Advanced liver fibrosis correlated with alcohol use
Kelly <i>et al</i> [91], 2017	WIHS cohort	FIB-4 <sup>2</sup>	Average number of drinks <i>per</i> week, past 6 mo	Light/moderate drinking was not associated with accelerated fibrosis progression
Sanvisens <i>et al</i> [25], 2018	Patients with AUD admitted for detoxification	FIB-4 <sup>2</sup> ; APRI <sup>1</sup> ; Forns	Past 6 mo, daily alcohol intake	Patients with HCV were two times as likely to present with advanced liver fibrosis

<sup>1</sup>Aspartate aminotransferase (AST)/platelet ratio index:  $\text{AST to platelet ratio index} = \{[\text{Patient AST}/\text{AST upper limit of normal (IU/L)}]/\text{platelet count (10}^9/\text{L)}\} \times 100$ .

<sup>2</sup>FIB-4 =  $\text{Age} \times \text{AST (IU/L)}/\text{platelet count (10}^9/\text{L)} \times \text{alanine aminotransferase (IU/L)}^{1/2}$ .

<sup>3</sup>AUDIT-C: Alcohol use disorders identification test. HCV: Hepatitis C virus; AST: Aspartate aminotransferase; APRI: AST/platelet ratio index; FIB-4: Fibrosis-4; AUD: Alcohol use disorder; HIV: Human immunodeficiency virus; LDH: Lifetime drinking History; WIHS: Women's interagency HIV study; VACS: Veterans aging cohort study.

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## Clinical indicators for progression of nonalcoholic steatohepatitis to cirrhosis

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### Abstract

Non-alcoholic fatty liver disease (NAFLD), is a disease spectrum characterized by fat accumulation in hepatocytes presenting as hepatic steatosis to advance disease with active hepatic inflammation, known as nonalcoholic steatohepatitis. Chronic steatohepatitis will lead to progressive hepatic fibrosis causing cirrhosis and increased risk for developing hepatocellular carcinoma (HCC). Fatty liver disease prevalence has increased at alarming rates alongside obesity, diabetes and metabolic syndrome to become the second most common cause of cirrhosis after alcohol related liver disease worldwide. Given this rise in prevalence, it is becoming increasingly more important to find non-invasive methods to diagnose disease early and stage hepatic fibrosis. Providing clinicians with the tools to diagnose and treat the full spectrum of NAFLD will help prevent known complications such as cirrhosis and HCC and improve quality of life for the patients suffering from this disease. This article discusses the utility of current non-invasive liver function testing in the clinical progression of fatty liver disease along with the imaging modalities that are available. Additionally, we summarize available treatment options including targeted medical therapy through four different pathways, surgical or endoscopic intervention.

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**Core Tip:** Fatty liver disease rates along with obesity, diabetes and metabolic syndrome continue to increase and now is the second leading cause of cirrhosis secondary to alcohol related liver disease. The need for consistent and readily available methods to accurately diagnose and stage hepatic fibrosis becomes increasingly necessary. With an up to date armamentarium to diagnose and treat the full spectrum of non-alcoholic fatty liver disease will decrease complications such as cirrhosis and hepatocellular carcinoma and will improve the likelihood for patients to have a higher quality of life.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) was first introduced by Schaffner and Thaler [1] in 1986. They assembled a group of non-alcoholic patients with liver diseases and biopsy specimens of liver pathology similar to that of alcoholic liver disease. They defined these subsets of patients as NAFLD. Over the last 20 years, the “non-alcoholic” portion of the diagnosis has been heavily criticized, as it carries an unfavorable connotation for patients that may negatively impact their overall care. In 2019, a group of international experts suggested the term metabolic (dysfunction) associated fatty liver disease “MAFLD” as a more appropriate diagnosis for NAFLD. As the underlying pathology is more related to metabolic dysfunction rather than the exclusion of alcohol [2]. Over the decades, NAFLD has grown to become the second most common cause of liver cirrhosis after alcohol related liver disease. The prevalence of NAFLD has grown every year in the United States secondary to a rise in diabetes, obesity and metabolic syndrome, with an incidence of 31% in 2012 as opposed to 18% in 1988–1991 [3,4]. NAFLD refers to a spectrum of liver injury due to accumulation of triglycerides in hepatocytes presenting as a spectrum of conditions, ranging from a simple hepatic steatosis characterized by fat accumulation in the absence of hepatic inflammation to a more severe disease form characterized by active hepatic inflammation, also known as nonalcoholic steatohepatitis (NASH). Progressive hepatic inflammation will lead to cirrhosis and increase the risk of developing hepatocellular carcinoma (HCC) as shown in (Figure 1). Up to 1/3 of NAFLD patients will have NASH which is a risk factor for fibrosis progression, and approximately 40% of NASH patients will experience fibrosis progression [5]. The recent estimated annual progression of fibrosis from NAFLD is up to 0.09% with an incidence of advanced fibrosis as 70 per 1000 patients [6]. The high prevalence of NASH among the biopsied patients could be explained secondary to the indication for biopsy in these patients with elevated liver function tests (LFTs), and the data cannot be extrapolated to the subset of NAFLD patients with normal LFTs where biopsy is not performed often. In the same study, the prevalence of NASH in patients without indication for biopsy was 6.7% [5]. The annual incidence of HCC in NAFLD patients is 44% per 1000 person-years. NAFLD-related HCC amounts to about 2% to 4% of annual cases [7].

Liver biopsy is not always performed and the diagnosis is often made with available non-invasive tests including blood tests and elastography [magnetic resonance elastography (MRE), Fibroscan]. Advantages of fibroscan, other than being a non-invasive modality that helps in sequential assessment of progression or regression of steatosis/fibrosis, include elimination of sampling error experienced by liver biopsy. Liver biopsy is the gold standard in confirming the diagnosis of NAFLD and allowing accurate hepatitis fibrosis staging. The major histologic features include steatosis, lobular inflammation, and cytological ballooning; these findings help in grading and



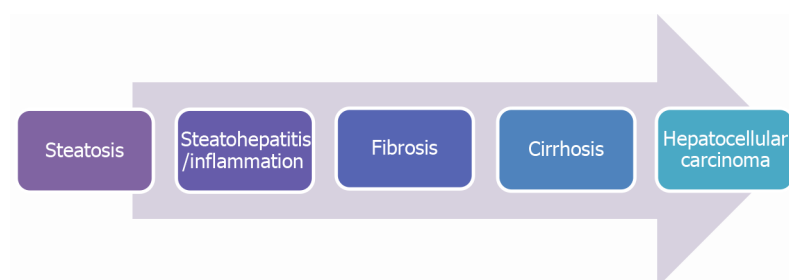


Figure 1 Histologic progression of nonalcoholic fatty liver disease from simple steatosis to cirrhosis.

staging the disease[8,9]. Moreover, the diagnostic tests especially non-invasive fibrosis assessment testing helps to monitor NAFLD stages to prevent disease progression and diagnose cancer early. Our review article discusses the indicators that help in understanding the progression of the disease including symptomatic worsening, liver function testing, imaging, and histopathological changes.

## REVIEW

NAFLD encompasses a spectrum of conditions which ranges from bland hepatic steatosis to steatohepatitis causing hepatic fibrosis which will lead to cirrhosis, liver failure and increase the risk of HCC. The risk factors of fatty liver disease are similar to those of metabolic syndrome which leads to insulin resistance[5]. This includes diabetes mellitus, dyslipidemia and elevated body mass index (BMI). It is important to distinguish simple hepatic steatosis, which carries very low risk of developing chronic disease and cirrhosis *vs* NASH which carries a risk of progressive fibrosis, cirrhosis, liver failure and HCC. Overall, one fifth of NASH patients can progress to advanced hepatic fibrosis[10-12]. Hence, the assessment of the degree of hepatic fibrosis with noninvasive diagnostic panels and imaging is important in monitoring disease progression. Several scoring systems and specialized biomarkers have been developed by combining various serologic and clinical parameters for the prediction of fibrosis in NAFLD[13-18]. Despite the advancement of many diagnostic noninvasive fibrosis assessment modalities, one fourth of advanced fibrosis NASH patients can be misclassified as mild hepatic fibrosis[14].

The mortality of NAFLD is not merely targeting the liver. The majority of NAFLD patients are at risk of developing atherosclerotic coronary artery disease carrying higher mortality rate approaching[19]. Understanding patients' risk factors and stage of hepatic fibrosis can help predict patients' clinical outcomes[10]. Multiple clinical indicators and serological markers of disease progression remains an area of intensive clinical and basic science research till this day (Tables 1 and 2).

## ROLE OF NONINVASIVE LIVER FUNCTION TESTING IN CLINICAL PROGRESSION OF NASH

### *Clinical assessment in progression of nash*

NASH is a histological diagnosis characterized by hepatocytic inflammation that may progress to fibrosis. Hepatic fibrosis divided into four stages. Stage I describes as mild hepatic fibrosis, stage II moderate hepatic fibrosis, stage III moderate to severe fibrosis, and stage IV severe or advanced fibrosis. It is crucial to identify advanced fibrosis stage as these patients are at-risk to develop decompensated cirrhosis and end-stage liver disease. A number of clinical factors help clinicians to predict the likelihood of the patient progressing into devastating categories of this disease.

### *Role of liver chemistry in the clinical progression of NAFLD*

Liver chemistry test identify active hepatic inflammation. This includes alanine aminotransferases (ALT) and aspartate aminotransferases (AST), alkaline phosphatase (ALP) and direct and indirect bilirubin. Other laboratory data should be monitored in NASH patients are platelet count and coagulation panel, fasting blood glucose and glycosylated proteins and lipid panel. Serum hyaluronic acid tissue metalloproteinase,

**Table 1 Role of noninvasive liver function testing in clinical progression of nonalcoholic steatohepatitis**

Sr. No.	Indicator	Ref.	Journal	Year	Results
1	Bilirubin	Demir <i>et al</i> [24]	<i>PLoS One</i>	2013	Total bilirubin was identified as a significant predictor of advanced fibrosis and used to construct the NIKEI score which can reliably exclude advanced fibrosis in subjects with NAFLD
		Ratziu <i>et al</i> [25]	<i>BMC Gastroenterol</i>	2006	FibroTest which includes total bilirubin in its panel is a simple and noninvasive quantitative estimate of liver fibrosis which reliably predicts advanced fibrosis
		Adams <i>et al</i> [11]	<i>J Hepatol</i>	2005	Hepascore, a model of 4 serum markers plus age and sex provides clinically useful information regarding different fibrosis stages among hepatitis C patients
2	Serum AST/ALT	Martin-Rodriguez <i>et al</i> [20]	<i>Medicine (Baltimore)</i>	2017	Serum ALT level is the most predictive laboratory investigation for the NAFLD. The AST-ALT Ratio (AAR) is higher in increasing liver fat content, fibrosis and other metabolic derangements like diabetes and dyslipidemia
		Enomoto <i>et al</i> [21]	<i>World J Gastroenterol</i>	2015	AAR > 1 is consistent with NASH
		Arora <i>et al</i> [22]	<i>J Clin Exp Hepatol</i>	2012	AAR > 1 may indicate the progression of NAFLD and aid in diagnosing liver fibrosis
		Shah <i>et al</i> [17]	<i>Clin Gastroenterol Hepatol</i>	2009	The FIB-4 score composed of age, AST and ALT and platelet counts is an invasive and inexpensive method which has shown superiority to BAAT (BMI, Age, ALT, Triglycerides) and BARD (BMI, AST: ALT, Diabetes) scores in monitoring the progress of NASH
		McPherson <i>et al</i> [18]	<i>Eur J Gastroenterol Hepatol</i>	2013	The FIB-4 score was reliable in ruling out advanced fibrosis in patients with histological evidence of NAFLD who had normal or increased levels of ALT, thus decreasing the need for invasive liver biopsy
3	Platelet Count	Enomoto <i>et al</i> [21]	<i>World J Gastroenterol</i>	2015	A reducing level of platelet count has been well documented in advancing liver diseases
		Kawamura <i>et al</i> [26]	<i>Hepatol Int</i>	2015	FSN score of 17 variables including platelet count could accurately predict fibrotic stage and discriminates patients with advanced fibrosis of NASH
		Kessoku <i>et al</i> [27]	<i>World J Gastroenterol</i>	2014	PLALA Score is a very unique scoring system as it has shown usefulness in distinguishing cirrhosis in NAFLD when compared with most fibrosis scoring systems
		Abdel-Razik A <i>et al</i> [47]	<i>Eur J Gastroenterol</i>	2016	MPV is a noninvasive novel marker to predict advanced disease as it was increased in NASH patients and advance liver fibrosis
		Cengiz <i>et al</i> [48]	<i>Eur J Gastroenterol</i>	2015	Red cell volume distribution width-to-platelet ratio was both correlated and able to predict liver fibrosis. It may reduce liver biopsy in NAFLD
4	Fasting blood glucose and glycosylated protein	Pelusi <i>et al</i> [49]	<i>PLoS One</i>	2016	Nonalcoholic steatohepatitis with greater degree of fibrosis was discovered in patients with insulin resistance. Type 2 diabetes in patients with NAFLD tends to drive the rate of fibrosis
5	Hyaluronic acid (hyluroante) tissue metalloproteinase	Arora <i>et al</i> [22]	<i>J Clin Exp Hepatol</i>	2012	European Liver Fibrosis score ELF scoring system has indicators for cellular matrix activities including Hyaluronic acid (hyluroante) tissue metalloproteinase which has been indicative of fibrosis
6	Type IV collagen	Nakamura <i>et al</i> [30]	<i>J Diabetes Investig</i>	2013	NAFIC Score including type IV collagen 7S and Modified NAFIC score were proven to be clinically useful in screening for NASH in NAFLD patients
7	Glycosylated Albumin to Glycosylated Hemoglobin Ratio	Hu <i>et al</i> [28]	<i>World J Gastroenterol</i>	2014	HOMA-IR score indicates NAFLD progression using a formula that involves insulin levels and fasting glucose to calculate insulin resistance. The score has a high sensitivity for NASH
		Stål[29]	<i>World J Gastroenterol</i>	2015	NAFLD fibrosis score, a non- invasive score which includes the presence of diabetes or impaired fasting glucose is the most predictive of mortality in NASH as compared to NAFL patients
8	Prothrombin time	Assy <i>et al</i> [31]	<i>World J Gastroenterol</i>	2005	Increase prothrombin time is usually associated with cirrhotic changes
9	Albumin	Bazick <i>et al</i> [5]	<i>Diabetes Care</i>	2015	Serum albumin gets reduced in patients progressing to NASH and fibrosis from NAFLD

ALT: Alanine aminotransferases; BMI: Body mass index; NAFIC: Nonalcoholic steatohepatitis, ferritin, insulin, and type IV collagen 7S; NASH:

Nonalcoholic steatohepatitis; AAR: Aspartate aminotransferases-alanine aminotransferases ratio; RPR: Red cell volume distribution width-to-platelet ratio; NFS: Non-alcoholic fatty liver disease fibrosis score; NIKEI: Non-invasive koeln-essen-index; NAFLD: Non-alcoholic fatty liver disease.

**Table 2** Role of imaging techniques in clinical progression of nonalcoholic steatohepatitis

Sr. No.	Imaging modality	Ref.	Journal	Year	Results
1	Ultrasound	Sanyal [32]	<i>Gastroenterology</i>	2002	US is currently the preferred method in United States for screening asymptomatic patients with elevated liver enzymes and suspected NAFLD with sensitivity in detecting steatosis varying between 60%-94%
2	Magnetic Resonance Elastography	Iijima <i>et al</i> [35]	<i>Hepatol Res</i>	2007	Magnetic resonance elastography has excellent diagnostic accuracy with sensitivity and specificity of 98% and 99%, respectively, for detecting all grades of fibrosis
		Huwart <i>et al</i> [36]	<i>Gastroenterology</i>	2008	Magnetic resonance elastography was associated with a higher technical success rate than US elastography
3	Fibroscan	Wong <i>et al</i> [39]	<i>Gut</i>	2012	Transient elastography had shown good results in patients with NAFLD. It is a non-invasive method of assessing liver fibrosis which can be performed at the bedside or in the outpatient clinic
		Wong <i>et al</i> [40]	<i>Hepatology</i>	2010	Transient elastography had shown good results in patients with NAFLD. It is a non-invasive method of assessing liver fibrosis which can be performed at the bedside or in the outpatient clinic
		Ratzu <i>et al</i> [43]	<i>Gastroenterology</i>	2005	Fibroscan has now been validated in NAFLD, and represents a useful tool for rapid, non-invasive assessment of liver fibrosis and determining the need for biopsy. Nonetheless, fibroscan values should be interpreted in consonance with clinical, biological, and morphological data

US: Ultrasonography; NAFLD: Non-alcoholic fatty liver disease.

and type 4 collagen are serological markers help in assessing fibrosis stage.

**AST and ALT:** In a cross-sectional study, Martin-Rodriguez *et al* [20] reported that serum ALT level is the most predictive laboratory investigation for NAFLD. The AST-ALT Ratio (AAR) is higher in increased liver fat content, fibrosis, and other metabolic derangements like diabetes and dyslipidemia. Steatosis or steatohepatitis can be observed, but nevertheless patients have normal serum ALT levels [8]. An AAR > 1 is consistent with a diagnosis of NASH. This forms the basis of several other laboratory combinations that may indicate the progression of NAFLD and diagnosing liver fibrosis including BAAT (which uses BMI, age, ALT, and triglycerides), BARD (which uses BMI, AST: ALT, and diabetes), and FIB-4 scores [21,22]. The FIB-4 score is a simple, noninvasive and inexpensive test superior to BAAT and BARD scores in monitoring the progress of NASH [17]. The FIB-4 score is reliable in ruling out advanced fibrosis in patients with histological evidence of NAFLD who had normal or increased levels of ALT, thus decreasing the need for invasive liver biopsy with sensitivity 84%-94% [18].

**ALP:** Few subsets of NASH patients present with an isolated ALP elevation [23]. Cholestasis also has been noted on histology in NASH [23]. Elevated ALP should be accompanied by an increase in  $\gamma$ -glutamyltransferase (GGT) enzyme suggesting hepatic inflammation. Otherwise elevated ALP without GGT elevation are seen in pregnancy, muscular disease and bone disease such as Paget's disease.

**Bilirubin:** Bilirubin is synthetic marker for liver function alongside with PT/INR. Also, it is a part of various scoring system used to estimate the degree of fibrosis. Demir *et al* [24] introduced the non-invasive koeln-essen-index (NIKEI) score which uses age, AST, AST/ALT ratio, and total bilirubin. In a prospective study by Ratzu *et al* [25], the diagnostic utility of FibroTest, a noninvasive marker of fibrosis, was determined in a sample of 170 patients with NAFLD. The FibroTest includes  $\alpha$ -2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin, and  $\gamma$ -glutamyl-transpeptidase. Ratzu concluded this simple and noninvasive quantitative estimate of liver fibrosis reliably predicts advanced fibrosis [25]. Hepascore, a combination of bilirubin,  $\gamma$ -glutamyl-transpeptidase, hyaluronic acid, and 2-macroglobulin together with age and sex, is an accurate and reliable panel in predicting different stages of fibrosis. However, the limitation of this study included validation of this score among

only patients with hepatitis C[21]. NIKEI had superior negative predictive value for advanced fibrosis compared to the FIB-4 score (which uses age, AST, ALT, and platelet counts)[24].

**Platelet count:** Platelet count has great value in assessing degree of fibrosis. Thrombocytopenia occurs in cirrhosis secondary to thrombopoietin deficiency and splenic sequestration from underlying splenomegaly occurring from portal hypertension. Platelet level has been used in combination with other biochemical parameters such as in AST/platelet ratio index and FIB-4 score to monitor liver disease progression[21]. Kawamura established the fibrosis score for NASH (FSN), a new scoring system specific to the fibrotic stage of NASH[26]. FSN can accurately predict the fibrotic stage and distinguishes patients with advanced fibrosis of NASH. The platelet albumin AAR (PLALA) score is unique in that it distinguishes cirrhosis in NAFLD compared to most other fibrosis scoring systems. Each factor (platelet count  $< 15.3 \times 10^4 / \mu\text{L}$ ; albumin  $< 4 \text{ g/dL}$ ; AAR  $> 0.9$ ) is awarded 1 point, and a PLALA score of 2 or 3 may be predictive of cirrhosis in patients with NAFLD[27]. The PLALA score may be an ideal scoring system for detecting cirrhosis in NAFLD patients with sufficient accuracy and simplicity for clinical use. Mean platelet volume (MPV) was elevated in NASH and advanced liver fibrosis (stages 3–4) patients, making MPV a noninvasive, novel marker to predict advanced disease. Another study looked into the performance of red cell volume distribution width-to-platelet ratio in predicting liver fibrosis in patients with NAFLD[27]. This ratio was both correlated and able to predict liver fibrosis.

**Fasting blood glucose and glycosylated protein:** In their observational cohort of 118 patients, assessing the clinical determinants of fibrosis progression rate in NAFLD patients with baseline and follow-up histological evaluation. Advanced fibrosis is more likely to be found in patients with underlying type 2 diabetes[28]. These patients had histological evidence of more inflammation in the fibrous portal areas in those already developing cirrhosis than those at an earlier stage of the disease. Furthermore, this study also observed that type 2 diabetes can drive fibrosis in the absence of hepatic inflammation.

**Glycosylated albumin to glycosylated hemoglobin ratio:** Glycosylated albumin (GA) and glycosylated hemoglobin (HbA1c), which are indicators of glycemic control, show a strong relationship with advanced liver fibrosis. The GA/HbA1c ratio, which is typically 3 in a healthy individual, is higher in liver fibrosis patients. Patients with chronic liver diseases have reduced albumin turnover resulting in an elevated level of GA. Also, they have a reduced erythrocyte lifespan which accounts for changes in the increased ratio. The GA/HbA1c ratio's accuracy in detecting liver fibrosis might be limited by other concurrent diseases that can affect plasma and hemoglobin levels[25]. The HOMA-insulin resistance score is a somewhat rigorous and reliable scoring system that indicates NAFLD progression using a formula that involves insulin levels and fasting glucose to calculate insulin resistance[28]. The HOMA-insulin resistance score has a high sensitivity for NASH. The NAFLD fibrosis score (NFS) is a noninvasive score (using age, albumin, AST/ALT ratio, BMI, the presence of diabetes or impaired fasting glucose, and platelet count) most predictive of mortality in NASH compared to NAFLD[29]. As of 2015, the NFS score was endorsed by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association as a screening guideline in clinical practice. These screening tools may be more important in detecting NASH in people with diabetes.

**Hyaluronic acid tissue metalloproteinase:** A high level of hyaluronic acid (hyaluronate) tissue metalloproteinase 1 has been indicative of fibrosis. The European liver fibrosis scoring system has indicators for cellular matrix activities including age, the amino-terminal peptide of procollagen III, tissue metalloproteinase 1 inhibitor, and hyaluronic acid[22].

**Type IV collagen:** The FSN score, which includes type IV collagen 7S, platelet count, AST, and ALT, has been more efficient in distinguishing the advanced fibrosis stages 3–4 of NASH compared to other scoring systems including APRI (AST to platelet ratio index), NAFLD Score, FIB-4 Index, BARD, and NIKEI[26]. The nonalcoholic steatohepatitis, ferritin, insulin, and type IV collagen 7S (NAFIC) score, and modified NAFIC score were proven to be clinically useful in screening for fatty liver patients[30].

**Albumin:** Bazick *et al*[5] demonstrated that serum albumin gets reduced drastically in patients presenting with NASH. Their clinical variable could be used to guide clinical



decision making about referring patients with diabetes and NAFLD to hepatologists [5].

**Prothrombin time:** In a study by Assy *et al* [31], up to 46% of patients with NAFLD showed thrombotic risk factors. The presence of thrombotic risk factors correlated with the extent of hepatic fibrosis. This is consistent with known coagulopathy in those with altered synthetic function due to hepatic fibrosis.

## ROLE OF IMAGING TECHNIQUES IN THE CLINICAL PROGRESSION OF NASH

Noninvasive techniques such as ultrasonography (US), computed tomography, magnetic resonance imaging, and proton magnetic resonance spectroscopy can detect hepatic steatosis but cannot reliably distinguish simple steatosis from NASH [32].

### Liver US

US is the preferred cost effective method in the United States for screening patients with suspected NAFLD. The findings on US include: diffuse increase in echogenicity of the liver parenchyma, hepatomegaly and vascular blunting [33]. The sensitivity of US in detecting hepatic steatosis up to 94%. The sensitivity decreases as the degree of steatosis dropped below 30% [33-35]. US cannot differentiate between simple hepatic steatosis *vs* NASH. Thus, laboratory serological and histological data is helpful in pointing towards NASH [22].

### MRE

MRE stands for magnetic resonance elastography which is an imaging technique that combines MRI imaging with low-frequency vibrations to measure hepatic stiffness. MRE equivalent of transient elastography has recently demonstrated excellent diagnostic accuracy. It has shown a sensitivity and specificity of 98% and 99%, respectively, for detecting all grades of fibrosis [35]. Huwart *et al* [36] conducted a prospective blind comparison of MRE, US elastography, and APRI (AST to platelet ratio index) in a study of 141 patients who underwent liver biopsy for chronic liver disease. They found MRE was associated with a higher technical success rate than US elastography [36].

### Fibroscan

Transient Elastography (Fibroscan, Echosens, Paris, France) is a noninvasive method of assessing liver fibrosis. It can be performed at the bedside or in the outpatient clinic. It employs US-based technology to measure liver stiffness and has been validated for use in patients with chronic hepatitis C and B [37,38]. However, studies have shown good results in patients with NAFLD [39,40]. In only 5% of the cases, it has failed to show any readings. This is mostly seen in obese patients. This limits the TE's utility in the NAFLD cohort. However, a recently introduced XL probe may reduce this problem [41]. In a meta-analysis for NASH with advanced fibrosis, pooled area under the receiver operating characteristic curve, sensitivity and specificity of NFS, and fibroscan are 0.85 (0.80-0.93), 0.90 (0.82-0.99), 0.97 (0.94-0.99), and 0.94 (0.90-0.99), 0.94 (0.88-0.99) and 0.95 (0.89-0.99), respectively [16]. Fibroscan is validated in NAFLD and represents a useful tool for rapid, noninvasive assessment of liver fibrosis and determining the need for biopsy. As this modality evaluates liver stiffness (related to fibrosis, inflammation, and portal hypertension), Fibroscan values should be interpreted in context of the morphological, biological, and clinical data.

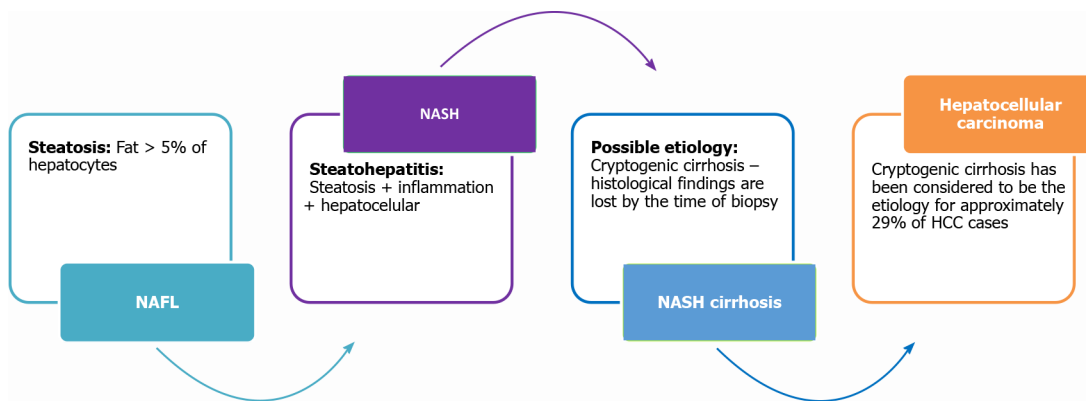
## ROLE OF LIVER BIOPSY IN THE CLINICAL PROGRESSION OF NASH

A percutaneous liver biopsy is currently the gold standard to assess hepatic fibrosis and inflammation in chronic liver disease [42]. However, liver biopsy is an invasive procedure with associated costs, complications, and inherent inaccuracy due to sampling error and inter-observer and intra-observer variability in histopathological interpretation [42,43]. Despite the criticism of liver biopsy's associated risks, there are 3 basic histological systems that can be used to monitor the progression of NASH (Figure 2). These systems are the steatosis activity and fibrosis score, the NASH

**Table 3** Nonalcoholic steatohepatitis activity score; steatosis, activity, and fibrosis score; and brunt grading and staging systems

NASH activity score	Steatosis, activity and fibrosis score	Brunt grading and staging
Steatosis grade 0-3	Steatosis S0-S3	Grade 1 (Mild)
Lobular inflammation 0-3	Activity A1-A3	Grade 2 (Moderate)
Ballooning 0-2	Lobular inflammation 0-2	Grade 3 (Severe)
Fibrosis 0-4 (grade 1 has subgrade A, B, C)	Ballooning 0-2	Stages fibrosis
	Fibrosis F0-F4	Stage 1-4

NASH: Nonalcoholic steatohepatitis.



**Figure 2** The spectrum of nonalcoholic fatty liver disease. NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma.

activity score, and the Brunt system that grades and stages NASH (Table 3)[28,44]. Due to the risks and limitations associated with liver biopsy, it is controversial to perform liver biopsy on every patient suspected of having NAFLD. Therefore, it cannot be considered a “screening” tool[45]. However, there are studies that support the importance of liver biopsy. An older study, by Skelly *et al*[46] showed that biopsy on 354 patients with abnormal liver tests-66% had fatty liver, 50% of those had steatohepatitis, and approximately 19% of the remaining biopsies had other treatable causes diagnosed by the pathology evaluation. This included autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis and alcoholic liver disease. An adequate liver biopsy, with appropriate clinical history, interpreted by a trained liver pathologist, is not only pivotal for an accurate and complete diagnosis (or exclusion) of NAFLD (or NASH), but also is optimal for obtaining detailed information regarding disease pattern, severity and fibrosis. It not only provides important information with respect to subtypes, potential future risks, possible etiology, and natural history of disease, but also sets the groundwork for future molecular studies and clinical trials, assisting clinical colleagues and patients with treatments and follow-up[47-49].

## CONCLUSION

NASH-related cirrhosis is the most common cause of chronic liver disease and indication for liver transplant. The increasing number of affected people imposes a strain on available organs. There are many comorbidities and risk factors implicated in NASH severity and progression to chronic liver disease.

Due to the increasing prevalence of NAFLD in the population, there is an increasing need to find non-invasive methods to diagnose and stage NAFLD. The ideal test should be reproducible, cheap, and able to diagnose full spectrum of NAFLD, predict fibrosis, and reflect changes that occur with treatment. Preliminary evaluation includes clinical presentation with consideration of comorbidities and liver function test in the blood. Noninvasive imaging such as MRE and fibroscan can provide objective measures of liver steatosis and stiffness in patients without advanced fibrosis or

cirrhosis. Due to the limitations, risks and cost of liver biopsy-it cannot be used as a screening test, although is typically relied upon to confirm the diagnosis. Several different methodologies including imaging modalities, serum markers and combined tests are currently being investigated.

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## Update on the management and treatment of viral hepatitis

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### Abstract

This review aims to summarize the current evidence on the treatment of viral hepatitis, focusing on its clinical management. Also, future treatment options and areas of potential research interest are detailed. PubMed and Scopus databases were searched for primary studies published within the last ten years. Keywords included hepatitis A virus, hepatitis B virus (HBV), hepatitis C virus, hepatitis D virus (HDV), hepatitis E virus, and treatment. Outcomes reported in the studies were summarized, tabulated, and synthesized. Significant advances in viral hepatitis treatment were accomplished, such as the advent of curative therapies for hepatitis C and the development and improvement of hepatitis A, hepatitis B, and hepatitis E vaccination. Drugs that cure hepatitis B, going beyond viral suppression, are so far unavailable; however, targeted antiviral drugs against HBV (immunomodulatory therapies and gene silencing technologies) are promising approaches to eradicating the virus. Ultimately, high vaccination coverage and large-scale test-and-treat programmes with high screening rates may eliminate viral hepatitis and mitigate their burden on health systems. The development of curative hepatitis C treatment renewed the enthusiasm for curing hepatitis B, albeit further investigation is required. Novel therapeutic options targeting HDV life cycle are currently under clinical investigation.

**Key Words:** Viral hepatitis; Hepatitis A virus; Hepatitis B virus; Hepatitis C virus; Hepatitis D virus; Hepatitis E virus

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**Core Tip:** Viral hepatitis is a major global public health problem due to the risk of progression to chronic hepatitis, cirrhosis, and hepatocellular carcinoma development. The clinical management and treatment of these infections have evolved over the last decade. Even though remarkable achievements have been accomplished, such as the development of curative hepatitis C treatment, drugs that cure hepatitis B are still missing. In addition, programmes to enhance viral hepatitis testing and treatment together with broad vaccination coverage are required. In this review, we summarize the current evidence on the treatment of viral hepatitis and detail future treatment options, and potential areas of research.

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## INTRODUCTION

Viral hepatitis is a major public health problem given that it can become chronic, and eventually lead to end-stage liver disease and/or hepatocellular carcinoma (HCC) development[1,2]. Consequently, viral hepatitis is one of the leading indications for liver transplantation and, thus, contributes to the discrepancy between donor organ supply and demand[3].

Whereas hepatitis A and E usually present with a self-limited course followed by complete recovery, hepatitis B and C often result in chronic infection and they are responsible for the most adverse consequences of this disease[4]. Worldwide, approximately 100 million people have the antibody against the hepatitis C virus (HCV) and 71 million present HCV viremia, according to the World Health Organization (WHO) [5]. In a multicentre international study, with the participation of 161 countries, the prevalence of the hepatitis B virus (HBV) surface antigen (HBsAg) was 3.61% [6]. Due to the high prevalence, WHO has set targets for eliminating hepatitis B and C by 2030. These targets include optimizing measures to prevent disease transmission and improving antiviral treatment offering[7]. In the last decade, rapid and significant advances in diagnosing and managing viral hepatitis have been made and have changed its treatment. Despite these advances, issues with screening, diagnosis, referral, and treatment of viral hepatitis still persist.

Therefore, due to the high prevalence of viral hepatitis and its serious consequences, research activity in this field has always been intense, and new and increasingly effective treatments have gradually emerged. This review aims to summarize the current evidence on the treatment of viral hepatitis, focusing on its clinical management. Also, future treatment options and areas of potential research interest are detailed.

## HEPATITIS A

Hepatitis A is caused by the hepatitis A virus (HAV), a ribonucleic acid (RNA) picornavirus. The virus is transmitted by the faecal-oral route and this is a major cause of acute viral hepatitis. Clinical manifestations range from asymptomatic infection to acute liver failure (ALF), occurring in less than 1% of cases, and there is no progression to chronic hepatitis[8]. Globally, an estimated 1.4 million cases of hepatitis A occur each year and 27731 deaths were registered in 2010[8]. This disease can occur sporadically or in an epidemic form and risk factors for transmission are mainly person-to-person contact related or *via* contaminated food or water[9,10].

Hepatic injury results from the host immune response to the HAV. Viral replication occurs in the hepatocyte cytoplasm and hepatocellular damage is caused by the destruction of infected cells mediated by human leukocyte antigen-restricted HAV-specific CD8<sup>+</sup> T lymphocytes and natural killer cells[8]. Exaggerated host response and marked reduction of circulation HAV RNA during acute infection are associated

with severe hepatitis. The development of symptomatic hepatitis is usually related to patient age as more than 70% of infected adults develop symptoms[8]. Full clinical and biochemical recovery is observed within two to three months in 85% of patients and complete recovery is observed by six months in nearly all patients[11]. The diagnosis is established by detection of serum immunoglobulin M antibody to HAV, which remains detectable for approximately three to six months. Serum immunoglobulin G antibodies appear early in the convalescent phase of the disease, remain detectable for decades, and are associated with lifelong protective immunity[8,11].

To date, there are no specific drugs against HAV infection available; thus, treatment consists mostly of supportive care[8,11]. Prevention of HAV infection includes vaccination, immune globulin, and attention to hygienic practices-handwashing, avoiding consumption of tap water and raw foods in areas with poor sanitation, and heating foods appropriately[12]. In summary, indications for vaccination include children aged 2-18 years who have not previously received hepatitis A vaccine, all persons aged more than one year infected with human immunodeficiency virus, and specific risk groups (individuals with chronic liver disease, travellers, men who have sex with men, *etc.*). Also, vaccination strategies may vary according to local public health policies in each country[8,12].

## HEPATITIS B

Although an effective preventive hepatitis B vaccine has existed for over 30 years, HBV infection is still a major cause of chronic liver disease worldwide[13]. HBV is a small deoxyribonucleic acid (DNA) virus of the Hepadnaviridae family. HBV infects hepatocytes and establishes its replication cycle *via* an RNA intermediate (through reverse transcription) and can integrate into the host genome, thus being able to persist in the nucleus of hepatocytes[13,14]. The viral envelope involves a nucleocapsid that contains a partially double-stranded and relaxed circular DNA genome (rcDNA)[15]. In the cytoplasm of infected hepatocytes, the nucleocapsid is transported to the nucleus and then the rcDNA is released and converted into a covalently closed circular DNA (cccDNA) by host factors, forming a stable minichromosome[15,16].

Chronic hepatitis B is a dynamic infectious disease with a pattern of progression strongly dependent on the interaction between the host immune response and the virus. Over two-thirds of patients with chronic hepatitis B are inactive carriers. They present a low viral replication rate and minimal or no liver necroinflammation, secondary to weak activation of the innate immunity and HBV-specific immunological response[17].

The definition of goals for HBV treatment is essential. A virological response during nucleos(t)ide analogue (NA) therapy is defined as a decrease in serum HBV DNA to undetectable levels by tests with a lower limit of detection of 10–20 IU/mL. If interferon (IFN) alpha is used for treatment, the virological response is defined as a serum level of HBV DNA below 2000 IU/mL, assessed at 6 mo after the start of treatment and at the end of the therapy[14]. The biochemical response is defined as the normalization of serum alanine aminotransferase. Biochemical response allied to a reduction in HBV viral load is an important goal to be achieved because they are both associated with a decreased risk of progression to cirrhosis and HCC[14,18].

Current key targets of HBV treatment are a functional cure and a complete or “sterilizing” cure[19,20]. A functional or partial cure is defined as a sustained loss of HBsAg with or without anti-HBs seroconversion, based on assays with a lower limit of HBsAg detection of 0.05 IU/mL. Complete cure is defined as the elimination of cccDNA together with sustained loss of HBsAg and undetectable serum HBV DNA[19,20]. Whilst liver biopsy is currently necessary to measure the intrahepatic activity of cccDNA, serum biomarkers that reflect this indicator have been examined for this purpose[21].

The persistence of cccDNA in the hepatocyte nucleus is the greatest therapeutic challenge in hepatitis B patient care. Even among patients who recover from acute infection, presenting HBsAg loss with HBsAg seroconversion, HBV may persist in a latent state. These patients are potentially at risk of reactivation if exposed to either cancer chemotherapy or immunosuppressive therapies (after transplantation, for example)[22,23].

Although lamivudine was used for many decades to treat chronic hepatitis B-due to its safety and low cost, the low genetic barrier and the risk of developing drug resistance resulted in this being a less effective therapy compared to other treatment agents. Currently, lamivudine therapy is reserved for specific situations, for example,



the unavailability of entecavir or tenofovir[24]. In addition, this treatment may still play a role in HIV-coinfected patients when used as part of an antiretroviral regimen [24].

The two formulations of IFN (conventional and pegylated) and five NAs [telbivudine, entecavir, tenofovir disoproxil fumarate (TDF), tenofovir alafenamide fumarate (TAF), and besifovir dipivoxil] are antiviral agents used for chronic hepatitis B treatment. Albeit these drugs strongly suppress HBV replication, reduce the risk of cirrhosis, and prevent further disease progression, they are not curative and have no proved positive impact on the existing viral hepatocyte reservoir[24]. According to major hepatology societies, entecavir, TDF, TAF, and pegylated (Peg) IFN alpha are currently the first-line anti-HBV agents recommended for chronic hepatitis B treatment [25-27].

Over the last few years, TAF was developed as a safer alternative to TDF because the latter is associated with both proximal renal tubular dysfunction and low bone mineral density. Due to the pharmacological properties of TAF, far more active drug is delivered to target cells while much less is measurable in the bloodstream, reducing systemic toxicity[28,29]. These properties are especially beneficial for elderly patients, patients with renal dysfunction, or osteoporosis[26,28-30].

NAs and IFN have different modes of action as well as particular advantages and disadvantages. On the one hand, compared to NA, IFN has the advantages of being a treatment with a finite duration, absence of resistance, and a higher chance of off-treatment sustained virological response (SVR); as well as potentially offering a greater opportunity for sustained loss of HBsAg/anti-HBs seroconversion. Yet, IFN has the disadvantage of moderate antiviral effects, low tolerability, and an increased risk of adverse events[24,31]. On the other hand, compared to IFN, NA therapy has higher rates of undetectable serum HBV DNA and transaminase normalization after treatment, whilst requiring long-term therapy-hardly envisioning withdrawal-due to the high rate of disease recurrence after discontinuing the medication[19,20].

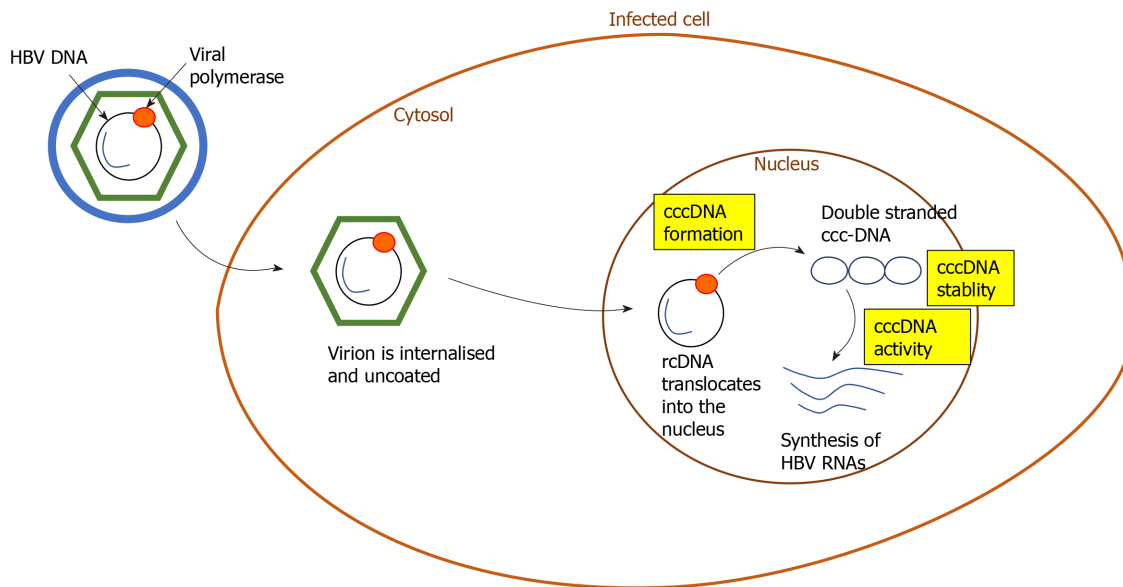
Importantly, proper patient selection for better clinical efficacy in HBV treatment with Peg-IFN alpha is essential. Female gender, young age, high level of transaminases, lower level of HBV DNA, high rate of liver inflammation on biopsy samples (at least METAVIR A2), HBV genotype A or B, and low viral load increase the chance of a more favourable response to treatment[19,20,24,31]. For patients treated with NA for a longer time without serum hepatitis B e antigen (HBeAg) seroconversion or loss of HBsAg, add-on or switch to Peg-IFN therapy is an option to enhance patient response, although a protocol for this has not been determined. Large randomized controlled trials are awaited to provide definitive evidence of these strategies[32-34].

Elimination or inactivation of HBV cccDNA is the central focus of HBV research nowadays. Figure 1 illustrates treatment options that target cccDNA to attack HBV persistence, and these include interventions aiming to prevent cccDNA formation, affect its stability or even its activity. Although mechanistically these therapies would potentially offer a cure for the infection, further basic research and more detailed molecular studies are needed to evaluate the translational potential of novel antiviral strategies. New drugs that target HBV are required and immunomodulatory therapies and gene silencing technologies are the most promising approaches to eradicate HBV without killing the infected hepatocytes[17,21,35]. The advent of curative therapies for hepatitis C has renewed enthusiasm for also curing hepatitis B, going beyond viral suppression. Currently, there are numerous drugs under investigation to enable the cure of HBV infection[36]. In addition, efforts to increase hepatitis B vaccination coverage must be a priority.

## HEPATITIS C

The Nobel Prize in Physiology or Medicine in 2020 was awarded to three scientists, Harvey Alter, Michael Houghton, and Charles Rice, for their efforts on the identification of HCV[37]. The discovery of HCV was a remarkable achievement, which saved millions of lives. It enabled the development of highly sensitive diagnostic blood tests and the rapid expansion of the pool of antiviral drugs directed at hepatitis C. Approximately 71 million people worldwide live with HCV and nearly half of them are currently unaware due to suboptimal screening programmes[5].

Hepatitis C infection is a silent systemic disease secondary to a hepatotropic and lymphotropic virus with a high chronicity rate. It promotes chronic systemic inflammation due to direct and indirect viral activities, characterised by increased levels of pro-inflammatory cytokines and chemokines. Chronic systemic inflammation is a well-known risk factor for insulin resistance; thus, it increases the risk for type 2 diabetes



**Figure 1 Diagrammatic summary of the therapeutic options targeting covalently closed circular deoxyribonucleic acid to prevent hepatitis B virus persistence.** After entering the cell, the virion is uncoated and the relaxed circular deoxyribonucleic acid (DNA) genome (rcDNA) translocates into the cell nucleus. Once there, the covalently closed circular DNA (cccDNA) formed resides in the nucleus of infected cells as a minichromosome and originate the new viruses. Drugs that prevent cccDNA formation, that affect its stability, or even cccDNA activity must stop hepatitis B virus persistence. cccDNA: covalently closed circular deoxyribonucleic acid; HBV: Hepatitis B virus; RNA: Ribonucleic acid.

mellitus and, for cardiovascular events[38].

Hepatic manifestations include steatosis, fibrosis, and, finally, cirrhosis. The complications of cirrhosis and the occurrence of HCC compose the indications for liver transplantation in this disease[39]. Due to its lymphotropic property, HCV is able to multiply inside B lymphocytes and cause chronic stimulation of these cells by the viral infection. This stimulation possibly triggers autoimmune disorders, such as cryoglobulinemia vasculitis, purpura or necrotizing acrodermatitis, membranoproliferative glomerulonephritis, peripheral neuropathies, and polyarthritis[39,40]. Ultimately, B lymphocyte infection or chronic antigenic stimulation may be associated with lymphoma, mainly non-Hodgkin, splenic lymphoma type, or diffuse lymphomas[39,40]. Thus, chronic hepatitis C can enter the consulting rooms of several medical specialties because it is a systemic disease with manifestations affecting different organs and systems.

In the last decades, there have been significant advances in the treatment of hepatitis C, which motivated the WHO in 2017 to set targets to eradicate HCV by 2030 [7]. For more than 20 years, IFN has been used to treat chronic HCV infection. Pegylation (Peg-IFN) allowed a reduction in the frequency of subcutaneous injections from three to once a week[41]. Whereas the combination of Peg-IFN with ribavirin significantly increased the effectiveness of the treatment, it was poorly tolerated and resulted in a cure rate of at most 50% in 24 to 48 wk[41,42].

In 2011, the first protease inhibitors (telaprevir and boceprevir) demonstrated significant benefits, but they were not well tolerated and resulted in a suboptimal cure rate. Later, the development of the first polymerase inhibitor (sofosbuvir) and the first inhibitor of nonstructural protein (NS) 5A (daclatasvir) changed hepatitis C history due to the excellent tolerance and a cure rate of approximately 95%[43].

Direct-acting antivirals (DAAs) are highly effective agents, regardless of genotype and high barrier to resistance, which revolutionized HCV treatment[44]. Multiple combinations of DAAs with high pangenotypic efficacy result in high SVR rates, excellent safety, and good tolerance, even for patients with advanced fibrosis and cirrhosis[44]. The strong antiviral potency of these pangenotypic treatments has withdrawn the factors of poor response and developed a 'simplified route', which allowed general practitioners to treat patients without hepatic comorbidity and liver dysfunction[45]. This HCV treatment decentralization strategy was shown to be effective and safe for most patients. For example, multiple combinations of drugs with high pangenotypic efficacy, easy to use (one to three capsules per day) for 8 to 12 wk provide a cure for the vast majority of patients; these include the combinations glecaprevir/pibrentasvir, sofosbuvir/velpatasvir with or without voxilaprevir[45,46].

Combinations of DAAs are also available for specific genotypes. For example, ledipasvir/sofosbuvir (Harvoni™, Gilead Sciences) is approved for genotypes 1, 4, 5, and 6; and elbasvir/grazoprevir (Zepatier™, Merck Sharp and Dohme) for genotypes 1 and 4[44,47-49].

Therapeutic failures occur in approximately 3%-5% of cases, secondary to non-adherence to treatment or drug resistance. Resistance-associated variants of HCV have been identified and they are mainly a consequence of mutations in the nonstructural proteins NS3 and especially NS5. Only in cases of therapeutic failure in the first regimen is it advisable to perform resistance genotyping[50].

Despite the existence of effective treatments, HCV still remains a threat to public health. Albeit differences between the effectiveness of the medicines in clinical trials and real-life being a contributing factor, the main challenges are the low awareness of the disease, lack of screening programs, loss of follow-up in health services, and high rate of reinfection in certain populations[51].

Extensive efforts are being made to create efficient HCV care programmes around the world, respecting the particularities of each country. Macro-elimination based on mass testing and treatment has started in several American and European countries. Other countries, aiming to improve the efficiency of the therapy and considering cost-effectiveness, have chosen to adopt micro-elimination, targeting smaller population groups at high risk of infection, such as those in hyper-endemic areas, prisons, and haemodialysis centres[52,53].

In the DAA era, optimisation of their use must be a top priority. Identifying factors predicting a high chance of SVR with an ultra-short DAA regimen could be of great value in the global goal of HCV eradication[54]. Also, specific care needs to be taken in the post-RVS phase: (1) surveillance every six months for both HCC and hepatic decompensation remains imperative in patients with advanced fibrosis, especially in those with comorbidities that increase the risk of fibrosis progression, such as obesity, diabetes mellitus, and alcohol abuse; (2) close monitoring of extrahepatic complications, such as cardiovascular diseases, diabetes, lymphoma, and cryoglobulinemia, the once beneficial effects of HCV elimination on these complications are not clear; and (3) annual screening for HCV reinfection, mainly for those at high risk, such as people who inject drugs and those in prisons[55,56]. Recent analyses investigated the effects of eliminating a long-term persistent infection on the immune system. Persistent HCV infection is known to cause profound changes in the immune system, which do not appear to be fully reversible after viral elimination[57].

It is expected that the efforts of several countries in extensive testing for HCV and the availability of oral treatments of acceptable cost and with few side effects will result in the successful elimination of HCV. Hopes for an eventual preventive HCV vaccine remain.

## HEPATITIS D

The hepatitis D virus (HDV) is a single-stranded circular RNA virus, first reported in 1977[58]. This is a defective virus, so HDV does not produce an envelope or capsid, requiring the use of HBV envelopes. Therefore, HBV infection is necessary for productive HDV infection in humans[58,59]. Although HDV infection is chronic in less than 5% of coinfecting patients in adulthood, chronic infection is more common in the neonatal period[60].

An estimated 15-20 million people are infected worldwide[60]. Due to the dependence of HDV on HBV, the presence of HBsAg is necessary for the diagnosis of HDV infection. Serum HDV RNA and the presence of serum delta antigen are useful for diagnosis[61,62]. HDV infection can be acute or chronic[60].

Acute HDV infection can occur through HBV coinfection (simultaneous infection with both viruses during the same exposure) or superinfection (HDV infection in an HBsAg-positive individual). The clinical course of an acute HDV/HBV coinfection resembles an acute HBV infection, but with an increased risk of ALF[60]. Characteristically, there is a biphasic course with two peaks of alanine aminotransferase, sometimes separated by weeks, since HBV infection must be established first to allow for subsequent HDV infection. Whereas acute HDV superinfection can be mistaken for an HBV flare in patients with previous HBV infection, in undiagnosed patients it can be misinterpreted as acute HBV infection[60,63]. Therefore, high suspicion of HDV infection is required in patients with identified risk factors, such as a history of intravenous drug use, high-risk sexual behaviour, first-degree relative infection, and immigration from HDV-endemic regions[63].

Chronic HDV/HBV coinfection commonly results in the most rapidly progressive form of hepatitis, with a higher likelihood of cirrhosis and its complications. Compared to HBV monoinfected patients, HDV/HBV coinfecting patients have a risk of HCC up to 3 times higher and that of liver decompensation up to 2 times higher[63, 64].

Although the guidelines recommend Peg-IFN alpha for the treatment of chronic HDV infection, this therapy is limited by poor tolerance. Also, it is usually avoided in patients with cirrhosis, active autoimmune disease, or certain psychiatric disorders[27, 63, 64].

Novel therapeutic options targeting HDV life cycle are currently under clinical investigation. HDV cell entry, replication, and viral assembly and release are targets for medications such as bulevirtide, telafarnibe, and REP3702139, respectively[65]. Among all the agents studied, bulevirtide (formerly known as Myrcludex-B) received conditional marketing authorization under the trade name Hepcludex® by the European Medicines Agency in 2020. The agency warns that administration should continue 'as long as the patient benefits' and until future clinical trial data indicate different therapeutic actions. Hepcludex® blocks the entry of viruses into hepatocytes and should be administered at a dose of 2 mg once daily by subcutaneous injection as monotherapy or co-administered with a nucleoside/nucleotide analogue for the treatment of underlying HBV infection. The ideal duration of treatment is unknown. Hepcludex® has also been tested in combination therapy with Peg-IFN[66].

Recently, Peg-IFN lambda has also been studied against HDV[65]. Despite having an antiviral effect equivalent to Peg-IFN alpha, patients had better tolerability to the drug[67]. The combination of Peg-IFN lambda and other drugs is also under clinical investigation[65].

## HEPATITIS E

Hepatitis E virus (HEV) is responsible for outbreaks in developing countries and zoonotic cases in both developing and developed countries, mainly transmitted enterically[68]. This virus is a member of the Hepeviridae family; within the genus Orthohepevirus, species Orthohepevirus A, which includes eight recognised HEV genotypes. Genotypes 1 and 2 HEV have only been detected in humans, and these infections frequently result in outbreaks of jaundice in areas traditionally considered endemic, which are resource-poor, where HEV is spread by the faecal-oral route often *via* contaminated water[68]. Other genotypes, including HEV3 and HEV4, have been detected in both humans and animals, with pigs being the main reservoir[68, 69].

Whilst most infections are acute and self-limiting or asymptomatic, there are situations wherein it can progress to ALF and even become chronic. Immunocompromised patients are at risk of developing chronic HEV infection, such as solid organ transplant recipients, patients with haematologic malignancy undergoing chemotherapy, and those with human immunodeficiency virus infection[68, 69]. Extrahepatic manifestations, mostly neurological and renal diseases, have also been described. Acute icteric hepatitis is a classic presentation that occurs in 5%–30% of infected patients. Pregnant women are particularly at risk and a large proportion of those in their second and third trimester of pregnancy can progress to ALF. Patients with underlying liver disease have a poor prognosis in developing and developed countries [68–70].

The mechanisms of pathogenesis appear to be substantially immune-mediated[71]. Several studies have suggested that the immune response, rather than viral damage to hepatocytes, may drive clinical manifestations of hepatitis E, including both self-limiting acute viral hepatitis and ALF. One of the reasons that pathogenesis may be mediated by the immune system rather than by the virus itself is that the onset of icteric symptoms typically coincides with a rise in antibodies and a decline in viral load[71]. Chronic HEV infections, which are rarely seen in otherwise healthy individuals, are increasingly being recognized in patients with impaired immune function[72].

Diagnostic assays with good sensitivity and specificity have only recently become commercially available. To facilitate global access to the tools necessary is vital to identify and respond to HEV infections, whether sporadic cases or nascent outbreaks. Clinical and field surveillance, coupled with laboratory investigations of viral strains isolated from human cases, will help advance our understanding of HEV genotypes' relative virulence, intergenotypic variation, and other features of the HEV global epidemiology[73].



There is no recommended treatment for acute HEV infections, which are usually self-limiting with spontaneous HEV clearance. Although a recent study suggested that ribavirin is effective in treating immunocompetent patients with severe hepatitis E, it is difficult to claim that the drug improved the course of the infection due to study limitations (*e.g.*, the absence of a control group)[74]. Sofosbuvir demonstrated antiviral activity against HEV *in vitro*, however, it had limited clinical efficacy. There are some studies on new anti-HEV drugs: NITD008, a broad-spectrum chain-terminating adenosine nucleoside analogue initially developed to treat the dengue virus; and GPC-N114, which binds to the RNA channels of picornavirus polymerases. These compounds are promising HEV antiviral candidates. Lastly, T cell therapy may be an alternative to conventional medicines[75,76].

Vaccines to combat HEV have been developed and tested, and one highly efficacious vaccine is now available to consumers in China[77]. Understanding the determinants of susceptibility and resistance to repeat infection and clinical disease is imperative. Identifying environmental factors, such as regional climatic patterns, water, and sanitation practices, farming, and food processing practices, which affect lifetime exposures to HEV may help both to explain regional differences in the age-specific incidence of the infection and the severity of the disease, which cannot be explained solely by genotypic variability. Identification of these determinants also may help to provide risk-based strategies for intervention.

## HURDLES AND OPPORTUNITIES IN VIRAL HEPATITIS TREATMENT

In the last decade, rapid and significant advances in diagnosing and managing viral hepatitis were made and changed its treatment. These advances include the development of DAAs for the treatment of chronic hepatitis caused by HCV[78]-with SVR rates greater than 95%, the improvement of HBV vaccination as well as enhancement of the immunogenicity of HBV vaccines[79,80], and the identification of antiviral therapies with low rates of viral resistance[27]. Table 1 summarises the current clinical management of viral hepatitis and areas of development for future treatments.

Immunomodulators have been investigated to strengthen the immune system to fight HBV. Medications that stimulate both innate and adaptive immune systems, overcome CD8+ T cell exhaustion by checkpoint blockade, and transfer HBV-specific engineered CD8+ T cells are some of the therapies under investigation[36]. Immunomodulators may present a future treatment to cure hepatitis B infection, even though further research is necessary for this treatment.

Acute hepatitis A and E, frequently self-limiting or asymptomatic, still have no treatment recommendations, although the development and enhancement of vaccines improved its prevention. A vaccine to combat HEV is already available and consistent indications for HAV vaccination are now defined[12,77,81].

Despite these advances, issues with screening, diagnosis, referral, and treatment of viral hepatitis still persist. Problems in accessing treatment are reported in the published literature and reinforce the need to establish appropriate public policies for patient referral[82]. In addition, the identification of patients with viral resistance to the new treatment regimens and those with a satisfactory viral response and liver fibrosis, who might need close monitoring, deserve further investigation[83].

## CONCLUSION

The treatment of viral hepatitis has evolved rapidly over the last decade with the remarkable introduction of curative therapies for hepatitis C. The development and improvement of HAV and HEV vaccination also constitute substantial advances in this field. Despite these advances, drugs that also cure hepatitis B, going beyond viral suppression, are so far not available. Targeted antiviral drugs against HBV are encouraging future treatments and immunomodulatory therapies and gene silencing technologies are the most promising approaches to eradicate the virus. The increase in the frequency of HDV cases leads to the development of targeted antiviral agents against HDV, currently under clinical investigation. Finally, optimal screening *via* extensive testing allied to broad vaccination and treatment coverage are fundamental goals to eliminate viral hepatitis and reduce the public health burden of these infections.

**Table 1** Current clinical management of viral hepatitis and areas of development for future therapies

Type	Current management	Areas of development
Hepatitis A	No specific drugs against HAV infection are available so far; thus treatment consists of supportive care; Prevention of HAV infection includes vaccination, immune globulin, and attention to hygienic practices	Public health campaigns to promote the prevention of hepatitis A; Raise awareness of indications for hepatitis A vaccination
Hepatitis B	Entecavir, tenofovir disoproxil fumarate, tenofovir alafenamide fumarate, and pegylated interferon alpha are currently the first-line anti-HBV agents recommended for chronic hepatitis B treatment; Prevention of HBV infection is focused on vaccination;	Elimination or inactivation of HBV cccDNA is the major focus of HBV research; Targeted therapies to HBV (immunomodulatory therapies and gene silencing technologies are promising approaches); Need to increase hepatitis B vaccination coverage
Hepatitis C	Multiple combinations of direct-acting antivirals with high pangenotypic efficacy result in high sustained virological response rates, excellent safety, and good tolerance, even for patients with advanced fibrosis and cirrhosis;	Increase awareness of the disease, develop screening programmes; Optimization of direct-acting antivirals use; Attention to specific care needs to be taken in the post-treatment phase
Hepatitis D	There are no satisfactory drugs for this disease; Pegylated interferon alpha recommended for the treatment of chronic HDV infection, although limited by poor tolerance is usually avoided in patients with cirrhosis, active autoimmune disease, or certain psychiatric disorders	Further research on novel targeted HDV antiviral medications is necessary due to the lack of effective therapeutic options
Hepatitis E	There is no recommended treatment for acute HEV infections because it is usually self-limiting with spontaneous HEV clearance	Ribavirin is suggested to be an effective treatment for immunocompetent patients with severe hepatitis E; New anti-HEV drugs are under investigation; T cell therapy may be an alternative to conventional medicines; Vaccines to combat HEV have been developed and tested

HAV: Hepatitis A virus; HBV: Hepatitis B virus; cccDNA: Covalently closed circular deoxyribonucleic acid; HDV: Hepatitis D virus; HEV: Hepatitis E virus.

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## Large-duct pattern invasive adenocarcinoma of the pancreas—a variant mimicking pancreatic cystic neoplasms: A minireview

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### Abstract

Pancreatic cancer currently has no subtypes that inform clinical decisions; hence, there exists an opportunity to rearrange the morphological and molecular taxonomy that guides a better understanding of tumor characteristics. Nonetheless, accumulating studies to date have revealed the large-duct type variant, a unique subtype of pancreatic ductal adenocarcinoma (PDA) with cystic features. This subtype often radiographically mimics intraductal papillary mucinous neoplasms (IPMNs) and involves multiple small cysts occasionally associated with solid masses. The “bunch-of-grapes” sign, an imaging characteristic of IPMNs, is absent in large-duct PDA. Large-duct PDA defines the mucin profile, and genetic alterations are useful in distinguishing large-duct PDA from IPMNs. Histologically, neoplastic ducts measure over 0.5 mm, forming large ductal elements. Similar to classic PDAs, this subtype is frequently accompanied by perineural invasion and abundant desmoplastic reactions, and *KRAS* mutations in codon 12 are nearly ubiquitous. Despite such morphological similarities with IPMNs, the prognosis of large-duct PDA is equivalent to that of classic PDA. Differential diagnosis is therefore essential.

**Key Words:** Large-duct pattern invasive carcinoma of the pancreas; Pancreatic ductal adenocarcinoma; Pancreatic cystic disease; Clinicopathological features of pancreatic cancer; Pancreatic cancer subtype

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**Core Tip:** This review integrates the current knowledge about large-duct pattern invasive adenocarcinoma of the pancreas [large-duct pancreatic ductal adenocarcinoma (PDA)]. This subtype is a rare exocrine pancreatic neoplasm and often mimics intraductal papillary mucinous neoplasms (IPMNs). However, its prognosis is notably different from that of IPMNs, and distinguishing this subtype from IPMNs preoperatively is crucial. We summarized the morphological features and genetic landscape of large-duct PDA, with a primary focus on its differences from other types of pancreatic cystic neoplasms. The information aid in making appropriate decisions when tackling atypical pancreatic cystic neoplasms.

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## INTRODUCTION

Pancreatic cancer is one of the most devastating and fatal diseases in humans, with pancreatic ductal adenocarcinoma (PDA) being the most common exocrine pancreatic neoplasm[1]. With the increase in the number of patients, PDA is expected to become the second leading cause of cancer-related mortality worldwide[2,3]. Advances in multidisciplinary treatment have improved patient survival and have enhanced the effectiveness of modalities for the early detection and identification of cancer subtypes. Despite all efforts to prolong survival, PDA represents one of the worst tumors, with a 5-year overall survival rate of only 2%-9%[4]. Molecular subtyping of this tumor based on genetic signatures has been developed over the past 10 years; nevertheless, the correlation of such information with clinical phenotypes has been somewhat evasive.

Macroscopically, PDA is characterized by yellowish-white masses, usually without hemorrhagic necrosis[5,6]. On histological examination, typical PDA exhibits duct-like glandular structures infiltrating the pancreatic parenchyma, eliciting a strong desmoplastic stromal response[7,8]. The neoplastic cells are columnar to cuboidal and produce various mucins, depending on differentiation and histotypes. Several genetic alterations such as *KRAS* (chromosome 12p), *SMAD4* (chromosome 18q), *CDKN2A* (chromosome 9p), and *TP53* (chromosome 17p) are responsible for causing PDA[9-11] that progresses from low-grade pancreatic intraepithelial neoplasia (PanIN) to higher-grade PanIN and invasive PDA[12-16].

PDA accounts for up to 90% of pancreatic neoplasms and is typically visualized as a solid mass accompanied by abundant desmoplasia[17]. However, tumors with cystic components occasionally compose a small subset in approximately 4%-5% of patients. Colloid carcinoma, a subtype of PDA that is often conjugated to intraductal papillary mucinous neoplasms (IPMNs), is characterized by extracellular mucin pools. Morphologically, colloid carcinoma tends to exhibit a large, well-demarcated cystic lesion[18]. This subtype of PDA originates, at least in part, from IPMNs, which are well-known pancreatic cystic neoplasms[19-21]. IPMNs can be categorized into two morphologically distinct types — namely, the branched type and main duct type[22,23]. Furthermore, multiple subsets, including gastric, intestinal, and pancreatobiliary types, are histologically defined[24]. These types have unique paths during development and progression to invasive tumors[25], and specific genetic alterations observed in IPMNs may be responsible for tumor phenotypes and ultimately influence the patients' outcome[26,27]. In most cases, other pancreatic lesions with cystic features, such as solid cystic neoplasm (SCN)[28,29], mucinous cystic neoplasm (MCN)[30], and solid pseudopapillary neoplasm (SPN)[28], can be easily distinguished from classic PDA[31]; however, atypical imaging findings of patients occasionally impair proper diagnosis. Knowledge and use of relevant genetic alterations unique to these cystic neoplasms may aid patients and physicians in making appropriate decisions.

Recent studies have revealed a histological subtype of PDA with cystic features mimicking pancreatic cystic diseases[32]. This PDA variant is referred to as large-duct pattern invasive adenocarcinoma of the pancreas (large-duct type) and is characterized by invasive PDA forming large, sometimes dilated glands. Nevertheless, this entity



is considered rare and often leads to the misdiagnosis of several pancreatic cystic neoplasms, including IPMNs. This review compares the clinical characteristics, gross morphology and histopathology, and genetic alterations of large-duct PDA and other pancreatic cystic neoplasms.

## CLINICAL MANIFESTATIONS

### **Epidemiology**

Large-duct PDA has been estimated to occur in < 7% of all PDA cases. It has been reported in only 63 patients in the past literature[33-35]. Large-duct PDA has been initially diagnosed as IPMN in most cases[34]; hence, the incidence of large-duct PDA has not been clearly recognized. Thus, regarding incidence, large-duct PDA of the pancreas is considered rare[36]. The median age of these patients with large-duct PDA was 63.0 years, and the sex ratio was female-dominant, with a female-to-male ratio of 1.74 (Table 1). The female-to-male ratio was higher in patients with large-duct PDA than in those without large-duct PDA[18]. A previous study reported no specific and unique risk factors.

### **Tumor location and symptoms**

Generally, 60% of classic PDAs develop in the pancreatic head[37], and the majority of the patients exhibit symptoms associated with obstructive jaundice. A summary of previously reported cases with large-duct PDA indicated similar tumor locations (Table 1). Kelly *et al*[34] reported that 2 out of 9 patients with large-duct PDA had jaundice. Other patients had acute pancreatitis ( $n = 1$ ) and abdominal pain ( $n = 5$ ).

## IMAGING FINDINGS

### **Computed tomography imaging**

Dynamic contrast-enhanced computed tomography imaging is an essential modality for assessing cystic lesions in the pancreas. Typical PDA presents as a solid and hypovascular tumor, and the most common radiographic finding of PDA is dilatation or stricture of the main pancreatic duct. In contrast, large-duct PDA usually presents as a cluster of small cystic lesions. The diameter of cysts generally measures 5-7 mm, although they sometimes exceed 10 mm[38]. However, large-duct PDA does not have a “bunch-of-grapes” appearance[39], commonly detected in IPMNs. Given the multiple small cysts visualized on cross-sectional images, distinguishing them from SCN is more complicated, particularly in cases with macrocystic-type cysts[40]. SCN typically has a spongy or honeycomb-like appearance; one previous report described a large-duct PDA case showing a honeycomb-like morphology[36]. Large-duct PDA lacks either a star-shaped scar or calcification at the center of the cyst, commonly observed in SCN[38,41,42]. Considering the female-dominated epidemiology, MCN is also a significant differential diagnosis. MCN has an orange-like appearance and sometimes forms cystic lesions measuring over 10 cm; these features are not typical in large-duct PDA[42,43].

Another critical finding suggestive of the large-duct pattern is the slightly diminished enhancement of the parenchyma surrounding cystic components (Figure 1). Similar to typical PDA, such enhancement gradually increases with time after contrast media injection. Furthermore, walled-off necrosis (WON) should be excluded. WON often contains debris and hemorrhage in cysts, resulting in a mixed density change in the fluid[44,45]. These findings are not usually observed in large-duct PDA. Retention cysts often occur along with PDA and are also important in distinguishing it from large-duct PDA. Retention cysts are typically unilocular and tend to be larger than the cysts observed in large-duct PDA[46,47] (Table 2).

### **Magnetic resonance imaging**

Magnetic resonance cholangiopancreatography is another essential modality for diagnosing pancreatic cystic diseases, except in claustrophobic individuals and those with implanted magnetic resonance imaging-unsafe foreign bodies. Crucial findings for large-duct PDA include small cysts in the pancreas[48], with each cyst usually having a diameter of 0.5-0.7 cm[49]. It should be noted that pancreatic duct stricture is more intense than that observed in other cystic neoplasms. Stricture of the main pancreatic duct and bile duct closely associated with a cluster of small cysts is another

**Table 1 Patient characteristics of large-duct pancreatic ductal adenocarcinoma**

Ref.	Number of cases	Median age	Sex		Tumor location	
			Female	Male	Head	Body or tail
Kelly <i>et al</i> [34], 2012	10	67	6	4	9	1
Bagci <i>et al</i> [33], 2012	28	67	19 <sup>1</sup>	8 <sup>1</sup>	16	11
Kosmahl <i>et al</i> [35], 2005	24	58	15	9	12 <sup>2</sup>	9 <sup>2</sup>
Total	63	63.0	40	23	37	21

<sup>1</sup>One case with missing information on sex.<sup>2</sup>Three cases with missing information on tumor location.

finding that we may consider when diagnosing large-duct PDA (Figure 2). In making a differential diagnosis of IPMNs, communication with the main pancreatic duct is crucial. Both typical main duct-type and branched duct-type IPMNs communicate with the main pancreatic duct and often show dilatation of the main pancreatic duct [41,50,51].

Moreover, diffusion-weighted imaging and apparent diffusion coefficient map on magnetic resonance cholangiopancreatography is helpful in diagnosing PDA. Considering the high intensity of cystic lesions on T2-weighted images[52,53], diffusion-weighted imaging may not help distinguish large-duct PDA from other pancreatic cystic diseases (Figure 2).

### Endoscopic findings

Endoscopic ultrasonography (EUS) has the highest sensitivity for detecting PDA lesions[54], specifically small-sized tumors. Irrespective of the size of PDA, non-neoplastic cystic changes associated with PDA include retention cysts caused by ductal obstruction, and pseudocysts attributable to tumor-associated pancreatitis may be considered to distinguish it from large-duct type tumors[55,56]. A “mucinous clot,” which is a hyperechoic lesion inside cysts, is sometimes visualized using EUS in patients with IPMNs, but not in those with large-duct PDAs[57,58]. Furthermore, large-duct PDAs typically lack mural nodules and papillary growth, signatures of high-risk stigmata, and worrisome features in the Fukuoka criteria[41,59]. Youn *et al* [38] described a case of large-duct PDA with a solid mass inside a dilated glandular cyst[38]. Such atypical findings make a differential diagnosis between large-duct PDAs and IPMNs with high-risk stigmata confusing among endoscopists; nonetheless, surgical resection should be performed for either disease. Considering the multifocality of IPMNs, performing EUS for multifocal lesions, which are not observed in large-duct PDA, is crucial (Table 2)[41,51]. The morphology of “cyst by cyst” appearance can be observed in typical IPMNs using EUS[41].

No report has specifically described EUS-guided fine-needle aspiration (EUS-FNA) in patients with large-duct PDA. In the case of pancreatic tumors with cystic components, concerns related to tumor cell dissemination from the cystic fluid (needle tract seeding)[60] may need to be pointed out (Figure 3). Thus, drawing a distinction between large-duct PDA and IPMN-associated cancer using EUS-FNA can become highly challenging, which will be explained well in the “Pathology” section. The requirements for preoperative histological diagnosis of PDA have increased in the neoadjuvant chemotherapy era; therefore, an ingenious solution for the safe and accurate diagnosis of large-duct PDA using EUS-FNA needs to be considered. No report regarding endoscopic retrograde cholangiopancreatography has described specific findings for large-duct PDA. For tumors developing in the pancreatic head, transpapillary biopsy for histological diagnosis can be useful if massive bile duct invasion is evident.

## PATHOLOGY

### Macroscopic features

Bagci *et al*[33] described 28 cases of large-duct PDA. Macroscopically, 10 patients had a cyst measuring < 10 mm, whereas 1 patient had a large cyst over 70 mm[33]. Pathologically, each small cyst is a dilated glandular carcinoma. Similarly, Kosmahl *et al*[35].

**Table 2 Clinical and morphological characteristics of pancreatic diseases**

Ref.	Diseases	Median age	Sex (% females)	Tumor location (% pancreatic head)	Multifocality	Gross appearance	Cyst diameter	Main pancreatic duct communication	Internal structure	Other features	Key genetic alterations
Bagci <i>et al</i> [33], 2012 Kelly <i>et al</i> [34], 2012 Kosmahl <i>et al</i> [35], 2005 Youn <i>et al</i> [38], 2018	Large-duct PDA	63	68.3	56.2	No	Multiple small cysts	0.5–0.7 cm in each cyst	No	Dilatated glandular cyst (duct)	Dilatated glandular cyst (duct) is > 0.5 mm in diameter. Easily invades to the perineural plexus and shows desmoplastic reaction	KRAS (codon 12)
Seidel <i>et al</i> [18], 2002 Adsay <i>et al</i> [24], 2016	Colloid carcinoma	61	52.9	66.7	Infrequent	Single or multiple cysts, well demarcated	1.2–16 cm	No	Well-defined pools of mucin, contained scanty malignant epithelial cells	Associated with intestinal-type IPMN	KRAS, BRAF, PIK3CA; MSIs are more frequently observed than non-colloid cancer PDA
Tanaka <i>et al</i> [41], 2012 Kim and Cho [46], 2015 Laurent <i>et al</i> [51], 2016	Branched duct-type IPMN	65–70	55.2	62.3	Yes	Bunch of grapes	Up to 30 mm for non-invasive IPMN; 30 mm or greater for IPMN with worrisome features	Yes	Cyst by cyst	Main pancreatic duct; normal or dilatated to > 5 mm, suggesting combined type with main duct IPMN	KRAS (codon 12), GNAS, RNF43
Hecht <i>et al</i> [19], 2021 Tanaka <i>et al</i> [41], 2012 Salvia <i>et al</i> [50], 2010	Main duct-type IPMN	60 s	41.3	59.6	Yes	Bunch of grapes	Up to 30 mm for non-invasive IPMN; 30 mm or greater for IPMN with worrisome features	Yes	Cyst by cyst	Main pancreatic duct; partial or diffuse dilatation > 5 mm	KRAS (codon 12), GNAS, RNF43, TP53
Ånonsen <i>et al</i> [42], 2019 Wu <i>et al</i> [80], 2011	SCN	60–70	70	50	No	Spongy or honeycomb-like	3.7–5.1 cm	No	Microcystic or macrocystic		VHL
Ånonsen <i>et al</i> [42], 2019 Yamao <i>et al</i>	MCN	40–50	95	5	Infrequent	Orange-like	1.0–26.4 cm	No	Cyst by cyst	Ovarian-like stroma	KRAS, RNF43

[43], 2011											
Wu <i>et al</i> [80], 2011											
Kim and Cho [46], 2015	Retention cyst	60 s	?	?	No	Unilocular	2.8–12 cm	Yes	No cellular dysplasia	Main pancreatic obstruction in should be observed downstream	?
Assifi <i>et al</i> [47], 2014											
Singhi <i>et al</i> [61], 2012	Pancreatic neuroendocrine neoplasms with cystic changes	50 s	42	24	Infrequent, except MEN1-related neuroendocrine neoplasms	Pinkish-tan to yellowish in color, well demarcated	0.8–18.0 cm	No	Well circumscribed and surrounded by a thin-to-thick fibrous capsule	Larger cysts tend to show hemorrhage	MEN1, PTEN, DAXX, ATRX, MUTYH, CHEK2
Halfdanarson <i>et al</i> [62], 2008											
Scarpa <i>et al</i> [86], 2017											
Tanaka <i>et al</i> [41], 2012	Walled-off necrosis	40–50	25	45	Rare	Variable	Variable	Yes	Unilocular	Main pancreatic duct; normal or irregularly dilated. Observed no later than 6 wk after the occurrence of acute pancreatitis	
Cohen <i>et al</i> [45], 2003											

PDA: Pancreatic ductal adenocarcinoma; IPMN: Intraductal papillary mucinous neoplasms; MCN: Mucinous cystic neoplasm; SCN: Solid cystic neoplasm.

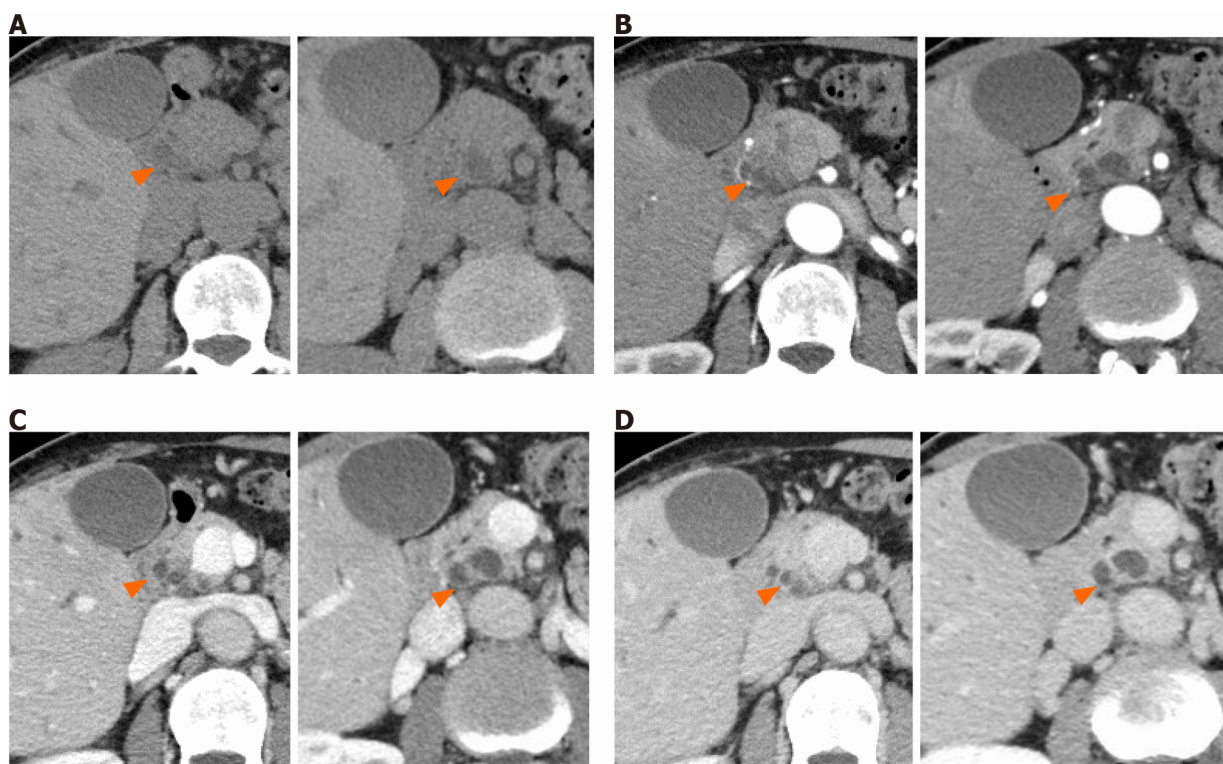
described 24 cases of large-duct PDA and reported a diameter ranging from 4 to 18 mm for each cyst; the gross tumor size was not different from ordinary PDA[35]. The entire cystic lesion size is crucial, and the cystic component of large-duct PDA is less than 100 mm generally. In comparison, the size of cysts in MCN, SCN, or pancreatic neuroendocrine neoplasms (PanNENs) sometimes exceeds 100 mm (Table 2)[61,62].

### Microscopic features

Microscopically, large-duct PDA shows dilated glands that range in size from 5 to 10 mm. The term “large duct” is defined as dilatation greater than 0.5 mm among  $\geq 50\%$  of glands in PDA[33]. Hematoxylin-eosin staining has revealed some essential pathological findings for large-duct PDA. The nuclei of the epithelial carcinoma (dilated glands) are irregular, wrinkled, and basally located. The cytoplasm exhibits a foamy or microvesicular pattern, and the glands are jagged and irregular[34]. While a papillary growth pattern is typically absent, discrimination of large-duct PDA from other cystic neoplasms, especially gastric-type IPMNs, is crucial[63,64]. In contrast to mucinous carcinoma, large-duct PDA lacks signet ring cells or mucinous lakes[65,66].

Perineural invasion is another important finding for diagnosing large-duct PDA to discriminate from other cystic neoplasms. Bagci *et al*[33] reported that perineural invasion occurred in 88% of large-duct PDAs[33] but was less commonly observed in





**Figure 1 Typical computed tomography imaging of large-duct pancreatic ductal adenocarcinoma.** The orange arrowheads show the cystic lesion in large-duct pancreatic ductal adenocarcinoma. A: Plain computed tomography indicates low attenuation area in the pancreas; B: Arterial phase reveals multiple cystic lesions in the pancreatic head without enhancement; C: Portal phase shows multiple cystic lesions in the pancreatic head with parenchymal enhancement; D: Equilibrium phase shows slight ring enhancement of the lesion.

IPMNs, even though the tumor had invasive compartments. In large-duct PDA, the stroma has a rich desmoplastic appearance[33,34]. These features are commonly observed in PDA compared to IPMNs; however, they are more remarkable in large-duct PDA. Additionally, hypercellular stroma or ovarian-like stroma is present in some cases. As for the differential diagnosis of colloid carcinoma or PanNENs, the cysts in these lesions are relatively well-demarcated relative to those in large-duct PDA. PanNENs sometimes have a thick fibrous capsule with hemorrhage[61]. WON usually shows necrotic debris in cysts. Macroscopically, this finding is usually not observed in large-duct PDA and can be a point to distinguish one from other cystic lesions[67].

### Immunohistochemistry

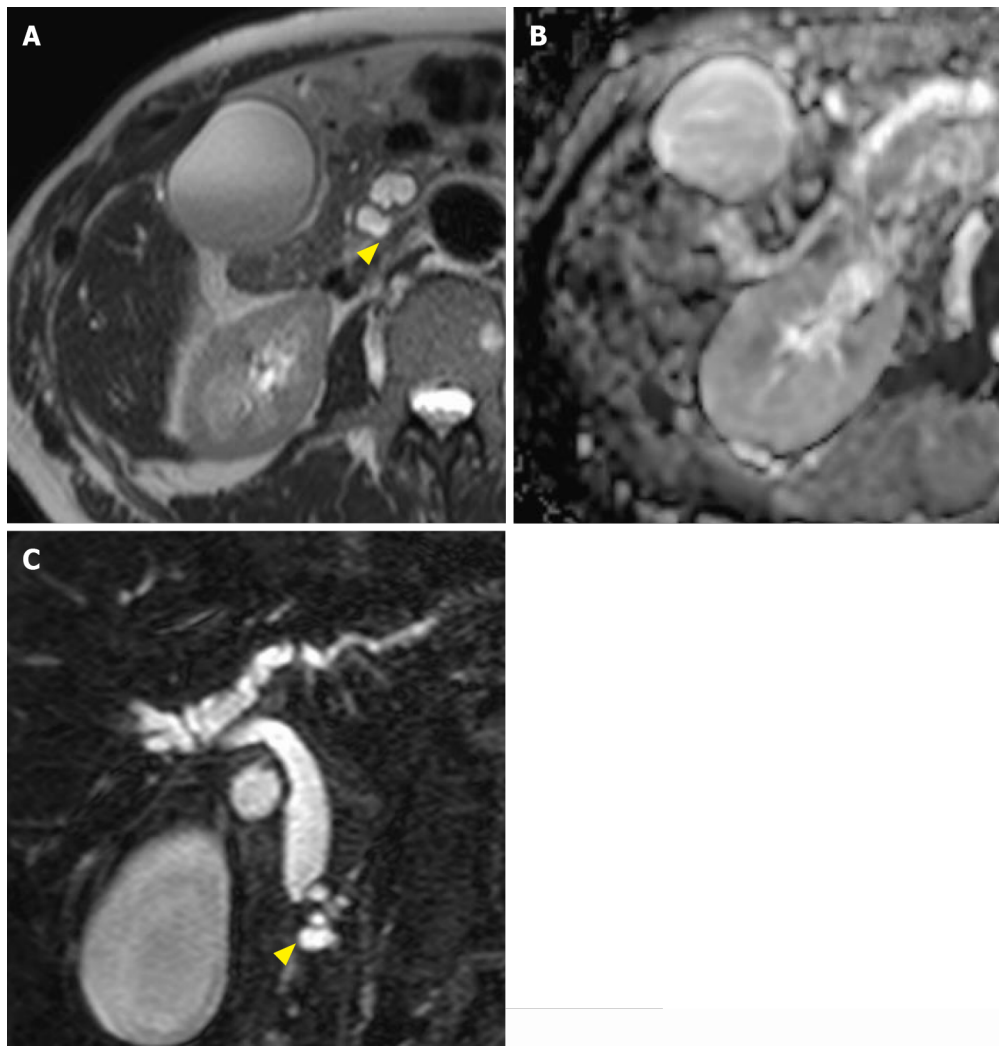
Immunohistochemistry (IHC) may be a crucial diagnostic tool that can be utilized to discriminate large-duct PDA from other pancreatic cystic neoplasms[36]. Among the immunostaining widely used in the diagnosis of tumors, mucin (MUC) staining is particularly important and may play a key role in diagnosing large-duct PDA. Gastric-type IPMN exhibits relatively low papillary growth[64] and is typically low grade. However, in tumors with high-grade gastric IPMN, distinction from the large-duct type PDA is sometimes uncertain pathologically. IPMNs present unique MUC staining patterns, depending on the epithelial subtypes[63]. Gastric-type IPMNs show MUC5AC-positive staining patterns and are negative for MUC1, MUC2, and MUC6. Kelly *et al*[34] and Kosmahl *et al*[35] reported MUC staining for large-duct PDAs (Table 3)[34,35]; the positivity rates of MUC1, MUC2, MUC5AC, and MUC6 immunostaining were 79.4%, 8.8%, 78.8%, and 60.6%, respectively. Thus, MUC1 and MUC6 IHC may help make to a differential diagnosis from IPMNs[68].

Furthermore, p53 IHC is beneficial in diagnosing lesions with malignant potential. Bagci *et al*[33] showed that 73% of all large-duct PDAs positively stained for p53. Another point of IHC for large-duct PDA is whether the dilated glands are dilated glandular carcinomas or dilated glands resulting from the occlusion of upper-stream pancreatic ducts. To presume the origin of anatomical localization of the dilated pancreatic ducts, Elastica-Masson staining may be suitable (Figure 4). In addition to the main pancreatic duct, elastin fibers in these normal pancreatic ducts can be stained with Elastica-Masson. Typically, the dilated glands in large-duct PDA are negative

**Table 3 Mucin staining profiles of large-duct pancreatic ductal adenocarcinoma**

Ref.	MUC1	MUC2	MUC5AC	MUC6
Kelly <i>et al</i> [34], 2012	10/10 (100.0%)	1/10 (90.0%)	9/10 (90.0%)	8/10 (80.0%)
Kosmahl <i>et al</i> [35], 2005	17/24 (70.8%)	2/24 (8.3%)	17/23 (73.1%)	12/23 (52.2%)
Total	27/37 (73.0%)	3/37 (8.1%)	26/36 (72.2%)	20/36 (55.6%)

No. of staining-positive cases/available cases. MUC: Mucin.

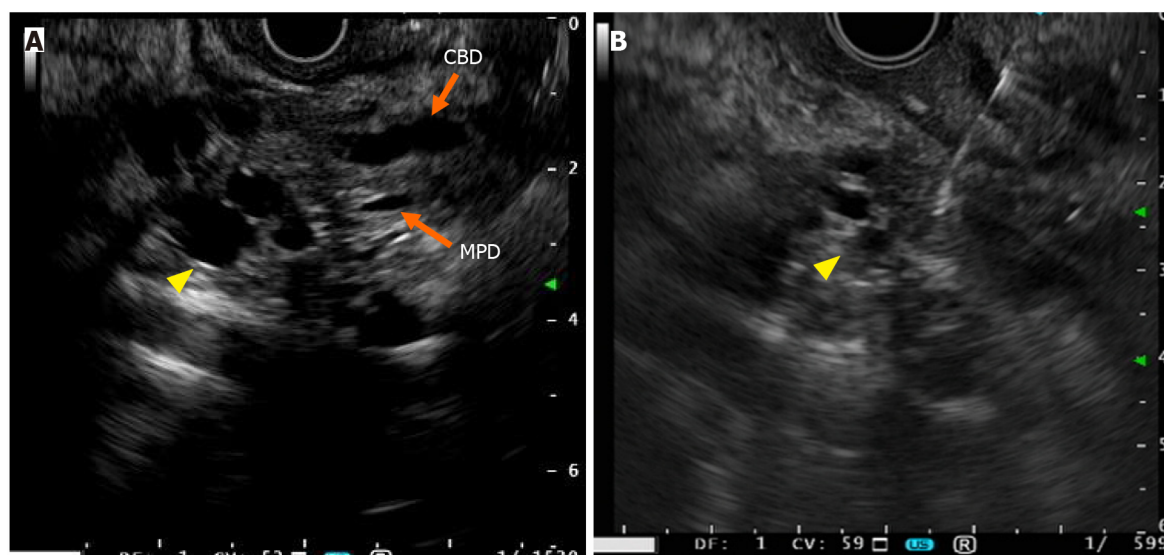


**Figure 2 Typical magnetic resonance imaging of large-duct pancreatic ductal adenocarcinoma.** The yellow arrowheads show the cystic lesion in large-duct pancreatic ductal adenocarcinoma (PDA). A: T2-weighted imaging reveals high intensity in the large-duct PDA lesion; B: Diffusion-weighted imaging shows no significant signal increase/decrease in the lesion; C: Magnetic resonance cholangiopancreatography (coronal view) reveals multiple cystic lesions in the pancreatic head and compressed bile duct.

for the immunostaining, suggesting they were derived from tumor glands rather than the secondary dilation of the normal main pancreatic duct and associated large branch duct[69].

## GENETIC ALTERATIONS

*KRAS* is mutated in the great majority of PDA, and approximately 90%-95% of PDA patients have major hot spot mutations in codons 12, 13, and 61[9]. This genetic event has been identified at the earliest stages of PanIN, and studies in genetically



**Figure 3 Endoscopic ultrasonography findings of large-duct pancreatic ductal adenocarcinoma.** The yellow arrowhead shows the cystic lesion in large-duct pancreatic ductal adenocarcinoma (PDA). A: Endoscopic ultrasonography (EUS) reveals multiple echoic lesions in the pancreatic head; B: EUS-fine-needle aspiration was performed in the parenchyma of the cystic lesion in large-duct PDA. The EUS model used was GF-UE260-AL5 (Olympus, Tokyo, Japan). The ultrasonic diagnostic equipment used was EU-ME2 (Olympus, Tokyo, Japan). CBD: Common bile duct; MPD: Main pancreatic duct.

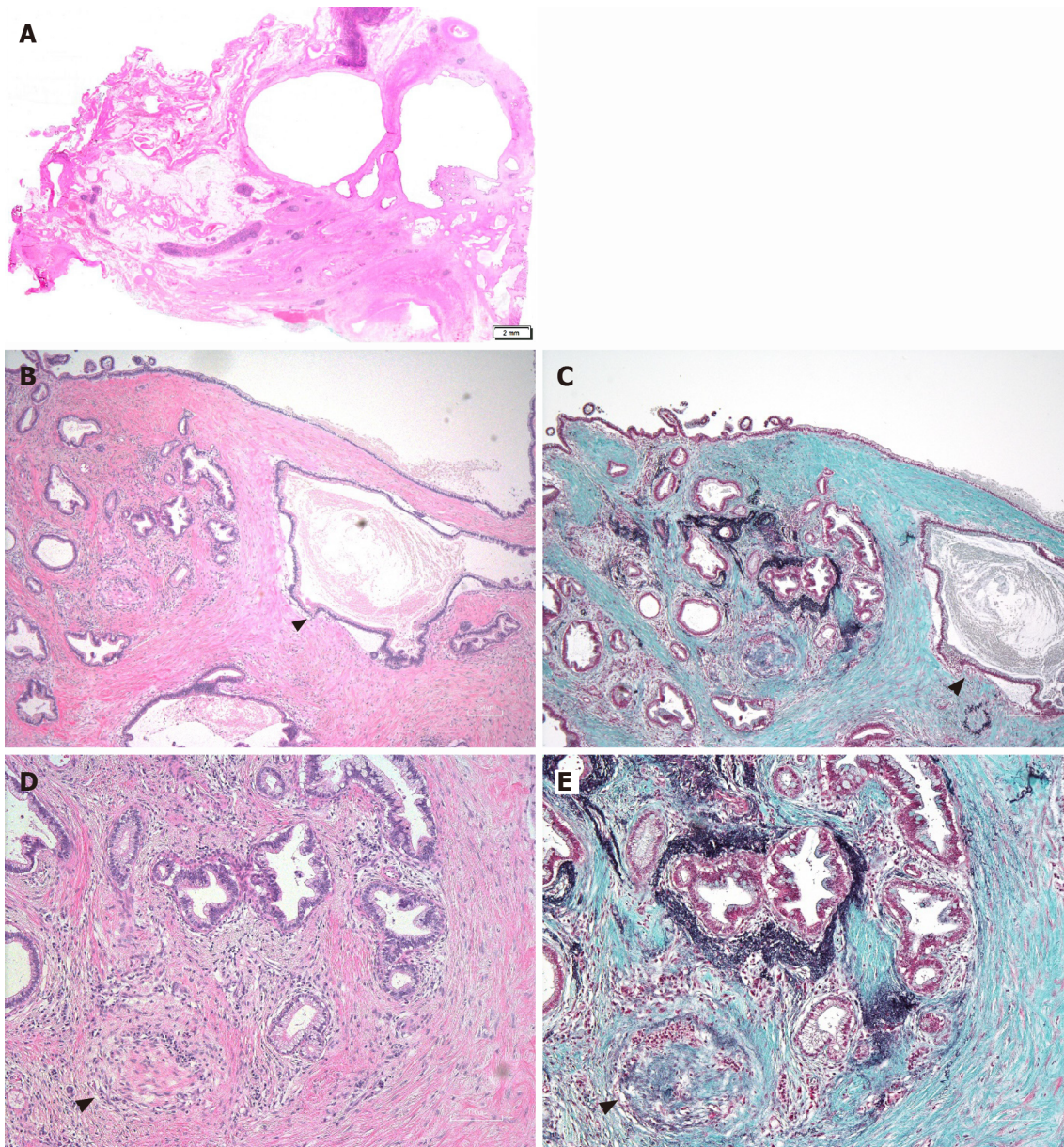
engineered mice models (GEMMs) indicate that PanINs arise from pancreatic acinar cells that incur *Kras* mutations (Ptf1a-induced *Kras* activation). In the *Kras*<sup>G12D</sup>-expressing mice, PanIN formation coincides with acinar-to-ductal metaplasia (ADM), characterized by the replacement of acinar cells with cells expressing ductal markers, such as CK19 and the ductal fate determinant Sox9[70]. Further, propensity of ductal and centroacinar cells to give rise to PanINs is demonstrated by GEMM with Sox9<sup>CreER</sup>-mediated *Kras* activation, although considerably less frequent relative to Ptf1a<sup>CreER</sup>-induced *Kras* activation[71].

Although observations in these mice models have not been validated in humans, multiple PanIN-like lesions with various grades of atypia can often be observed in the “normal area” of the resected specimens of pancreatic cancer. These lesions are usually solitary and localized in the normal acinar compartment without pancreatitis. However, PanINs associated with a cluster of ADM can also be seen[9]. Nevertheless, it remains to be determined if the PDA, including large-duct type, originated from either differentiated duct or duct-like cells associated with ADM. Such hypothesis may be clarified based on the particular mutation profiles and molecular signature related to cellular origin.

Moreover, genetic analysis is valuable in establishing a differential diagnosis between large-duct PDA and IPMNs. Bagci *et al*[33] conducted a mutation analysis of *KRAS* codon 12, which is the most frequent mutation point in PDA, using specimens from 28 large-duct PDA patients compared to ordinary ductal adenocarcinoma of the pancreas[33]. The results indicated 82% of large-duct PDAs harbored *KRAS* codon 12 mutations, a bit less frequency observed in classic PDAs (generally over 90%)[33,72]. Since the authors utilized slot-blot Southern analysis of polymerase chain reaction-amplified samples, *KRAS* mutation in codon 12 might be missed in case of the tumor with low tumor cellularity, and latest methods may also identify other hotspots at codons 13 and 61. Additionally, IPMN can be initiated by *KRAS* mutation[9], although less frequently than PanIN[73,74]. *KRAS* mutations are observed in 53%-87% of gastric-type IPMNs and approximately 40% in intestinal-type IPMNs. In contrast to PDAs including large-duct type, IPMNs frequently harbor multiple different types of *KRAS* mutations, suggesting poly-clonal diseases[25]. Therefore, specifying the variation in *KRAS* mutation types may be valuable in determining a subset of patients with large-duct PDA.

*TP53* gene is also frequently mutated around 75% of pancreatic carcinoma patients [75], and Bagci *et al*[33] reported that abnormal immunostaining of p53 was observed in 73% of large-duct type PDA, a higher frequency relative to classic PDA (60%). Moreover, *TP53* mutation can be seen in 10%-40% of high-grade IPMNs and 40%-60% of IPMN-associated invasive carcinomas[73,76]. Given the strong gain-of-function of particular missense mutations in *TP53* relative to the truncating mutations[77,78], further studies will be required if such *TP53* mutation types are associated with the





**Figure 4 Pathological findings of large-duct pancreatic ductal adenocarcinoma.** A: Hematoxylin–eosin (HE) staining of the lesion ( $\times 4$ ); B: HE staining of the lesion ( $\times 200$ ) shows a dilated glandular carcinoma  $> 0.5$  mm in diameter (black arrowhead); C: Elastica–Masson immunohistochemistry ( $\times 200$ ) reveals that the dilated glands lack elastic fibers (black arrowhead); D: HE staining of the carcinoma invading the myelin sheath ( $\times 200$ ; black arrowhead); E: Elastica–Masson immunohistochemistry ( $\times 200$ ) reveals that the dilated glands invading the myelin sheath lack elastic fibers (black arrowheads show the myelin fiber).

tumor phenotypes. *SMAD4* is also inactivated in 55% of PDA cases; of these, 35% are inactivated by homozygous deletion, whereas 20% show loss of one allele[10]. Inactivation of *SMAD4* enhances the rapid progression of *Kras*<sup>G12D</sup>-initiated mice pancreatic neoplasms, showing IPMN-like phenotypes[79], and a significant fraction of IPMN-associated human pancreatic cancer harbors *SMAD4* mutation and the abnormal immunostaining[25]. Whether *SMAD4* inactivation can influence the progression or promotion of large-duct PDA remains unclear.

*GNAS* mutations are unique to IPMNs[80,81] and are more frequently identified in intestinal-type IPMNs than in gastric- and pancreatobiliary-type IPMNs[9]. The mutation can progress to IPMN-associated PDA, and recent studies demonstrated that about 8%-11% of pancreatic cancers[9], including cases considered not related explicitly to IPMN, harbor *GNAS* mutations or amplifications. Since *GNAS* mutation can lead to entirely distinct transcriptional and metabolic reprogramming to *KRAS*-driven circuitry[82], identification of *GNAS* mutations may discriminate large-duct PDA from IPMN-related pancreatic cancer.

*RNF43* encodes an E3 ubiquitin ligase. *RNF43* mutations are observed in IPMNs and MCNs and are detected in approximately 50% of all IPMNs[83]. Nonetheless, a recent

meta-analysis has suggested that *RNF43* mutations are not associated with clinicopathologic parameters in patients with IPMN[83,84]. Approximately 50% of MCNs present *RNF43* mutations, including loss of heterozygosity[85]. Other cystic neoplasms in the pancreas exhibit specific genetic mutations. Genetic alterations such as *VHL* mutations and loss of heterozygosity in SCN, *CTNNB1*, and *PIK3CA* mutations in SPN and PanNEN-associated mutations (*MEN1*, *PTEN*, *DAXX*, and *ATRX*) have been specifically identified in SCNs and PanNENs, particularly in cases with cystic components resembling retention cysts[85,86] (Table 2).

Further genetic analysis should be performed in the large-duct PDA, considering whether the cellular origin of the large-duct PDA was the branch or main pancreatic duct and pathways of carcinogenesis[87,88]. Whole-genome sequencing may be helpful in determining the genes responsible for forming large-duct glands[87,89].

## TREATMENT AND PROGNOSIS

Large-duct PDA has been recognized as an uncommon subtype of PDA. Hence, in previous reports, large-duct PDA was mainly diagnosed from surgically resected specimens[33,34]. Preoperative diagnosis was variable, including IPMN, MCN, and "PDA with solid and cystic mass." Furthermore, the patients in these reports were diagnosed at an advanced stage, and the period of survival after resections was about 7-16 mo. These reports were published in the era when adjuvant or neoadjuvant chemotherapy for PDA was not established. The outcome is worse than that of surgically resected IPMNs, reported as 37.0 mo[90], and chemotherapy is not generally considered if the invasive component in IPMN-associated cancer is not evident. Therefore, distinguishing large-duct PDA from other cystic neoplasms, including IPMNs, is essential. Accurate histological and genomic information related to the tumor will help decide between appropriate therapeutic options before surgery[91-93]. Currently, how standard chemotherapy against PDAs, including both adjuvant and neoadjuvant chemotherapy, can effectively eradicate large-duct PDAs remains unclear.

## CONCLUSION

Large-duct PDA is a subtype of PDA that mimics IPMNs or other pancreatic cystic neoplasms. Given the rarity of this disease, the diagnostic approach is sometimes challenging. Considering the estimated prognosis of the patients, it is crucial to distinguish large-duct PDAs from IPMNs using macroscopic and pathological findings. When an atypical cystic lesion is identified in the pancreas, both symptomatic and asymptomatic, the differential diagnosis should include large-duct PDA. Given their advantages, genetic analysis of PDAs and IPMNs, exploration of *KRAS* mutations, and mutation profiling of other cancer-related genes may help establish an accurate diagnosis.

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## Chronic hepatitis B in pregnant women: Current trends and approaches

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### Abstract

Chronic hepatitis B (CHB) is a significant public health problem worldwide. The aim of the present review is to summarize the actual trends in the management of CHB in pregnant women. The prevalence of hepatitis B virus (HBV) infection in pregnant women is usually comparable to that in the general population in the corresponding geographic area. All women have to be screened for hepatitis B surface antigen (HBsAg) during pregnancy. Additional examinations of pregnant women with CHB may include maternal hepatitis B e antigen, HBV viral load, alanine aminotransferase level, and HBsAg level. The management of pregnancy depends on the phase of the HBV infection, which has to be determined before pregnancy. In women of childbearing age with CHB, antiviral therapy can pursue two main goals: Treatment of active CHB, and vertical transmission prevention. During pregnancy, tenofovir is the drug of choice in both cases. A combination of hepatitis B immunoglobulin and vaccine against hepatitis B should be administered within the first 12 h to all infants born to mothers with CHB. In such cases, there are no contraindications to breastfeeding.

**Key Words:** Chronic hepatitis B; Hepatitis B viral load; Pregnancy; Antiviral treatment;

s/by-nc/4.0/

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**Core Tip:** All women have to be screened for hepatitis B surface antigen (HBsAg) during pregnancy. Additional examinations of pregnant women with chronic hepatitis B (CHB) may include maternal hepatitis B e antigen, hepatitis B virus (HBV) viral load, alanine aminotransferase level, and HBsAg level. The management of pregnancy depends on the phase of the HBV infection, which has to be determined before pregnancy. During pregnancy, tenofovir is the drug of choice both for active CHB treatment and vertical transmission prevention. A combination of hepatitis B immunoglobulin and vaccine against hepatitis B should be administered within the first 12 h to all infants born to mothers with CHB.

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## INTRODUCTION

Chronic hepatitis B (CHB) is a significant public health problem worldwide. According to the current estimation by the World Health Organization (WHO), in 2015 about 257 million people in the world were living with CHB[1,2]. The geographic distribution of CHB is highly heterogeneous. There are regions with high (more than 8%), medium (2%-8%) and low (less than 2%) levels of hepatitis B (HB) prevalence. The course of CHB varies from asymptomatic carriage of hepatitis B surface antigen (HBsAg) to severe, active variants with progression of fibrosis, formation of liver cirrhosis, and the development of hepatocellular carcinoma (HCC). Despite the successes achieved by the introduction of mass vaccination against hepatitis B, the vertical route of transmission remains an important factor. Every year, 4-5 million children in the world are infected from mothers with CHB[3]. In endemic regions, more than 50% of patients with CHB become infected at birth or in early childhood[4]. The problem of HBV mother-to-child transmission (MTCT) is important because patients infected in early childhood develop CHB in most cases, while the risk of CHB development in patients infected in adulthood is not higher than 20%. Without prophylaxis, MTCT rates vary significantly depending on the mother's hepatitis B e antigen (HBeAg) status: the transmission rate for HBeAg-positive mothers is about 70%-90%, vs 10%-40% for HBeAg-negative mothers[5]. In 2016, the WHO set the goal of eliminating viral hepatitis as a major public health threat by 2030[6]. However, this goal cannot be achieved without solving the problem of vertical transmission of HBV. In this context, in order to reduce the HBV MTCT risk, it is important to apply different approaches to the management of pregnancy in women with CHB.

## CURRENT LIMITATION ON SCREENING FOR HBSAG IN PREGNANT WOMEN

In most developed and developing countries, all pregnant women are screened for HBsAg. Examining pregnant women only from the so-called risk groups (intravenous drug use, promiscuous sex, work in sex industry, sexual contact with HBsAg carriers) was not enough, since such an examination leaves up to 50% of pregnant women with CHB undetected[7].

Particular attention should be given to women who are diagnosed with CHB for the first time during pregnancy. In these patients, acute hepatitis B has to be excluded. Additional examinations of pregnant women with CHB may differ depending on the region. Table 1 presents the recommendations of the main hepatological communities



**Table 1 Examination of pregnant women**

	APASL 2016[8]	EASL 2017[9]	AASLD 2018[10]
All pregnant women	Pregnant female (preferably during the first trimester to vaccinate unprotected mothers) should be tested for HBV infection	Screening for HBsAg in the first trimester of pregnancy is strongly recommended	All pregnant women should be screened for HBV infection
Examination of HBsAg-positive women during pregnancy	Maternal HBeAg, HBV DNA status, and ALT level should be checked during pregnancy	ALT, HBV DNA level, and HBsAg level	ALT level, HBV DNA or imaging for HCC surveillance if indicated

APASL: Asia-Pacific Association Society for the Study of the Liver; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; HCC: Hepatocellular carcinoma.

for the examination of pregnant women with CHB[8-10].

Most recommendations agree that viral load determination is necessary to understand the advisability of antiviral treatment during pregnancy. Recommendations differ as to the timing of therapy initiation and timing of the examination. The viral load determination should be performed no later than week 30 of gestation.

Determination of the HBsAg level during pregnancy is currently prescribed only in the European clinical guidelines for the management of patients with CHB[9]. Meanwhile, available studies indicate a significant correlation between the level of HBsAg during pregnancy and the risk of vertical transmission[11-13]. During pregnancy, HBsAg level is a more stable parameter than viral load, and its measurement is cheaper. Therefore, it can be recommended as a predictor of the vertical transmission of HBV infection, especially in a resource-limited setting. In a pregnant woman with a low HBsAg level, HBV viral load testing is not necessary.

## PREVALENCE OF HEPATITIS B IN PREGNANT WOMEN

The prevalence of HBV infection in pregnant women is usually comparable to that in the general population in the same geographic area. In China the prevalence of HBV infection among women of childbearing age is 2%-8%[14,15], while in the United States it is only 0.4%[16].

The prevalence of HBsAg positive patients among pregnant women in several countries is shown in Table 2.

At present, a high HBV prevalence among pregnant women persists in African countries, while the rate of HBsAg-positive pregnant women in Europe and America is low. Even in China, where the prevalence of HBV was very high in the past, a significant reduction in the rate of HBsAg-positive pregnant women is now observed.

## COURSE OF CHB AND VERTICAL TRANSMISSION RISK

As agreed by most researchers, there are five phases of the natural course of CHB.

The first phase, called the phase of immune tolerance, usually occurs during perinatal infection and is characterized by a prolonged and low-symptom course, normal serum alanine aminotransferase (ALT) level and minimal changes in liver tissue. As shown in Table 3, patients in this phase of CHB are seropositive for HBeAg and have mostly a high viral load ( $10^8$ - $10^9$  IU/mL HBV DNA)[30,31]. In patients infected in adulthood, the duration of this phase is usually short[32].

The second phase, known as the immunoreactive phase, occurs in patients infected at birth or in early childhood. It starts after two or three decades and is characterized by occasionally increasing ALT values. The anti-HBV immune response results in a moderate (as compared to the first phase) decrease in HBV DNA level. The age of patients when this phase occurs depends on the HBV genotype and varies by geographic region. In Taiwan, 90% of HBeAg seroconversion occurs in patients under the age of 40 years, with genotype B seroconversion occurring earlier than with genotype C[30]. In the European region, no more than 30% of patients remain HBeAg-positive after the age of 40 years[30]. This is important, because the earlier pregnancy occurs, the higher the chances that the woman is in the first phase of CHB, with high viral replication, and, accordingly, a high risk of vertical transmission of HBV

**Table 2 Prevalence of hepatitis B surface antigen among pregnant women**

Ref.	Country	Years	Number	HBsAg-positive (%)
Kirbak <i>et al</i> [17], 2017	Republic of South Sudan	2013-2014	280	11
Fouelifack <i>et al</i> [18], 2018	Cameroon	2016	360	9.4
Bittaye <i>et al</i> [19], 2019	Gambia	2015	426	9.2
Tanga <i>et al</i> [20], 2019	South Western Ethiopia	2017	253	7.9
Kishk <i>et al</i> [21], 2020	Egypt	2018-2019	600	5
Fessehayye <i>et al</i> [22], 2018	Eritrea	2016	5009	3.2
Sheng <i>et al</i> [23], 2018	China	2016	14314	3.1
Cetin <i>et al</i> [24], 2018	Turkey	2016	475	2.1
Mishra <i>et al</i> [25], 2017	India	2016	3567	1.09
Biondi <i>et al</i> [26], 2020	Canada	2012-2016	651745	0.63
Lembo <i>et al</i> [27], 2017	Italy	2010-2015	7558	0.5
Ruiz-Extremera <i>et al</i> [28], 2020	Spain	2015	21870	0.42
Harris <i>et al</i> [29], 2018	United States	2011-2014	870888	0.14

HBsAg: Hepatitis B surface antigen.

**Table 3 Clinical features and vertical transmission risk in different phases of chronic hepatitis B**

Phase of CHB	ALT	Fibrosis (Metavir score)	HBV DNA level	Markers of HBV-infection	Vertical transmission risk
Phase of immune tolerance	Normal	F0	Very high ( $10^8$ - $10^9$ IU/mL)	HBsAg+; HBeAg+; HBeAb-; HBcorAb+	Very high
Immunoreactive phase	Elevated	F1-F4	High ( $10^6$ - $10^7$ IU/mL)	HBsAg+; HBeAg+/-; HBeAb-/+; HBcorAb+	High
Inactive carriage of HBsAg	Normal	F0	Less than 2000 IU/mL	HBsAg+; HBeAg-; HBeAb+; HBcorAb+	Low
Phase of HBeAg-negative CHB	Elevated	F1-F4	Middle ( $10^3$ - $10^7$ IU/mL)	HBsAg+; HBeAg-; HBeAb+; HBcorAb+	Depends on HBV viral load
Occult CHB	Normal	F1-F4	+/-, HBV DNA in liver+	HBsAg-; HBeAg-; HBeAb-; HBcorAb+/-	Low

CHB: Chronic hepatitis B; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody; HBcorAb: Hepatitis B core antibody.

infection.

The third phase—the phase of inactive carriage of HBsAg—is characterized by the presence of HBsAg, the absence of HBeAg, and a low (less than 2000 IU/mL) or undetectable HBV viral load. The ALT level is normal in this phase, and no fibrosis progression is observed. Spontaneous HBsAg seroconversion is possible. This phase can continue for decades. The risk of vertical transmission at this stage is low.

The fourth phase, referred to as the HBeAg-negative CHB phase, is characterized by an undulating course, with periodic ALT increases. The HBV viral load can vary significantly, while HBsAg level is a more stable indicator[33]. HBeAg is absent during this phase. There is a gradual progression of fibrosis, and the risk of developing HCC increases. In this phase, the vertical transmission risk depends on HBV viral load.

The fifth phase, called the HBsAg-negative phase or "occult" CHB, is characterized by the disappearance of HBsAg, although the virus continues to replicate in the liver. Clinical symptoms are usually not pronounced, the ALT level remains normal. There is a possibility of CHB reactivation, especially due to immunosuppression, for example a physiological immunosuppression during pregnancy. A few cases of CHB reactivation during pregnancy are reported[34,35]. The vertical transmission risk in

such situations is low.

Management of pregnancy depends on the phase of HBV-infection. Unfortunately, women frequently only learn about their CHB diagnosis during pregnancy. Thus, it is advisable to examine all women for markers of viral hepatitis before pregnancy. During pregnancy, there are limitations for reliably determining the stage of CHB, since several indicators change significantly from the beginning of pregnancy. The level of alpha-fetoprotein increases as early as in the first weeks of pregnancy. Some pathological conditions (toxemia of the first half of pregnancy, excessive vomiting of pregnant women, *etc.*) can lead to significant changes in cytolytic indicators. In such cases, it is sometimes difficult to determine whether an increase in ALT is caused by these conditions or by CHB activity. Some standard examinations are unreliable during pregnancy. For example, a significant change in circulating blood volume during pregnancy can lead to inaccurate data on liver fibrosis obtained using transient elastography. For this reason, it is preferable to determine the stage of CHB before pregnancy.

Typically, women of childbearing age do not have significant liver fibrosis and cirrhosis. However, due to the increasing age of primiparous women and to the fact that before mass vaccination of newborns against hepatitis B was introduced, one of the main routes of transmission was the vertical route, CHB with advanced fibrosis is not unique. Pregnancy at the stage of liver cirrhosis is also associated with an increased risks of complications for the mother[36].

## EFFECT OF PREGNANCY ON THE COURSE OF CHB

In most cases, no exacerbation of CHB occurs during pregnancy, and the cytolytic activity indicators are usually normalized. Nevertheless, a few cases of CHB exacerbation during pregnancy, including development of fulminant liver failure[37,38]. The level of HBV viral load during pregnancy may vary. Cases of CHB reactivation during pregnancy have been known. In one study, in mothers without detectable HBV DNA in the first trimester, HBV DNA was detected in 19.6% of cases in the second trimester and in 30.4% of cases in the third trimester[39]. In another study, the viral load in women with CHB increased during pregnancy and decreased after childbirth[34]. In addition, some studies describe exacerbation of hepatitis in the first months after childbirth[34,40,41]. In the majority of women, the ALT level decreases during pregnancy, but after childbirth there is a significant increase in the cytolytic activity. For example, an increase in ALT level of three times or more was observed in 45% of women within 6 mo after childbirth[34]. Cases of HBeAg seroconversion during pregnancy have also been described in 12.5%-17% of patients[40,41].

Clinical manifestations of CHB in pregnant women are characterized by the predominance of asthenic and dyspeptic syndromes (63%). Hemorrhagic syndrome, such as bleeding gums, was observed in 15% of pregnant women, and hepatomegaly occurred in 10% of cases[42].

## PREGNANCY OUTCOMES IN HBV INFECTED WOMEN

The effect of chronic maternal HBV infection on pregnancy outcome has not been well studied. Published works on this topic contradict each other. Some studies show that there is no association between pregnancy outcomes and maternal CHB[43]. Other studies have shown that chronic HBV infection does not result in negative perinatal outcomes, except for lower Apgar scores in newborns[44,45]. However, some studies indicate a high rate of diseases such as fetal distress syndrome, preterm labor and meconium peritonitis among HBV infected women and their newborns[41,46]. A large cohort study carried out in China showed that HBsAg positive pregnant women had a higher risk of gestational diabetes mellitus, postpartum hemorrhage, and intrahepatic cholestasis[44]. A recent study showed a significant correlation between HBV viral load and blood glucose level (fasting blood glucose, 2-h postprandial blood glucose and hemoglobin A1c)[47]. No statistical associations were found between HBsAg positivity and pre-eclampsia, as well as between HBsAg positivity and placenta previa. HBsAg positivity during pregnancy was associated with a higher risk of multiple adverse maternal outcomes.

In a large case-control study in China[48], it was shown that maternal HBsAg carriage was associated with several adverse pregnancy outcomes. In particular, it was correlated with an increased risk of pregnancy-induced hypertension, fetal distress,

cesarean delivery and macrosomia. This study also demonstrated a statistically significant association between high maternal viral load in the second trimester and a high risk of preterm birth. Other previous studies have also reported that maternal HBV infection was associated with an increased risk of preterm birth[49,50], although there are also studies showing the opposite results[51,52].

Some studies indicate a more frequent development of bleeding during childbirth in women with CHB[53]. It was also reported that women with CHB are less likely to have hypertension and pre-eclampsia during pregnancy[51].

## CHB THERAPY DURING PREGNANCY

At present, the therapy of CHB cannot yet achieve complete HBV elimination in patients. Therefore, depending on the status of the patient, the goals of CHB therapy may be the following: (1) Suppression of virus replication; (2) Reduction of the inflammatory process in the liver; (3) Reverse the development of fibrosis; (4) Prevention of cirrhosis and HCC development; and (5) Reduction of the HBV vertical transmission risk.

When choosing a therapy, it is necessary to take into account the safety and effectiveness of antiviral drugs, as well as the possibility of drug resistance developing. In women of childbearing age with CHB, antiviral therapy can pursue two main goals: the treatment of women with active CHB and the prevention of vertical transmission (see Table 4). At present, the necessity to treat inactive HBsAg carriers[54] is being discussed, but currently it is recommended only by the Asia-Pacific Association for the Study of the Liver (APASL)[8], while the European Association for the Study of the Liver (EASL)[9] and American Association for the Study of Liver Diseases (AASLD)[10] societies refrain from such recommendations.

A large trial[55] has reported reduced HBV transmission and HBsAg-positivity in infants born to telbivudine or lamivudine treated HBsAg-positive mothers. A systematic review[56] has shown that antiviral therapy of pregnant women with nucleoside analogues (NAs), such as lamivudine, telbivudine or tenofovir, significantly decreases maternal HBV viral load. During pregnancy, tenofovir is the drug of choice, due to its profile of antiviral activity and a low risk of developing resistance. Tenofovir in pregnancy is well tolerated and reduces viral load prior to parturition[57].

NA prophylaxis is also useful in HBeAg-negative women with a high HBV DNA level but normal ALT level[11,55].

The administration of NAs at 28-30 wk of gestation leads to a rapid decrease in the viral load by the time of delivery[58], and, as a consequence, to a significant reduction in vertical transmission risk. However, if the drug intake is discontinued, the viral load quickly returns to its original level. It is reported[58] that a prescription of telbivudine in the third trimester to women with a high viral load leads to an HBV DNA decrease up to an undetectable level at the time of delivery in 33% of patients. In the control group, no such decrease was observed. In the same study, it was shown that there were no cases of vertical transmission in the group of women who received telbivudine in the third trimester, while in the control group, 8% of children 7 mo after delivery were HBsAg-positive. Another large prospective study of 450 HBeAg-positive women with high viral load also showed no vertical transmission in women receiving telbivudine, while in the control group HBsAg was detected in 14.7% of newborns 6 mo after birth[59].

If antiviral therapy was administered in order to prevent MTCT, it is usually discontinued after delivery. However, there is no common opinion how soon after delivery this can be done. As shown in Table 5, according to the AASLD recommendations, the drug can be discontinued soon after delivery; according to EASL – at delivery or within the first 3 mo; while APASL recommends continuing drug intake for 4-12 wk.

## HBV PROPHYLAXIS IN NEWBORNS

HBV vaccination reduces the vertical transmission risk from 90% to 21% in HBeAg-positive women and from 30% to 2.6% in HBeAg-negative women[60]. With the addition of hepatitis B immunoglobulin (HBIG), the risk of MTCT is decreased to 6% in HBeAg-positive women and to 1% in HBeAg-negative women[60]. This prophylaxis has to be administered within 12 h after birth (see Table 6).



**Table 4 Treatment of pregnant women with chronic hepatitis B**

	APASL 2016[8]	EASL 2017[9]	AASLD 2018[10]
Therapy	In pregnant females with chronic HBV infection who need antiviral therapy, tenofovir is the drug of choice for mothers indicated for antiviral treatment during the first through third trimester of pregnancy	Tenofovir is recommended for pregnant women with CHB and advanced fibrosis. Therapy with tenofovir should be continued, and if the woman was receiving other drugs, these other drugs should be replaced with tenofovir	Women who meet standard indications for HBV therapy should be treated. HBV-infected pregnant women with cirrhosis should be managed in high-risk obstetrical practices and treated with tenofovir to prevent decompensation
To prevent vertical transmission	For reduction of risk of mother-to-infant transmission that occurs during the perinatal period, short-term maternal NAs starting from 28 wk to 32 wk of gestation is recommended using either tenofovir or telbivudine for those mothers with HBV DNA above 6-7 log <sub>10</sub> IU/mL. Since, the HBV transmission could occur even with lower maternal HBV DNA level, NAs could be administered after discussion with the patient, even in patients with lower DNA level. The NA could be stopped at birth and when breastfeeding starts, if there is no contraindication to stopping NA	In all pregnant women with high HBV DNA level (> 200000 IU/mL) or HBsAg level > 4 log <sub>10</sub> IU/mL, antiviral prophylaxis with tenofovir disoproxil fumarate should start at week 24-28 of gestation and continue for up to 12 wk after delivery	Women without standard indications but who have HBV DNA > 200000 IU/mL in the second trimester should consider treatment to prevent mother-to-child transmission

APASL: Asia-Pacific Association Society for the Study of the Liver; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; HBV: Hepatitis B virus; CHB: Chronic hepatitis B; NA: Nucleoside analogues; HBsAg: Hepatitis B surface antigen.

**Table 5 Cessation of nucleoside analogues treatment after delivery**

APASL 2016[8]	EASL 2017[9]	AASLD 2018[10]
Cessation of NA therapy (at delivery or 4-12 wk after delivery) is recommended in females without ALT flares and without pre-existing advanced liver fibrosis/cirrhosis. Continuation of NA treatment after delivery may be necessary according to maternal liver disease status	If NA therapy is given as prophylaxis, <i>i.e.</i> , only for the prevention of perinatal transmission, its duration is not well defined (stopping at delivery or within the first 3 mo after delivery)	HBV-infected pregnant women who are not on antiviral therapy as well as those who stop antiviral at or early after delivery should be monitored closely for up to 6 mo after delivery for hepatitis flares and seroconversion. Long-term follow-up should be continued to assess need for future therapy

APASL: Asia-Pacific Association Society for the Study of the Liver; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; NA: Nucleoside analogues; ALT: Alanine aminotransferase.

**Table 6 Hepatitis B virus prophylaxis in newborns**

APASL 2016[8]	EASL 2017[9]	AASLD 2018[10]
HBIG and hepatitis B vaccine can be given to newborns from HBsAg-positive mothers immediately after delivery	The combination of HBIG and vaccination is administered within 12 h of birth	HBIG and HBV vaccine should be administered to the newborn < 12 h after delivery

APASL: Asia-Pacific Association Society for the Study of the Liver; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; HBIG: Hepatitis B immunoglobulin; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

The 3-dose vaccine against hepatitis B produces a protective antibody response (anti-HBs  $\geq$  10 mIU/mL) in approximately 95% of healthy infants[61].

## BREASTFEEDING

It is known that in many women infected by HBV, HBsAg can be detected in the breast milk[62]. Moreover, there is evidence that HBV DNA can also be found in breast milk and colostrum[63]. As a result, there are frequent concerns that breastfeeding may facilitate MTCT, although the studies available so far have not confirmed this. No statistically significant differences between breastfed and artificially fed perinatally infected children were detected, and provided a timely vaccination[64-66]. A recent study showed that the frequency of vertical transmission in mothers with similar HBV DNA level is independent of the type of feeding[67]. Thus, HBV infection is not currently considered to be a contraindication to breastfeeding infants receiving HBIG

Table 7 Breastfeeding of newborns

APASL 2016[8]	EASL 2017[9]	AASLD 2018[10]
Breastfeeding is not recommended while the woman is receiving antiviral therapy	Breastfeeding is not contraindicated in women not receiving antiviral therapy and during treatment with tenofovir	Breastfeeding is not prohibited for women with or without antiviral therapy

APASL: Asia-Pacific Association Society for the Study of the Liver; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases.

and HBV vaccine. In addition, there are several studies showing that breastfeeding does not affect the child's immune response to vaccination[68]. The current recommendations of major societies are shown in Table 7.

## CONCLUSION

Despite the continuously decreasing prevalence of CHB achieved after the introduction of vaccination against hepatitis B, this disease remains a significant public health problem worldwide. In the present study, we summarized the major trends in the management of CHB in pregnant women and provided recommendations for clinical practice necessary to achieve the elimination of hepatitis B as a public health threat, as proposed by the WHO. The most important of these recommendations are: (1) All women have to be screened for HBsAg during pregnancy. Additional examinations of pregnant women with CHB may include maternal HBeAg, HBV viral load, ALT level, and HBsAg level; (2) The management of pregnancy depends on the phase of the HBV infection, which has to be determined before pregnancy; (3) In women of childbearing age with CHB, antiviral therapy can pursue two main goals: treatment of active CHB, and vertical transmission prevention. During pregnancy, tenofovir is the drug of choice in both cases; and (4) A combination of HBIG and vaccine against hepatitis B should be administered within the first 12 h to all infants born to mothers with CHB. In such cases, there are no contraindications to breastfeeding.

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## Viscoelastic tests in liver disease: where do we stand now?

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### Abstract

Hemostasis is a complex physiological process based on the balance between pro-coagulant and anticoagulant systems to avoid pathological bleeding or thrombosis. The changes in standard coagulation tests in liver disease were assumed to reflect an acquired bleeding disorder, and cirrhotic patients were considered naturally anticoagulated. In the light of the new evidence, the theory of rebalanced hemostasis replaced the old concept. According to this model, the hemostatic alteration leads to a unique balance between pro-coagulant, anticoagulant, and fibrinolytic systems. But the balance is fragile and may prone to bleeding or thrombosis depending on various risk factors. The standard coagulation tests [INR (international normalized ratio), platelet count and fibrinogen] only explore parts of the hemostasis, not offering an entire image of the process. Rotational thromboelastometry (ROTEM) and thromboelastography (TEG) are both point of care viscoelastic tests (VET) that provide real-time and dynamic information about the entire hemostasis process, including clot initiation (thrombin generation), clot kinetics, clot strength, and clot stability (lysis). Despite prolonged PT/INR (international normalized ratio of prothrombin time) and low platelet counts, VET is within the normal range in many patients with both acute and chronic liver disease. However, bleeding remains the dominant clinical issue in patients with liver diseases, especially when invasive interventions are required. VET has been shown to assess more appropriately the risk of bleeding than conventional laboratory tests, leading to decrimal use of blood products transfusion. Inappropriate clotting is common but often subtle and may be challenging to predict even with the help of VET. Although VET has shown its benefit, more studies are needed to establish cut-off values for TEG and ROTEM in these populations and standardization of transfusion guidelines before invasive interventions in cirrhotic patients/orthotopic liver transplantation.

**Key Words:** Liver diseases; Viscoelastic tests; Portal vein thrombosis; Acute-on-chronic

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**Core Tip:** Despite having specific alterations in all hemostasis phases and, thus, considered naturally anticoagulated, cirrhotic patients have, in fact, balanced hemostasis. However, this balance may be disturbed by different factors, and the result may vary from devastating bleeding to massive thrombosis. Conventional laboratory tests failed to predict these events. Viscoelastic tests appear to offer a better, global view of hemostasis in these patients. They have been used to assess bleeding risk before invasive interventions and for a precocious use of blood product transfusions.

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## INTRODUCTION

### *Hemostasis in advanced liver disease*

The hematological changes encountered in cirrhosis have shown a great interest in the last two decades. The misconception of the cirrhotic patient being naturally anticoagulated has changed with the new concept of balanced hemostasis.

The new cell-based model of hemostasis elaborated by Hoffman and Monroe[1] in 2001 led to a better understanding of hemostasis's complex process. In Hoffman's conception, three phases simultaneously cooperate for adequate hemostasis: primary hemostasis, which involves the activated platelets with the formation of platelet-plug; coagulation with the fibrin mesh construction and clot fortification, involving plasma procoagulant proteins and, finally, clot fibrinolysis by plasma anticoagulant proteins.

In liver cirrhosis, all these three phases are affected by hepatic synthetic dysfunction and portal hypertension[2].

In hemostasis, platelets have a dual role[3,4]. Through the adhesive protein von Willebrand factor (vWF), they adhere to the subendothelium and aggregate to initiate thrombus formation and, by assembling vitamin K dependent coagulation factors on their surface, they support thrombin generation. The most common abnormality in cirrhotic patients is the thrombocytopenia-numerical decrease of circulating platelet count[2-4]. The etiology of thrombocytopenia is multifactorial: platelet spleen sequestration, low thrombopoietin levels from impaired hepatic synthesis, immune destruction.

However, there is controversy over the qualitative changes in platelet function in chronic liver disease[2,3]. vWF, activated by cleavage into smaller subunits (high molecular weight multimers) by the endothelial-derived metalloproteinase ADAMTS13, mediates platelet adhesion and aggregation[3]. Lisman *et al*[5] have shown *in vitro* that cirrhotic patients' plasma may support the adhesion of normal or cirrhotic platelets. This is possible due to the increased level of vWF and, at the same time, a decrease in vWF collagen binding capacity, as well as a reduction in vWF and ADAMTS13 multimers. These results indicate that increased levels of VWF contribute to the induction of primary hemostasis by maintaining the platelets adherence despite the functional or numerical alteration of them. Tripodi *et al*[6] found in an *in vitro* study that thrombocytes from cirrhotic patients were qualitatively able to support thrombin generation if their range was over  $50-60 \times 10^9/L$ .

A reduction in the synthesis of procoagulant factors (FII, FV, FVII, FIX, FX, FXI) characterizes chronic liver disease[2,4]. The exception makes FVIII, whose level is elevated secondary to synthesis induced by cytokines, released from necrotic tissue, and reduced clearance[2,7]. Fibrinogen level is normal or increased in most patients with cirrhosis, and dysfibrinogenemia occurs in 50%-78% of patients with chronic liver disease[4]. Despite the reduction in hepatic synthesis of procoagulant factors, patients with liver cirrhosis do not experience spontaneous bleeding similarly to those with congenital deficiency of coagulation factors do (*e.g.*, haemarthrosis)[2]. The decreased

protein C synthesis, a potent anticoagulant, protein S, and antithrombin III, also contributes to the coagulations' normality[2-4,7].

All profibrinolytic and antifibrinolytic factors are synthesized by hepatic cells, except tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI 1), which are produced by endothelial cells. Liver cirrhosis is associated with hyperfibrinolysis secondary to high levels of t-PA and low levels of plasma inhibitor and thrombin-activatable fibrinolysis inhibitor (TAFI), but also with hypofibrinolysis secondary to increased PAI and plasminogen levels[7]. Lisman *et al*[8] pointed out that a parallel decrease in antifibrinolytic factors counterbalances the low levels of profibrinolytic factors that occur in cirrhosis. However, Colucci *et al*[9] showed contradictory results, demonstrating that the reduction in TAFI level is associated with hyperfibrinolysis.

All of these changes (Figure 1) support the new theory of rebalanced hemostasis in patients with liver cirrhosis[2,4,7,10]. However, various circumstantial risk factors can quickly destabilize this balance, increasing the risk of bleeding or thrombosis[3,10].

## HEMOSTASIS TESTING IN ADVANCED LIVER DISEASES

The problems appear when it comes to exploring hemostasis. The major inconvenience of conventional laboratory tests [platelet count, PT/INR (international normalized ratio of prothrombin time), procoagulant/anticoagulant factors, profibrinolytic/antifibrinolytic factors] is that they test parts of hemostasis, and they do not offer a global view of the process.

The INR appeared as a necessity in the standardization of anticoagulant therapy with vitamin K antagonists (VKA). Still, it is not calibrated to the specific changes of cirrhotic coagulation[2,3]. There were two attempts[11,12] to introduce a new liver dedicated INR ( $INR_{liver}$ ) by recalibrating ISI (International Sensitivity Index). The method requires replacing plasma from the patients treated with VKA with cirrhotic patients' plasma. This technique has technological limits, so it remains more theoretical than a practical one[2]. Moreover, the usual lab tests are not useful for appreciating the hemorrhagic risk related to invasive maneuvers in cirrhotic patients[2].

There is no evidence that a prolonged PT/INR is an indicator of hemorrhagic risk during or following invasive procedures[13].

The platelet count seems to correlate better with the bleeding risk, but a cut-off value below which the risk is increased has not been demonstrated.

Some studies have associated values below 60.000-75.000 with increased hemorrhagic risk following invasive procedures[14].

The fibrinogen level is variable in hepatic diseases[15]. A correlation with bleeding risk has not been defined, except for evident disseminated intravascular coagulation syndrome, sepsis, and different liver transplant stages[16].

Thrombin generation tests measure the entire quantity of thrombin that is generated during hemostasis. Using this assay, several studies[17,18] have shown that compensated cirrhotic patient plasma can produce normal or increased quantities of thrombin, despite prolonged PT/INR.

Thromboelastography (TEG) and Rotational thromboelastometry (ROTEM) are tools based on Hartert's invention, which assesses overall hemostasis, reflecting the interaction between plasma, platelets, and blood cells[4].

ROTEM and TEG are both point of care viscoelastic tests (VET) of hemostasis in whole blood providing real-time, dynamic information about the entire coagulation process, including clot initiation (thrombin generation), clot kinetics, clot strength, and clot stability (lysis). The force exerted on a small metal pin suspended in whole blood during clot formation is measured while the cup (TEG) or the pin is rotated. Data are processed and analyzed with dedicated software and exposed as graphical and numerical values. Table 1 and Figure 2 represents the principal parameters from both VETs.

It should be highlighted that PT/INR correlates poorly with R/CT (reaction time/clotting time) VET parameters[19,20]. However, an excess of anticoagulants or low coagulation factors (less than 30%) would prolong the R/CT time. In contrast, a lot of tissue factor, high factor VIII, or low protein C would shorten these parameters[21]. An increase in maximum clot firmness (MCF) or amplitude could be explained by a combination of increased fibrinogen levels and platelet reactivity[22].

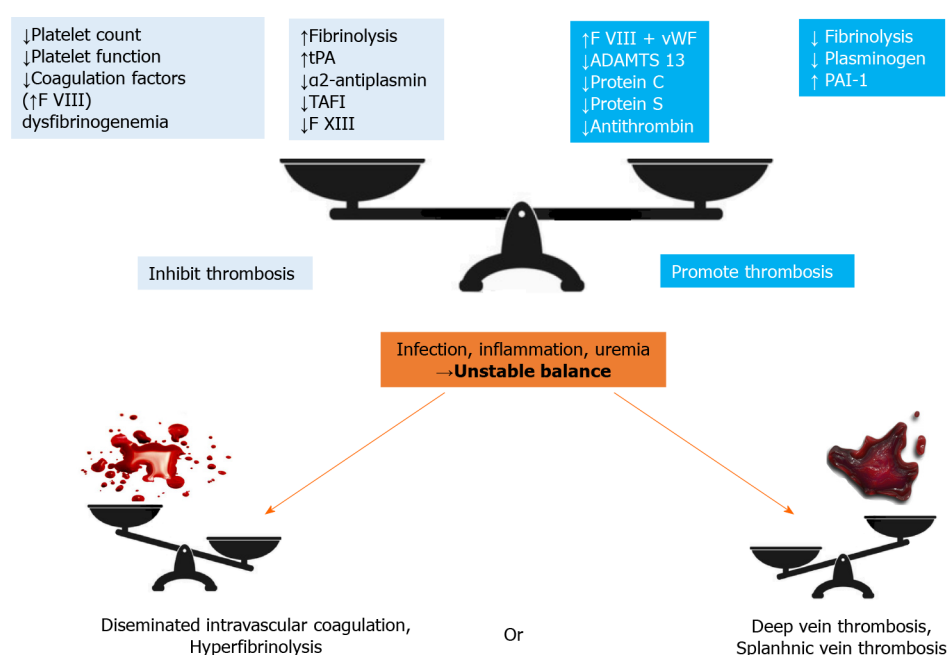
Since TEG/ROTEM are global hemostasis tests, they are more used to evaluate coagulopathy in chronic liver disease[23]. Consistent with the new vision of the rebalanced hemostasis, patients with compensated liver cirrhosis often have normal



**Table 1 Parameters of viscoelastic tests numerical**

	ROTEM	TEG	
Clotting initiation	CT (clotting time)	R (reaction time)	Enzymatic coagulation
Clot kinetics	CFT (clot formation time); $\alpha$ angle	K (K time); $\alpha$ angle	Speed to reach a certain level of clot strength; Rapidity of fibrin synthesis
Clot strength	MCF (maximum clot firmness)	MA (maximum amplitude)	Ultimate strength of the fibrin clot
Clot stability	CLI30 (clot lysis index at 30 min after MCF); CLI60 (clot lysis index at 60 min after MCF)	Ly30 (clot lysis at 30 min after MA); Ly60 (clot lysis at 60 min after MA)	Clot lysis

TEG: Thromboelastography; ROTEM: Rotational thromboelastometry; MCF: Maximum clot firmness; MA: Maximum amplitude; CT: Clotting time; CFT: Clot formation time.



**Figure 1 Rebalanced hemostasis in liver cirrhosis.** In primary hemostasis, high levels of von Willebrand factor and low levels of disintegrin and metalloproteinase with a thrombospondin type 1 motif 13 counteract numerical or functional abnormalities of platelets. In the coagulation phase, low levels of procoagulant proteins are balanced by reduced synthesis of anticoagulant factors. In fibrinolysis, parallel changes are seen in profibrinolytic and antifibrinolytic proteins. The balance is though fragile, and various factors, as inflammation, infection, uremia may unstable it, leading to bleeding or thrombosis. tPA: Tissue plasminogen activator; TAFI: Thrombin-activatable fibrinolysis inhibitor; vWF: von Willebrand factor; ADAMTS13: Disintegrin and metalloproteinase with a thrombospondin type 1 motif 13; PAI-1: Plasminogen activator inhibitor-1.

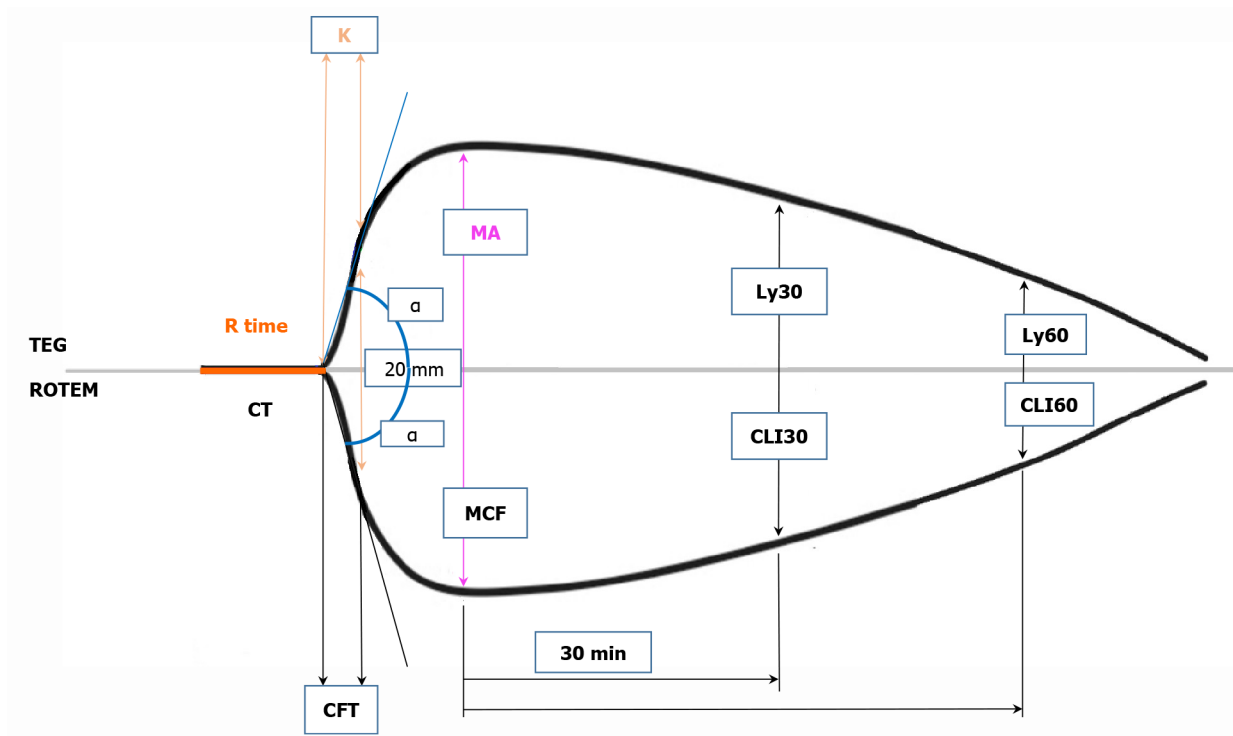
TEG parameters[24].

We will discuss further the importance of VET in the most frequent settings from the hepatology field.

## HYPERCOAGULABILITY, THROMBOSIS AND VET IN LIVER CIRRHOSIS

Despite the "natural anticoagulation" concept that marked the diagnosis of cirrhosis, portal vein thrombosis (PVT) is a relatively frequent complication of patients with cirrhosis (up to 25% of patients with decompensated cirrhosis)[25]. All the elements of Virchow's triad are present in patients with cirrhosis: decreased velocity (through the presence of portal hypertension), vessel-wall abnormalities (endothelial dysfunction, fibrotic mechanical distortion), and hypercoagulation[7]. However, using the conventional coagulation test, the hypercoagulation status is difficult to demonstrate.

Moreover, the hypercoagulation could vary among different etiologies of cirrhosis. Compared to only 5% of non-cholestatic cirrhosis, 28% of patients with primary biliary cholangitis (PBC) and 43% of patients with primary sclerosing cholangitis (PSC)



**Figure 2 Parameters of viscoelastic tests graphical.** TEG: Thromboelastography; ROTEM: Rotational thromboelastometry; CFT: Clot formation time; CT: Clotting time; R: Reaction time; K: K time; MA: Maximum amplitude; MCF: Maximum clot firmness; CLI30: Clot lysis index at 30 min after maximum clot firmness; Ly30: Clot lysis at 30 min after maximum amplitude; CLI60: Clot lysis index at 60 min after maximum clot firmness; Ly60: Clot lysis at 60 min after maximum amplitude.

demonstrated hypercoagulation status on TEG parameters[26]. Notably, the conventional coagulation tests did not identify this hypercoagulability. Pihusch *et al*[27] also found a hypercoagulable state in noncirrhotic patients with PBC/PSC.

Among various etiologies, non-alcoholic fatty liver disease (NAFLD) has a higher risk of thrombosis. Using TEG, patients with NAFLD had a significantly stronger clot development than healthy controls [maximum amplitude (MA)  $58.3 \pm 6.3$  vs  $52 \pm 10$  mm,  $P = 0.01$ ][28]. The platelet contribution to overall clot strength was higher in NAFLD patients with a trend to reduced inducible clot lysis ( $P = 0.03$ ). Based on shortened TEG's R and increased net clot strength, Krzanicki *et al*[29] found a high rate of hypercoagulation in patients with PBC (42.9%), patients with PSC (85.7%), patients with fulminate hepatic failure (50%), and patients with NAFLD (37.5%). Similar findings were also reported by Hugenholtz *et al*[30] in a large ( $n = 270$ ) prospective study where 43% of patients with cholestatic liver disease had hypercoagulability MA values beyond the normal range. Contrary to what would be expected, 80% of patients with obstructive jaundice had hypercoagulable status on TEG analysis (increased MA), which was independent of prolonged PT[31]. However, three weeks after a biliary drainage procedure, all TEG parameters had returned to normal range.

It is still not clear whether a hypercoagulable status increases the risk of PVT[32]. It is tempting to assume that hypercoagulability in cirrhotic patients puts them at a higher risk of thrombosis. However, scarce data is supporting this hypothesis. Moreover, among the thrombotic risk factors, the lower portal velocity is the only independent factor of PVT [odds ratio of 44.9 (95% confidence interval (CI): 5.3-382)] [33]. The existing evidence is contradictory. Hugenholtz *et al*[30] found no difference in TEG parameters at baseline between patients who developed PVT (8 out of 270 patients followed almost three years). In another study[34], in patients with cirrhosis and gastroesophageal varices, TEG's R was significantly lower in the group with PVT ( $5.20$  vs  $6.00$ ,  $P = 0.009$ ), a sign of enhanced coagulation activity.

The evidence is more apparent in patients with hepatocellular carcinoma (HCC). In HCC patients, the fibrinogen and the MCF in the FIBTEM module were higher in patients who developed PVT during follow-up than those who did not ( $24$  mm vs  $16$  mm,  $P = 0.04$ ). An increased baseline MCF FIBTEM ( $0.25$  mm) was linked to a higher risk of developing PVT in HCC patients [risk ratio: 4.8 (95%CI: 2-11.3),  $P = 0.0001$ ][35]. These findings might be valid for cirrhosis patients and no HCC, which still needs to be proved.

Regarding the treatment of PVT, which is still a matter of debate, it would be ideal that the VET's parameters would predict which patients will recanalize with anticoagulation treatment and who will experience spontaneous recanalization. TEG use to guide antithrombotic therapy has been reported in Budd-Chiari syndrome (BCS)[36]. However, the problem is much more complicated as TEG's hemostasis proved to be heterogeneous in BCS. Contrary to the general belief that all patients with BCS have a hypercoagulable status, 20% of patients have a hypocoagulable status based on TEG [37]. However, large-scale prospective studies are mandatory to evaluate the impact of VET in managing PVT in cirrhotic patients.

Another real clinical dilemma is the ability to monitor the efficacy and safety of anticoagulant therapy in patients with liver diseases. First, patients with cirrhosis can develop vein thrombosis despite a prolonged INR[23]. Second, monitoring the efficacy of LMWH using anti-factor Xa is not readily available[38]. Third, TEG was shown to be a sensitive method for monitoring LMWH efficacy in non-cirrhotic patients[39]. And last, the use of LMWH in 70 patients with advanced cirrhosis completely abolished the risk of PVT compared to 17% in the control group[40]. Altogether, the use of point of care VET could be a solution for this scenario.

### **Coagulation in acute-on-chronic liver failure**

When it comes to acute multisystem imbalances, such as acute-on-chronic liver failure (ACLF), available data is relatively scarce, and reliable reports are rare.

It is essential to recognize the distinctive features of ACLF, to understand better its impact on coagulation and why precise assessment is needed[41]. Along with the classic liver failure features, the clinics are dominated by a marked systemic inflammatory response syndrome, often associated with bacterial infections, sequentially leading to multiple organ failure and, ultimately, death[42]. The typical ACLF patient is either treated in a high-dependency or an intensive care unit, requiring multiple invasive procedures[41,42]. In this light, an adequate assessment of their coagulation status appears to be particularly important. Based on prior experience with compensated and decompensated liver disease, the validity of standard coagulation tests (SCTs) in accurately assessing coagulation and bleeding risk in this clinical setting may yet again stand on shaky grounds.

Most of the VETs' data is relatively recent. It comprises monocentric reports, typically including less than one hundred patients with ACLF, assessing coagulation *via* ROTEM or TEG.

To this point, three available published reports are assessing the role of TEG in ACLF, all on Asian populations. In 2018, Goyal *et al*[43], comparing the coagulation profile of 68 ACLF patients with non-ACLF acutely decompensated patients and healthy controls, revealed a stark increase in SCT alteration with liver disease severity. Yet, the dynamic assessment was mostly normal, except for the reduced MA in ACLF, entailing a minimally altered coagulation profile. These findings might suggest that SCTs better reflect liver failure, rather than *per se* coagulation failure, as the diagnostic criteria for ACLF would imply. However, conclusions drawn from this dataset are in relative discordance with the other two available reports.

The patients who developed sepsis had a worse coagulation profile, tilted towards hypocoagulation, expressed by a higher R time[44]. In addition, among the enrolled ACLF patients, those with a hypocoagulation TEG profile had a significantly higher risk of bleeding [hazard ratio (HR) 2.1; CI: 1.6-4.9;  $P = 0.050$ ] and short-term mortality (HR 1.9; CI: 1.3-7.9;  $P = 0.043$ ). A more recent Chinese report[45] compared 51 hepatitis B virus-related cases of ACLF with healthy controls and patients with fully compensated chronic hepatitis B. They found that the coagulation dynamics were significantly altered in ACLF, with higher R and K times and lower  $\alpha$  angles and MAs, corresponding to a marked hypocoagulable state. However, in this case, the comparison groups may not be ideal because healthy subjects are at the opposite spectrum of the disease than ACLF patients. Here, SCTs and TEG variables appeared to follow a concordant trend line. Furthermore, 90-day mortality was significantly associated with hypocoagulation within the ACLF group, as patients with ACLF and low MA were prone to a worse outcome.

While the studies were significantly different in design, a fragile common ground seems to emerge. While not all patients appear to have a marked coagulation imbalance, those who do tend to be in a hypocoagulable state appear to have a worse outcome.

Studies using ROTEM for assessing the coagulation profile have reached similar conclusions to those using TEG. One report comparing 36 ACLF patients to 24 non-ACLF acutely decompensated patients estimated transfusion requirement, bleeding events, and short-term mortality[46]. On admission, patients with ACLF had a more

hypocoagulable state, and the parameters worsened at 72 h, contrasting to the control group, which had an improved coagulation profile. Hypocoagulation was associated with a marked pro-inflammatory status and led to increased 28-d mortality. However, there was no association with an increased risk of bleeding events or transfusion requirements despite a worse coagulation profile. Of note, while SCTs and ROTEM variables followed the same trend line, ROTEM was a better outcome predictor. A second study, published in 2020, compared 22 ACLF patients with a compensated control group[47]. In this small dataset, the agreement between SCTs and ROTEM was slightly better in the ACLF group, which had a more hypocoagulable state. Besides, bleeding events were more frequent among ACLF patients with a worse coagulation function.

### **VET in liver transplantation**

Historically, orthotopic liver transplantation (OLT) was associated with significant blood loss and the need for massive blood product transfusions[48]. Recently, with the new concept of rebalanced hemostasis, a more conservative attitude towards transfusion of red blood cells (RBC), fresh frozen plasma (FFP), or platelets is proposed. VET-guided transfusion algorithms to treat coagulopathy in OLT were first proposed by Kang *et al*[16] in the 1980s. They evaluated the blood coagulation system of 66 consecutive patients undergoing liver transplantation using TEG or standard liver transplantation monitoring and assessed the first clinical use of TEG in OLT. The use of TEG contributed to a 33% reduction of RBC, FFP, and platelet transfusion, whereas blood loss was comparable in all patients.

Comparing the standard management with ROTEM-guided hemostatic control results in a significant reduction of transfused RBC, FFP, and platelets in the ROTEM group[49]. Moreover, the number of blood product-free transplantations increased from 5% to 24% ( $P < 0.001$ ). Secondary endpoints like reintervention for bleeding, acute kidney failure, or hemodynamic instability were significantly lower in the ROTEM group.

During liver transplantation, enhanced physiological fibrinolysis can occur, especially during the anhepatic period due to lack of tPA clearance. Immediately after reperfusion, there is a substantial increase in tPA, which can lead to hazardous primary hyperfibrinolysis resulting in diffuse uncontrolled bleeding. Suppose the graft has a good function the hyperfibrinolysis after reperfusion is self-limiting and does not require treatment. Hyperfibrinolysis in OLT has been reported very frequently (range 5%-84%), mainly during the transplanted liver[50,51]. However, most fibrinolysis is self-limiting and shall only be treated when it occurs concomitantly with excessive bleeding[50].

In the context of OLT, VETs are particularly useful for detecting the presence of systemic fibrinolysis[52] and also to detect poor clot strength that is often the result of low fibrinogen levels[52,53]. Therefore, the VET parameters for fibrinogen (FIBTEM or TEG functional fibrinogen) are essential to avoid over transfusion of platelets to increase the MA or MCF[52], which is associated with higher mortality[54].

The risk-benefit balance of the routine use of prophylactic antifibrinolytic agents (*e.g.*, tranexamic acid 1-2 g) shifted to a more precocious use of antifibrinolytics, in high-risk patients or treatment only, since massive bleeding is less frequent. Because hyperfibrinolysis-induced bleeding may manifest in the postreperfusion stage of surgery and depends on the donor liver's quality, the assessment is more difficult[55]. Treatment with antifibrinolytics is recommended only when there is evidence of microvascular ooze or documented fibrinolysis ( $CLI > 15$ ) on TEG/ROTEM[56].

Conventional coagulation tests give no information where the balance in the coagulation lies since they do not provide a composite picture of the interaction of plasma, blood cells, and platelets. Some data suggest that VET detected hypercoagulability increases individual patients' risk for both venous and arterial thrombotic events[57,58] and is associated with high morbidity and mortality rates[59].

In a systematic review[60] to predict postoperative thromboembolic events by TEG, the most relevant parameter was MA. However, there was significant inhomogeneity among the included studies regarding the definition of hypercoagulability, and the majority of them were underpowered. It seems that hypercoagulation is more common in alcoholic and viral cirrhosis, and most often during the anhepatic phase (28%)[61]. Moreover, there is an association between hypercoagulation TEG profile and intracardiac thrombi. Despite conventional tests proving hypocoagulation, more than 70% of cases demonstrated TEG parameters compatible with hypercoagulation[62].



## THE HEMOSTASIS ASSESSMENT BEFORE INVASIVE PROCEDURES IN PATIENTS WITH CIRRHOSIS

Traditionally, due to the presence of thrombocytopenia and hypoprothrombinemia, it was considered that the patients with cirrhosis have an increased risk of bleeding after interventional procedures[14,63]. Consequently, guidelines have recommended the correction of INR and platelets deficits through FFP or platelet transfusion before invasive procedures to prevent bleeding complications[64]. Arguments against this fear were raised by liver transplantation, which can be easily performed without blood product replacement[63,65]. Moreover, recent studies show little evidence for a higher prevalence of post-procedural bleeding following invasive procedures[63]. In 6 trials studying the prevalence of severe bleeding after interventional procedures (of low, high, and intermediate-risk) was 0.69%, range 0%-2.75% (50 out of 7146)[63]. In one prospective Italian study, the incidence of bleeding among 380 cirrhotic with or without abnormal coagulation parameters (defined as an INR  $\geq 1.5$  and/or platelet count  $\leq 50 \times 10^9/L$ ) was zero for low-risk procedures like paracentesis[66]. High-risk procedures like percutaneous liver biopsy and percutaneous ablation were associated with higher bleeding rates in the abnormal coagulation group than the normal coagulation group. When analyzing in detail, the presence of sepsis and Child-Pugh C cirrhosis was associated with a higher incidence of bleeding in the abnormal coagulation group[67]. One large retrospective single-center study from the United States analyzed bleeding complications from 3357 liver biopsies and found a bleeding rate of 0.6%[68]. The median pre-biopsy platelet count, PT, and APTT (activated partial thromboplastin time) did not differ between patients that experienced or not bleeding complications; however, multivariate logistic regression identified a combination of APTT  $> 35$  s and platelet count  $\leq 100 \times 10^9/L$ , as independent predictors of bleeding risk[67]. Seeff *et al*[68] found a bleeding rate of 0.6% in 2740 cirrhotic patients undergoing liver biopsy. In this study, a platelet count of less than  $60 \times 10^9/L$  was associated with a higher risk of bleeding meanwhile, an INR above 1.5 was not[68].

Therefore, the INR is not an accurate predictor of bleeding events in patients with cirrhosis. However, most of the studies have shown that severe thrombocytopenia was associated with a higher risk of bleeding, although the cut-off values were different in several studies ( $50 \times 10^9/L$ - $75 \times 10^9/L$ )[14,63]. Reflecting the recent findings showing that cirrhotic patients are more often on a procoagulant slope[14,63], conventional coagulation tests are limited in predicting bleeding risk in cirrhosis because they do not account for the true *in vivo* coagulation status[69,70]. A systematic review comparing cirrhotic patients with a prolonged INR to those with normal INR found no difference in bleeding between the groups[13].

### Role of VET before invasive procedures in cirrhosis

Conventional SCTs (PT and aPTT) omit thrombomodulin, which activates protein C and, thus, downregulating *in vivo* the thrombin generation. Therefore, the SCTs are not suitable to investigate acquired deficiency of both pro- and anticoagulants as occurs in cirrhosis[64].

Four studies have assessed the role of TEG before invasive procedures in cirrhosis (Table 2). All four studies reported a statistically significant reduction in overall blood product use with TEG guided transfusion[64,71-73]. The trials reported different outcomes regarding the transfusion of specific blood products such as FFP, platelets, and cryoprecipitate. A statistically significant reduction in platelet transfusion was reported in all studies[64,65,71-73]. The most striking difference was in the study of Vuyyuru *et al*[71], where only 10.3% of patients with cirrhosis undergoing interventional procedures needed platelet transfusion when guided by TEG compared to 75.9% when guided by conventional methods. The number of platelets used for transfusion was significantly lower in 3 of 4 studies[64,72,73]. Interestingly the number of platelets transfused was significantly lower for high-risk procedures (6 units *vs* 78 units,  $P < 0.001$ ) but not low risk[64].

Three out of four studies reported a statistically significant reduction in FFP use[64,72,73]. In the study of De Petri 0% required FFP in the TEG arm compared to 53.3% in the conventional arm ( $P = 0.001$ )[64]. The absolute volume of FFP transfused was also markedly reduced in the TEG arm, where 4400 mL of FFP was transfused compared with 17550 mL in the control arm[64]. The difference was also maintained in upper gastrointestinal bleeding (both variceal and non-variceal)[72,73]. The amount of cryoprecipitate transfused was also lower with TEG in non-variceal bleeding (4 units in the TEG group compared with 16 in the standard of care group)[73].

**Table 2** Role of thromboelastography prior invasive procedures in cirrhosis

Ref.	Type of invasive procedure	Threshold for intervention	Transfusion	Blood products transfused (%) / total amount of FFP (mL) and PLT (units)	Bleeding complications (%)	No. of death
De Pietri <i>et al</i> [64] (2016)	All invasive procedures (low and high risk)	TEG: FFP R > 40 min; PLT MA < 30 mm. SOC: FFP INR > 1.8; PLT transfusion PLT < 50000/mm <sup>3</sup>	TEG guided ( <i>n</i> = 30); SOC ( <i>n</i> = 30)	TEG guided/SOC: All % 16/100; FFP % 0/53.3; PLT % 6.7/33.3; FP + PLT % 3/13.3. Low risk procedures: FFP (mL) 4000/11050; PLT (unit) 22/28. High risk procedures: FFP (mL) 0/6500; PLT (unit) 6/78	TEG 0; SOC 3.3	TEG 8; SOC 7; 90 d
Vuyyuru <i>et al</i> [71] (2020)	All invasive procedures (low and high risk)	TEG: FFP R > 14 min; PLT MA < 33 mm. SOC: FFP INR > 1.8; PLT transfusion PLT < 50000/mm <sup>3</sup>	TEG guided ( <i>n</i> = 29); SOC ( <i>n</i> = 29)	TEG guided/SOC: All % 27.6/96.6; FFP % 24/27; PLT % 10.3/75.9; FFP + PLT % 3.4/3.4	TEG 0; SOC 0	TEG 0; SOC 1; 28 d
Rout <i>et al</i> [72] (2020)	Procedures for treating variceal bleeding	TEG: FFP R > 15 min; PLT MA < 30 mm, SOC: FFP INR > 1.8; PLT transfusion PLT < 50000/mm <sup>3</sup>	TEG guided ( <i>n</i> = 30); SOC ( <i>n</i> = 30)	TEG guided/SOC: All % 13.3/100; FFP % 13.3/46.7; PLT % 10/70; FFP + PLT % 10/16.7; FFP (mL) 4000/11050; PLT (mL) 450/3450	Rebleeding 5 d; TEG 3.3; SOC 13.3. Rebleeding 42 d; TEG 10; SOC 36.7	TEG 13; SOC 26
Kumar <i>et al</i> [73] (2020)	Procedures for treating nonvariceal bleeding	TEG: FFP R > 10 min; PLT MA < 55 mm; CryoP angle < 45. SOC: FFP INR > 1.8; PLT transfusion PLT < 50000/mm <sup>3</sup> . CryoP Fibrinogen < 80 mg%	TEG guided ( <i>n</i> = 49); SOC ( <i>n</i> = 47)	TEG guided/SOC: All % 26.5/87.2; FFP 4.1/0; PLT % 4.1/0; FFP + PLT % 14.3/0; Cryo % 12.2/0; Cryo + PLT % 8.2/4.3; CryoP + FFP % 16.3/8.5; None % 14.3/0; FFP (mL) 440/880; PLT (unit) 1/2; CryoP (unit) 4/16	Failure to control bleeding at 5 d. TEG 22.4; SOC 29.8. Failure to prevent bleeding after 5 d. TEG 50; SOC 57	TEG 22.4; SOC 29.8; 5 d. TEG 55; SOC 66; 42 d

TEG: Thromboelastography; INR: International normalized ratio; SOC: Standard of care; FFP: Fresh frozen plasma; PLT: Platelets; CryoP: Cryoprecipitate.

There was no statistically significant difference in blood loss and bleeding events in the two trials, which examined the use of TEG before an invasive procedure [64,71]. It is important to emphasize that the bleeding rates were low in both arms [64,71].

There was no difference in the control of initial bleeding between the TEG and conventional hemostasis assessment in the two trials in cirrhotic patients with upper gastrointestinal bleeding [72,73]. In those with variceal bleeding, the re-bleeding rate at 42 d was lower in the TEG guided transfusion group (10% *vs* 26.7%, *P* = 0.012) [72]. This advantage is not surprising since the over transfusion was associated with worse bleeding control and prognostic in cirrhosis patients [74].

Still, when considering overall mortality [64,71-73], length of stay in the intensive care unit [73], and the number of days in the hospital, there is no difference between the two guiding modalities [73].

One of the main advantages of using TEG for hemostasis assessment may be reducing transfusion-related adverse effects, 30.6% in the TEG group *vs* 74.5% in the control arm [73].

## CONCLUSION

Recently, VETs of hemostasis are increasingly used for “point-of-care” assessment of complex hemostatic abnormalities. These tests' advantages lie in providing real-time, dynamic information about the whole coagulation process, including clot initiation (thrombin generation), clot kinetics, clot strength, and clot stability (lysis). In cirrhosis, SCTs are reliable tools in assessing liver function, but they fail to evaluate the hemostasis correctly. VET based assessment of bleeding risk and VET-guided transfusion strategies have been shown to reduce blood product use in cirrhotic patients who require invasive procedures and those presenting with variceal and non-variceal gastrointestinal bleeding. The reduction in blood product use was not associated with an increased risk of bleeding, the difference in controlling bleeding, morbidity, or mortality compared to standard care. The main disadvantage is related to the lack of extended validation in cirrhosis using more robust endpoints. By now, in the majority of the interventional randomized validation studies, the primary endpoint was the transfusion reduction, with eventual benefit extended to clinical endpoints as bleeding or survival. Therefore, all these studies were underpowered for reliable validation of some robust endpoints, and, thus, the VETs use is not widely available.

However, the standardization of TEG cut-off is mandatory to ensure a more reproducible evaluation of bleeding risk in cirrhosis patients.

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## Gastrointestinal involvement in paediatric COVID-19 — from pathogenesis to clinical management: A comprehensive review

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### Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), is responsible for the first pandemic of the 21<sup>st</sup> century. As found in adults, signs and symptoms related to the disease mainly involve the respiratory tract in the paediatric population. However, a considerable number of children present with gastrointestinal symptoms such as vomiting, abdominal pain, and diarrhea. The purpose of this review is an accurate description, from pathogenesis to clinical presentation, diagnosis and treatment, of COVID-19 effects on the gastrointestinal system at a paediatric age. SARS-CoV-2 can be identified in stool specimens of affected children by real-time polymerase chain reaction techniques. Positivity can last for several weeks after the end of the symptomatic phase. Gastrointestinal signs and symptoms are generally self-limited, can correlate with blood tests and imaging alterations, and may require supportive treatment such as hydration. However, they can precede severe disease manifestations such as the COVID-19-related multisystem inflammatory syndrome. Children belonging to risk categories such as those affected by celiac disease, inflammatory bowel disease, and hepatic disease seem to not have a more severe course than the others, even if they are undergoing immunosuppressant treatment. Medical follow-ups of patients with chronic diseases need to be revised during the pandemic period in order to postpone unnecessary tests, mainly endoscopic ones.

**Key Words:** SARS-CoV-2; COVID-19; Paediatric; Gastrointestinal; Diarrhea; Hydration

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coronavirus disease 2019 (COVID-19) compared to adults. Manifestations are generally self-limited, and may only require supportive treatment. In a minority of children, gastrointestinal involvement may precede severe forms such as the multisystem inflammatory syndrome. Conversely to what is expected, the COVID-19 impact on paediatric patients with chronic gastrointestinal diseases is limited, with no need for therapeutic regimen changes. However, the severe acute respiratory syndrome coronavirus 2 pandemic determined multiple variations in routine practice. The use of telemedicine and telehealth can be a solution in order to continue to provide regular follow-up to chronic patients, avoiding the risk of viral transmission.

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## INTRODUCTION

The coronavirus disease 2019 (COVID-19) is caused by single-stranded RNA severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The disease was first described in Wuhan, China, in December 2019. Then the virus spread rapidly worldwide, making the World Health Organization declare the first pandemic of the 21<sup>st</sup> century in March 2020[1]. On March 4, 2021, there were more than 114 million confirmed cases of COVID-19 worldwide, with nearly 3 million registered deaths[2].

The clinical spectrum of COVID-19 in children and adolescents ranges from asymptomatic/pauci symptomatic infection to severe disease. Digestive symptoms related to COVID-19 such as vomiting, diarrhea, and abdominal pain have emerged as extrapulmonary manifestations, with SARS-CoV-2 RNA detected in the faeces of people affected, suggesting faecal-oral transmission. Children with gastrointestinal (GI) chronic diseases, including those undergoing immunosuppressive or biological treatment, need to be strictly evaluated for the risk of developing severe forms of COVID-19. GI involvement may play a role in the presentation of symptoms in children affected by the multisystem inflammatory syndrome (MIS-C) associated with COVID-19[3].

The purpose of this review is to summarise what is known about COVID-19 GI manifestations in children, from diagnosis to symptoms and treatment, including the peculiar aspects of patients with GI chronic diseases.

## PATHOGENESIS

There are many mechanisms through which SARS-CoV-2 can interact and damage the GI system.

The first is a virus-induced cytopathic effect. SARS-CoV-2 interacts with membrane receptors of the host cells through the spike protein, which mediates the fusion of the virus and the cell membrane[4]. Angiotensin-converting enzyme receptor 2 (ACE-2) and transmembrane protease serine 2 (TMPRSS2) are both essential for the cellular entry process of the virus. They are co-expressed at a high level both in the type II alveolar cells of the lung, in the glandular cells of the gastric, duodenal, and rectal epithelium, and in the enterocytes of ileum and colon[4]. After viral entry, new virions are synthesised in the cytoplasm of the GI cells and then are released in the GI tract, causing direct disruption of enterocytes and the viral shedding in the stool[5,6]. As absorptive enterocytes are destroyed by SARS-CoV-2, this changes the intestinal permeability, leading to malabsorption and unbalanced intestinal secretion, resulting in the genesis of diarrhea[6-8].

As ACE-2 is equally expressed in liver and pancreas, the virus cytopathic effect can also be detected in these organs. Hepatic distribution of ACE-2 is peculiar; it is highly expressed in the endothelial layer of small blood vessels but not in the sinusoidal



endothelium[9]. Its concentration on cholangiocytes' surface is higher than of the hepatocyte surface and is similar to the type II alveolar cells of the lungs. SARS-CoV-2 may have the ability to infect cholangiocytes *via* the ACE-2 receptor and directly dysregulate liver function[7,9]. However, there is no evidence of active virus replication in hepatocyte cells[6]. A histological examination of a liver biopsy obtained from a deceased COVID-19 patient showed no viral inclusions, but rather a microvesicular steatosis and mild lobular activity[7].

SARS-CoV-2 can affect both the exocrine and endocrine pancreas; an abnormal elevation of amylase and lipase, together with glucose dysregulation and acute diabetes are described in patients with severe COVID-19 pneumonia, with development of acute pancreatitis in a few cases[10].

The second mechanism depends on immune-system activation[11]. SARS-CoV-2-infected cells release a large number of inflammatory mediators and chemokines such as interleukin (IL)-2, IL-7, granulocyte colony-stimulating factor, interferon- $\gamma$  inducible protein 10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1- $\alpha$  and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ). This "cytokine storm" causes neutrophil aggregation and activates type 1 helper cells. This promotes the accumulation of immune cells in the GI system[7]; a large number of infiltrating plasma cells and lymphocytes together with interstitial oedema have been found in the inherent layers of the stomach, duodenum, and rectum in adult COVID-19 patients[8]. The cytokine overproduction correlates with disease severity and multiple organ insufficiency development outside the lung, liver, and pancreas.

SARS-CoV-2 may alter the intestinal microbiome even when only the respiratory mucosa is involved (the "gut-lung axis") [5,6]. Increased inflammatory mediators lead to lung hyperpermeability so that the virus and inflammatory mediators migrate to the intestine *via* circulation. SARS-CoV-2 and the inflammatory mediators disrupt the intestinal permeability leading to the leakage of gut microbes and associated metabolites into circulation. The leaked microbes and products migrate to organs including lungs and produce abnormalities.

Other hypothesised mechanisms of liver and pancreatic damage include hypoxic injury due to respiratory distress and drug-induced injury[5-7,10]. Kidneys can also be involved, as they are fundamental in eliminating amylases and lipases from the circulatory stream: Their malfunction can lead to a transient increase in pancreatic enzymes[10].

## CLINICAL MANIFESTATIONS

Children and adolescents with GI symptoms such as nausea, vomiting, or diarrhea should be seriously evaluated for COVID-19, as the faecal-oral route transmission of SARS-CoV-2 is extensively described (Figure 1). Oba *et al*[12] reported that GI signs and symptoms may affect 3% to 79% of children, adolescents and adults with COVID-19. Various paediatric systematic reviews evidenced similar results[4,6,13], curiously with different frequencies in the United States and Europe compared to China (21.1% *vs* 12.9%)[4]. Manifestations include diarrhea (2%-50%), anorexia (40%-50%), vomiting (4%-67%), nausea (1%-30%), abdominal pain (2%-6%) and GI bleeding (4%-14%). Diarrhea and vomiting are the most common GI symptoms described, sometimes as the first symptoms of disease, even before or in absence of respiratory manifestations. Diarrhea, often watery, occurs from 1 d to 8 d after the onset of COVID-19, with a median time of 3.3 d, and lasts for a mean of 4 d[12]. Vomiting is more often reported in the paediatric population than in the adult one.

GI symptoms may correlate with severe COVID-19 in children admitted to hospital. A Spanish multicentre study involving 101 paediatric inpatients noticed that patients presenting with GI symptoms tended to have higher C-reactive protein (CRP), procalcitonin (PCT), ferritin and aspartate aminotransferase values, and to receive antibiotics, lopinavir-ritonavir, corticosteroids and immunoglobulins more frequently than the others. Moreover, they had a higher risk of paediatric intensive care unit (ICU) admission, regardless of age, gender, immunosuppressive therapy and previous underlying conditions[14]. Similar findings were evidenced in studies among adults, where those with diarrhea had a higher risk of ICU admission regardless of the age, sex and comorbidities[15,16].

GI symptoms such as vomiting, abdominal pain and/or diarrhea are typically present and considered diagnostic criteria in children with the COVID-19-related MIS-C (71%-84% of the cases), along with fever lasting more than 3 d, evidence of mucocutaneous inflammation (rash, conjunctivitis, oromucosal changes), lymphopenia and

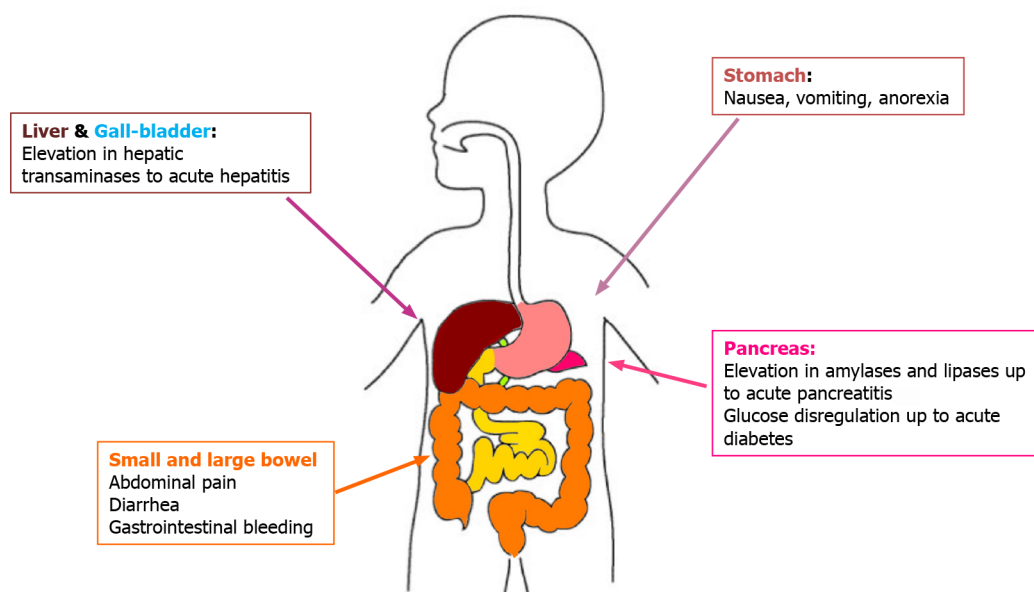


Figure 1 Gastrointestinal involvement in coronavirus disease 2019.

high levels of circulating inflammation[3,17-19]. Abdominal pain may be so relevant that few children with a final diagnosis of MIS-C initially present with acute surgical abdomen; they may undergo exploratory laparotomy with intra-operative findings of mesenteric lymphadenitis and peritonitis[20]. Evolution to severe disease including cardiac involvement, hypotension and shock is often described in children with MIS-C [19].

## DIAGNOSIS

Prompt diagnosis of SARS-CoV-2 infection can determine treatment strategies and influence the outcome of the disease in children. Suggestive symptoms together with the history of a close contact with a COVID-19 patient are the most useful criteria for a suspicion of infection[21]. Diagnosis is confirmed by SARS-CoV-2 isolation in patient samples, while auxiliary examinations are useful to determine the severity of the disease and organ involvement in infected children.

Leukopenia with neutropenia and lymphocytosis or lymphopenia are the most common findings at blood count. Erythrocyte sedimentation rate is elevated in a great number of children, as well as IL-10 and IL-6. CRP and PCT levels can be normal. However, a PCT value > 0.5 ng/mL may be suggestive of bacterial coinfection or of a severe autoinflammatory state such as the MIS-C[22,23].

A mild increase in liver enzymes is well described in COVID-19, with various percentages among studies, ranging from 13% to 50% in paediatric patients; however, serious liver dysfunction is uncommon[6,24]. Elevated aspartate aminotransferase levels (> 50 UI/L; 20.4%-50% of cases) are observed more frequently than alanine aminotransferase levels (> 45 UI/L; 9%-35% of cases). Increased transaminases are often accompanied by high creatinine kinase and lactate dehydrogenase, suggesting the possibility of a viral myositis[25,26].

Considerable alteration in liver enzymes is common in those with severe spectrum of disease (40%-60%) compared to those who are asymptomatic or have mild manifestations (18%-25%). Nevertheless, every child with COVID-19 and raised transaminases should be investigated for other causes of liver pathology[5].

Patients with MIS-C present with a remarkable elevation in transaminases levels (52.3%) together with a mild decrease in albumin rates. Elevation of lipase levels > 3 times the normal limit was observed in one paediatric patient with MIS-C[3,25]. In fact, pancreatic involvement in adults with COVID-19 is well described, with a lipase increase in 7.9% of cases. Bilirubin levels are also more than doubled in those with severe infection, when compared to those with mild disease[10].

Nucleic acid testing is the method of choice for virus identification. SARS-CoV-2 RNA can be detected in sputum, lower respiratory tract secretions, urine, stool, tears,

and blood samples by real-time polymerase chain reaction (RT-PCR) technology or by viral gene sequencing. RT-PCR on nose-pharyngeal swab (NPS) is the diagnostic method of choice in children. However, paediatric patients tested for SARS-CoV-2 by RT-PCR on a rectal swab or stool returned a positive result in 89% of cases, despite not presenting with any GI symptoms. Moreover, COVID-19 children may have a stool SARS-CoV-2 RT-PCR positive result more frequently than adults in spite of a negative respiratory swab; RT-PCR on stool samples seems to be as accurate as those performed on the NPS in order to identify SARS-CoV-2[27,28].

As the faecal-oral route is confirmed as a way for SARS-CoV-2 transmission, all children with digestive tract symptoms should be tested for SARS-CoV-2 on faeces[12,29]. RT-PCR on stool becomes positive from 2 d to 2 wk after the respiratory specimen ones, and 23%-82% of patients continue to have positive faecal test for approximately 1-16 d after their NPS turn negative[4,28]. The interval to stool negativisation may be prolonged, exceeding 70 d in healthy children, longer in patients treated with corticosteroids[4].

Therefore, despite major evidence being necessary to consider a negative RT-PCR on faeces as one of the discharge criteria, it may be important to recommend isolation at home for at least 2 wk after hospital discharge[4].

Little is known about typical imaging findings in COVID-19 paediatric patients with GI symptoms. Abdominal ultrasonography to computed tomography or magnetic resonance imaging may be taken into consideration in patients with a severe course of disease or relevant blood test alterations (*e.g.*, increased transaminases and/or lipases). Miller *et al*[3] described a paediatric population of 44 cases with MIS-C and GI symptoms, with abdominal images collected in 15 patients. Common findings were mesenteric adenitis (2 patients), biliary sludge or acalculous cholecystitis (6) and ascites (6). Normal abdominal imaging was found in 20% of cases. In 3 patients, ultrasonography or magnetic resonance imaging evidenced bowel wall thickening analogous to that of inflammatory bowel disease (IBD). One child had severe clinical manifestations (fever, abdominal pain, rash) with evidence of concentric mural thickening, oedema and hyperenhancement of a short segment of terminal ileum, with similar findings in the rectosigmoid colon. Two patients had nonspecific ultrasonography imaging, with thickened bowel loops on the right iliac site associated with highly elevated inflammatory index and mildly decreased albumin levels[3].

## THERAPY

Generally, COVID-19 paediatric patients require symptomatic care, both because the great majority of them has mild symptoms and also because all virus-targeted therapies are employed exclusively in clinical trial settings[30]. Supportive care included fever treatment, oxygen therapy in patients with respiratory complications with or without airway management, and nasogastric or intravenous hydration in children unable to tolerate oral fluids such as those with severe GI symptoms[30].

COVID-19 children are at higher risk of developing malnourishment during critical illness, which has been associated with increased morbidity and mortality; therefore, nutritional therapy plays a significant role in these children[12]. The European Society of Paediatric and Neonatal Intensive Care cornerstones for nutrition recommend commencing early enteral feeding within 24 h of hospital admission in critically ill children unless contraindicated. Energy requirements need not exceed resting energy expenditure during the acute phase and an increase in enteral nutrition in a stepwise fashion is recommended until the goal for delivery is achieved. Overfeeding harms critically ill children, especially during the acute phase[31].

In children with severe GI COVID-19 and MIS-C, enteral nutrition support may be continued for a long time into the recovery phase until sufficient oral intake is consistently achieved to support physical and nutritional rehabilitation[32]. Enteral nutrition is also recommended in critically ill children on hemodynamic support with a stable clinical condition; parenteral nutrition has to be withheld during the first 7 d of admission[12,31]. However, in children who continue to require fluid resuscitation or escalating doses of vasoactive agents with evidence of severe GI dysfunction and MIS-C, enteral nutrition may be withheld for up to 7 d[32].

It may be useful to consider providing enteral feeds *via* a post-pyloric tube in critically ill children with COVID-19 with severe GI symptoms or cardiac manifestations or inotrope resistance shock in which early gastric enteral feeding is not possible [12,32].

There is no evidence to support supra-physiological doses of micronutrients supplementation, including zinc during the acute phase[32].

Recent literature suggests the role of probiotics in manipulating the gut microbiota, as they may play a fruitful role as a therapeutic strategy for GI COVID-19 and its comorbidities. Focused clinical trials are needed to support this hypothesis[33].

Based on currently available limited data, children with comorbidities or pre-existing chronic diseases, such as IBD or liver disease, do not seem to carry a higher risk of COVID-19 infection compared to the general population (see below)[34,35].

## PARTICULAR CASES

### Celiac disease

Celiac disease (CeD), also known as celiac sprue or gluten-sensitive enteropathy, is a common immune-mediated inflammatory disease of the small intestine resulting from sensitivity to dietary gluten and related proteins in genetically predisposed individuals[36]. It is estimated that CeD affects approximately 0.5% to 1% of the general population[37]. CeD therapy consists in a strict gluten-free diet (GFD) to achieve complete resolution of symptoms and mucosal healing for most individuals. It also reduces the risk of long-term adverse health outcomes including intestinal lymphoma[38].

During the COVID-19 pandemic, it has been postulated that some patients with chronic GI disease may be at an increased risk of a more severe illness due to COVID-19. Potential risk factors in these patients include their chronic inflammatory disease, comorbidities (*e.g.*, diabetes mellitus), and the use of glucocorticoids[39].

Considering that CeD subjects have an increased risk of both bacterial and viral infections, such as pneumococcal pneumonia[40], sepsis[41], and complications from influenza virus, probably due to malnutrition and increased mucosal permeability [42]. Various studies have investigated the possibility of a higher risk of SARS-CoV-2 infection in CeD patients compared to the general population.

Data on patients with chronic GI disease who have been infected by SARS-CoV-2 are accumulating, and disease-specific patient registries include Surveillance Epidemiology of Coronavirus Under Research Exclusion (SECURE-Celiac). This is an international, paediatric and adult database to monitor and report the outcomes of COVID-19 in patients with CeD. On January 17, 2021 a total number of 111 cases was reported; 4 cases < 18 years of age, nobody was hospitalised, and no death was registered (SECURE-Celiac Database, available at: <https://covidceliac.org/data>). Several studies evidenced that the risk of severe COVID-19 is not increased in patients with CeD both in adulthood and childhood[43-48]. A large-scale study, performed on 18000 participants from different Countries, including Argentina, Australia, Canada, Italy, Mexico, New Zealand, Spain, Uruguay, and the United States, examined the risk of COVID-19 in CeD compared with the nonceliac population. They found that patients with CeD had similar odds to contract SARS-CoV-2 infection compared to the control subjects. The presence of comorbidities, which were identified as an important predictor of morbidity and mortality associated with COVID-19, were more frequent in CeD than the control subjects. However, they did not determine higher odds for SARS-CoV-2 infection in CeD. The authors observed that the sole factor increasing the odds of a positive test was exposure to a COVID-19 contact. In this study, patients with CeD were less exposed to COVID-19 than the control subjects, probably due to a careful prevention related to their chronic condition[46]. Moreover, a cross-sectional study conducted by a paediatric celiac centre in Central Italy did not show any significant increase in SARS-CoV-2 infection prevalence among the group of children with CeD compared to the general population[48].

A relevant aspect of the COVID-19 pandemic is the impact of restrictive measures on primary health care. In this new scenario, all elective diagnostic procedures such as paediatric digestive endoscopy have been suspended and were allowed only in emergency cases[49,50].

As esophagogastroduodenoscopy is still necessary for CeD diagnosis in children with low antibodies titres and this elective procedure has been substantially shut down during the COVID-19 pandemic, many children remained undiagnosed and therefore untreated for a long time. The European Society of Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) analysed the feasibility and accuracy of a biopsy-free approach in suspected CeD children with tissue transglutaminase-immunoglobulin A (TGA-IgA) values < 10 times upper the normal limit (ULN) during the COVID-19 outbreak. A temporary reduction of the TGA-IgA threshold seems



feasible in antiendomysial antibodies (EMA) positive children with TGA-IgA between 5 and 10 ULN for a biopsy-sparing approach[51].

The global impact of the lockdown on the compliance to GFD in CeD patients was investigated in a cross-sectional survey conducted in Italy on a total of 1983 patients with CeD, 1614 (81.4%) adults and 369 (18.6%) children. The compliance to GFD was unchanged for 70% of the subjects, and even improved for 29%, in particular for those with a previous worse disease control, due to reduced opportunities for contamination and transgression and an increased use of naturally gluten-free ingredients[52].

An observational study on 71 paediatric patients with CeD examined the prevalence of functional GI disorders (FGIDs) in children during the COVID-19 lockdown. The Authors observed a reduced prevalence of FGIDs in these patients, probably due to a good quality of life and a low state of anxiety related to the positive effects of reducing stressful events, such as school, as well as to an increased parental closeness, which may have influenced the occurrence of GI symptoms and the related FGIDs. Psychosocial aspects may play a particularly important role in the genesis of the FGIDs, despite the presence of a residual low-grade chronic inflammatory process[53].

### **IBD**

IBD, including Crohn's disease (CD) and ulcerative colitis, are chronic pathologies of the GI system characterised by a dysregulated immune response with an over production of pro-inflammatory cytokines. To control the disease, patients require frequent treatment with corticosteroids, immunosuppressants, and/or biological drugs with the consequence of an increased risk of infections. IBDs tend to be more extensive and severe in children than in adults, with a consistently greater need for immunomodulators and biological agents to maintain remission in this population [54].

The IBD-related immunosuppressive treatment has raised concerns regarding the management and the risk of SARS-CoV-2 infection severity. On the other hand, it can be assumed that immunosuppressive medications may be associated with a decreased risk of poor COVID-19 outcomes by limiting the cytokine storm typical of severe COVID-19.

The first document on the global impact of SARS-CoV-2 infection on paediatric IBD was published in March 2020 by the Paediatric IBD Porto Group of ESPGHAN. It was a survey among 102 Paediatric IBD centres affiliated with the Porto and Interest-group of ESPGHAN, with external paediatric experts from China and South Korea invited to participate. All cases of COVID-19 in IBD paediatric patients enrolled were mild despite they were under immunosuppressive treatment. The study expert group suggested that IBD children, with or without immunosuppressive and biological therapy, do not seem to have a greater risk of severe SARS-CoV-2 infection compared to the general population. Due to this, they recommend not stopping standard IBD treatments[35].

A total of 522 IBD patients were enrolled in one of the Italian regions with greater COVID-19 incidence and were followed for a 1-mo period between February and March 2020. Fifty-nine (11%) paediatric patients (7-18-years-old) were included. In this IBD cohort, the Authors did not report any case of COVID-19 despite the fact that the enrolled patients continued to receive immunosuppressive treatments such as thiopurines or methotrexate and steroids[55].

Surveillance Epidemiology of Coronavirus Under Research Exclusion (SECURE-IBD) is an international, paediatric and adult database to monitor and report on outcomes of COVID-19 occurring in IBD patients. On January 5, a total number of 4280 cases was reported; 441 cases < 19 years of age, only 22% hospitalised, and no death evidenced[56].

Brenner *et al*[57] described the course of the disease of COVID-19 in a sample of 209 paediatric IBD patients (age 18 years and younger) from the 2 international databases (The SECURE-IBD and the COVID-19 database of the Paediatric IBD Porto group of ESPGHAN). They reported a hospitalisation rate of 7%; only 2 patients (1%) required mechanical ventilation, one for a MIS-C and one for a secondary infection. There were no deaths. Risk factors for hospitalisation included other comorbidities, moderate/severe IBD disease activity, and GI symptoms.

In March 2020 Paediatric IBD Porto group of ESPGHAN generated guidance points for paediatric gastroenterologists in the era of the COVID-19 pandemic. These points are mainly focus on therapy indications. There is no evidence that any of the drugs used in IBDs, including immune-modulators and biological drugs, increases the severity of COVID-19. IBD patients should stay on IBD medications prescribed before the SARS-CoV-2 pandemic[35]. Newly diagnosed patients should be treated according to the standard protocols as before the spread of the virus[5,12].

SARS-CoV-2 enters cells *via* the ACE-2 receptor, widely expressed in the GI tract. Its expression is upregulated during inflammation and this overexpression may therefore increase host susceptibility. There are two forms of ACE-2: full-length ACE-2 with a structural transmembrane domain, which anchors its extracellular domain to the cellular membrane, and a soluble form of ACE-2 that lacks the membrane anchor and circulates in the blood. Some authors have speculated that the soluble form may act as a competitive interceptor for SARS-CoV-2, preventing the binding of the viral particle to the surface full-length ACE-2[58]. It can explain why children with IBD have a COVID-19 course similar to healthy individuals.

By contrast, immune dysfunction in untreated IBD children may increase the risk of a severe inflammatory response to SARS-CoV-2 infection. Pro-inflammatory cytokines, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 are overproduced in IBD patients[59], and serum concentration of TNF- $\alpha$  and IL-6 are associated with severe COVID-19 illness[60]. Dolinger *et al*[61] described a case of MIS-C associated with COVID-19 in a recently diagnosed paediatric CD patient. He was treated with infliximab for both clinical entities with a successful result. The authors speculated that anti-TNF agents could play a role in the therapy of patients with active IBD and MIS-C temporally related to COVID-19.

A COVID-19 Risk Calculator is on the SECURE-IBD website and it is intended for use by physicians caring for patients with IBD to calculate the risk of hospitalisation, ICU admission, mechanical ventilation, or death in IBD patients with COVID-19. It is available at: <https://covidibd.org/covid-19-risk-calculator/>.

Regarding CeD, the COVID-19 pandemic determined essential healthcare changes, mainly during the lockdown period, which could alter IBD follow-up in children. These include postponed elective appointments, reduced access to diagnostic endoscopy, and difficulties in continuing infusion therapy, with potential clinical and psychological negative impact on the course of the disease.

A recent survey, conducted in the United Kingdom, outlined that more than 50% of children and young people presenting with a suspected diagnosis of IBD were diagnosed without a histological examination due to restrictions placed on endoscopy at over 90% of centres across the United Kingdom. The authors observed that “diagnosing children and young people with IBD, without a histological confirmation, is controversial and only acceptable given the special circumstances we are currently finding ourselves in”[62].

A multicentre investigation of the Italian Society of Paediatric Gastroenterology, Hepatology and Nutrition (SIGENP) analysed the impact of COVID-19 related lockdown on the levels of care offered to paediatric IBD patients all over the Italian territory[63]. In total, 2291 children affected by IBD were regularly followed by the 21 participating referral centres; a total of 6 cases of SARS-CoV-2 infections of 2291 (0.2%) patients were identified. In 5 of 6 (83.3%) cases, the clinical course of the SARS-CoV-2 infection resulted as mild, without the need for hospitalisation. The authors observed a reduction of hospitalisations for new diagnosis and endoscopic re-evaluation, while the number of hospitalisations for relapse and surgical procedure remained substantially unchanged. The number of outpatients' visits were significantly decreased. Biologics' infusions did not significantly vary and Italian paediatric IBD centres did not modify their therapeutic approach in the majority of cases, as recommended by the ESPGHAN guidelines.

Differently from the United Kingdom data, the multicentre Italian survey found a drop in the new diagnoses during the lockdown.

The fear of SARS-CoV-2 infection could determine the risk of inappropriate management of IBD with consequent significant impact on the health of IBD patients [35]. As the decrease of face-to-face consultations, telemedicine represents a promising opportunity. In the Italian survey, telemedicine services for children with IBD were activated in 52.3% of the participating centres.

Recently, members of the SIGENP IBD group study drafted a position paper with the aim of providing guidance for the management of paediatric IBD on the basis of the existing evidence[64].

In addition to specific recommendations regarding diagnostic procedures and therapies, the position paper provides general indications for a safer and gradual restarting of routine clinical activities after the COVID-19 peak, with particular attention also on psychological issues.

### Chronic liver diseases

At present, there is no concrete evidence that the SARS-CoV-2 infection causes significant worsening in underlying chronic liver disease. Children undergoing treatment for pathologies like Wilson disease, autoimmune hepatitis, hepatitis B and C should continue their treatment protocols[5]. Elevated transaminases in COVID-19 are

not a contraindication for antiviral therapy in viral chronic hepatitis, even if regular monitoring of liver function is needed[65].

### **Solid organ transplantation**

Reports of critical disease in adults and children with cancer have raised concerns about the risk of a severe COVID-19 course in patients with immune system impairment, including recipients of solid organ transplants on long-term immune suppression[66].

Data on the COVID-19 course and outcome in children needing liver solid organ transplantation (SOT), or already transplanted, are limited. Reports by the team of the Transplantation Unit of Papa Giovanni XXIII Hospital in Bergamo (Italy) are reassuring; liver-transplanted young patients did not experience any severe respiratory infection, despite residing in one of the earliest and hardest-hit areas world-wide, where first cases likely dated long before any distancing or isolation measure [67].

Six cases of children awaiting transplantation and eight cases of paediatric transplant recipients infected by SARS-CoV-2 were reported by Doná *et al*[68] None of the candidates in the SOT waiting list, nor any of the SOT recipients, presented severe COVID-19.

It seems that the immunosuppressive treatment in immunocompromised children may not significantly increase the risk of severe COVID-19, as its complications are mainly driven by a well-documented pro-inflammatory state[69].

Post-liver transplant patients need particular emphasis on preventive measures, such as frequent hand washing, frequent cleaning of touched surfaces and social distancing. Generally, as the cell injury in COVID-19 disease is thought to be immune-mediated, immunosuppression and mycophenolate should not be reduced or stopped in asymptomatic post-transplant patients. In an established COVID-19, the continuation of calcineurin inhibitors targeting a lower trough levels and lowering of the dose of mycophenolate or azathioprine is recommended. Patients on high-dose steroids should have them reduced to a minimum dose based on body weight in order to prevent adrenal insufficiency. At present, there is no recommendation for any antivirals or hydroxychloroquine prophylaxis either in post-liver transplant children or those with COVID-19 associated acute liver disease[5].

The emergence of COVID-19 has had a profound impact on transplantation worldwide, both for issues regarding donors and recipients viral transmission and for healthcare resources, as the magnitude of COVID-19 cases in certain regions exceeds the available capacity of the health system[70].

Members of the European Reference Network of Paediatric Transplantation investigated the impact of the COVID-19 outbreak on paediatric transplant activity and healthcare practices in both SOT and hematopoietic stem cell transplantation: transplantation activity as well as outpatient visits were negatively affected by the COVID-19 pandemic across Europe[68]. The risk of SARS-CoV-2 transmission and the shortage of hospital bed capacity and staff might be the main determinants of this reduction. Significant extension of these limitations in healthcare resources may have severe consequences both for children on the transplant waiting list and for transplanted patients as the access to close monitoring and diagnostic testing would be significantly reduced. In this emerging public health context, there are no reasons to delay or interrupt oncological treatments or withdraw immune suppression, to postpone life-saving treatments in liver-transplanted patients, or to suspend transplant programs as an a priori preventative measure[71].

### **Abdominal surgery implications in paediatrics during COVID-19 pandemic**

The COVID-19 pandemic determined diagnosis delay and higher complication rates among common paediatric medical conditions[72].

An example may be represented by the increased incidence of complicated appendicitis among children: no changes in patient demographics nor in the rate of appendicitis itself could explain this phenomenon. A considerable extension in the average time between the onset of symptoms and surgery was identified as the causative agent: normally, surgical intervention time is influenced by other factors such as elective activity and availability of theatre and staff. The fear of contracting SARS-CoV-2 infection leads the parents to avoid hospital access of their child at the early stages of the disease, doubling the time before the patient's access to the Emergency Department (ED). Delayed access to ED may explain the higher prevalence of complicated appendicitis[73]. At the same time and for the same reasons, a statistically significant increased rate of appendiceal perforation during the COVID-19 pandemic was reported. In a cross-sectional study, appendiceal perforation also

resulted in pelvic abscess, bowel obstruction, and sepsis[74]. Delayed presentation of children with acute appendicitis at the ED may increase the morbidity related to a common childhood condition, leading to increased complications and poor outcomes [75,76].

Although complicated courses have arisen, an Italian study noticed a reduction in the total number of acute appendicitis cases. A decrease in social contacts during the lockdown period, a reduction of respiratory and GI infections, healthy food intake due to the permanence at home and increased domestic hygiene could have played a crucial role, bringing mild appendicitis to a spontaneous resolution cases with or without domestic treatments[77,78].

## CONCLUSION

This review describes the spectrum of GI manifestations in paediatric COVID-19, from signs and symptoms of disease to laboratory and imaging tests alterations. All the organs can be involved, ranging from mild to severe alterations. Pathogenesis is outlined, however further clarifications of virus-induced damages are needed. Although generally self-limited, GI signs and symptoms are closely related to complicated courses of disease: As a matter of fact, GI involvement is a diagnostic criteria for the MIS-C. Close monitoring of infected children will permit to delineate potential predictors of severe COVID-19 evolution.

Conversely to what is expected, the COVID-19 impact on children with chronic GI diseases appears to be limited, with no need for therapeutic regimen changes. Ongoing studies may confirm these initial observations. Certainly, the SARS-CoV-2 pandemic determined multiple variations in routine practice of these patients. The use of telemedicine and telehealth can be a solution in order to continue to provide regular follow-up to chronic patients, avoiding the risk of viral transmission.

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## Can control of gut microbiota be a future therapeutic option for inflammatory bowel disease?

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### Abstract

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract encompassing two main clinical entities, Crohn's disease and ulcerative colitis. Accumulated evidence indicates that an aberrant immune activation caused by the interplay of genetic susceptibility and environmental impact on the gut microbiota may be involved in the pathogenesis of IBD. Rapid advances in next-generation sequencing technology have enabled a number of studies to identify the alteration of the gut microbiota, termed dysbiosis, in IBD. Moreover, the alteration in the metabolites derived from the gut microbiota in IBD has also been described in many studies. Therefore, microbiota-based interventions such as fecal microbiota transplantation (FMT) have attracted attention as a novel therapeutic option in IBD. However, in clinical trials, the efficacy of FMT for IBD remains controversial. Additional basic and clinical studies are required to validate whether FMT can assume a complementary role in the treatment of IBD. The present review provides a synopsis on dysbiosis in IBD and on the association between the gut microbiota and the pathogenesis of IBD. In addition, we summarize the use of probiotics in IBD and the results of current clinical trials of FMT for IBD.

**Key Words:** Inflammatory bowel disease; Dysbiosis; Fecal microbiota transplantation; Short chain fatty acid; Probiotics

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**Core Tip:** In this review, we discuss the gut microbiota in inflammatory bowel disease and gut microbiota-derived metabolites, especially short chain fatty acids. The anti-inflammatory function of short chain fatty acids on the mucosal immune system in the

and hepatology

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gastrointestinal tract is also discussed. In addition, we review the efficacy of probiotics on inflammatory bowel disease and the current clinical trials on the effectiveness of fecal microbiota transplantation on inflammatory bowel disease.

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## INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), which are known as inflammatory bowel diseases (IBD), are chronic and relapsing inflammatory disorders of the gastrointestinal tract[1,2]. The precise etiology and pathogenesis remain to be elucidated. Genome-wide association studies have identified over 200 IBD associated-susceptible genes, some of which are known to be involved or implicated in mediating host responses to the gut microbiota[3]. This has evoked the possibility that the gut microbiota is implicated in the pathogenesis of IBD[4-7].

The human digestive tract is inhabited by more than 100 trillion commensal bacteria, which exceeds the total number of human cells of 37 trillion[8,9]. The concentration of human intestinal bacteria has been estimated to be from  $10^{11}$  to  $10^{12}$  cells per gram of luminal contents[10,11]. The intestinal bacteria regulate the balance between each bacterium by interacting with each other, thereby maintaining the homeostasis of the intestinal environment. More than 99% of the bacteria that live in the human intestine belong to the four major phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, of which Firmicutes is the most predominant phylum followed by Bacteroidetes[10,12].

Recent studies have shown that the composition of human gut microbiota is closely linked to health and disease[13,14]. Moreover, the gut microbiota contributes to the differentiation and maturation of intestinal epithelial cells and immune cells, the supply of energy to the host through metabolic processes, and protection against infection by pathogens[10]. Humans, on the other hand, provide the gut microbiota with an anaerobic place to reside. In this way, a symbiotic relationship is established between the gut microbiota and human host[15].

Developments in gene sequencing technologies as well as the increased availability of powerful bioinformatic tools have enabled novel insights into the microbial composition of human gut microbiota and the effect of microbial communities on human physiology and disease[16,17]. Recent studies using these technologies have indicated that abnormal microbiota composition, known as "dysbiosis," and a decreased complexity of the gut microbial ecosystem are common features in patients with IBD. Moreover, it has been demonstrated that dysbiosis is involved in the pathophysiology of non-gastrointestinal diseases such as obesity and diabetes[17,18], in addition to gastrointestinal diseases such as IBD and irritable bowel syndrome [6,7, 19].

Recently, it has been widely recognized that the gut microbiota plays a vital role in human immunity, metabolism, and diseases[4,6,20-22], leading to the idea of considering it as an organ. Based on this idea, the gut microbiota is sometimes called a "superorganism" or "forgotten organ"[23]. Considering these various functions of the gut microbiota on human biological functions, controlling its composition and diversity might allow us to treat or cure human diseases.

In this review, we will discuss the relationship between the gut microbiota and IBD, the efficacy of probiotics on IBD, and the current status of fecal microbiota transplantation (FMT) as a therapeutic option for IBD.

## THE GUT MICROBIOTA IN IBD

Various alterations of the gut microbiota have been reported in patients with IBD. The most consistent findings of the gut microbiota in IBD patients are a reduction of

diversity and a reduction of Firmicutes compared to healthy individuals[6,7,16]. Some studies on IBD patients have reported an increase in the phyla Proteobacteria and Bacteroidetes, while others have reported a decrease in these phyla[5,7].

*Faecalibacterium prausnitzii* (*F. prausnitzii*), which belongs to *Clostridium* cluster IV, has been reported to have an anti-inflammatory effect by producing short-chain fatty acids (SCFAs: C2-C6), especially butyrate[24]. It has been reported that there is a reduced abundance of *F. prausnitzii* in patients with CD and that this deficiency is associated with postoperative recurrence of CD[25]. It has been demonstrated that *F. prausnitzii*, *Blautia faecis*, *Roseburia inulinivorans*, *Ruminococcus torques*, and *Clostridium lavalense* are decreased in patients with CD when compared to healthy subjects[25,26] and that the number of *F. prausnitzii* is correlated with the risk of relapse of ileal CD after surgery[27]. Deficient colonization of *F. prausnitzii* was observed in UC patients during remission, and the recovery of the *F. prausnitzii* population after relapse is associated with the maintenance of clinical remission[27]. Moreover, Sokol *et al*[28] showed that human peripheral blood mononuclear cells stimulated with *F. prausnitzii* induce the production of interleukin (IL)-10 and inhibit the production of inflammatory cytokines, such as IL-12 and interferon- $\gamma$ . Furthermore, a significant decrease of *Roseburia spp.* was shown in the gut microbiota of healthy individuals with a high genetic risk for IBD[29].

Another consistent finding from a number of reports is the relative increase in the phylum Proteobacteria, especially *Escherichia coli* (*E. coli*), in CD patients. The CD-associated[30] proinflammatory *E. coli* is known as adhesion-invasive *E. coli*, which is a bacterium isolated from CD patients. Adhesion-invasive *E. coli* has been reported to increase the permeability of the intestinal epithelium and induce intestinal inflammation by adhering directly to it[31].

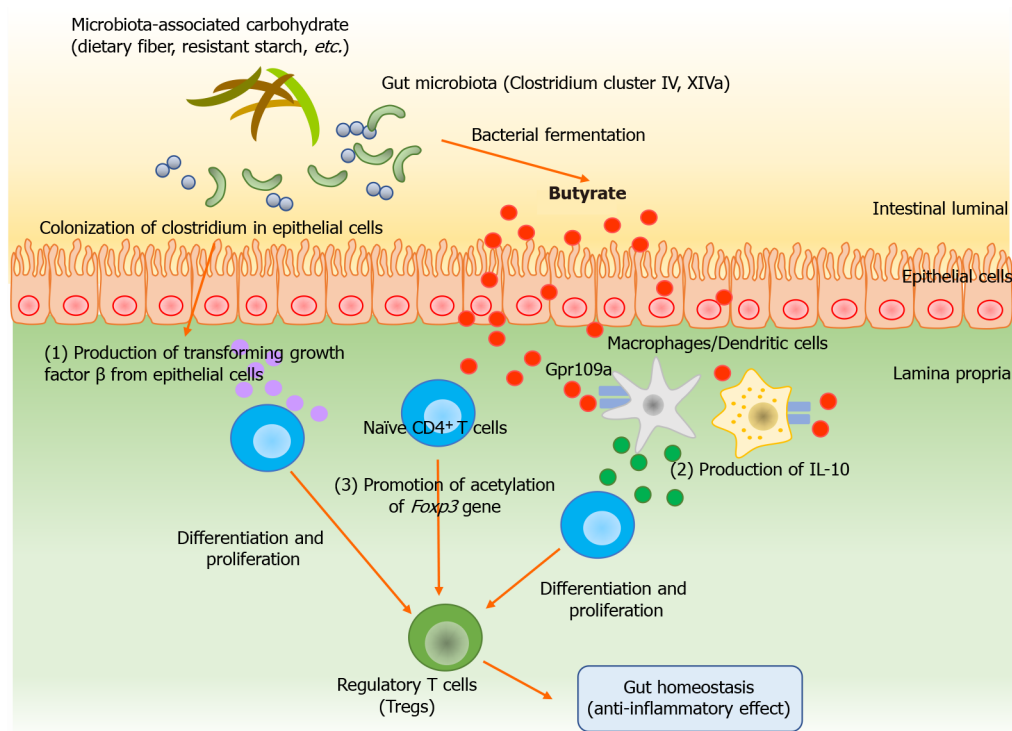
Thus, in IBD enterobacteria, along with a decrease in diversity, a decrease in anti-inflammatory bacteria (Firmicutes phylum) and an increase in proinflammatory bacteria (Proteobacteria phylum) were observed, and these changes have also been observed in IBD. It is possible that these factors contribute to chronic inflammation of the intestinal tract. It is possible that it contributes to chronic inflammation of the intestinal tract.

## ROLE OF SHORT-CHAIN FATTY ACIDS IN THE INTESTINAL TRACT

Although most nutrients are digested and absorbed in the duodenum and small intestine, dietary fiber remains intact until it reaches the colon. Dietary fibers are complex carbohydrates of plant origin broken down by specialized enzymes produced by gut bacteria but indigestible by the host[32]. They have recently been redefined as microbiota-accessible carbohydrates (MACs) and represent the major energy source for colonic bacteria[33]. MACs favor an increase in beneficial bacteria. The composition and function of the gut microbiota are dependent on the availability of MACs[34]. In preclinical studies, low dietary MACs have been shown to aggravate the development of inflammatory diseases, including autoimmune diseases, infections, and allergies [35-38] (Figure 1).

SCFAs, namely, acetate, propionate, and butyrate, are produced in the intestinal tract by the gut microbiota during fermentation of dietary fibers under anaerobic conditions[17]. Among these SCFAs, butyrate is the main energy source for the intestinal epithelial cells. Butyric acid positively modulates mitochondrial function, such as enhancing oxidative phosphorylation and  $\beta$ -oxidation, leading to an increase in oxygen consumption of colonic epithelial cells[32,33,39,40]. As a result, the concentration of oxygen in the intestinal tract decreases, and the number of obligate anaerobic bacteria, including those Firmicutes phylum that produce butyrate, increases[41]. As mentioned above, there is a decrease in butyrate-producing bacteria, such as *F. prausnitzii*, *Clostridium* cluster IV and XIVa, and a decrease in the concentration of butyrate in the gut microbiota in IBD patients[26,42]. Therefore, the decrease of both butyrate-producing bacteria and concentration of butyrate may be involved in the development of IBD and the persistence of chronic intestinal inflammation.

It has been reported that the genus *Clostridium* is important for the induction of regulatory T cells with an immunosuppressive function by producing butyrate from MACs, and in turn, the butyrate suppresses inflammatory cytokines *via* mucin and antimicrobial peptides from intestinal epithelial cells[22,43-45]. Moreover, it has been reported that the genus *Clostridium* promotes the differentiation and proliferation of regulatory T cells by enhancing the production of transforming growth factor  $\beta$  from intestinal epithelial cells[43]. In addition to the direct function of butyrate on T cells,



**Figure 1 Function of butyrate in intestinal mucosa.** Butyrate contributes to the maintenance of gut homeostasis by multiple mechanisms. Butyrate is mainly produced in the intestinal tract by bacteria of the Firmicutes phylum during fermentation of dietary fibers under anaerobic conditions. Butyrate is the main energy source for the intestinal epithelial cells. (1) The genus *Clostridium* promotes the differentiation and proliferation of regulatory T cells by enhancing the production of transforming growth factor  $\beta$  from intestinal epithelial cells; (2) Butyrate enhances the production of the anti-inflammatory cytokine, interleukin-10, produced by macrophages and dendritic cells through GPR109a, which is the G protein-coupled receptor for butyrate; and (3) Butyrate upregulates histone H3 acetylation at regulatory regions of the *Foxp3* gene and promotes the differentiation of naïve CD4<sup>+</sup> T cells into regulatory T cells. IL: Interleukin.

butyrate suppresses the induction of the inflammatory cytokine, IL-6, and enhances the production of the anti-inflammatory cytokine, IL-10, produced by macrophages and dendritic cells through GPR109a, which is the G protein-coupled receptor for butyrate[43,44]. Butyrate is also known to regulate gene expression epigenetically by inhibiting histone deacetylases. Recent studies have demonstrated that butyrate upregulates histone H3 acetylation at regulatory regions of the *Foxp3* gene and promotes the differentiation of naïve CD4<sup>+</sup> T cells into regulatory T cells[45].

Based on these findings, SCFAs play an important role in maintaining intestinal homeostasis through their anti-inflammatory properties. Thus, the decreased concentration of SCFAs in the feces of IBD patients may be involved in the pathophysiology of IBD through multiple points of action.

## THE EFFECT OF PROBIOTICS ON IBD

Probiotics are defined as “live microorganisms that when administered in adequate amounts confer a health benefit on the host”[46]. Over the past decade, there has been a great interest in the use of probiotics as a therapeutic option in IBD. However, highly reliable scientific evidence regarding the efficacy of probiotics in IBD has been lacking.

There is a large double-blind clinical trial to investigate the efficacy of *E. coli* Nissle 1917 on maintaining remission in comparison to mesalamine (1500 mg/d) in UC patients in clinical remission ( $n = 120$ ). The clinical trial showed the similar relapse rate between *E. coli* Nissle 1917 and mesalamine (*E. coli* Nissle 1917 group: 14%, mesalamine group: 16%)[47]. This group conducted a second large trial to examine the efficacy of *E. coli* Nissle 1917 on maintaining UC remission compared to mesalamine (1500 mg/d). This study revealed a comparable clinical relapse rate (*E. coli* Nissle 1917 group: 36%, mesalamine group: 34%)[48].

There is one randomized clinical trial that examined whether the addition of *E. coli* Nissle 1917 to standard therapy increased the rate of remission of patients with active UC. While undergoing the induction therapy, subjects were randomized to *E. coli* Nissle 1917 group and mesalamine group (2400 mg/d). After remission, patients were



maintained on either mesalamine or *E. coli* Nissle 1917. The remission rates were similar in the mesalamine group (75%) and *E. coli* Nissle 1917 group (68%). Moreover, the relapse rates were also similar in mesalamine group (73%) and *E. coli* Nissle 1917 group (67%). Notably, it is the only probiotic mentioned in the European Crohn's and Colitis Organization guidelines as an effective alternative to mesalamine in maintenance of remission in UC patients[49]. Collectively, the efficacy of *E. coli* Nissle 1917 on maintenance of remission was comparable to mesalamine.

To date, the evidence of the use of VSL#3 in UC patients has been accumulated. VAL#3 is a combination of four strains of *Lactobacillus* (*Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbruekii* subsp. *bulgaricus*), three strains of *Bifidobacteria* (*Bifidobacteria longum*, *Bifidobacteria breve*, and *Bifidobacteria infants*) and *Streptococcus salivarius* subsp. *Thermophilus*. Sood *et al*[50] conducted a randomized, double-blind clinical trial to investigate the efficacy of VSL#3 on mild-to-moderately active UC compared to placebo. By week 12, more patients given VSL#3 achieved remission (43%) as compared with those given placebo (15.7%;  $P < 0.001$ ). Furthermore, by week 12, more patients in the VSL#3 group achieved mucosal healing (32%) as compared to the placebo group (15%;  $P < 0.03$ ). Another study with similar design conducted by Tursi *et al*[51] also showed that the remission rate in VSL#3 group was higher than in the placebo group (44% *vs* 32%;  $P = 0.13$ ). Collectively, these results suggest that the use of VSL#3 has a benefit in inducing remission in active UC.

There has been a lack of large clinical trials assessing the efficacy of probiotics in both inducing and maintaining the remission for patients with CD. A Cochrane review published in 2020 assessing the efficacy of probiotics to induce remission in CD patients found two studies that met criteria for inclusion[52]. One study had 11 subjects with mild-to-moderately active CD that randomized assignment to *Lactobacillus rhamnosus* strain GG or placebo. The other study had 35 subjects with active CD, whose CD activity index score of 150 to 450 randomized to receive a symbiotic treatment (freeze-dried *Bifidobacterium longum* and a commercial product) or placebo. The review concluded that there was no evidence to support the use of probiotics for the induction of remission in CD.

A Cochran systematic review published in 2006 assessing the efficacy of probiotics to maintain the remission in CD found seven small controlled studies worthy of inclusion in their review[53]. The review concluded that there was no evidence to suggest that probiotics are beneficial for the maintenance of remission in CD. In summary, large clinical trials in the efficacy of probiotics on active and quiescent CD should be conducted to change these conclusions in the future.

## THE EFFECT OF FMT ON IBD

FMT aims to restore the intestinal microbiota in diseased individuals by transplanting intestinal microbiota from healthy donors[10]. FMT has been reported to be highly effective against recurrent *Clostridium difficile* infection[54]. The success of FMT in treating *Clostridium difficile* infection has attracted attention as a new therapeutic option for IBD. Several clinical studies have been conducted to examine the effect of FMT on IBD, but the results have been inconsistent so it cannot be stated with confidence whether or not the treatment is effective. Furthermore, in these studies, the protocols including donor selection, method of stool administration, and method of stool preparation are not consistent. Collectively, at present FMT has not yet been used clinically as a therapeutic option.

To date, the findings of four randomized controlled trials (RCTs) of FMT for UC patients have been published: two in Gastroenterology in 2015[55,56], one in The Lancet in 2017[57], and the other in the Journal of the American Medical Association in 2019[58] (Table 1). In a report from Canada in 2015, 50 mL of donor stool was administered six times by enema in an FMT group, and 50 mL of water was administered in the same manner in a placebo group. The remission rate in the FMT group was significantly higher than the placebo group [9/38 (24%) *vs* 2/37 (5%);  $P = 0.03$ ][55]. On the other hand, according to a report from the Netherlands in 2015, donor stool was administered to patients at day 0 and 3 wk later using a nasoduodenal tube in an FMT group, and autologous stool was administered to patients in the same manner in a control group. In this study, there was no significant difference in the effect of FMT between the two groups[56]. More recently, two RCTs in patients with mild-to-moderately active UC were reported in 2017 and in 2019. Paramsothy *et al*[57] reported in 2017 that 81 UC patients were randomly assigned FMT or placebo and that the primary outcome was defined as steroid-free clinical remission with endoscopic

**Table 1 Randomized controlled studies of fecal microbiota transplantation in ulcerative colitis**

Ref.	Moayyedi <i>et al</i> [55]	Rossen <i>et al</i> [56]	Paramsothy <i>et al</i> [57]	Costello <i>et al</i> [58]
Date of publication	2015	2015	2017	2019
Reference number	55	56	57	58
Number of patients	38	23	41	38
Number of controls	37	25	40	35
Severity of UC	Mayo 4-12 (mild to severe)	SCCAI 4-11 (mild to moderate)	Mayo 4-10 (mild to moderate)	Mayo 3-10 (mild to moderate)
Donor and Donor stool	6 volunteers fresh or frozen	15 donors fresh	Multi-donors (3-7 donors), frozen	Multi-donors (3-4 donors), frozen
Mode of FMT	Retention enema	Nasoduodenal tube	Colonoscopy and enema	Colonoscopy and enema
Number of FMT	6 1/wk × 6 wk	2 0 and 3 wk	41 First infusion by colonoscopy + 5/wk for 8 wk by enema	3 3/wk (colonoscopy followed by 2 enemas)
Follow-up	6 wk	12 wk	8 wk	8 wk
Pretreatment with antibiotics	No	No	No	No
Primary endpoint	Remission (Mayo ≤ 2 with an endoscopic score of 0)	Remission (SCCAI ≤ 2) combined with ≥ 1-point decrease in Mayo endoscopic score	Steroid-free clinical remission with endoscopic remission or response (Mayo ≤ 2, all subscores ≤ 1, and ≥ 1-point reduction in endoscopy subscore)	Steroid-free remission with endoscopic remission (Mayo ≤ 2 with endoscopic subscore ≤ 1)
Subjects who achieved the primary endpoint	9/38 (24%) treated with FMT <i>vs</i> 2/37 (5%) control ( <i>P</i> = 0.03)	7/23 (30.4%) treated with FMT <i>vs</i> 5/25 (20.0%) control ( <i>P</i> = 0.51)	11/41 (27%) treated with FMT <i>vs</i> 3/40 (8%) control ( <i>P</i> = 0.021)	12/38 (32%) treated with FMT <i>vs</i> 3/35 (9%) control ( <i>P</i> = 0.03)

SCCAI: Simple Clinical Colitis Activity Index; FMT: Fecal microbiota transplantation; UC: Ulcerative colitis.

remission or response at week 8. The rate of primary outcome of the FMT group was significantly higher than the placebo group [11/41 (27%) *vs* 3/40 (8%); *P* = 0.02]. Costello *et al*[58] reported that 73 patients with active UC were enrolled to an FMT group or autologous FMT group (placebo group). The steroid-free remission rate of the FMT group was significantly higher than that of the placebo group [12/38 (32%) *vs* 3/35 (9%); *P* = 0.03].

Since these four RCTs differ in donor selection, method of fecal administration, and the number of fecal administrations, it is difficult to make a direct comparison. It is presumed that increasing the number of FMT will be effective for UC. Based on the clinical data, FMT for UC may remain in clinical trials but not be adopted in practice. More high-quality RCTs are needed to optimize the protocol for FMT.

Sood *et al*[59] reported the effect of FMT in maintenance of remission in UC patients who had achieved clinical remission by FMT. In this pilot study, 61 patients with UC in clinical remission achieved after multisession FMT were randomized to an FMT group (*n* = 31) or placebo group (*n* = 30). There was no significant difference in the rate of steroid-free clinical remission between the FMT group and placebo group [27/31 (87.1%) *vs* 20/30 (66.7%); *P* = 0.111]. Secondary endpoints of endoscopic remission [FMT group: 18/31 (58.1%) *vs* placebo group: 8/30 (26.7%); *P* = 0.026] and histological remission [FMT group: 14/31 (45.2%) *vs* placebo group: 5/30 (16.7%); *P* = 0.033] were achieved in a significantly higher number of patients with FMT. This pilot study suggested that maintenance FMT therapy may be one of the therapeutic options for UC patients in clinical remission.

To date, one pilot randomized controlled study has reported the effects of a single FMT administered *via* colonoscopy in patients with colonic or ileo-colonic CD who achieved clinical remission with systemic corticosteroids[60]. In this pilot study, 8 patients received FMT, and 9 patients received sham transplantation. The primary endpoint was the implantation of the donor microbiota at week 6. None of patients reached the primary endpoint. There was no significant difference in the steroid-free remission rate at week 10 between the FMT group and the sham group Stood [7/8 (87.5%) *vs* 4/9 (44.4%); *P* = 0.13]. The CD Endoscopic Index of Severity decreased significantly 6 wk after FMT [8.5 (4.6; 13.0) *vs* 3.5 (1.0; 8.9); *P* = 0.03] but not after sham

[2.4 (0.0; 8.3) *vs* 2.7 (0.7; 10.0);  $P = 0.8$ ]. Moreover, 6 wk after FMT, C-reactive protein levels remained stable [3.0 (3.0; 3.0) *vs* 3.0 (3.0; 14.2) mg/L;  $P = 0.05$ ], while they had already started to increase in the sham group [3.0 (3.0; 4.2) *vs* 6.9 (4.0; 8.7) mg/L;  $P = 0.008$ ]. This pilot study indicated that the benefit of FMT over sham transplantation was observed for several clinically relevant endpoints, including CD Endoscopic Index of Severity and C-reactive protein level. Several reports have been published on the effects of FMT for CD on a small number of cases, but the results have been inconsistent. A recent systematic review and meta-analysis of 11 studies (four case reports and seven prospective uncontrolled cohort studies) on FMT for CD that included 83 CD patients showed an overall clinical remission rate of 50.5% (42/83) [61]. However, the result of one large study had a great influence on the remission rate. The authors indicated that their overall findings should therefore be treated with caution because of the potential bias from this one study [30].

## CONCLUSION

A convergence of technology, data from clinical studies, and experimental insights have revealed that the gut microbiota and its metabolites markedly contribute to the pathogenesis of IBD. However, it remains unclear whether the dysbiosis of the gut microbiota observed in IBD is a cause or a consequence of chronic intestinal inflammation. To answer this question, more basic approaches to reveal the precise effects of the gut microbiota on intestinal functions, including immune system and barrier system, will be essential. Moreover, intestinal microorganisms other than bacteria, such as viruses, archaea, and fungi should be investigated. The results from these basic studies may help to clarify the situation.

Although there is great promise for novel probiotics, we need to be circumspect when there was a lack of proof in efficacy of probiotics on IBD. There are promising results for *E. coli* Nissle 1917 in maintenance of quiescent UC and VSL#3 in active UC. There is no evidence available to support the use of probiotics in CD. The same results were found in a recently published systematic review [62]. In the future, large clinical trials should be essential to determine the effect of probiotics on IBD.

FMT has attracted attention as a new therapeutic option for IBD. However, despite its significant effect in the treatment of *Clostridium difficile* infection, its therapeutic efficacy in IBD still remains limited. There are many factors that should be taken into consideration to increase the success rate of FMT in IBD, including disease state, selection of stool donors, route of fecal administration, number of infusions, and use of antibiotic pretreatment. Therefore, further basic research as well as clinical studies are required to understand the mechanisms of action of FMT and to improve FMT preparations, modes of administration, and donor selection.

Recently, the techniques of genetic engineering have been introduced into treatments with gut microbiota. Various genetic platforms to deliver therapeutic molecules have been developed and transformed into microorganisms [63]. For disease treatment, the microbial delivery of various therapeutic molecules has been tested [64]. Host proteins, bacterial therapeutic proteins, antigens, and synthetic metabolic pathways have been introduced into the host through heterogeneous production in microbes [64]. As part of another microbiome-based therapy, engineered microbes may open new avenues for the treatment for IBD.

In the future, the combination of gut microbiology, gastroenterology, and epidemiology with advances in the rapid analysis of the gut microbiota, metabolites, molecular signals, and genetic engineering promises the development of novel therapeutic strategies in IBD.

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## Basic Study

# Oncogenic tuftelin 1 as a potential molecular-targeted for inhibiting hepatocellular carcinoma growth

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## Abstract

### BACKGROUND

Abnormal tuftelin 1 (TUFT1) has been reported in multiple cancers and exhibits oncogenic roles in tumor progression. However, limited data are available on the relationship between TUFT1 and hepatocellular carcinoma (HCC), and the exact biological mechanism of TUFT1 is still poorly understood in HCC.

### AIM

To investigate TUFT1 expression in HCC and how interfering *TUFT1* transcription affects HCC growth.

### METHODS

TUFT1 in HCC and non-HCC tissues based on databases of the Cancer Genome Atlas and Oncomine were analyzed, and TUFT1 in human HCC tissues on microarray were detected by immunohistochemistry for clinicopathological features, overall survival, and disease-free survival. HCC cells were transfected with constructed vectors of *TUFT1* that interfere or over-express TUFT1 for analyzing the biological behaviors of HCC cells. Proliferation, invasion, migration, and apoptosis of cells were detected by cell counting kit-8, scratch assay, transwell

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tests, and flow cytometry and confirmed by Western blotting, respectively.

## RESULTS

Abnormal TUFT1 levels in databases expressed in HCC at messenger RNA (mRNA) level and HCC tissues were mainly located in cytoplasm and membrane. The level of TUFT1 expression in the HCC group was significantly higher ( $\chi^2 = 18.563$ ,  $P < 0.001$ ) than that in the non-cancerous group, closely related to clinical staging, size, vascular invasion of tumor, hepatitis B e-antigen positive, and ascites ( $P < 0.01$ ) of HCC patients, and negatively to HCC patients' overall survival and disease-free survival ( $P < 0.001$ ). After interfering with *TUFT1* transcription at mRNA level in the MHCC-97H cells by the specific TUFT1-short hairpin RNA, cell proliferation, invasion, and metastasis were significantly inhibited with increasing apoptosis rate. In contrast, proliferation, invasion, and migration were significantly enhanced after over-expression of TUFT1 mRNA in Hep3B cells *in vitro*.

## CONCLUSION

Oncogenic TUFT1 was associated with the progression of HCC and could be a potential molecular-target for inhibiting HCC growth.

**Key Words:** Hepatocellular carcinoma; Tuftelin 1; Prognosis; Molecular-target; Growth

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**Core Tip:** Tuftelin1 (TUFT1) has been reported to be regulated by hypoxia and involved in the Hedgehog signaling pathway, with over-expression in hepatocellular carcinoma (HCC) tissues or cell lines. Abnormal TUFT1 level was significantly related to tumor size, vascular invasion, positive hepatitis B e-antigen, advanced tumor-node-metastasis stage of HCC, patients with ascites, and shorter overall survival and disease-free survival. Interfering *TUFT1* transcription could markedly suppress the growth and metastasis of high TUFT1 MHCC-97H cell lines *in vitro* through accelerating apoptosis. Moreover, increasing TUFT1 expression might promote the growth and metastasis of low TUFT1 Hep3B cell lines *in vitro*. The data suggested that TUFT1 is involved in HCC progression *via* the mechanism of inhibiting apoptosis and might serve as a potential therapeutic target for inhibiting HCC growth.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is usually lethal because of late diagnosis and early metastasis[1]. HCC is still one of the most common cancers with increasing incidence in the inshore area of the Yangtze River[2]. Aberrant up-regulated or down-regulated expression of many genes was identified during malignant transformation of hepatocytes in patients with chronic inflammation or damage by hepatitis B viruses (HBV), hepatitis C viruses (HCV), toxic intake, liver cirrhosis, and non-alcoholic fatty liver disease[3,4]. Given the limited effective therapies available for HCC patients with advanced stages, surgical resection and radiofrequency ablation are still the most common curative treatments for early stage patients, but the prognosis as overall survival (OS) or disease free survival (DFS) rate is very poor[5,6]. Therefore, the pathogenesis and feasible therapeutic treatments of HCC need urgent investigation[7-9]. Recently, tuftelin 1 (TUFT1) has been reported to be elevated during multiple cancer types, and the abnormal expression of TUFT1 has attracted medical attention [10,11]. TUFT1 was first identified and sequenced from a bovine ameloblast-enriched complementary DNA library with a highly conserved gene localized in chromosome



1q21-31 that contains 13 exons encoding an acidic, phosphorylated glycoprotein of 390 amino acids and plays a critical role in the development and mineralization of enamel [12].

TUFT1 belongs to the enamel associated teeth proteins and is thought to play a role in enamel mineralization. Also, TUFT1 is expressed in embryonic stem cells, neuronal cells, and non-mineralizing tissues like eyes, brain, adrenal gland, lung, liver, kidneys, testis, and tumor tissues[13-15]. TUFT1 can modulate the Rab GTPase-regulated process to activate the mammalian target of rapamycin complex 1 signaling that is activated in many cancers[16] and associated with poor prognosis in breast or pancreatic cancer and thyroid carcinoma[10,11,14]. In addition, TUFT1 is correlated with the metastasis and epithelial-mesenchymal transition[10], which could be regulated by hypoxia that enhanced TUFT1 by down-regulating miR-671-5p *via* interaction with 3'-ultranslated region of TUFT1 messenger RNA (mRNA) rather than by directly promoting hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) binding to TUFT1 promoter, and the Hedgehog signaling pathway[17-19]. Abnormal TUFT1 has been reported in multiple cancers and exhibits oncogenic roles in tumor progression[17,20]. However, limited data are available on the relationship between TUFT1 and HCC, and the exact biological mechanism of TUFT1 in HCC is still poorly understood. In this present study, the landscape of TUFT1 expression in human HCC tissues was investigated, and its molecular mechanism was confirmed through intervening or over-expressing TUFT1 activation *in vitro*.

## MATERIALS AND METHODS

### Bioinformatics analysis

Differential expressions of TUFT1 mRNA between HCC and normal liver tissues were collected according to the bioinformatics data from the Gene Expression Profiling Interactive Analysis (GEPIA) public Website[21] (<http://GEPIA.cancer-pku.cn/detail.php>) and the Oncomine database (<https://www.Oncomine.org/resource>).

### Liver tissues and clinical data

This study was approved by the Ethics Committee permission (TDFY2018-025) at the Affiliated Hospital of Nantong University, China from Jan 2009 to Dec 2014, and the prior written informed consent was obtained from HCC patients according to the Helsinki Declaration of World Medical Association. A total 132 pairs of self-controlled HCC tissues (HCC group) and their para-cancerous tissues (2 cm to cancer, Para-C group) were collected from patients with HCC after post-operation, frozen in liquid nitrogen, and kept at -85 °C until used. According to their medical records, the patients were 111 males and 21 females with 21-79-years-old (average 60.04  $\pm$  15.8 years). Serum alpha-fetoprotein (AFP) concentrations were 104 cases with  $\geq$  20 ng/mL and 28 cases with < 20 ng/mL. There were 120 cases with positive HBV surface antigen and 45 cases with positive hepatitis B e-antigen (HBeAg). There were 106 cases with tumor size  $\geq$  3.0 cm, 58 cases with vascular invasion, and 32 cases with ascites.

According to the 2019 edition of the diagnosis and treatment of primary liver cancer, there were three groups: Well (I), medium (II), and poor (III) differentiation. HCC patients were divided into I-IV stages, with 59 cases at I-II, and 73 cases at III-IV staging on the tumor-node-metastasis (TNM) classification of the International Union Against Cancer. All patients underwent histopathology and did not receive radiation or chemotherapy prior to surgery. HCC patients had regular follow-up from operation to death until Jan 2020. Diagnosis of HCC was confirmed with the criteria set by the Chinese National Collaborative Cancer Research Group[22].

### Tissue microarray

Tissue microarrays (TMA) were constructed by the Departments of Pathology, Affiliated Hospital of Nantong University, China, containing formalin-fixed and paraffin-embedded specimens from 132 HCC tissues and their non-cancerous tissues. Tissue cores (1.5 mm) from the representative areas were constructed into the TMA slices.

### Immunohistochemistry

Immunohistochemistry staining was determined for assessing TUFT1 expression by GTVision™ III Detection System/Mo&Rb (GK600710) according to the manufacturer's protocol. TMA slices were dewaxed and rehydrated, underwent antigen retrieval, and were blocked endogenous peroxidase and antibody nonspecific binding with 3% H<sub>2</sub>O<sub>2</sub>.

and 10% goat serum, respectively. Slices were incubated with the primary anti-human TUFT1 antibody (1: 250, Abcam, Cambridge, United Kingdom) overnight at 4 °C. The slices after incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (Everest Biotech, Oxford, United Kingdom) were stained with diaminobenzidine, and counterstained with hematoxylin, cleared in xylene, and covered. The levels of TUFT1 expression were calculated with the Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, United States). Blinded evaluations of TUFT1 immunostaining and independent observation by two independent pathologists were carried out simultaneously, and the score was calculated by the sum of staining intensities and the rates of 100 positive cells. TUFT1 level in HCC tissues was divided into low and high expression. The expressing intensities of TUFT1 were divided into four categories: 0, negative; 1, weakly positive; 2, moderate positive; and 3, strongly positive.

### Cell culture

All human HCC (Hep3B, MHCC-97L, MHCC-97H, BEL-7404, HepG2, HCCLM3, and SMMC-7721) cells and LO2 cell line were purchased from the Zhongqiao Xinzhou Science and Technology Co. (Shanghai, China). HCC cells were divided into three groups: Control, negative control (sh-NC or NC), and intervening (sh-TUFT1 or over-expressing) groups. HCC (MHCC-97L, MHCC-97H, BEL-7404, HepG2, and HCCLM3) cells in high glucose Dulbecco's modified Eagle's Medium with 10% fetal bovine serum (FBS, Gibco Laboratories, Gaithersburg, MD, United States), SMMC-7721 and LO2 cell lines in Roswell Park Memorial Institute-1640 medium with 10% FBS, and Hep3B cells in Minimal Essential Medium with 15% FBS were cultured containing 100 U/mL of penicillin/streptomycin at 37 °C with 5% of CO<sub>2</sub>. Cell density to about 85% was used to next cell passage.

### Plasmid coding short hairpin RNA against TUFT1

Specific short hairpin RNA (shRNA) targeting sites of *TUFT1* sequence (GenBank: AH009496.2)[23] were designed and synthesized (Guangzhou Cyagen Biotechnology Co., Ltd., China) and used to construct lentivirus LV-U6 > Scramble-shRNA-PGK > enhanced green fluorescent protein (EGFP)/T2A/Puro vector. Inserted interfering sequences in plasmids were TUFT1-shRNA1: 5'-AGAAGCTCCGG GAGGATA-TAACTCGAGTTATATCCTCCCGGAGCTCT-3', TUFT1-shRNA2: 5'-TGA GGTGGACACCTGTATAAACTCGAGTTTATACAGGTGTCCACCTCA-3', and TUFT1 -shRNA3: 5'-GATTCACGAGAAGAATATTAACCTCGAGTTAATA-TTCTTCTCGTGAA TC-3', respectively. TUFT1-negative plasmid was constructed similarly with a shRNA sequence that does not suppress TUFT1 expression, and the Scramble vector (Cyagen, Santa Clara, CA, United States) as a control. All inserted sequences were confirmed by sequencing.

### Expressing TUFT1 plasmid

The exon sequence of *TUFT1* gene (GenBank: AH009496.2)[23] was inserted into the Scramble vector (GenePharma, Shanghai, China) to construct pEX-4 (pGCMV/MCS/T2A/EGFP/ Neo) vector. Negative or control plasmids were equal to above vectors.

### Cell transfection

Human MHCC-97H cell line in high glucose Dulbecco's modified Eagle's Medium with 10% of FBS or Hep3B cell line in MEM with 15% of FBS was cultured at 37 °C with 5% CO<sub>2</sub>. When Hep3B cells up to optimal confluence over 70% were transfected at time, the cells of blank, negative control, and TUFT1 over-expressing groups were transfected with corresponding plasmids using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. MHCC-97H cells were also divided into blank, negative control, and sh-TUFT1 groups. They were transfected with corresponding plasmids using polybrene (Cyagen) according to the manufacturer's instructions.

### Western blotting

Western blot analysis was performed as described previously[24]. Briefly, 50 µg of purified protein from transfected cells was separated on electrophoresis of 10% sodium dodecyl sulfate-polyacrylamide gel, transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA, United States), blocked in 5% of nonfat dry milk in Tris-buffered saline (pH 7.5, 100 mmol/L NaCl, 50 mmol/L Tris, 0.1% Tween-20), and incubated with anti-human TUFT1 antibodies (Abcam) overnight at 4 °C, followed by HRP conjugated immunoglobulin G (Everest Biotech, Oxford, United

Kingdom). Alterations of proteins were analyzed by Quantity-one software (Bio-Rad, Laboratories, Hercules, CA, United States) with Immobilon ECL Chemiluminescence HRP Substrate (Millipore) and quantified using Gel-Pro Analyzer software.

### **CCK-8 assay**

Cell counting kit (CCK-8) was employed to evaluate HCC cell proliferation. There were three groups in the experiment: Blank, negative control, sh-TUFT1 intervention or TUFT1 over-expressing groups. Cells from different treatment groups were counted, adjusted to concentration  $4 \times 10^4$ /mL, and seeded in a 96-well plate with 100  $\mu$ L/per well. Cells were seeded in triplicate for each group. The 96-well plate was placed in the incubator (37 °C and 5% CO<sub>2</sub>), and cells were cultured till the appropriate time. Then, 10  $\mu$ L CCK-8 solution was added in each well, and the culture plate was incubated for 1-4 h. The absorbance (A) at 450 nm was detected using a plate reader.

### **Detection of apoptosis**

Cell culture and experiment groups were set up as described above. Cells were cultured in a 6-well plate at about 70% confluence. Cells were aspirated and placed in a centrifuge tube, centrifuged at 1000 rpm for 5 min, rinsed with phosphate-buffered saline twice, and centrifuged again at 1000 rpm for 5 min. Cells ( $1 \times 10^6$ ) with 5  $\mu$ L of Annexin V Alexa Fluor 647 staining solution were mixed in 100  $\mu$ L binding buffer, collected, and placed in dark at room temperature for 10 min. Then, 400  $\mu$ L binding buffer and 10  $\mu$ L propidium iodide were mixed and placed in the dark at room temperature for 10 min. Fluorescent microscope observation or flow cytometry was conducted within 1 h. FL2 channel was used to detect the yellow fluorescence of propidium iodide with excitation wavelength of 488 nm. FL4 channel was used to detect the red fluorescence of Alexa Fluor 647 with emission wavelength of 640 nm. Cells without apoptotic induction were used as control for fluorescence compensation to remove spectral overlap and determine the position of cross-over.

### **Cell invasion**

Transwell assays were performed to examine the invasion of cells transfected with small interfering RNA that targeted TUFT1 or a scrambled negative control. Cell invasion was defined using Transwell polycarbonate membrane inserts (Millipore). Cells ( $1 \times 10^5$ ) in serum-free medium were placed into the upper chamber of an insert coated with Matrigel (TheWell Bioscience Inc., North Brunswick, NJ, United States). Medium containing 10% FBS was added to the lower chamber to stimulate invasion, and the cells remaining on the upper membrane were removed with cotton wool after cultivation for 48-72 h. Cells that had invaded through the membrane were stained with methanol and 0.1% crystal violet, imaged, and counted using an IX71 inverted microscope (Olympus, Tokyo, Japan). Each assay was repeatedly conducted three times.

### **Scratch migration assay**

To measure the logarithmic growth period, cells were seeded on plates for scratch analysis. When the cell growth density was about 90%, the scratch migration assay was performed to test cell migration. Using the ruler perpendicular to the ix-hole plate, with the 200  $\mu$ L spear head along the ruler, a scratch was made, keeping strength as far as possible. A picture was taken, and the cells were allowed to continue to grow; a picture was taken every 24 h. The images were then compared, and the data analyzed.

### **Statistical analysis**

Statistical analysis was performed by GraphPad Prism 8.0 software (GraphPad Co. Ltd., San Diego, CA, United States) and SPSS statistical package Version 25.0 (SPSS Inc., Armonk, NY, United States). The Student's *t* test, a rank-sum test,  $\chi^2$  test, and Fisher's exact probability test were used for categorical variables[25]. Cumulative survival curves of OS and DFS were calculated using the Kaplan-Meier method and compared by the log-rank test. The Cox proportional hazards model was used to determine independent factors of recurrence based on the variables selected by univariate analysis. Estimated 95% confidence intervals (CIs) with hazard ratio (HR) were combined to evaluate the prognostic and clinicopathologic values according to the Tierney method[26]. All the significant predictors of recurrence in the univariate analysis were analyzed in a logistic regression model to show an independent value at the multivariate analysis. A two-tailed value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### *TUFT1 in bioinformatics database*

The comparative analysis of TUFT1 mRNA box diagrams between HCC ( $n = 594$ ) and normal liver ( $n = 270$ ) tissues from the bioinformatics websites are shown in [Figure 1](#). In the Cancer Genome Atlas database ([Figure 1A](#)) from the GEPIA public website, the levels of TUFT1 mRNA expression in the HCC group were significantly higher ( $P < 0.05$ ) than those in the normal group. Based on the Oncomine database ([Figure 1B](#)), the levels of TUFT1 mRNA expression in the HCC group were also significantly higher ( $P < 0.05$ ) than those in the normal group. The data from online databases indicated that hepatic TUFT1 should be over-expressing status in HCC progression.

### *TUFT1 expression in Human HCC tissues*

The immunohistochemistry analysis of hepatic TUFT1 expression in 132 cases of HCC and their non-HCC tissue microarray with clinical staging and prognosis is shown in [Figure 2](#). The cellular distribution of hepatic TUFT1 expression was mainly located in cytoplasm and cell membrane. The intensity of TUFT1 staining was stronger in the HCC group ([Figure 2A](#) and [A1](#)) than that in the non-HCC group ([Figure 2B](#) and [B1](#)). The positive rate of TUFT1 in the HCC group (87.1%, 115/132) was significantly higher ( $\chi^2 = 18.563$ ,  $P < 0.001$ ) than that in the non-HCC group (64.4%, 85/132). Comparative analysis of TUFT1 expressions in the HCC or non-HCC tissues is summarized in [Table 1](#). The significant difference ( $Z = 4.911$ ,  $P < 0.001$ ) of TUFT1 staining scores was found between HCC and non-HCC tissues by Z test, with higher expression in 50.0% HCC tissues (2-3 scores, 66/132), and low or no expression in 76.5% their non-HCC tissues (0-1 scores, 101/132). Regarding the relationship between TUFT1 and HCC staging ([Figure 2C](#) and [D](#)), there was lower/no TUFT1 staining in non-HCC tissues ([Figure 2C1](#) and [D1](#)) with brown staining of TUFT1 gradually increasing from stage I to IV ([Figure 2C2-5](#) or [Figure 2D2-5](#)).

### *Clinicopathologic features and prognostic value of TUFT1*

The clinicopathological features of TUFT1 expression in 132 HCC patients are shown in [Table 2](#). The higher TUFT1 level was associated with tumor size, vascular invasion, HBeAg, advanced TNM stage of HCC, and ascites of patients. However, no significant difference was found between TUFT1 and age, sex, AFP level, HBV surface antigen, and Edmondson-Steiner grading. Analysis of univariate or multivariate Cox regression of TUFT1 in HCC tissues ([Table 3](#)) was significantly correlated with HBeAg, tumor size, vascular invasion, ascites, TNM stage, and AFP level with OS of patients, and further confirmed that TUFT1, tumor size, vascular invasion, ascites, and TNM stage were independent predictive factors for OS. On the contrary, TUFT1 and TNM stage should be independent prognostic factors for DFS of HCC. The OS or DFS of HCC with high or low TUFT1 was analyzed at early or advanced HCC ([Figure 3A](#) or [Figure 3B](#)). Compared to low TUFT1 group, the cases with high TUFT1 expression had significantly decreased OS and DFS, suggesting that TUFT1 should be a promoter or prognostic marker for HCC.

### *Effects of interfering TUFT1 transcription on HCC cells*

Screening TUFU1 and interfering TUFT1 mRNA transcription on effects of HCC cell lines are shown in [Figure 4](#). Differential expressions of TUFT1 among different HCC cell lines were screened ([Figure 4A](#)). The relative ratio from TUFT1 to glyceraldehyde 3-phosphate dehydrogenase showed Hep3B cells with lowest and MHCC-97H cells with strongest level of TUFT1 expression ( $P < 0.001$ , [Figure 4A1](#)). Based on the status of TUFT1 expression in the MHCC-97H or Hep3B cells, the MHCC-97H cells were chosen to be used for interfering TUFT1 mRNA transcription with shRNA1-3 ([Figure 4B](#) left), and the most significantly inhibiting effect was shRNA3 (sh-3) plasmid for TUFT1 ([Figure 4B1](#) left). The Hep3B cells were selected to over-express TUFT1 with constructed pEX-4 (pGCMV/MCS/T2A/EGFP/Neo) plasmid ([Figure 4B](#) right) and showed markedly increasing TUFT1 expression ([Figure 4B1](#) right). Using a different style of TUFT1 interference, the TUFT1-shRNA3 significantly suppressed proliferation of MHCC-97H cells ( $P < 0.001$ , [Figure 4C](#)), and TUFT1 activation markedly increased proliferation of Hep3B cells ( $P < 0.001$ , [Figure 4D](#)). The results confirmed that TUFT1 could promote HCC cell proliferation.

### *Effects of TUFT1 on healing, invasion, and migration of HCC cells*

According to the above results, the effects of higher or lower level of TUFT1 on the healing, invasion, and migration of HCC cells were further observed. The effects of



**Table 1 Comparative analysis of hepatic tuftelin 1 immunohistochemical staining and expressing intensity between hepatocellular carcinoma and their para-cancerous tissues**

Group	n	TUFT1		$\chi^2$ value	P value	TUFT1 score				Z value	P value
		Neg.	Pos.			0	1	2	3		
HCC	132	17	115	18.563	< 0.001	17	49	55	11	4.911	< 0.001
Non-HCC	132	47	85			47	54	23	8		

HCC: Hepatocellular carcinoma; Neg: Negative; Non-HCC: Non-cancerous tissue; Pos: Positive; TUFT1: Tuftelin 1.

**Table 2 Relationship between tuftelin 1 and clinicopathological characteristics in hepatocellular carcinoma patients**

Group	n	TUFT1 expression		Pearson $\chi^2$	P value
		Low	High		
AFP (ng/mL)				2.901	0.089
< 20	28	18	10		
$\geq$ 20	104	48	56		
HBsAg				3.300	0.069
Neg.	12	9	3		
Pos.	120	57	63		
HBeAg				4.080	0.043
Neg.	87	49	38		
Pos.	45	17	28		
Tumor size (cm)				9.388	0.002
< 3	26	20	6		
$\geq$ 3	106	46	60		
Differentiation degree				3.013	0.083
Well-Med.	19	13	6		
Poor	113	53	60		
Vascular invasion				14.885	< 0.001
With	58	18	40		
Without	74	48	26		
Ascites				5.940	0.015
With	32	10	22		
Without	100	56	44		
TNM staging				13.516	< 0.001
I-II	59	40	19		
III-IV	73	26	47		

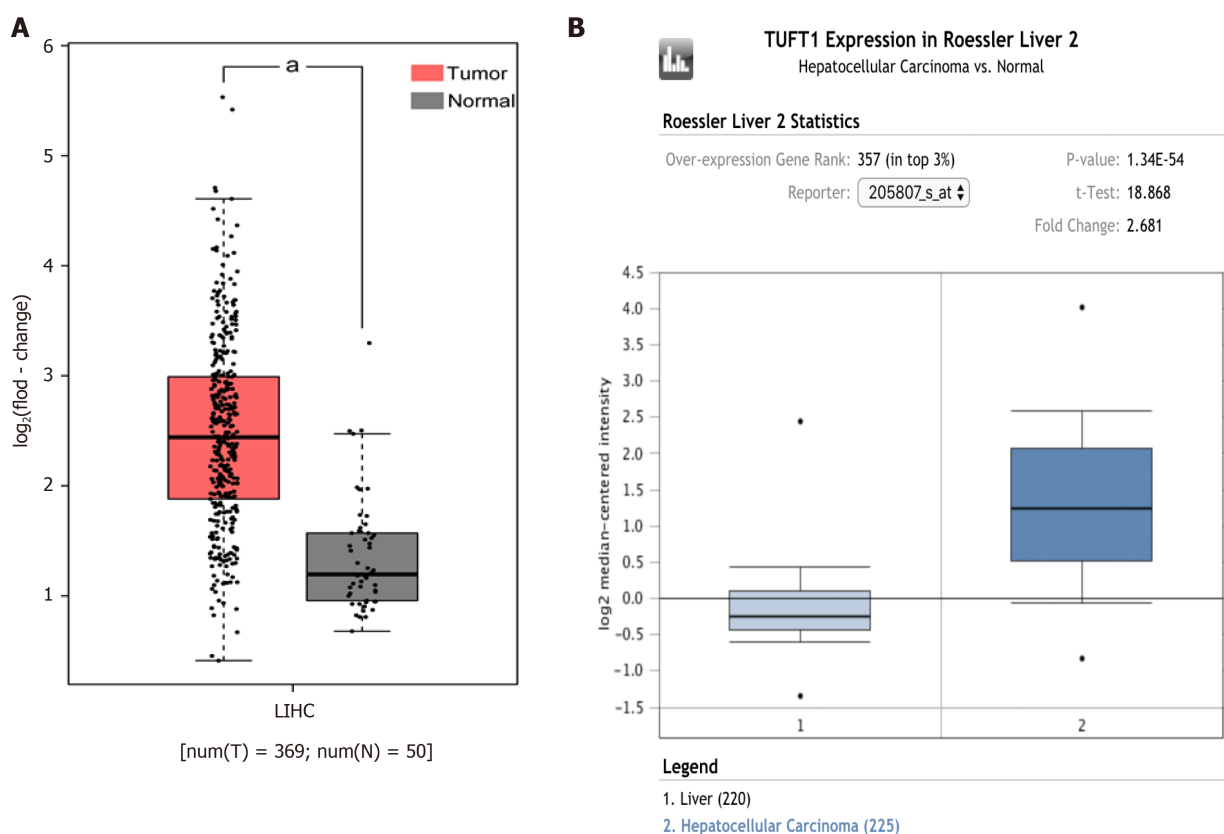
AFP: Alpha-fetoprotein; HBeAg: Hepatitis B virus e antigen; HBsAg: Hepatitis B virus surface antigen; HCC: Hepatocellular carcinoma; Neg: Negative expression; Pos: Positive expression; TUFT1: Tuftelin 1.

different TUFT1 levels on the healing, invasion, and migration of the MHCC-97H or Hep3B cells are shown in [Figure 5](#). The healing abilities of the MHCC-97H cells transfected with the TUFT1-shRNA3 plasmid were averaged down to 50% in the sh-NC group ([Figure 5A](#)), and the healing abilities in the sh-RNA3 group were significantly inhibited compared with the sh-NC group ( $P < 0.001$ , [Figure 5A1](#)). However, the healing abilities of the Hep3B cells transfected with the over-expressing

**Table 3 Univariate and multivariate analysis of tuftelin 1 in the prognosis of hepatocellular carcinoma**

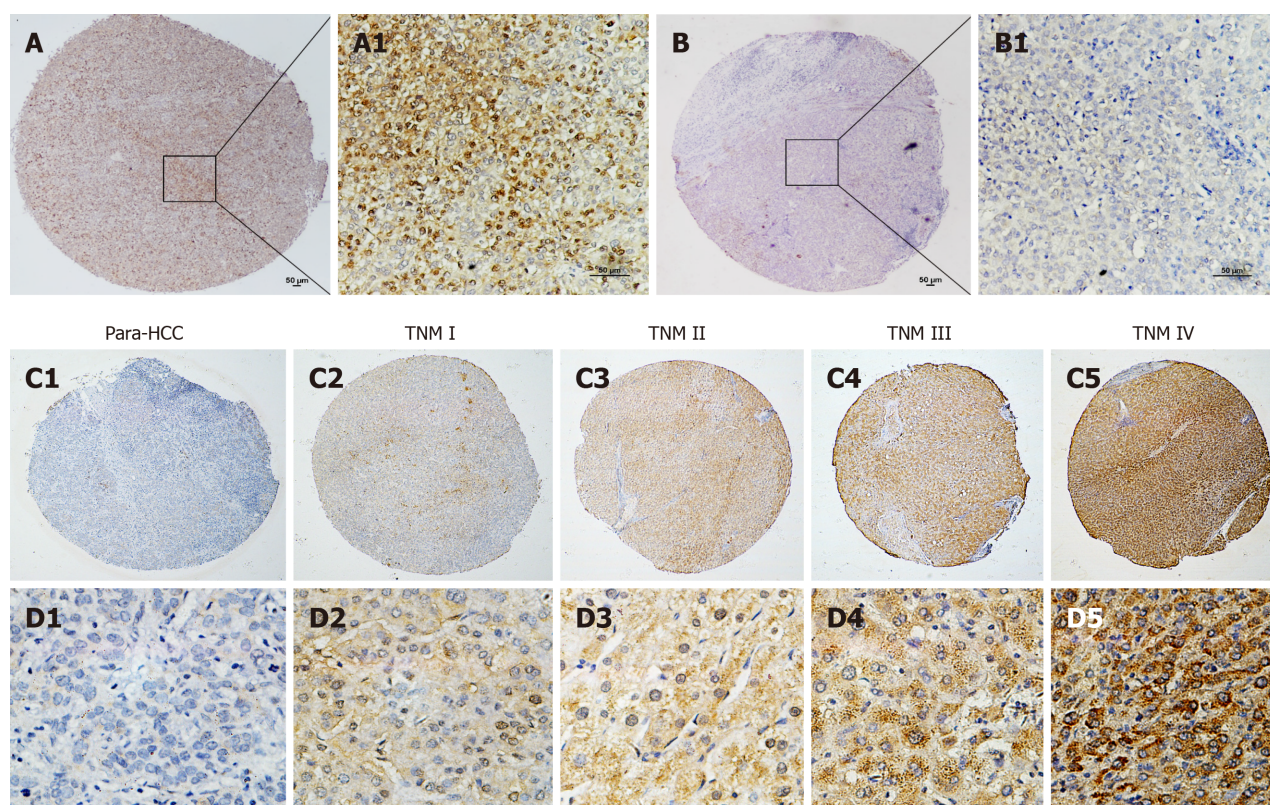
Group	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
TUFT1 expression, high <i>vs</i> low	3.026	1.927-4.752	< 0.001	1.743	1.044-2.910	0.034
AFP (ng/mL), $\geq 20$ <i>vs</i> < 20	1.866	1.066-3.266	0.027	1.482	0.835-2.630	0.178
HBsAg, pos. <i>vs</i> neg.	1.495	0.652-3.431	0.339			
HBeAg, pos. <i>vs</i> neg.	1.670	1.084-2.574	0.019	1.070	0.659-1.739	0.784
Tumor size (cm), $\geq 3$ <i>vs</i> < 3	3.680	1.837-7.370	< 0.001	2.235	1.043-4.485	0.039
Differentiation degree, Poor <i>vs</i> well-med.	1.642	0.869-3.100	0.123			
Vascular invasion, with <i>vs</i> without	3.297	2.133-5.094	< 0.001	2.018	1.186-3.434	0.010
Ascites, with <i>vs</i> without	2.592	1.648-4.076	< 0.001	1.929	1.177-3.161	0.009
TNM staging, III-IV <i>vs</i> I-II	2.411	1.532-3.796	< 0.001	1.110	0.614-2.005	0.731

AFP: Alpha-fetoprotein; HBeAg: hepatitis B virus e antigen; HBsAg: hepatitis B virus surface antigen; HR: Hazard ratio; Neg: Negative expression; Pos: Positive expression; TNM: Tumor-node-metastasis.



**Figure 1 Comparative analysis of tuftelin 1 messenger RNA between hepatocellular carcinoma and normal livers based on bioinformatics databases.** A: The expression of tuftelin 1 (TUFT1) messenger RNA in the cancerous tissues of hepatocellular carcinoma (HCC) patients ( $n = 369$ , red,  $^aP < 0.05$ ) and normal liver tissues ( $n = 50$ , grey) from the Cancer Genome Atlas database; B: The expression of TUFT1 messenger RNA in the cancerous tissues of HCC patients ( $n = 225$ , dark blue,  $^aP < 0.05$ ) and normal liver tissues ( $n = 220$ , light blue) from the Oncomine database. The horizontal line represents the median of the two groups. Vertical bars indicate the range of data. LIHC: Liver hepatocellular carcinoma.

TUFT1 plasmid were averaged up to 200% in the sh-NC group (Figure 5B); and the healing abilities of the Hep3B cells were significantly improved compared with the sh-NC group ( $P < 0.001$ , Figure 5B1). Compared with the sh-NC group, the migration or invasion abilities of the MHCC-97H cells were significantly suppressed (Figure 5C); and the migration or invasion abilities of the MHCC-97H cells were down to 50% or 30% less than those in the sh-NC group ( $P < 0.001$ , Figure 5C1). However, the



**Figure 2 Tuftelin 1 expression with clinical staging of hepatocellular carcinoma.** A, A1: Hepatocellular carcinoma (HCC) tissue with strongly positive staining; B, B1: Adjacent non-HCC tissue with negative staining, original magnifications of  $\times 40$  (scale bar: 50  $\mu\text{m}$ ) in A and B, and  $\times 200$  (scale bar: 50  $\mu\text{m}$ ) in A1 and B1; C, D: Tuftelin 1 (TUFT1) expression in different HCC staging; C1, D1: Low TUFT1 expression in the non-HCC tissues; C2-C5: The brown staining of TUFT1 gradually increases in the HCC tissues from stage I to IV (original magnification  $\times 40$ ); D2-D5: The staining of TUFT1 in the non-HCC tissues from stage I to IV (original magnification  $\times 400$ ). TNM: Tumor-node-metastasis.

migration or invasion abilities of the Hep3B cells were significantly increased compared with the sh-NC group (Figure 5D); and the migration and invasion abilities of the Hep3B cells were up to 200% or 300% more than those in the sh-NC group [ $P < 0.001$ , (Figure 5D1)].

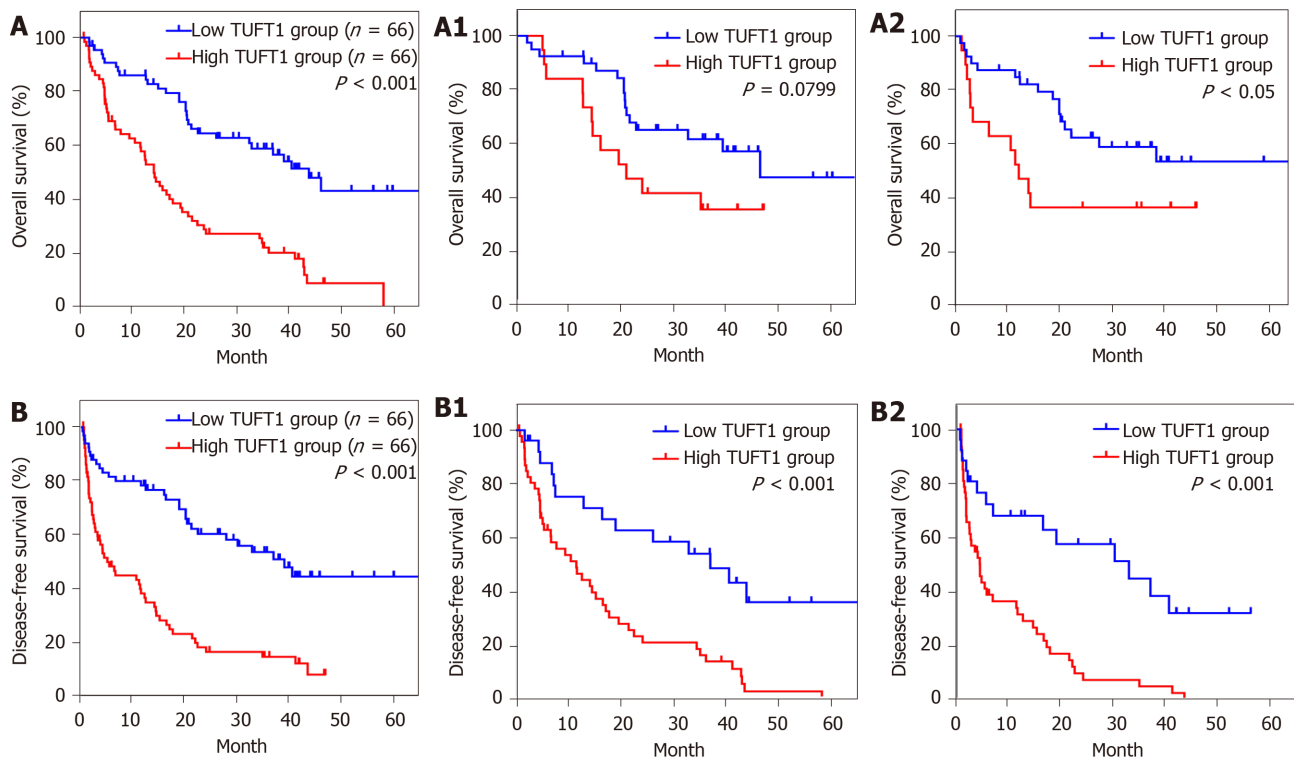
### Anti-apoptotic mechanism of TUFT1

After the MHCC-97H cells were transfected with the most effective TUFT1-shRNA3 plasmid, the relationship between TUFT1 expression and apoptosis of MHCC-97H cells was determined (Figure 6). After the MHCC-97H cells were transfected with the more specific TUFT1-shRNA3 plasmid, the numbers of the apoptotic cells in the sh-TUFT1 group (Figure 6A2) were markedly increased than those in the control (Figure 6A) or sh-NC groups (Figure 6A1). The ratio of the apoptotic cells in the sh-TUFT1 group was significantly higher ( $P < 0.001$ , Figure 6B) than those in the control or sh-NC groups.

Speculations based on the above data and the possible mechanism of TUFT1 promoting the progression of HCC are shown in Figure 6C. Aberrant up-regulating or down-regulating expressions of TUFT1 was associated with the biological features of HCC. Increased hepatic TUFT1 expression promoted the proliferation, growth, and metastasis of HCC by inhibiting tumor cell apoptosis.

## DISCUSSION

Carcinogenesis of hepatocytes is a multi-factor, multi-step, and complex process with major risk factors including chronic infections with HBV or hepatitis C virus, exposure to dietary toxin, hereditary liver disease, and non-alcoholic fatty liver disease of any etiology[2,27]. Despite the urgent need for molecular targeted anti-HCC agents, the therapeutic strategies are difficult to be identified. Accumulating data have demonstrated that many kinds of abnormal signaling pathways are associated with HCC by interacting with highly complex genetic aberrations such as abnormal



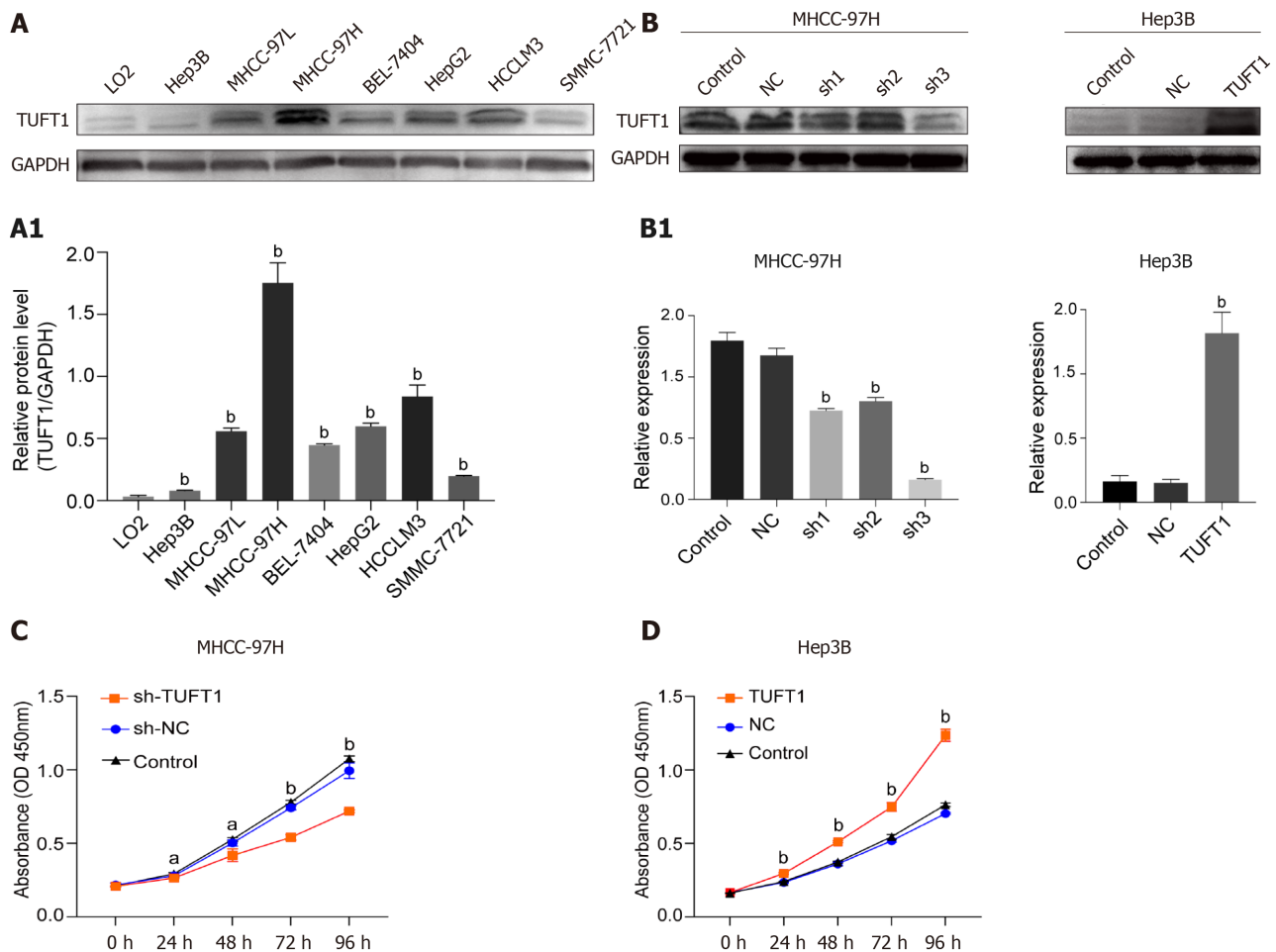
**Figure 3 Relationship between tuftelin 1 expression and prognosis of hepatocellular carcinoma.** A: Kaplan-Meier analysis was performed to compare the overall survival (OS) between hepatocellular carcinoma (HCC) with high ( $n = 66$ ) and low ( $n = 66$ ) TUFT1; A1: The high ( $n = 19$ ) and low ( $n = 40$ ) tuftelin 1 (TUFT1) with OS at early HCC; A2: The high ( $n = 47$ ) and low ( $n = 26$ ) TUFT1 with OS at advanced HCC; B: Kaplan-Meier analysis was performed to compare the disease-free survival (DFS) between HCC with high ( $n = 66$ ) and low ( $n = 66$ ) TUFT1; B1: The high ( $n = 19$ ) and low ( $n = 40$ ) TUFT1 with DFS at early HCC; B2: The high ( $n = 47$ ) and low ( $n = 26$ ) TUFT1 with DFS at advanced HCC.

methylation, histone modification, and chromosome remodeling[28]. Recently, TUFT1 has been shown to exist in many tissues and has been identified as a useful biomarker associated with poor prognosis in several cancers[29-31]. However, only limited studies were available on the relationship between TUFT1 and HCC. In this study, the alterations of TUFT1 expression in HCC tissues and if the oncogenic TUFT1 might promote HCC growth and metastasis *via* an anti-apoptotic mechanism were investigated.

TUFT1 was originally discovered and mostly studied in the tooth, where it plays an important role in the initial stages of the mineralization of ectodermal enamel[12,29]. Even though TUFT1 is widely distributed and expressed in different embryonic and adult tissues, TUFT1 has been shown to be associated with cellular adaptation to hypoxia and recently even with cell differentiation[32]. TUFT1 activation is related to the progression of tumors, and abnormal TUFT1 has been reported in breast, lung, pancreatic, and thyroid carcinomas[10,11,13]. TUFT1 expression was significantly higher in the HCC tissues than in non-HCC tissues, with invasion and metastasis of tumors[14]. In this study, the comparative analysis of TUFT1 in HCC and non-HCC tissues showed high TUFT1 expression related to tumor size, vascular invasion, advanced TNM stage of HCC, and HBV replication or ascites formation of HCC patients, suggesting that abnormal TUFT1 activation could play oncogenic roles in the progression of HCC.

TUFT1 expression in tissue hypoxia was associated with activations of signaling pathways and poor outcome of patients[18,19]. Previous studies have indicated high HIF-1 $\alpha$  level in the hypoxic microenvironment of HCC[33,34] because of tissue hypoxia inducing HIF-1 $\alpha$  expression and binding to TUFT1 promoter, thereby promoting tumor progression *via* the activation of the Rac1/ $\beta$ -catenin, mitogen activated protein kinase, calcium/phosphatidylinositol 3 kinase/AKT, Akt-mammalian target of rapamycin/glycogen synthase kinase 3 $\beta$ , and HIF1-SNAIL signaling pathway[11,14,18]. In this study, TUFT1 level was shown to be positively correlated with tumor size, histological grade, and lymph node metastasis rate, and elevated TUFT1 levels were related to unfavorable clinicopathologic characteristics and poor survival. Furthermore, the univariate and multivariate analysis of clinical data indicated that TUFT1 could be an independent prognostic marker for HCC.



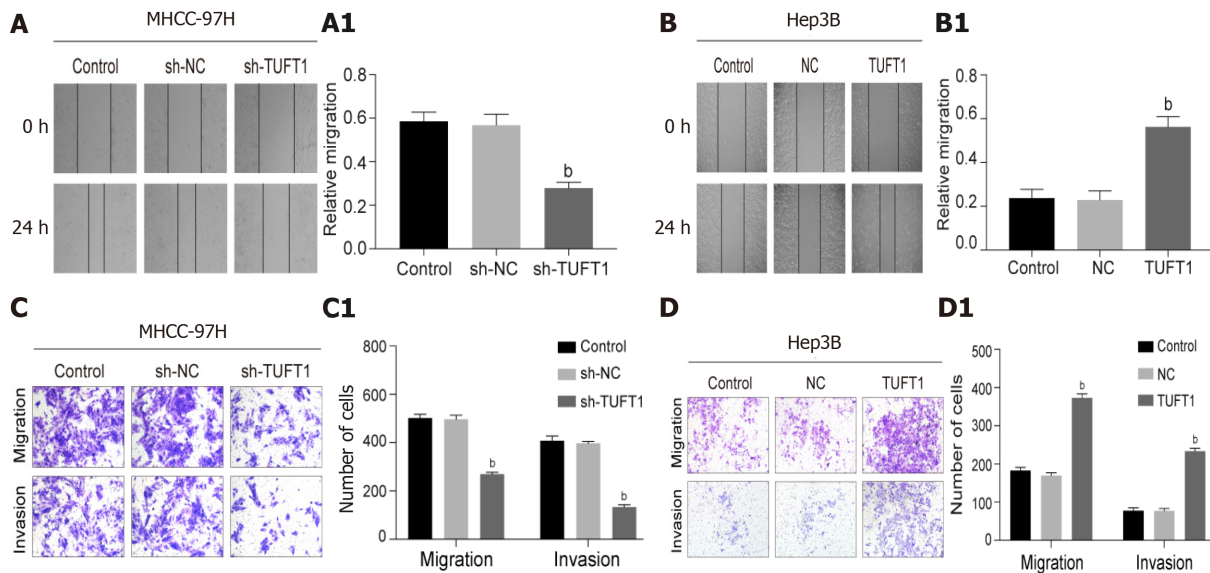


**Figure 4 Expression, interfering of tuftelin 1 with proliferation of hepatocellular carcinoma cells.** A: Expressions of tuftelin 1 (TUFT1) among different hepatocellular carcinoma (HCC) cell lines were detected by Western blotting, and the expressing levels of all HCC cells were significantly higher than that of the control LO2 cells ( $^bP < 0.001$ ); A1: The relative ratio from TUFT1 to glyceraldehyde 3-phosphate dehydrogenase with the MHCC-97H cells showed the strongest TUFT1 expression, and the Hep3B cells showed the lowest TUFT1 expression; B: The MHCC-97H cells and interfering with TUFT1 short hairpin RNA (sh-RNA)1-3 (left); the TUFT1 was over-expressed after the Hep3B cells transfected with the constructed pEX-4 (pGCMV/MCS/T2A/EGFP/Neo) plasmid (Right); B1: The TUFT1 expression was significantly inhibited by the TUFT1-shRNA transfection ( $^bP < 0.001$ , left); the markedly increasing TUFT1 Level was compared with the control cells ( $^bP < 0.001$ , right); C: The TUFT1 activation interfering by TUFT1-shRNA3 significantly inhibited the proliferation of MHCC-97H cells ( $^aP < 0.05$ ,  $^bP < 0.001$ ); D: the over-expression of TUFT1 significantly enhanced the proliferation of the Hep3B cells ( $^bP < 0.001$ ). Data are presented as mean  $\pm$  standard error of the mean of at least three independent experiments. NC: Blank control; sh-NC: Negative sh-RNA for tuftelin 1 mRNA.

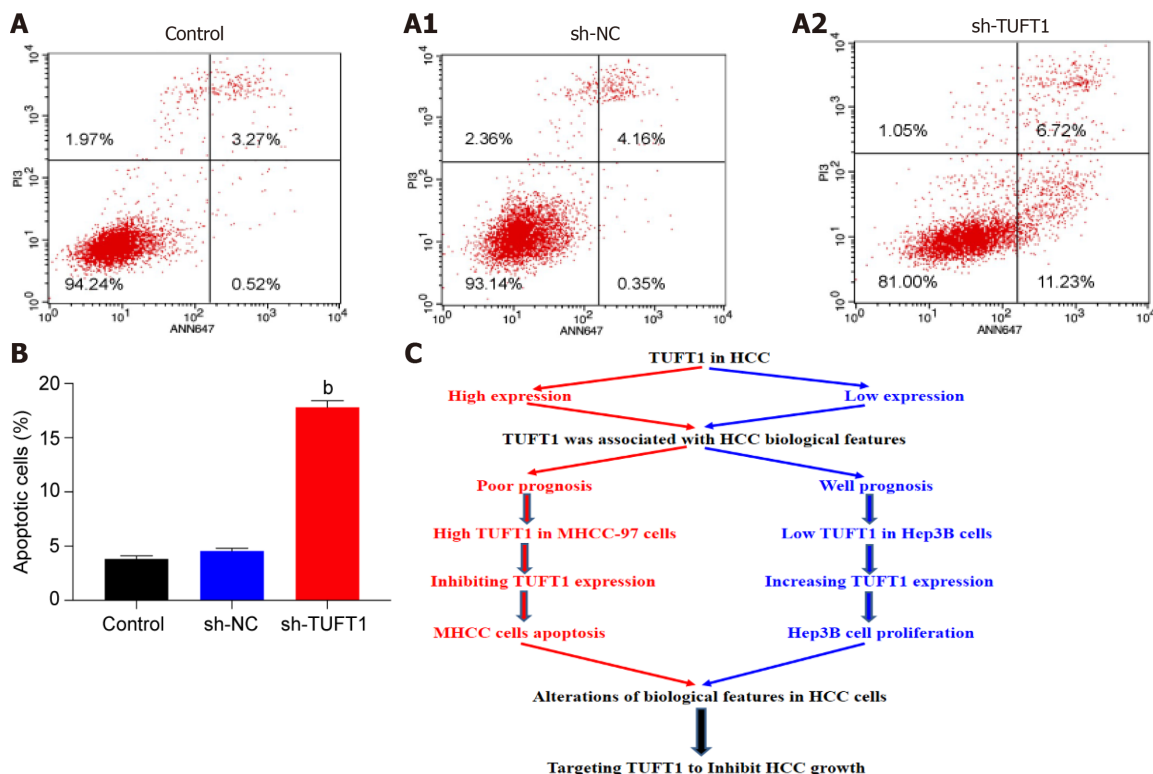
The levels of inhibiting or expressing TUFT1 might alter the biological features of HCC cells[12,23]. In this study, evidence from different cell line studies *in vitro* suggested that the higher or lower TUFT1 level could affect the biological behaviors of HCC cells. The MHCC-97H cells with high TUFT1 expression were transfected with the specific interfering shRNA3 plasmid, and the proliferation, healing, invasion, and migration abilities of the cells were significantly inhibited less than those in the sh-NC group. However, the Hep3B cells with low TUFT1 expression were transfected with the over-expressing TUFT1 plasmid, and those abilities of the cells were significant increased compared to those in the sh-NC group. These results have confirmed that TUFT1 could promote the growth and metastasis of HCC cells *in vitro* via an anti-apoptosis mechanism, suggesting that TUFT1 might function as a potential therapeutic target for inhibiting HCC growth.

## CONCLUSION

In conclusion, high TUFT1 levels based on human specimen studies were associated with HCC progression. Further inhibiting TUFT1 *in vitro* could significantly alleviate the proliferation and metastasis of HCC cells. Moreover, TUFT1 over-expression could promote the proliferation and metastasis of HCC cells *in vitro*. Furthermore, TUFT1 inhibition led to significant changes of cell apoptosis rate. More studies in future are



**Figure 5** Effects of tuftelin 1 on healing, invasion, and migration of hepatocellular carcinoma cells. A: The scratch test of the MHCC-97H cells, the cell healing ability was decreasing after the high tuftelin 1 (TUFT1) cells were transfected with the TUFT1-shRNA3 plasmid, and A1, the relative cell healing ability was significantly inhibited compared with the control or negative sh-RNA for tuftelin 1 mRNA (sh-NC) cells ( $^bP < 0.001$ ); B: The scratch test of the Hep3B cells, the cell healing ability was increased after the cells were transfected with the over-expressing TUFT1 plasmid; B1, the relative cell healing ability was significantly improved compared with the control or blank control (NC) cells ( $^bP < 0.001$ ); C: The migration and invasion abilities of the MHCC-97H cells were analyzed by the transwell test; C1: The histograms of the cell migration and invasion abilities among the different groups ( $^bP < 0.001$ ); D: The migration and invasion abilities of the Hep3B cells were analyzed by the transwell test; D1: The histograms of the cell migration and invasion abilities among the different groups ( $^bP < 0.001$ ). Data are presented as mean  $\pm$  standard error of the mean of at least three independent experiments. sh-RNA: sh-RNA3 for tuftelin 1 mRNA.



**Figure 6** Possible mechanism of tuftelin 1 expression in hepatocellular carcinoma cells progression. A: The MHCC-97H cells were transfected with the tuftelin 1 (TUFT1)-short hairpin RNA (sh-RNA)3 plasmid, and the apoptotic cells in the sh-TUFT1 group (A2) were increased more than those in the blank control (NC) (A) or negative sh-RNA for tuftelin 1 mRNA (sh-NC) group (A1); B: The histograms of the apoptotic cells among the different groups, the rate of the MHCC-97H cell apoptosis in the sh-TUFT1 group was significantly higher ( $^bP < 0.001$ ) than those in the NC or sh-NC group. Data are presented as mean  $\pm$  standard error of the mean of at least three independent experiments; C: Speculation of possible mechanisms of TUFT1 promotion of the progression of hepatocellular carcinoma (HCC). Aberrant TUFT1 activation promoted the proliferation and metastasis *via* anti-apoptosis of HCC cells.

needed to clarify the molecular mechanisms underlying the TUFT1 up-regulation con

#### ARTICLE HIGHLIGHTS

### Research background

Tuftelin 1 (TUFT1) has been reported to be elevated in multiple cancer types, and its abnormal expression has attracted medical attention as a novel oncogenic biomarker. However, the relationship between TUFT1 and hepatocellular carcinoma (HCC) remains to be identified. In this study, the levels of TUFT1 in HCC tissues were investigated and the roles of TUFT1 expression were explored *in vitro* by intervening in TUFT1 activation of HCC cell lines.

### Research motivation

TUFT1 was first identified and sequenced from a bovine ameloblast-enriched complementary DNA library with a highly conserved gene localized in chromosome 1q21-31 that contained 13 exons, encoding an acidic, phosphorylated glycoprotein of 390 amino acids, and it plays a critical role in the development and mineralization of enamel. Recently, TUFT1 has been reported to be elevated in multiple cancers, and abnormal TUFT1 has attracted medical attention for HCC. However, limited data is available on the relationship between TUFT1 and HCC, and the exact biological mechanism of TUFT1 is still poorly understood in HCC.

### Research objectives

The pathogenesis and feasible therapeutic treatments of HCC need urgent investigation. Abnormal TUFT1 has been reported in multiple cancers, and it exhibits oncogenic roles in tumor progression. In this study, the landscape of TUFT1 expression in human HCC tissues was investigated, and its mechanism was confirmed through intervening or over-expressing TUFT1 activation *in vitro*.

### Research methods

TUFT1 on databases of the Cancer Genome Atlas and Oncomine or in HCC tissues were analyzed for clinicopathological features, overall survival, and disease-free survival. High and low expressing HCC cell lines were screened among different HCC cell lines and transfected with constructed vectors that interfere or over-express TUFT1 to analyze biological behaviors. Proliferation, invasion, migration, and apoptosis of cells were detected by CCK-8, scratch assay, transwell tests, and flow cytometry, respectively, and confirmed by Western blotting.

### Research results

The research results confirmed that TUFT1 was over-expressed in HCC tissues, and its expression was significantly related to tumor size, vascular invasion, positive hepatitis B e-antigen, advanced tumor-node-metastasis staging, ascites, shorter overall survival, and disease-free survival of HCC patients. Novel findings were that interfering with TUFT1 gene transcription could markedly suppress the proliferation and metastasis of the higher TUFT1 MHCC-97H cell lines through an apoptosis mechanism. Moreover, over-expressing TUFT1 promoted the growth and metastasis of the lower TUFT1 Hep3B cell lines *in vitro*.

### Research conclusions

Based on human specimen studies, high TUFT1 levels were associated with HCC progression, inhibiting TUFT1 affected proliferation and metastasis, over-expressing TUFT1 promoted proliferation and metastasis, and interfering activation of TUFT1 led to significant increase in cell apoptosis. TUFT1 could be a novel useful target for HCC effective therapy.

### Research perspectives

Basic and clinical studies have confirmed that the alterations of oncogenic TUFT1 expression might promote HCC growth and metastasis *via* an anti-apoptotic mechanism, and abnormal TUFT1 level could be a new prognostic marker or potential molecular target for HCC therapy. More studies in the future are needed to clarify the molecular mechanisms underlying TUFT1 up-regulation contribution to the progression of HCC.

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## Basic Study

# Conditioned secretome of adipose-derived stem cells improves dextran sulfate sodium-induced colitis in mice

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**Author contributions:** Lee S, Ahn EK, Park SJ and Heo J designed the study; Lee S, Ahn EK, Chang HK, Kim J, Park SJ and Heo J performed the research; Lee S, Ahn EK, Kim JH, Kim YH, Lee SJ, Park SJ and Heo J participated in the interpretation of the data; Lee S, Ahn EK, Park SJ and Heo J wrote paper; Chang HK, Kim YH, Lee SJ, Kim J, Park SJ and Heo J supervised the analysis; all authors approved the final version.

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## Abstract

### BACKGROUND

Inflammatory bowel diseases (IBD) is related to uncontrolled immune response. Currently, there is no successful treatment for significant improvement in IBD. Stem cells display their therapeutic effects through their repopulating capacity or secreting factors.

### AIM

To investigate the effects of conditioned mouse adipose-derived stem cells (mADSCs) secretome on colitis-induced mice.

### METHODS

mADSCs were isolated from adipose tissue of C57BL/6 mice. Conditioned mADSCs secretome was obtained by culturing of mADSCs with lipopolysaccharides (LPS, 1 µg/mL) for 24 h. Acute colitis was induced by 2% dextran sulfate

College of Medicine (IACUC protocol number: Kosin15-12).

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None of the authors have any conflicts of interest to declare.

**Data sharing statement:**

No additional data are available.

**ARRIVE guidelines statement:**

The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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sodium (DSS) drinking water for 7 d and then normal drinking water for 4 d. The mice were treated with normal culture medium (NM group), conditioned mADSCs secretome (CM group) or mADSCs (SC group). The length of colon and histopathology of colon tissues were evaluated. The mRNA expression levels of inflammatory cytokines in colon tissue and the serum interleukin (IL)-6 levels were determined.

## RESULTS

The isolated mADSCs maintained the mADSCs specific gene expression profiles during experiment. The conditioned mADSCs secretome released by the treatment of mADSCs with LPS contained mainly inflammatory chemokines, colony-stimulating factors and inflammatory cytokines. The loss of body weight and reduction in colon length were ameliorated in the CM group. The conditioned mADSCs secretome reduced the histological score in colon tissue. The expression of IL-1b and IL-6 mRNAs in colon tissues significantly inhibited in the CM group compared to SC group and NM group, respectively. The elevation of serum IL-6 levels was also ameliorated in the CM group. These results indicate that the conditioned mADSCs secretome suppressed the synthesis of inflammatory cytokines in damaged colon tissue and the elevation of serum IL-6 concentration in DSS-induced mice

## CONCLUSION

Conditioned mADSCs secretome might play regenerative roles by the suppression of IL-6 in serum and tissue during acute colitis, and may be more effective than stem cells themselves in the regeneration of colon tissue.

**Key Words:** Adipose-derived stem cells; Conditioned secretome; Cytokines; Interleukin-6; Colitis; Regeneration

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**Core Tip:** The therapeutic ability of mesenchymal stem cells (MSCs) is mostly mediated by their paracrine effects. Cell free therapy using MSCs secretome could be more promising strategy than stem cells based therapy. The present study demonstrates that the conditioned secretome of adipose-derived stem cells (ADSCs) has more efficient effects for improving acute colitis in dextran sulfate-sodium-induced mouse model. The effects by the conditioned secretome of ADSCs were mediated by suppression of interleukin (IL)-6 mRNA synthesis in colon tissue and serum IL-6 protein levels, which suggests that the stem cell secretome may have efficient therapeutic potential for incurable inflammatory bowel diseases.

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## INTRODUCTION

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease (CD), are multifactorial disorders characterized by chronic inflammation, visceral hypersensitivity and diarrhea in the gastrointestinal tract[1]. Although the etiology of IBD remains unknown, it is well accepted that IBD is related to a dysregulated immune response, genetic susceptibility, and the environment[2]. Uncontrolled production of inflammatory cytokines and chemokines by infiltrating immune cells ultimately lead to damage of colon tissue. Current treatments for IBD are intended to control the inflammatory intestinal process using immunosuppressive agents; however, these current treatments are not entirely effective to maintain remission of intestinal inflammation and can result in multiple adverse effects due to their

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inefficacy or toxicity[3]. The use of certolizumab perol in patients with moderate-to-severe CD triggered a mild improvement in therapeutic response rates but no significant improvement in remission rates[4]. Therefore, it is necessary to develop novel therapeutic approaches that are effective, feasible and safe for IBD patients.

Stem cells are immature tissue precursor cells that have the ability to self-renew and to differentiate into various cell lineages[5]. Due to these properties, stem cells have emerged as attractive therapeutic tools for incurable diseases such as IBD[6]. Mesenchymal stem cells (MSCs) are mesoderm-derived fibroblast-like stem cells that have been isolated from various adult tissues including adipose tissue[7], skin[8], muscle[9], and peripheral blood[10]. The characteristics of MSCs are identified by the adherence of cells to plastic dishes in standard culture conditions, the expression profiles of specific cell surface molecules and their differentiation potential into osteogenic, chondrogenic and adipogenic lineage[11,12]. Besides their differentiation potential, MSCs also regulate the immune response including *in vitro* suppression of T-cell proliferation, B-cell function and dendritic cell maturation[13,14]. The three main actions of MSCs with regard to therapeutic potential are their homing action for migration of MSCs into damaged sites[15], differentiation action for replacing damaged tissues[16], and paracrine actions for secreting bioactive factors[17]. Generally, the protocol of stem cell therapy requires hundreds of millions of MSCs *per* treatment. However, the overall quantity of MSCs in the body is scarce, and *in vitro* cell expansion is necessary for obtaining enough cells before implantation. *In vitro* manipulation of MSCs for expansion or differentiation affects the quality of the cells such as their senescence, differentiation capacity and survival of the administered MSCs *in vivo*, which may influence homing and engraftment of MSCs into injured sites [18,19]. Although MSCs have become a promising therapeutic strategy for IBD because of their immunosuppressive and tissue healing ability[20], MSCs transplantation in IBD patients had yield inconsistent results, which may be due to the different sources of MSCs with distinct differentiation and regenerative potential, and the variety of protocols for treatments[21].

It has been known that the therapeutic ability of MSCs is mostly mediated by their paracrine effects. MSCs secrete a variety of protective bio-active factors (it is called secretome) such as cytokines, chemokines, growth factors, lipid mediators, hormones, and exosomes[22], which play important roles in the cross-talk between cells and the surrounding tissues for tissue repair and regeneration by their paracrine actions[23]. Many studies have shown that a range of bioactive factors play an important role in the modulation of the immune response. interleukin (IL)-6 and IL-10 secreted by MSCs inhibited the differentiation of macrophage into dendritic cells[24]. Therefore, MSCs secretome has received attention for concerning its potential use in tissue repair and regeneration[25]. The use of MSCs secretome as cell-free therapy may provide several advantages over direct stem cell-based therapy such as safety associated with immunocompatibility and tumorigenicity by stem cells, evaluation for dosage in a manner analogous to conventional pharmaceutical agents, storage for a long time without loss of potency, avoidance of invasive cell collection procedures, and mass production with less time and cost[26,27]. The effectiveness of MSCs secretome as therapeutic applications has been demonstrated in a variety of diseases such as colitis, gastric mucosal injury, osteoarthritis, spinal cord injury and cardiovascular disease[28-31]. It has been suggested that the therapeutic effectiveness of MSCs secretome has been attributed to the mixture of bioactive factors with their attendant paracrine activities[32]. These studies suggest that cell free therapy using the MSCs secretome could be more promising strategy than stem cell based therapy in regenerative medicine. Therefore, the purpose of this study was to investigate whether the conditioned mouse adipose-derived stem cells (ADSCs) secretome has a regenerative effect on damaged colon tissue in an acute colitis mouse model. The results showed that the conditioned mouse ADSCs (mADSCs) secretome recovered the colon tissue damaged by acute colitis in the mouse model. Moreover, the effects of the conditioned ADSCs secretome were more potent than those of ADSCs themselves in the regeneration of damaged colon tissue in acute colitis.

## MATERIALS AND METHODS

### Animals

C57BL/6 mice (female, 8-10 wk-old) were purchased from the 'KOATECH' laboratory animal company. The animals were kept in in pathogen-free facility at controlled conditions (20-23 °C, 12 h/12 h light/dark cycle, 50% humidity) with free access of



sterilized regular mouse chow and drinking water. Mice were housed 2 wk before the experiment for adaptation. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at Kosin University College of Medicine (IACUC protocol No. Kosin15-12).

### **Isolation and identification of mADSCs**

mADSCs were isolated from subcutaneous, inguinal, epididymal and mesenteric fat of mice. Briefly, animals were euthanized with using CO<sub>2</sub>. Fresh adipose tissues were washed with phosphate-buffered saline (PBS; Hyclone, Logan, UT, United States) and minced for 5 min with the sterilized fine scissors. The minced adipose tissues were digested with 0.1% collagenase type I (Invitrogen, Carlsbad, CA, United States) for 40 min at 37 °C in a shaking bath and then centrifuged at 260 × g for 7 min to obtain a pellet. The pellet was incubated in a 1:1 ratio of culture medium (StemX VIVO; R&D systems, Minneapolis, MN, United States) and DMEM/F12 (Invitrogen) along with 5% FBS (Hyclone, Logan, UT, United States) and 1 × penicillin-streptomycin (Invitrogen) overnight at 37 °C and 5% CO<sub>2</sub>. The residual non-adherent red blood cells were removed by washing with the medium after 24 h. The cells grown until 80%-90% confluence were subcultured. The medium was changed every 2 d. For all experiments, cells at passage 4 to 6 were used.

To identify the isolated mADSCs, fluorescence-activated cell sorting analysis was performed. Approximately 5 × 10<sup>5</sup> cells were incubated with the conjugated antibodies, CD29-PE, CD31-FITC, CD34-PE, CD45-FITC, CD73-PE and CD90-FITC (Beckman Coulter, Brea, CA, United States), in the dark for 30 min at room temperature. After washing with PBS twice, the cells were resuspended in PBS and analyzed using a flow cytometer (Beckman Coulter, Brea, CA, United States).

### **Preparation of mADSCs secretome**

To prepare the mADSCs secretome, mADSCs (1 × 10<sup>5</sup> cells/well) were seeded onto 6-well plates in culture medium and incubated for 24 h. And then serum-free medium with or without 1 µg/mL lipopolysaccharides (LPS; Sigma, Saint. Louis, MO, United States) replaced the culture medium of the cells. After 24 h, the supernatants were filtered using a 0.45 µm sieve and frozen in -80 °C. To concentrate the supernatants, the frozen supernatants were lyophilized using the lyophilizer (SFDSM12; SamWon Freezing Engineering Co., Busan, South Korea). The lyophilized powders were resuspended in PBS solution, transferred into Amicon Ultra-15 Centrifugal Filter Devices (3000 MWCO; Millipore, Burlington, MA, United States) and then centrifuged at 4000 × g for 50 min in 4 °C condition. The aliquots of concentrated mADSCs secretome were stored in -80 °C deep freezer until use. The conditioned mADSCs secretome stimulated with LPS were used for the animal experiment. Untreated mADSCs secretome were used as a control for the analysis of cytokine antibody array.

### **Cytokine antibody array analysis of mADSCs secretome**

To characterize the mADSCs secretome, cytokine antibody array was performed with a Proteome Profiler mouse cytokine array kit (R&D systems), following the manufacturer instructions. Briefly, the mADSCs secretome were mixed with reconstituted detection antibody cocktail and incubated at room temperature for 1 h. Then the mixture of sample/antibody was incubated with the supplied membrane overnight at 4 °C on a rocking platform. After washing the membrane in wash buffer for 10 min on a rocking platform, Streptavidin-horse radish peroxidase (HRP) was added to the membrane and incubated for 30 min at room temperature. After washing, the membrane was labeled with Chemi Reagent Mix and exposed to X-ray film. The intensity of each spot was analyzed using Gel Documentation System Software (FluorChem HD2; Alpha Innotech, Santa Clara, CA, United States). The intensity of each spot was normalized with the intensity of positive reference spot on each membrane. The normalized intensity values were used to compare the cytokine profiles in untreated mADSCs secretome and the conditioned mADSCs secretome used in this study.

### **Induction of mouse colitis and treatment**

To induce acute colitis, C57BL/6 mice (female, 8 wk old) were supplied with 2% dextran sodium sulfate (DSS; MW = 36000-50000; MP Biomedicals, Solon, OH, United States) for 7 d and then with normal drinking water for 4 d. Animals were divided into 4 groups, control (CON) (*n* = 4), normal culture medium (NM) (*n* = 6), mADSCs (SC) (*n* = 6) and conditioned mADSCs secretome (CM) (*n* = 6) groups. Animals in CON group received normal drinking water during the experimental period, and animals in

the NM, SC and CM groups received 2% DSS with intraperitoneal injection of 100  $\mu$ L normal medium solution three times (on 4, 6 and 8 d; NM group), 1  $\times$  10<sup>5</sup> cells/100  $\mu$ L ADSCs once (on 4 d; SC group) or 100  $\mu$ L mADSCs secretome three times (on days 4, 6, and 8; CM group), and then received normal drinking water *ad libitum*. Animals were monitored daily for the body weight, the appearance of diarrhea, and the presence of blood in the stool. The length of the colon was measured after sacrificing animals on 11 d.

### **Histopathological analysis**

To evaluate the histopathology of colon tissues, colons were incised longitudinally, fixed with 10% buffered formalin and embedded in paraffin. The paraffin-embedded colon tissue sections (4  $\mu$ m-thickness) were stained with hematoxylin-eosin (H&E) to evaluate the damage of colon tissues. The damage of colon tissues was determined by four histological scoring parameters including inflammation severity (score 0-3), inflammation extent (score 0-3), tissue injury (score 0-4) and crypt damage (score 0-4) as described previously[33]. The histological score was defined as the sum of the four parameter scores.

### **Quantitative real-time polymerase chain reaction assay**

To determine the expression levels of inflammatory cytokines in colon tissue, total RNA was extracted from each colon segment using Trizol reagent (Invitrogen). After assessing the quality and concentration, 3  $\mu$ g of total RNA was subjected to cDNA synthesis using the TOPscript™ cDNA synthesis Kit (Enzynomics, Daejeon, Korea). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, United States) and 500 nM of each primer as follows: IL-1b, F: 5'-GAAATGC-CACCTTTTGACAGTG-3', R: 5'-TGGATGCTCTCATCAGGACAG-3'; IL-6, F: 5'-CTGCAAGAGACTTCCATCCAG-3', R: 5'-AGTGGTATAGACAGGTCTGTTGG-3'; tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), F: 5'-CTGAAGTTCGGGGTGATCGG-3', R: 5'-GGCTTGTCACCTCGAATTTTGAGA-3'; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), F: 5'-TGGCCTTCCGTGTTCTTCTAC-3', R: 5'-GAGTTGCTGT-TGAAGTCGCA-3'. The following conditions were used: 95 °C/15 min, followed by 40 cycles of 95 °C/30 s, 60 °C/30 s, and 72 °C/30 s in the RT-PCR (7300 RT-PCR System; Applied Biosystems, Foster City, CA, United States). The levels of mRNA expression were normalized according to the internal control of the housekeeping gene GAPDH and represented as the levels of mRNA expression in NM, SC and The CM groups relative to those in CON group.

### **Enzyme-linked immunosorbent assay for serum IL-6**

To determine the concentration of serum IL-6, blood was obtained from the inferior vena cava using a 21-gauge syringe and centrifuged at 100  $\times$  g for 3 min, then serum was collected and stored at -20 °C until use. The concentration of IL-6 in serum was measured using mouse IL-6 enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go (eBiosciences, San Diego, CA, United States) according to the manufacturer's protocols. Briefly, an ELISA plate was coated with anti-mouse IL-6 and incubated overnight at 4 °C. After washing 3 times with wash buffer, the plate was blocked with diluent solution at room temperature for 1 h. Then, serum sample was added to the plate and incubated overnight at 4 °C. After washing, the plate was incubated with detection antibody at room temperature for 1 h and then with Avidin-HRP for 30 min. After incubating with TMB solution for 5 min, the reaction was stopped with stop solution. The absorbance was measured at 450 and 570 nm. The concentrations of serum IL-6 were determined with the standard curve and represented as pg/mL.

### **Statistical analyses**

Data values were expressed as mean  $\pm$  SEM for each group. Statistical analysis was performed with the Kruskal-Wallis test using SPSS version 18. Differences with  $P < 0.05$  or  $P < 0.005$  were considered to be statistically significant.

## **RESULTS**

### **Characterization of isolated mADSCs**

mADSCs were isolated from abdominal adipose tissue of 7 week-old C57BL/6 mice. To characterize the isolated mADSCs, flow cytometry analysis was performed to

validate the surface antigens of mADSCs at passage 3 or 4. The isolated mADSCs were positive for CD90, CD73, and CD29, but negative for CD45, CD34, CD31 (Figure 1A). The expression profiles of the surface antigens of mADSCs were not changed during passages 3-7 (data not shown). Therefore, mADSCs at passage 3 through 5 were used for all experiments. These results indicate that the isolated mADSCs maintain the gene expression profile specific for mADSCs during the experiment.

### ***Cytokine profiles in conditioned mADSCs secretome***

Conditioned mADSCs secretome were prepared from the conditioned medium in which mADSCs were cultured with 1 µg/mL of LPS for 24 h and compared with mADSCs secretome cultured without LPS. Cytokine profiles in mADSCs secretome and the conditioned mADSCs secretome were determined using mouse cytokine array kit containing 40 kinds of cytokine antibody (Figure 1B). 21 antibodies out of 40 antibodies were detected as strong positive spots with more than 1000 intensity in the conditioned mADSCs secretome (Table 1). The conditioned mADSCs secretome contained a high amount of colony-stimulating factors [granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage-colony stimulating factor], inflammatory chemokines [(C-X-C motif) ligand (CXCL) 1, CXCL2, carbon tetrachloride (CCL)3, CCL5, and CCL2] and inflammatory cytokines (TNF-α and IL-6). The amount of CCL4, G-CSF, CXCL2 and CXCL10 increased more than 10-folds in the conditioned mADSCs secretome compared to mADSCs secretome. These results indicate that the conditioned mADSCs secretome by the stimulation of LPS contains high amount of colony-stimulating factors, inflammatory chemokines and cytokines.

### ***Effects of the conditioned mADSCs secretome on body weight and colon length in DSS-induced mice***

To investigate the effects of conditioned mADSCs secretome in the DSS-induced colitis model of mice, mice in the NM, SC, and CM groups were treated with 2% DSS in drinking water from days 0 to 7 and then with normal drinking water thereafter until sacrifice. The NM mice and CM mice were injected intraperitoneally on days 4, 6, and 8 with normal medium and conditioned mADSCs secretome, respectively. SC mice were injected with mADSCs ( $5 \times 10^5$  cells/100 µL/mouse) through the tail vein on day 4. In all treatment groups, body weight started to decrease on day 5 (Figure 2A). The NM and SC groups kept the loss of body weights until sacrificed on day 11. However, the body weight in the CM group started to be recovered on day 7 and similar to body weight in untreated CON group on day 11. On day 11 mice were sacrificed and colon length was measured without tension from cecum to rectum (Figure 2B). Colon length in NM and SC groups significantly ( $P < 0.05$ ) decreased compared to that in the CON group, but colon length in the CM were longer than that in NM and SC groups (Figure 2C). These results indicate that the conditioned mADSCs secretome improved the recovery of body weight and colon length in DSS-induced mice.

### ***Effects of the conditioned mADSCs secretome on damage of colon tissues in DSS-induced mice***

To investigate the effects of conditioned mADSCs secretome on the damage of colon tissues in DSS-induced mice, the damage of colon tissues was assessed by histological score using H&E stained colon tissue sections. To assess the overall damage to colon tissue, macroscopic appearances of the opened whole colon tissues were examined after sacrificing the mice on day 11 (Figure 3A). Colon tissues from the NM and SC groups showed striking hyperemia and a little hyperemia, respectively. Meanwhile, colon tissues from the CM group did not show hyperemia. In H&E stained tissues (Figure 3B), the CM group showed no loss of mucosa without crypt damage whereas the NM and SC groups showed severe loss of mucosa with crypt damage. The NM and SC group also showed the significant infiltration of immune cells, but the CM group showed a little infiltration of immune cells. The damage to colon tissues was assessed using histological scores determined by the severity of inflammation, tissue injury and crypt damage (Figure 3C). Histological scores in the CM group were significantly lower than in the NM group ( $14.4 \pm 1.2$  in the NM group *vs*  $6.3 \pm 1.5$  in the CM group,  $P < 0.05$ ). These results indicate that the conditioned mADSCs secretome improved the recovery of damaged colon tissue in DSS-induced mice.

### ***Effects of the conditioned mADSCs secretome on expression of inflammatory cytokines***

The mRNA expression levels of inflammatory cytokines IL-1b, IL-6, and TNF-α were

**Table 1 Cytokine expression profiles in the secretomes of mouse adipose-derived stem cells and mouse adipose-derived stem cells stimulated with lipopolysaccharides ( $\mu\text{g/mL}$ ) for 24 h**

Cytokine	mADSCs	MADSCs w/LPS	Ratio <sup>1</sup>
CCL4	218	4516	20.8
G-CSF	218	4138	19.0
CXCL2	467	4811	10.3
CXCL10	433	4342	10.0
GM-CSF	335	3198	9.5
CCL3	629	4986	7.9
TNF- $\alpha$	836	5863	7.0
IL-1ra	515	3610	7.0
CXCL1	1583	6263	4.0
CCL1	307	1178	3.8
CCL5	1137	4194	3.7
CD54	243	796	3.3
CXCL9	399	1180	3.0
M-CSF	1530	3147	2.1
IL-6	3197	4642	1.5
IFN- $\gamma$	512	559	1.1
CXCL12	4711	4798	1.0
TIMP-1	3735	3553	1.0
CCL2	5228	3646	0.7
IL-1 $\alpha$	852	482	0.6

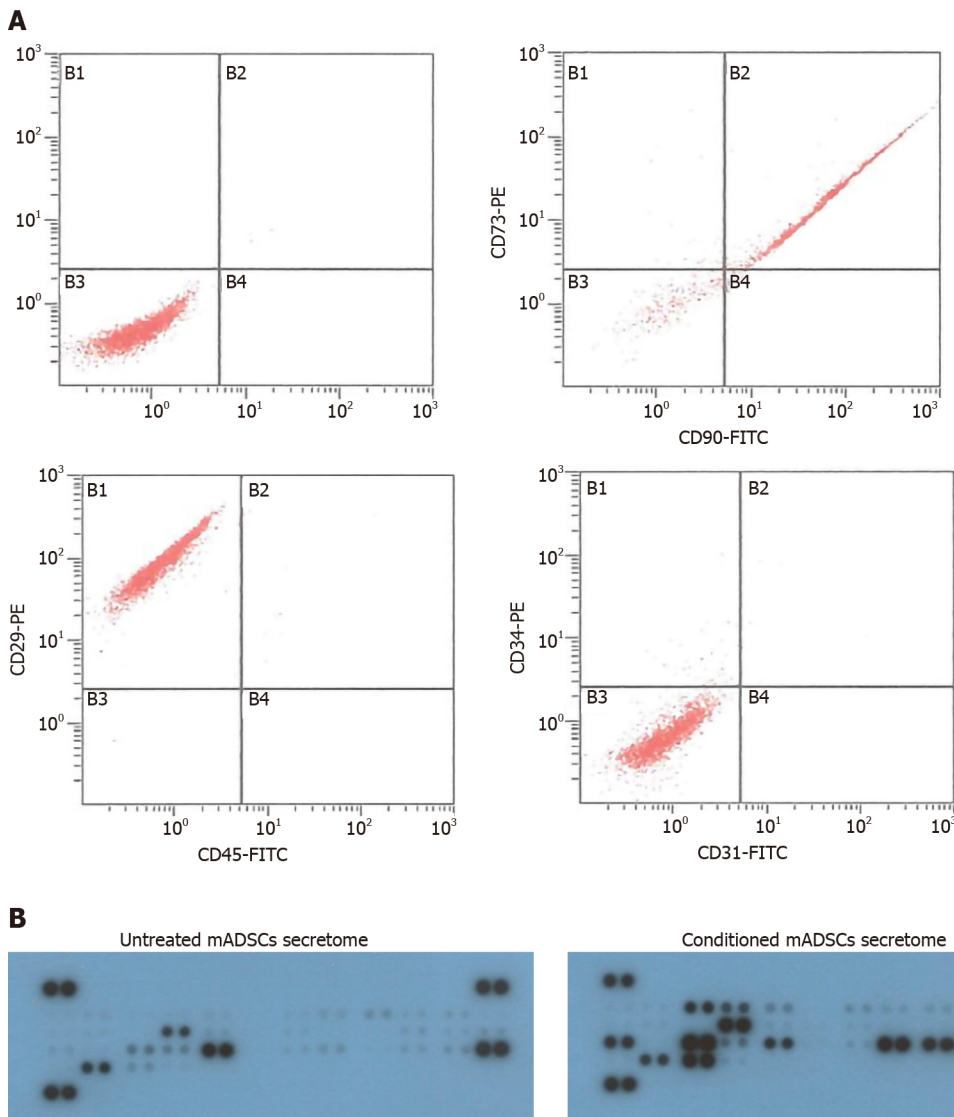
<sup>1</sup>Ratio represents the intensity value of mouse adipose-derived stem cells (mADSCs) w/lipopolysaccharides relative to the intensity value of mADSCs used as a control. mADSCs: Mouse adipose-derived stem cells; LPS: Lipopolysaccharides; CCL: Carbon tetrachloride; G-CSF: Granulocyte colony-stimulating factor; CXCL: (C-X-C motif) ligand; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; IL: Interleukin; M-CSF: Macrophage-colony stimulating factor; IFN- $\gamma$ : Interferon- $\gamma$ ; TIMP-1: Tissue inhibitor of metalloproteinase 1.

evaluated in colon tissues of DSS-induced mice on day 11 after treatment using qRT-PCR (Figure 4). The expression levels of mRNAs in all groups were expressed as the relative levels compared to the CON group. The expression of IL-1b mRNAs increased in the NM group, SC group and CM group compared to the CON group. The expression of IL-1b mRNA in the CM group was significantly lower ( $P < 0.05$ ) than in the SC group. The expression of IL-6 mRNA increased in the NM group and SC group. The expression levels of IL-6 in the CM group were significantly lower than in both of the NM group ( $P < 0.005$ ) and SC group ( $P < 0.05$ ). The expression level of TNF- $\alpha$  mRNAs was significantly higher in the SC group than in the CON group ( $P < 0.005$ ) and the CM group ( $P < 0.05$ ). However, the expression level of TNF- $\alpha$  mRNAs in the NM and CM groups did not differ from that in the CON group. These results indicate that the conditioned mADSCs secretome suppressed the upregulation of IL-6 mRNA expression in damaged colon tissue of DSS-induced mice.

#### **Effects of the conditioned mADSCs secretome on serum IL-6 concentration of DSS-induced mice**

To investigate the effects of conditioned mADSCs secretome on serum IL-6 concentration of DSS-induced mice, the DSS-induced mice were sacrificed on 11 d after DSS treatment. The concentration of IL-6 protein in serum was determined by ELISA (Figure 5). The concentration of serum IL-6 in DSS-induced mice was significantly higher in NM group (207.40 pg/mL) than in the SC group (38.01 pg/mL,  $P < 0.05$ ) and the CM group (5.94 pg/mL,  $P < 0.05$ ). The concentration of serum IL-6 in the CM group was not much different from that in the CON group compared to the NM group and SC group. These results indicated that the conditioned mADSCs secretome





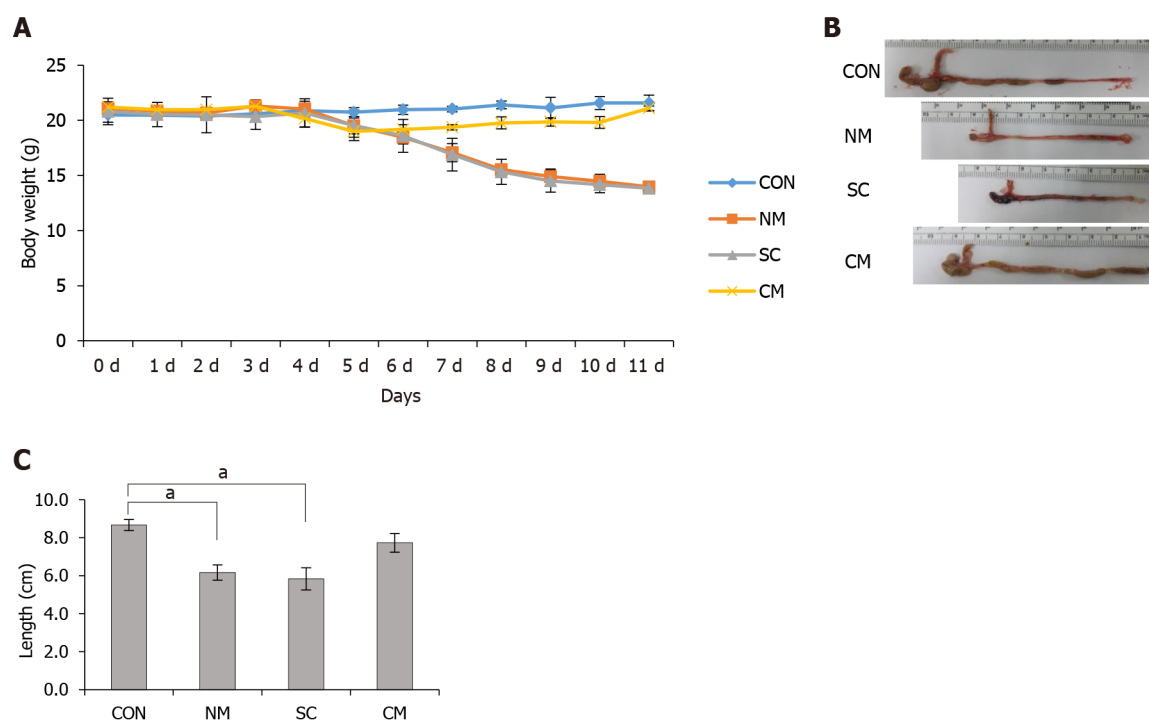
**Figure 1** Characterization of mouse adipose-derived stem cells and secretome of mouse adipose-derived stem cells. A: Flow cytometric analysis of surface antigens of mouse adipose-derived stem cells (mADSCs) isolated from C57BL/6 mice. They were positive for Crohn's disease (CD)90, CD73 and CD29, and negative for CD45 and CD34, and CD31; B: Cytokine antibody arrays for secretome of mADSCs. Untreated mADSCs secretome was isolated from mADSCs without lipopolysaccharides (LPS) treatment, and conditioned mADSCs secretome was isolated from mADSCs cultured with LPS (1  $\mu$ g/mL) for 24 h. Cell culture supernatants were subjected to cytokine antibody array. mADSCs: Mouse adipose-derived stem cells; CD: Crohn's disease.

suppressed the elevation of serum IL-6 concentration in DSS-induced mice.

## DISCUSSION

ADSCs have emerged as a promising therapeutic tool for tissue inflammation and injury. The majority of clinical trials for MSC therapy have used ADSCs than other type of MSCs because of their availability, less invasiveness and cell yield from adipose tissue[34,35]. Although the regenerative effects of ADSCs may be the result of their ability to migrate and engraft into injured sites, their immune-modulatory activity is also mediated by soluble paracrine factors secreted from ADSCs. However, it remains unclear whether the therapeutic efficacy of soluble paracrine factors is better than ADSCs themselves in inflammation-related disease. This study showed that the conditioned ADSCs secretome had more potent therapeutic effects than ADSCs themselves in the regeneration of damaged colon tissue in acute colitis through the suppression of IL-6 production.

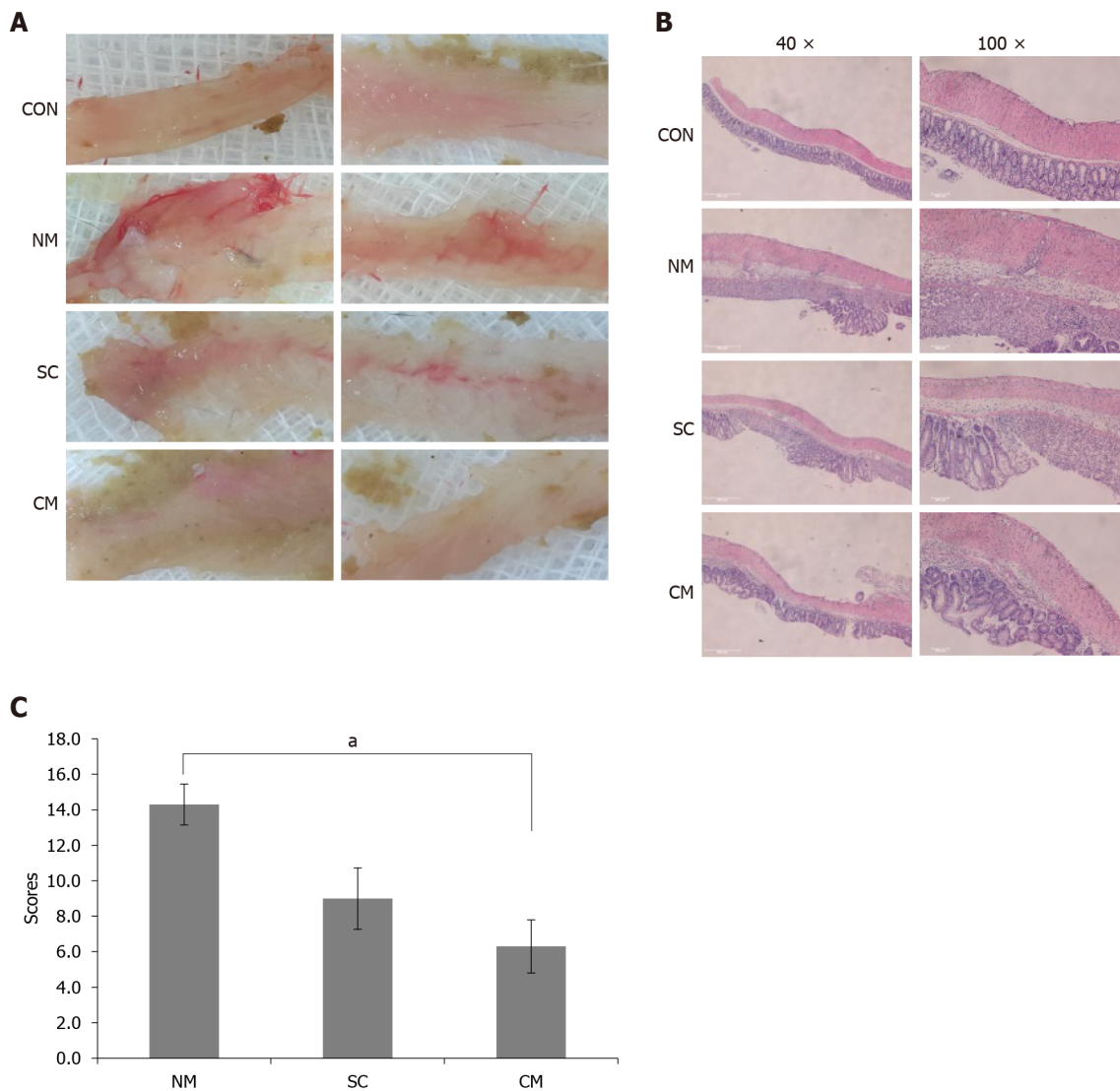
In this study, mADSCs isolated from abdominal adipose tissue of 7 w-old C57BL/6 mice were used for the transplantation and preparation of conditioned mADSCs secretome. The mADSCs expressed the stromal-associated markers (CD90, CD73 and



**Figure 2** Effects of conditioned mouse adipose-derived stem cells secretome on body weight and colon length in dextran sulfate sodium-induced mice. A: The treatment of Conditioned mouse adipose-derived stem cells (mADSCs) secretome (CM) ameliorated the loss of body weight of dextran sulfate sodium-induced mice. Normal culture medium or CM was injected intraperitoneally into mice on days 4, 6 and 8. mADSCs was injected *via* tail vein on day 4. Mice were sacrificed on day 11; B and C: The treatment of CM ameliorated the shortening of colon length. Values are mean  $\pm$  SEM ( $n = 4-6$  mice per group). <sup>a</sup> $P < 0.05$ . CON: Control; NM: Normal culture medium; SC: Mouse adipose-derived stem cells (mADSCs); CM: Conditioned mADSCs secretome.

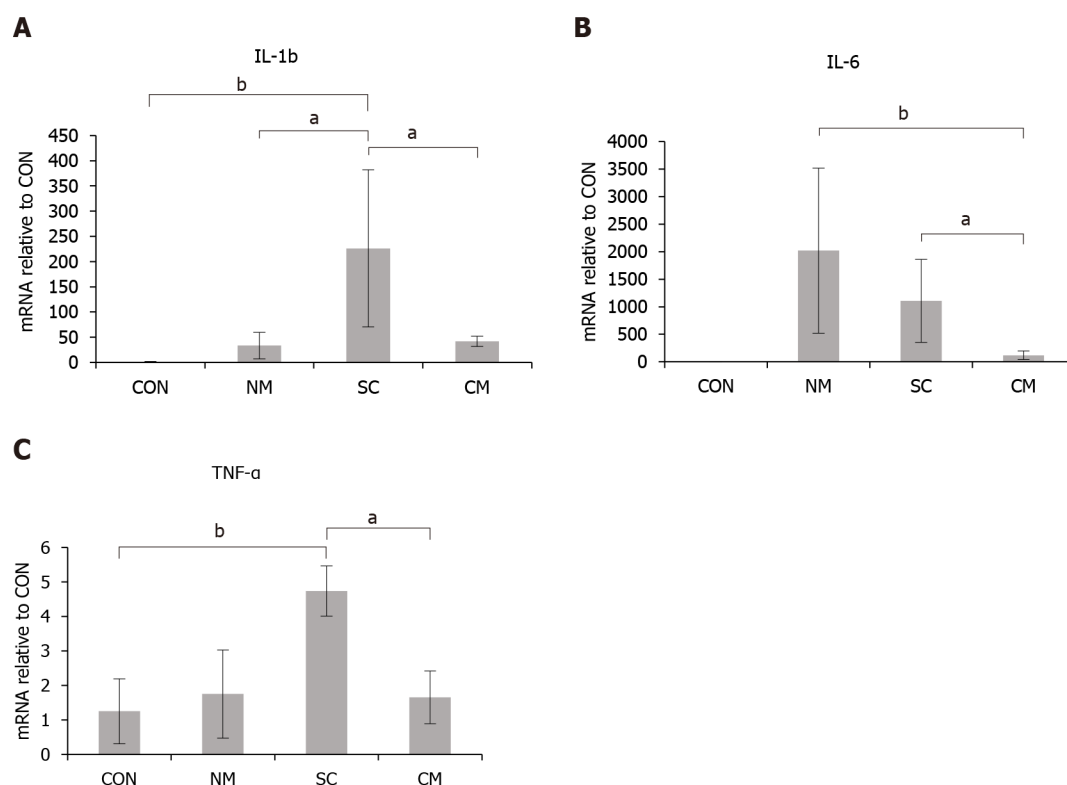
CD29) known as positive markers of mADSCs, and did not expressed hematopoietic markers (CD34 and CD45) or endothelial cell marker (CD31), which are known as negative markers for mADSCs. The results of this study are consistent with the result of the previous study[36], which confirms the characteristics of the isolated mADSCs. It has been shown that ADSCs secretome consists of the growth factors for wound healing, cytokines for modulation of immune system, and chemokines for cell migration and engraftment[26,37]. A variety of culture conditions for ADSC culture affects the compositions of ADSCs secretome, which may influence the biological functions for tissue repair and regeneration[38]. It has been reported that the MSC secretome contains pro-inflammatory cytokines (IL-1b, IL-6, IL-8 and IL-9) as well as anti-inflammatory cytokines (IL-10, IL-13, IL-17 and IL-1ra), and the balance between these pro-inflammatory and anti-inflammatory cytokines may influence the final effect [26]. Moreover, the exposure of human ADSCs to LPS for 24 h increase the secretion of hematopoietic factors (G-CSF, GM-CSF and M-CSF) and the pro-inflammatory cytokines (IL-6, IL-8, IL-11 and TNF- $\alpha$ )[39]. In this study, we treated mADSCs with 1  $\mu$ g/mL LPS to prepare the conditioned mADSCs secretome. The exposure of mADSCs to LPS for 24 h increased the secretion of pro-inflammatory cytokines (IL-6, IL-17 and TNF- $\alpha$ ), anti-inflammatory cytokines (IL-1ra and IL-5), hematopoietic factors (G-CSF, GM-CSF and M-CSF), cell recruitment-related chemokines (CCL1, CCL3, CCL4 and CCL5) and inflammatory-related chemokines (CXCL1 and CXCL2), which is consistent with the previous studies[37,39]. Taken together, these results show that the conditioned mADSCs secretome used in this study contains various components involved in the immunomodulation and regeneration, which suggests that the conditioned mADSCs secretome may display more efficient regenerative effects in the recovery of damaged tissues.

Many studies have shown the therapeutic potent of ADSCs in several inflammatory diseases such as acute colitis, thyroiditis, and arthritis[36,40,41]. The injection of mADSCs reduced the histopathologic severity of colitis, body weight loss, diarrhea and inflammation in trinitrobenzene sulfonic acid (TNBS)- or DSS-induced colitis mouse models[42]. However, the clinical results of ADSCs therapy for inflammatory diseases are inconsistent, which may be due to the different cell sources and cell preparation methods[43,44]. Moreover, MSCs therapy requires a large number of cells and is influenced by uncertain factors such as administration routes and culture

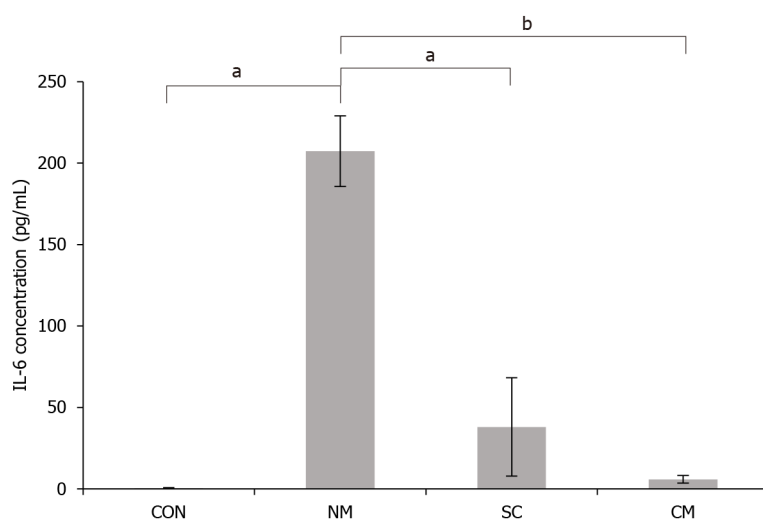


**Figure 3** Effects of conditioned mouse adipose-derived stem cells secretome on damage of colon tissues in dextran sulfate sodium-induced mice. A: Macroscopic appearance of colon tissues; B: Representative hematoxylin and eosin stained sections of damaged colon tissues are shown on magnification, 40 × or 100 ×; C: The damage of colon tissues was assessed by histological score. Values are mean ± SEM ( $n = 4-6$  mice per group). <sup>a</sup> $P < 0.05$ . CON: Control; NM: Normal culture medium; SC: Mouse adipose-derived stem cells (mADSCs); CM: Conditioned mADSCs secretome.

conditions, which indicates that there is a difficulty in standardizing MSCs therapy protocols. Therefore, recent studies have focused on the secretome from MSCs as an alternative therapeutic option for tissue regeneration[26]. In this study, we compared the therapeutic effects of conditioned mADSCs secretome with mADSCs in DSS-induced acute colitis mouse model. The injection of conditioned mADSCs secretome intraperitoneally into mice significantly improved the recovery of body weight, colon length and histopathological scores, but the intraperitoneal injection of mADSCs did not show any improvement in the recovery of mice. In a previous study, the intraperitoneal injection of mADSCs recovered body weight and significantly reduced histopathological signs in mice with TNBS-induced colitis[42]. Another study, however, showed that the intraperitoneal injection of mADSC did not improve the recovery of body weight, colon length and histological score in mice with DSS-induced colitis[45]. The inconsistent results in the effect of mADSCs on acute colitis may result from the different ADSCs injection protocols including variation in cell dose, injection timing and cell preparation method. However, our results showing the achievement of significant improvement by the treatment of mADSCs secretome in DSS-induced colitis is consistent with the results of a previous study reporting a reduction in DSS-induced colitis by the intraperitoneal injection of umbilical cord-derived MSC extract [46]. Therefore, the present study indicates that mADSCs secretome might be a more efficient and manageable therapeutic tool in regenerative medicine than the direct injection of mADSCs themselves.



**Figure 4** Effects of the conditioned mouse adipose-derived stem cells secretome on the expression of inflammatory cytokines in colon tissues of dextran sulfate sodium-induced mice on day 11. The mRNA expression levels of inflammatory cytokines interleukin (IL)-1b, IL-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were evaluated by quantitative real-time polymerase chain reaction analysis. The mRNA expression levels in normal culture medium, mouse adipose-derived stem cells (mADSCs) or conditioned mADSCs secretome were represented as the relative values to the expression levels in control. Values are mean  $\pm$  SEM ( $n = 4-6$  mice per group). A: IL-1b; B: IL-6; C: TNF- $\alpha$ . <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.005$ . CON: Control; NM: Normal culture medium; SC: Mouse adipose-derived stem cells (mADSCs); CM: Conditioned mADSCs secretome; IL: Interleukin; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ .



**Figure 5** Effects of conditioned mouse adipose-derived stem cells secretome on the concentration of interleukin-6 in serum of dextran sulfate sodium-induced mice on day 11. The concentration of interleukin-6 in serum was evaluated by enzyme-linked immunosorbent assay. Values are mean  $\pm$  SEM ( $n = 4-6$  mice per group). <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.005$ . CON: Control; NM: Normal culture medium; SC: Mouse adipose-derived stem cells (mADSCs); CM: Conditioned mADSCs secretome; IL-6: Interleukin-6.

Cytokines are key regulators in the intestinal immune response including inflammation. It has been known that IL-1b, IL-6 and TNF- $\alpha$  are important pro-inflammatory cytokines promoting inflammation in the innate immune response[47]. Previous studies have reported that ADSC or BMSC decreased the expression of IL-1b, IL-6 and TNF- $\alpha$  mRNA in colon tissues of mice treated with TNBS or DSS in comparison with



untreated mice[41,48]. In our results, mADSC treatment decreased the expression of IL-6 mRNA in colon tissue, but did not decrease the expression of IL-1b and TNF- $\alpha$  mRNA in colon tissue of DSS-treated mice. However, treatment with mADSC secretome decreased the expression of IL-1b, IL-6 and TNF- $\alpha$  mRNAs in colon tissue of DSS-treated mice. Moreover, the serum levels of IL-6 protein were much less with treatment of the conditioned mADSC secretome than with mADSCs themselves. Our results indicate that treatment using conditioned mADSCs secretome is more effective in suppressing inflammation than in treatment with mADSCs themselves, which is mediated through the inhibition of pro-inflammatory cytokine synthesis in colon tissue as well as the reduction of serum IL-6 levels.

## CONCLUSION

In conclusion, conditioned mADSCs secretome could inhibit the synthesis of pro-inflammatory cytokines in colon tissue and reduce serum IL-6 levels more effectively than mADSCs themselves. This effective suppression of proinflammatory cytokines in colon tissue and serum might mainly contributes to the recovery of damaged colon tissue in a DSS-induced acute colitis model. Further studies are needed to identify the factors in conditioned mADSCs secretome involved in the suppression of pro-inflammatory cytokines in damaged colon tissue.

## ARTICLE HIGHLIGHTS

### Research background

Inflammatory bowel diseases (IBD) causing chronic and destructive inflammation of the gastrointestinal tract is mainly related to uncontrolled immune response. Current treatment for IBD using immunosuppressive agents is not successful for significant improvement in remission rates. It is necessary to develop effective, feasible and safe therapeutic strategy for IBD. Stem cells having the ability to regulate immune response have emerged as attractive therapeutic tools for incurable IBD.

### Research motivation

Mesenchymal stem cells (MSCs) including adipose-derived stem cells (ADSCs) has been known as a promising therapeutic for IBD. However, the transplantation of MACs in IBD patients showed inconsistent results because of their distinct differentiation and regenerative potential, and the variety of protocols for treatment. In addition, the therapeutic ability of MSCs is mostly mediated by their secretome including cytokines, chemokines, growth factor, and hormones. Therefore, it has been suggested that the MSCs secretome may have therapeutic potential for IBD treatment.

### Research objectives

Although the immune-modulatory activity of ADSC is mediated by soluble paracrine factors, it is still unclear whether the therapeutic efficacy of soluble factors secreted from ADSCs is better than ADSCs themselves in inflammation-related diseases. Therefore, the purpose of this study was to investigate the effects of conditioned mouse ADSCs (mADSCs) secretome on colitis-induced mice.

### Research methods

mADSCs were isolated from C57BL/6 mice (female, 8-10 wk-old) and identified using fluorescence-activated cell sorting. The conditioned mADSCs secretome was obtained by culturing mADSCs in serum-free medium with lipopolysaccharide (1  $\mu$ g/mL) for 24 h, and characterized using a Proteome Profiler mouse cytokine array kit. For induction of mouse acute colitis, mice (C57BL/6, female, 8-wk-old) were supplied with 2% dextran sodium sulfate for 7 d and then normal drinking water for 4 d. Animals were divided into 4 groups, control (CON) group receiving normal drinking water during the experimental period, normal culture medium (NM) group receiving 100  $\mu$ L normal culture medium three times (on 4, 6 and 8 d), mADSCs (SC) group receiving  $1 \times 10^5$  cells/100  $\mu$ L ADSCs once (on 4 d) and conditioned mADSCs secretome (CM) group receiving 100  $\mu$ L mADSCs secretome three times (on days 4, 6, and 8). The length of the colon and histopathology of colon tissues were evaluated after sacrificing animals on 11 d. The mRNA expression levels of inflammatory cytokines in colon

tissue were determined by quantitative real-time polymerase chain reaction assay, and the serum interleukin (IL)-6 levels were determined by enzyme-linked immunosorbent assay.

### Research results

The isolated mADSCs maintained the mADSCs specific gene expression profiles during experiment. The conditioned mADSCs secretome obtained by culturing mADSCs with 1 µg/mL of lipopolysaccharides for 24 h contained high amounts of colony-stimulating factors, inflammatory chemokines and cytokines. The conditioned mADSCs secretome in dextran sulfate sodium (DSS)-induced mice ameliorated the loss of body weight and reduction of colon length, and improved the recovery of damaged colon tissue. The expression of IL-1b mRNA and IL-6 mRNA in colon tissues was significantly lower ( $P < 0.05$ ) in the CM group than in the SC group ( $P < 0.05$ ) and/or NM group ( $P < 0.005$ ). However, the expression level of tumor necrosis factor- $\alpha$  mRNAs in the NM and CM groups did not differ from that in the CON group. The concentration of serum IL-6 in DSS-induced mice was significantly lower in the CM group (5.94 pg/mL,  $P < 0.005$ ) and the SC group (38.01 pg/mL,  $P < 0.05$ ) than in NM group (207.40 pg/mL), which indicates that the conditioned mADSCs secretome suppressed the elevation of serum IL-6 concentration in DSS-induced mice.

### Research conclusions

This study suggests that conditioned mADSCs secretome may inhibit the synthesis of pro-inflammatory cytokines in the damaged colon tissue, and reduce serum IL-6 Levels more effectively than mADSCs themselves in DSS-induced mice. This effective suppression of proinflammatory cytokines in colon tissue and serum might mainly contributes to the recovery of damaged colon tissue in DSS-induced mice, which suggests the therapeutic potential of the conditioned mADSCs secretome for IBD treatment.

### Research perspectives

The conditioned mADSCs secretome may serve as a novel therapeutic tool for IBD. Therefore, it is needed to investigate the factors in conditioned mADSCs secretome involved in the effective regeneration of damaged colon tissue.

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## Case Control Study

# Pancreatic enzymes and abdominal adipose tissue distribution in new-onset prediabetes/diabetes after acute pancreatitis

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**Institutional review board statement:** The study was approved by the Health and Disability Ethics Committee (New Zealand).

**Informed consent statement:** All study participants or their legal guardians provided informed written consent about personal and medical data collection prior to study enrolment.

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**Data sharing statement:** No additional data are available.

**STROBE statement:** The authors have read the STROBE Statement – checklist of items, and

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## Abstract

### BACKGROUND

New-onset prediabetes/diabetes after acute pancreatitis (NODAP) is the most common sequela of pancreatitis, and it differs from type 2 prediabetes/diabetes mellitus (T2DM).

### AIM

To study the associations between circulating levels of pancreatic amylase, pancreatic lipase, chymotrypsin and fat phenotypes in NODAP, T2DM, and health.

### METHODS

Individuals with NODAP ( $n = 30$ ), T2DM ( $n = 30$ ), and sex-matched healthy individuals ( $n = 30$ ) were included. Five fat phenotypes (intra-pancreatic fat, liver fat, skeletal muscle fat, visceral fat, and subcutaneous fat) were determined using the same magnetic resonance imaging protocol and scanner magnet strength for all participants. One-way analysis of covariance, linear regression analysis, and relative importance analysis were conducted.

### RESULTS

Intra-pancreatic fat deposition (IPFD) was higher in NODAP ( $9.4\% \pm 1.8\%$ ) and T2DM ( $9.8\% \pm 1.1\%$ ) compared with healthy controls ( $7.8\% \pm 1.9\%$ ) after adjusting for covariates ( $P = 0.003$ ). Similar findings were observed in regards to visceral fat volume ( $P = 0.005$ ), but not subcutaneous fat volume, liver fat, or skeletal muscle fat. Both IPFD ( $\beta = -2.201$ ,  $P = 0.023$ ) and visceral fat volume ( $\beta = -0.004$ ,  $P = 0.028$ ) were significantly associated with circulating levels of pancreatic amylase in NODAP, but not in T2DM or healthy individuals. Of the five fat phenotypes, IPFD explained the highest amount of variance in pancreatic amylase concentration ( $R^2 = 15.3\%$  out of  $41.2\%$ ). None of the phenotypes contributed meaningfully to the variance in pancreatic lipase or chymotrypsin.

the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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## CONCLUSION

Both NODAP and T2DM are characterized by increased IPFD and visceral fat volume. However, only NODAP is characterized by significant inverse associations between the two fat phenotypes and pancreatic amylase.

**Key Words:** Amylase; Lipase; Chymotrypsin; Pancreatitis; Diabetes; Intra-pancreatic fat; Visceral fat; Liver fat

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**Core Tip:** Intra-pancreatic fat deposition and visceral fat volume are significantly inversely associated with circulating levels of pancreatic amylase in individuals with new-onset prediabetes/diabetes after acute pancreatitis, but not in healthy individuals or those with type 2 prediabetes/diabetes mellitus.

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## INTRODUCTION

Individuals after acute pancreatitis often develop metabolic sequelae such as post-pancreatitis diabetes, which accounts for 80% of cases of diabetes of the exocrine pancreas—the second most common type of adult-onset diabetes[1,2]. There is a circulating biomarker, involved in the regulation of exocrine pancreatic function, that distinguishes post-pancreatitis diabetes from type 2 diabetes[3]. Also, epidemiological data have shown that post-pancreatitis diabetes leads to worse clinical outcomes compared with type 2 diabetes. A population-based study found that individuals with post-pancreatitis diabetes were more likely to have poor glycemic control and to require more insulin than individuals with type 2 diabetes[4]. Another population-based study demonstrated that individuals with post-pancreatic diabetes (versus type 2 diabetes) were at a higher risk of mortality from cancer, gastrointestinal diseases, and infectious diseases, as well as hospitalization for chronic pulmonary disease, renal disease, and infectious disease[1]. The reasons for the above differences between post-pancreatic diabetes and type 2 diabetes are not fully understood but are worth investigating with a view to optimizing the management of both types of diabetes.

Excess deposition of body fat increases the risk of diabetes and has a deleterious effect on its clinical outcomes[5-9]. However, little evidence exists on the difference in excess body fat between post-pancreatitis diabetes and type 2 diabetes. A 2017 population-based study of people with new-onset diabetes showed that the proportion of individuals with obesity was higher in type 2 diabetes (48%) *vs* diabetes of the exocrine pancreas (35%)[4]. A 2020 population-based study of individuals with a history of clinically resolved acute pancreatitis demonstrated that the risk of new-onset diabetes was higher among individuals with normal body mass index (BMI) than those in the overall cohort (adjusted odds ratios of 3.1 and 2.1, correspondingly)[10]. BMI is a commonly used proxy for general adiposity but it may be suboptimal in quantifying excess abdominal fat[1,2]. Given that the effect of excess abdominal fat on metabolic functions depends on not only the degree of fat deposition but also its distribution[9,11-13], data on various abdominal fat phenotypes [determined with the use of magnetic resonance imaging (MRI)] are likely to provide useful insights[14-17]. For example, intra-pancreatic fat deposition (IPFD) and visceral fat volume were significantly higher in individuals with post-pancreatitis diabetes compared with healthy individuals[1]. Also, individuals with type 2 diabetes had a significantly higher IPFD compared with healthy individuals[18]. However, to date, no study has compared head-to-head abdominal fat phenotypes in post-pancreatitis diabetes *vs* type 2 diabetes. Also, individuals with metabolic disorders (including type 2 diabetes and obesity) not infrequently have within-normal but significantly lower circulating levels

of pancreatic enzymes (amylase, lipase, and trypsin) compared with healthy individuals[19], and exocrine pancreatic dysfunction often develops after acute pancreatitis[2]. Hence, it is conceivable that the relationship between abdominal adipose tissue distribution and pancreatic enzymes may differ in post-pancreatitis diabetes *vs* type 2 diabetes.

The primary aim was to compare the differences in MRI-derived abdominal fat phenotypes between healthy individuals and the two types of diabetes. The secondary aim was to investigate the associations between abdominal fat phenotypes and circulating levels of pancreatic enzymes in the study groups.

## MATERIALS AND METHODS

### Study design

The present case-control study was nested into prospective cohort study of individuals after an attack of acute pancreatitis (ARIES project) and was approved by the Health and Disability Ethics Committee (13/STH/182) of New Zealand. Individuals with new-onset prediabetes or diabetes after acute pancreatitis (NODAP) were randomly selected and 1:1 matched on sex with individuals with type 2 prediabetes or diabetes mellitus (T2DM) from the same cohort. Individuals with fasting plasma glucose (FPG)  $\geq 100$  mg/dL ( $\geq 5.6$  mmol/L) and/or glycated hemoglobin A1c (HbA1c)  $\geq 5.7\%$  (39 mmol/mol) beyond three months after an attack of acute pancreatitis constituted the NODAP group, in line with the published recommendations[1]. Individuals with HbA1c  $\geq 5.7\%$  (39 mmol/mol) before, during hospitalization for AP, or within three months after it constituted the T2DM group. FPG  $\geq 100$  mg/dL ( $\geq 5.6$  mmol/L) during hospitalization was not considered as an eligibility criterion for the T2DM group due to the possibility of stress-induced hyperglycemia during acute illness[1]. All cases were at least 18 years old, provided informed consent, had a primary diagnosis of mild acute pancreatitis established prospectively at the time of hospitalization, and met the American Diabetes Association criteria for prediabetes or diabetes[6].

Individuals were excluded from the study if they had a recurrent attack of acute pancreatitis within three months of the enrollment date, chronic pancreatitis, post-endoscopic retrograde cholangiopancreatography pancreatitis, pancreatic cyst, pancreatic lipomatosis or lipomatous pseudohypertrophy, congenital anomalies of the pancreas, hereditary pancreatitis, cystic fibrosis, malignancy, cognitive disability, received surgical, endoscopic or radiological interventions involving the pancreas, had metallic foreign body implantations, heart pacemakers, or other electronic device implantations, received steroid therapy, or were pregnant.

The control group included healthy volunteers who were 1:1 matched on sex with the two case groups. These participants were at least 18 years old, provided informed consent, had no personal and family history of diseases of the exocrine pancreas and diabetes, had no family history of cystic fibrosis or coeliac diseases, had no upper abdominal symptoms in the 12 mo preceding the study, had no history or evaluation for infectious or inflammatory diseases in the 6 mo preceding the study, and had no history of cancer.

### Quantification of fat phenotypes

**Imaging protocol:** Abdominal MRI for all participants was performed at the Centre of Advanced MRI (The University of Auckland) using 3.0 Tesla MAGNETOM Skyra scanner (Siemens, Erlangen, Germany). All participants underwent MRI wholly and exclusively for the purpose of the ARIES project. During the MRI, participants lied down in supine position and were asked to hold their breath during end-expiration. Axial T1-weighted volumetric interpolated breath-hold examination Dixon sequence was applied with the following parameters: true form abdomen shim mode; field of view, 420 mm; base resolution, 320; echo time, 1.27 ms, 2.5 ms; repetition time, 3.85 ms; flip angle, 9; pixel bandwidth, 920 Hz; slice thickness, 5 mm. All but liver fat phenotypes were quantified independently by two observers and average values of two independent MRI measurements were used for statistical analyses. The observers were blinded to the group allocation.

**Intra-pancreatic fat:** Intra-pancreatic fat was quantified using the 'MR-opsy' technique, as described in detail elsewhere[20]. In brief, two candidate slices with clear visualization of the pancreas were selected from a series of abdominal scans. Three regions of interest were placed in the head, body, and tail region of the pancreas for quantification of IPFD. Further, to prevent possible inclusion of non-parenchymal tissues

within the selected area of interest, a thresholding range of 1%-20% was applied, as recommended[21]. The intra-pancreatic fat percentage was calculated as the average pancreatic fat fraction of both slices.

**Liver fat:** Single-voxel spectroscopy was used to quantify liver fat. A voxel (20 mm × 20 mm × 20 mm) was placed in the right lobe of the liver, away from the blood vessels and bile ducts and at least 10 mm away from the edge. Automated shimming was performed prior to signal acquisition to improve B0 homogeneity. Spectra were acquired using a free-breathing navigator-triggered spin echo acquisition with repetition time 3000 ms, echo time 33 ms, 50 averages. Acquisition duration was 853 ms. Both water-suppressed and non-water-suppressed spectra were taken, with the non-water-suppressed spectrum acting as a reference for liver fat quantification. Spectra were processed and analyzed using SIVIC software (University of California-San Francisco, California, United States)[22]. The magnetic resonance spectroscopy fat fraction was defined as fat fraction = area under fat peak/area under fat and water peaks × 100%.

**Skeletal muscle fat:** Total muscle area and intra-muscular fat area of erector spinae muscles were measured using a single axial slice at the lower endplate of L3 vertebra, as it had been demonstrated that the L3 level is optimal for determination of skeletal muscle fat[23]. The free-hand tool of ImageJ software (National Institutes of Health, United States) was used to outline the left and right erector spinae muscles followed by measurement of total pixel content[23,24]. Further, to calculate the intra-muscular fat area, the threshold-function of ImageJ was used to convert grayscale pixels into binary images, using global histogram-derived method. Care was taken not to include extra-muscular fat (*i.e.*, beyond the fascial layer of the erector spinae muscles). Total muscle area and intra-muscular fat area were calculated by multiplying the selected total pixel content with pixel surface area. The ratio of fat-free cross-sectional muscle area to total cross-sectional muscle area was determined by subtracting intra-muscular fat area from the total muscle area and dividing this value by the total muscle area. Skeletal muscle fat percentage was defined as (1-fat-free cross-sectional muscle area to total cross-sectional muscle area ratio) × 100%.

**Subcutaneous and visceral fat:** Visceral fat volume and subcutaneous fat volume were quantified manually using ImageJ software. Identical fat-phase images (L2-L5) from the selected series were used for segmentation of visceral and subcutaneous fat compartments[25]. The threshold-function of ImageJ was used to convert grayscale pixels into binary images, using the global histogram-derived method[24]. Using the free-hand tool, visceral and subcutaneous fat regions were delineated from the abdominal musculature and measured separately. The non-adipose tissue, soft organs, and blood vessels were excluded from the measurement of visceral fat. The final step for all the above measurements involved summation of the pixel contents of all the slices in series and multiplied by the pixel area and slice thickness to obtain the total volume.

### **Measurement of pancreatic enzymes**

Venous blood samples were obtained from each participant after at least 8 h of fasting to assess pancreatic enzymes. These blood samples were centrifuged 4000 g for 5.5 min and plasma was separated into aliquots and stored at -80 °C until further use. The active form of pancreatic amylase was measured in plasma using the Reflotron® Plus reflectance photometer (Roche®, Basel, Switzerland) and results were expressed in U/L. The active forms of pancreatic lipase and chymotrypsin were measured using sandwich enzyme-linked immunosorbent assay (ELISA) kits. Pancreatic lipase was measured using the Cloud-Clone Corporation ELISA kit (Houston, Texas, United States) and results were expressed in ng/mL. The intra- and inter-assay variations of the assay were < 10% and < 12%, respectively. Chymotrypsin concentration was measured using the Cusabio ELISA kit (Wuhan, Hubei Province, China) and results were expressed in ng/mL. The intra- and inter-assay variations of the assay were < 8% and < 10%, respectively. Absorbance was detected at 450 nm. Concentrations in each sample were estimated using a standard curve.

### **Covariates**

Anthropometric data (height, weight, and waist circumference) of all study participants were recorded to calculate BMI and waist-height ratio. All measurements were taken over the light clothing of participants, and height and weight were measured in a standing position without shoes and headgear. Waist circumference



was measured at the level of the umbilicus. Blood samples for lipids (triglycerides, total cholesterol, high-density lipoproteins cholesterol, and low-density lipoprotein cholesterol) were measured at LabPlus-a tertiary referral medical laboratory at Auckland City Hospital. The same laboratory measured HbA1c, using the boronate affinity chromatography assay (Trinity Biotech, Wicklow, Ireland) that is certified by the National Glycohaemoglobin Standardisation Program and standardized to the Diabetes Control and Complications Trial reference assay. Fasting insulin was measured using chemiluminescence sandwich immunoassay (Roche Diagnostics, Auckland, New Zealand).

A standardized questionnaire was administered at the time of the study. For information on the use of antidiabetic medications, participants were asked, 'Are you currently on antidiabetic medication?'. If the answer was 'yes', they were classified as antidiabetic medication user, otherwise they were classified as non-user. For information on smoking status, participants were asked, 'Have you ever smoked cigarettes?'. If the answer was 'yes', they were classified as ever-smokers, otherwise, they were classified as never-smokers[26]. For information on alcohol consumption, participants were asked, 'On average, how much alcohol do you consume in a week?'. A reference diagram for drink volumes *per* unit was provided. The response to this question was presented as grams *per* week and used to determine the average amount of alcohol consumption[26].

### Statistical analysis

All analyses were performed using SPSS for Windows Version 25 (SPSS Inc., Illinois, United States). A two-sided  $P < 0.05$  was deemed to be statistically significant. The mean and standard deviation of the five studied abdominal fat phenotypes (*i.e.*, intra-pancreatic fat, liver fat, skeletal muscle fat, visceral fat, and subcutaneous fat) in the three groups (NODAP, T2DM, and healthy controls) were compared using one-way analysis of variance (ANOVA).

To examine the differences in the five studied abdominal fat phenotypes between the three groups, one-way ANOVA and one-way analysis of covariance (ANCOVA) were conducted. ANCOVA enabled the reduction of within-group variance while adjusting for covariates. The following four models were constructed: (1) Unadjusted; (2) Adjusted for age and sex; (3) Adjusted for age, sex, triglycerides, and HbA1c; and (4) Adjusted for age, sex, triglycerides, HbA1c, BMI, use of antidiabetic medications, alcohol consumption, and smoking status. The Fisher's least significant difference method was used for post-hoc pair-wise comparisons.

To investigate the associations between the five fat phenotypes and pancreatic enzymes (pancreatic amylase, pancreatic lipase, and chymotrypsin) in each study group, linear regression analyses were conducted. In these analyses, each abdominal fat phenotype was entered as an independent variable and concentrations of pancreatic enzymes were treated as the dependent variable. In addition, relative importance of each abdominal fat phenotype in explaining the variance of pancreatic enzymes concentrations was determined in each study group. Using the 'relaimpo' package in R Studio Version 3.6.1 (RStudio Inc., Massachusetts, United States), a multivariable linear regression model was constructed in each study group, including the five abdominal fat phenotypes as independent variables and pancreatic enzymes concentrations as the dependent variable[27]. The resulting individual  $R^2$  values of all the independent variables were obtained and plotted.

## RESULTS

### Characteristics of study participants

A total of 90 individuals were included (30 NODAP, 30 T2DM, and 30 healthy controls). The median time since the last attack of pancreatitis was 29 mo (interquartile range, 15.7-42.8 mo) and 29 mo (interquartile range, 21.1-36.2 mo) in the NODAP group and T2DM group, respectively. Other characteristics are presented in Table 1.

### Abdominal fat phenotypes in the study groups

The intra-pancreatic fat percentage was  $9.4 \pm 1.8\%$ ,  $9.8 \pm 1.1\%$ , and  $7.8 \pm 1.9\%$  in the NODAP group, T2DM group, and healthy controls group, respectively. The difference between the three groups was statistically significant in both the unadjusted ( $P < 0.001$ ) and all the adjusted models ( $P = 0.002$  in model 2;  $P = 0.001$  in model 3;  $P = 0.003$  in model 4).

**Table 1 Characteristics of the study groups at the time of magnetic resonance imaging**

Characteristic	Healthy controls (n = 30)	T2DM (n = 30)	NODAP (n = 30)	P value <sup>1</sup>
Age (yr)	50.0 (36.5-68.8)	55.5 (41.8-66.3)	58.5 (48.5-67.3)	0.213
Men, n (%)	21 (70.0)	21 (70.0)	21 (70.0)	1.000
Body mass index (kg/m <sup>2</sup> )	24.0 (21.8-28.1)	30.3 (26.4-35.4)	27.5 (24.1-32.7)	< 0.001
Waist-height ratio	0.5 (0.5-0.5)	0.6 (0.5-0.6)	0.6 (0.5-0.6)	< 0.001
Triglycerides (mmol/L)	1.0 (0.6-1.2)	1.7 (1.3-3.7)	2.2 (1.3-3.6)	0.035
Total cholesterol (mmol/L)	4.5 (3.4-5.6)	5.1 (3.9-5.8)	5.1 (4.1-5.7)	0.388
HDL cholesterol (mmol/L)	1.2 (0.8-1.8)	1.2 (0.9-1.4)	1.2 (1.1-1.4)	0.680
LDL cholesterol (mmol/L)	2.7 (2.0-3.4)	2.7 (1.6-3.3)	2.6 (2.1-3.5)	0.542
Fasting insulin (mIU/L)	11.8 (4.8-16.6)	12.2 (8.2-17.2)	13.3 (7.4-18.3)	0.523
HOMA-IR (mIU/L mmol/L)	2.8 (1.2-3.3)	3.3 (2.1-5.1)	3.3 (1.9-5.3)	0.244
Smoking status <sup>2</sup>	0 (0-1)	0 (0-1)	1 (0-1)	0.016
Alcohol consumption (g/wk)	39 (4.5-96)	12 (6-144)	12 (0-108)	0.279
Amylase (U/L)	29.0 (20.2-33.9)	20.2 (14.0-31.5)	28.7 (19.4-33.3)	0.158
Lipase (pg/mL)	7.2 (5.5-8.7)	7.6 (5.9-10.5)	6.5 (5.5-8.6)	0.340
Chymotrypsin (U/L)	6.2 (5.2-7.1)	4.6 (2.9-6.7)	5.9 (4.9-6.6)	0.314

Data are presented as median and interquartile range or percentage.

<sup>1</sup>P values were from one-way analysis of variance.

<sup>2</sup>Smoking status was classified as either ever-smokers or never-smokers.

T2DM: Type 2 prediabetes/diabetes mellitus; NODAP: New-onset prediabetes/diabetes after acute pancreatitis; HDL: High-density lipoproteins; LDL: Low-density lipoprotein; HOMA-IR: Homeostasis model assessment of insulin resistance.

The liver fat percentage was  $12.0 \pm 9.7\%$ ,  $11.3 \pm 11.1\%$ , and  $9.3 \pm 7.7\%$  in the NODAP group, T2DM group, and healthy controls group, respectively. The difference between the three groups was not statistically significant in all the models.

The skeletal muscle fat percentage was  $14.9 \pm 6.1\%$ ,  $15.5 \pm 6.0\%$ , and  $14.1 \pm 7.0\%$  in the NODAP group, T2DM group, and healthy controls group, respectively. The difference between the three groups was not statistically significant in all the models.

The visceral fat volume was  $2205.0 \pm 1098.1 \text{ cm}^3$ ,  $2622.5 \pm 1172.2 \text{ cm}^3$ , and  $1208.8 \pm 808.1 \text{ cm}^3$  in the NODAP group, T2DM group, and healthy controls group, respectively. The difference between the three groups was statistically significant in both the unadjusted ( $P < 0.001$ ) and all the adjusted models ( $P = 0.010$  in model 2;  $P = 0.001$  in model 3;  $P = 0.005$  in model 4).

The subcutaneous fat volume was  $3011.4 \pm 1432.2 \text{ cm}^3$ ,  $3463.0 \pm 1323.7 \text{ cm}^3$ , and  $2523.8 \pm 1437.7 \text{ cm}^3$  in the NODAP group, T2DM group, and healthy controls group, respectively. The difference between the three groups was statistically significant in the unadjusted ( $P = 0.013$ ) and two adjusted models ( $P = 0.038$  in model 3;  $P = 0.034$  in model 4).

Results of all the pair-wise comparisons between the study groups are presented in Table 2.

### **Associations between abdominal fat phenotypes and pancreatic enzymes in the study groups**

In the NODAP group, the five abdominal fat phenotypes altogether explained 41.2% of the variance in pancreatic amylase, 4.5% of the variance in pancreatic lipase, and 11.1% of the variance in chymotrypsin. Of the fat phenotypes studied, the variance in pancreatic amylase concentration was explained the most by IPFD ( $R^2 = 15.3\%$ ) (Figure 1A); the variance in pancreatic lipase concentration was explained the most by IPFD ( $R^2 = 3.0\%$ ) (Figure 2A); the variance in chymotrypsin concentration was explained the most by liver fat ( $R^2 = 6.5\%$ ) (Figure 3A). IPFD and visceral fat volumes were significantly associated with pancreatic amylase concentration ( $\beta = -2.201$ ,  $P = 0.023$ ; and  $\beta = -0.004$ ,  $P = 0.028$ , correspondingly). The other abdominal fat phenotypes were not significantly associated with the studied pancreatic enzymes (Table 3).

**Table 2** Differences in abdominal fat phenotypes between the study groups

Fat phenotype	Overall <sup>1</sup>	T2DM vs NODAP	T2DM vs healthy controls	NODAP vs healthy controls
Intra-pancreatic fat (%)				
Model 1	< 0.001	0.290	< 0.001	0.001
Model 2	0.002	0.555	0.001	0.004
Model 3	0.001	0.440	0.001	0.001
Model 4	0.003	0.187	0.002	0.012
Liver fat (%)				
Model 1	0.416	0.806	0.322	0.211
Model 2	0.681	0.457	0.983	0.465
Model 3	0.556	0.998	0.393	0.312
Model 4	0.612	0.428	0.329	0.801
Skeletal muscle fat (%)				
Model 1	0.348	0.148	0.516	0.420
Model 2	0.329	0.137	0.455	0.489
Model 3	0.585	0.319	0.692	0.544
Model 4	0.477	0.243	0.551	0.460
Visceral fat (cm <sup>3</sup> )				
Model 1	< 0.001	0.093	< 0.001	0.001
Model 2	0.010	0.596	0.005	0.012
Model 3	0.001	0.249	< 0.001	0.003
Model 4	0.005	0.787	0.012	0.003
Subcutaneous fat (cm <sup>3</sup> )				
Model 1	0.013	0.214	0.003	0.082
Model 2	0.740	0.488	0.508	0.987
Model 3	0.038	0.245	0.012	0.081
Model 4	0.034	0.063	0.009	0.301

<sup>1</sup>Overall *P* values were from one-way analysis of variance in model 1 (unadjusted) and one-way analysis of covariance in models 2–4 (adjusted as described below). Data are presented as *P* values. Fisher's least significant difference method was used in pair-wise comparison. Model 2: Adjusted for age and sex; Model 3: Adjusted for age, sex, triglycerides, and glycated hemoglobin A1c; Model 4: Adjusted for age, sex, triglycerides, glycated hemoglobin A1c, use of antidiabetic medications, average weekly alcohol consumption, and smoking status. T2DM: Type 2 prediabetes/diabetes mellitus; NODAP: New-onset prediabetes/diabetes after acute pancreatitis.

In the T2DM group, the five abdominal fat phenotypes altogether explained 4.0% of the variance in pancreatic amylase, 14.7% of the variance in pancreatic lipase, and 29.9% of the variance in chymotrypsin. Of the fat phenotypes studied, the variance in pancreatic amylase concentration was explained the most by skeletal muscle fat ( $R^2 = 1.5\%$ ) (Figure 1B); the variance in pancreatic lipase concentration was explained the most by skeletal muscle fat ( $R^2 = 10.7\%$ ) (Figure 2B); the variance in chymotrypsin concentration was explained the most by IPFD ( $R^2 = 8.9\%$ ) (Figure 3B). None of the abdominal fat phenotypes were significantly associated with the studied pancreatic enzymes (Table 3).

In the healthy controls group, the five abdominal fat phenotypes altogether explained 17.8% of the variance in pancreatic amylase, 31.3% of the variance in pancreatic lipase, and 7.5% of the variance in chymotrypsin. Of the abdominal fat phenotypes studied, the variance in pancreatic amylase concentration was explained the most by liver fat ( $R^2 = 7.1\%$ ) (Figure 1C); the variance in pancreatic lipase concentration was explained the most by skeletal muscle fat ( $R^2 = 13.0\%$ ) (Figure 2C); the variance in chymotrypsin concentration was explained the most by visceral fat ( $R^2 = 4.0\%$ ) (Figure 3C). Skeletal muscle fat percentage was significantly associated with

**Table 3 Associations between abdominal fat phenotypes and pancreatic enzymes**

Fat phenotype	Healthy			T2DM			NODAP		
	$\beta$	S.E.	P value	$\beta$	S.E.	P value	$\beta$	S.E.	P value
Intra-pancreatic fat (%)									
Pancreatic amylase	-0.832	2.220	0.712	0.573	1.709	0.741	-2.201	0.899	0.023
Pancreatic lipase	0.258	0.421	0.546	-0.631	0.397	0.876	1.343	1.462	0.367
Chymotrypsin	-0.137	0.179	0.452	-0.579	0.610	0.343	0.164	0.270	0.550
Liver fat (%)									
Pancreatic amylase	0.685	0.444	0.140	-0.022	0.157	0.890	-0.493	0.331	0.151
Pancreatic lipase	-0.136	0.089	0.141	-0.134	0.351	0.709	-0.098	0.280	0.730
Chymotrypsin	0.002	0.040	0.967	-0.071	0.069	0.314	0.059	0.051	0.259
Skeletal muscle fat (%)									
Pancreatic amylase	-0.570	0.595	0.351	-0.241	0.474	0.619	-0.185	0.125	0.151
Pancreatic lipase	0.195	0.093	0.047	-1.345	0.912	0.161	-0.071	0.446	0.875
Chymotrypsin	-0.023	0.044	0.609	-0.129	0.105	0.238	-0.014	0.086	0.868
Visceral fat (cm <sup>3</sup> )									
Pancreatic amylase	0.002	0.005	0.664	0.000	0.002	0.841	-0.004	0.002	0.028
Pancreatic lipase	-0.588	0.839	0.490	-3.247	0.462	0.493	0.533	0.250	0.833
Chymotrypsin	-0.314	0.362	0.394	0.287	0.566	0.619	0.240	0.458	0.606
Subcutaneous fat (cm <sup>3</sup> )									
Pancreatic amylase	0.003	0.002	0.224	0.000	0.002	0.821	-0.002	0.001	0.232
Pancreatic lipase	-0.573	0.482	0.246	-2.032	3.444	0.564	-0.036	1.931	0.985
Chymotrypsin	-0.030	0.215	0.890	-0.527	0.534	0.338	-0.091	0.350	0.797

Data are presented as beta coefficients, standard errors, and *P* values (from linear regression). T2DM: Type 2 prediabetes/diabetes mellitus; NODAP: New-onset prediabetes/diabetes after acute pancreatitis.

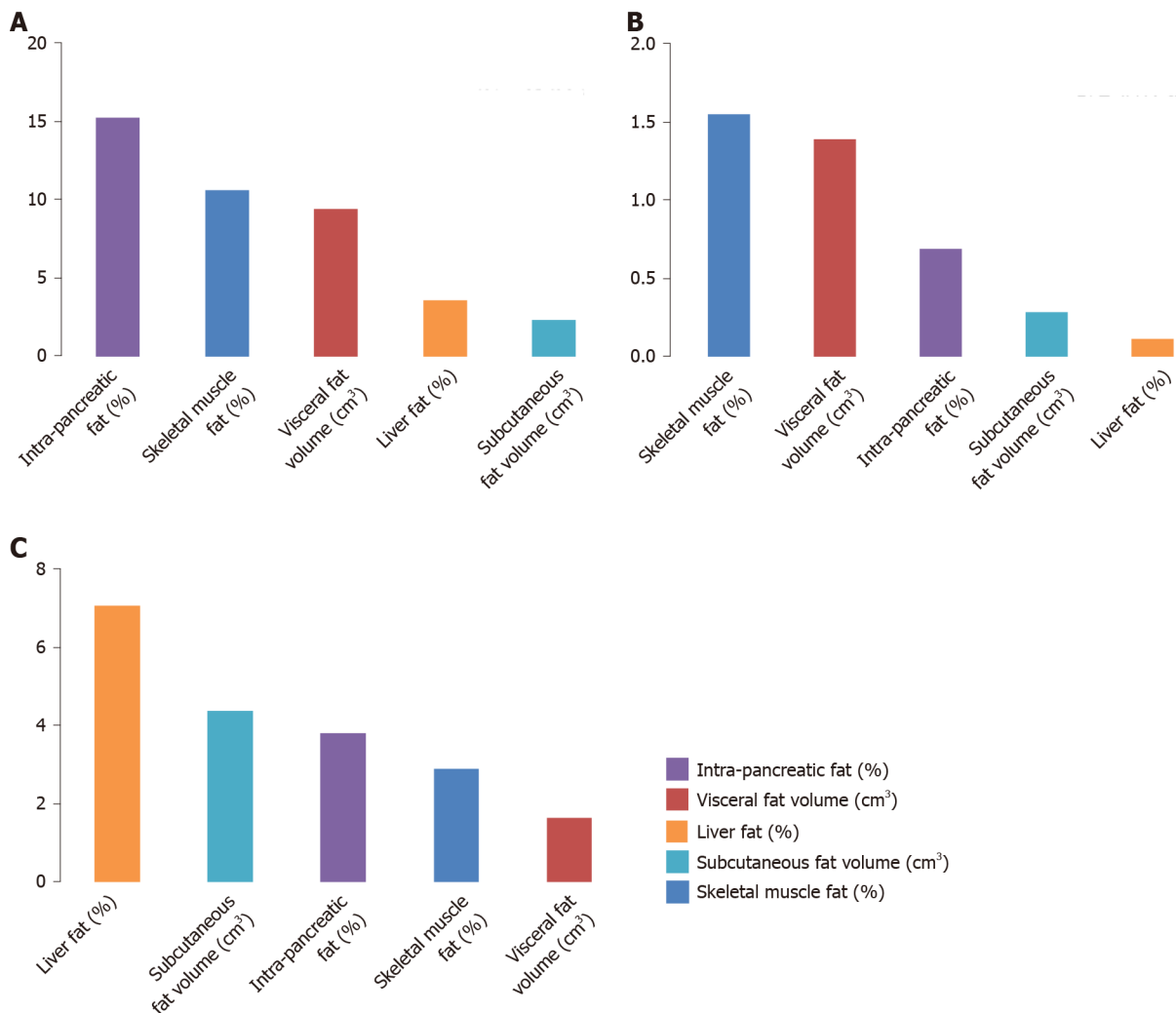
pancreatic lipase concentration ( $\beta = 0.195$ ,  $P = 0.047$ ). The other abdominal fat phenotypes were not significantly associated with the studied pancreatic enzymes (Table 3).

## DISCUSSION

In the present study, a uniformed MRI protocol on a single 3T scanner was used to comprehensively compare, for the first time, abdominal fat phenotypes in 90 matched individuals with NODAP, T2DM, and healthy controls. An important finding of the study was that both NODAP and T2DM were characterized by a significantly larger amount of intra-pancreatic fat and visceral fat (but not liver fat, skeletal muscle fat, or subcutaneous fat) compared with healthy controls, consistently in both unadjusted and all the adjusted analyses. In addition, IPFD and visceral fat volume were significantly inversely associated with circulating levels of pancreatic amylase in the NODAP group (but not the other two groups). The relative importance analyses revealed that the five studied abdominal fat phenotypes altogether explained 41% of the variance in pancreatic amylase concentration in individuals with NODAP. By contrast, only 4% and 13% of the variance in this pancreatic enzyme was explained by the five abdominal phenotypes in individuals with T2DM and healthy controls, respectively.

Abdominal fat phenotypes are often used to differentiate between type 1 diabetes and the much more common type 2 diabetes. Several studies have compared head-to-head these two types of diabetes and have agreed that individuals with type 1 diabetes typically have lower levels of adiposity (as evidenced by both BMI and visceral

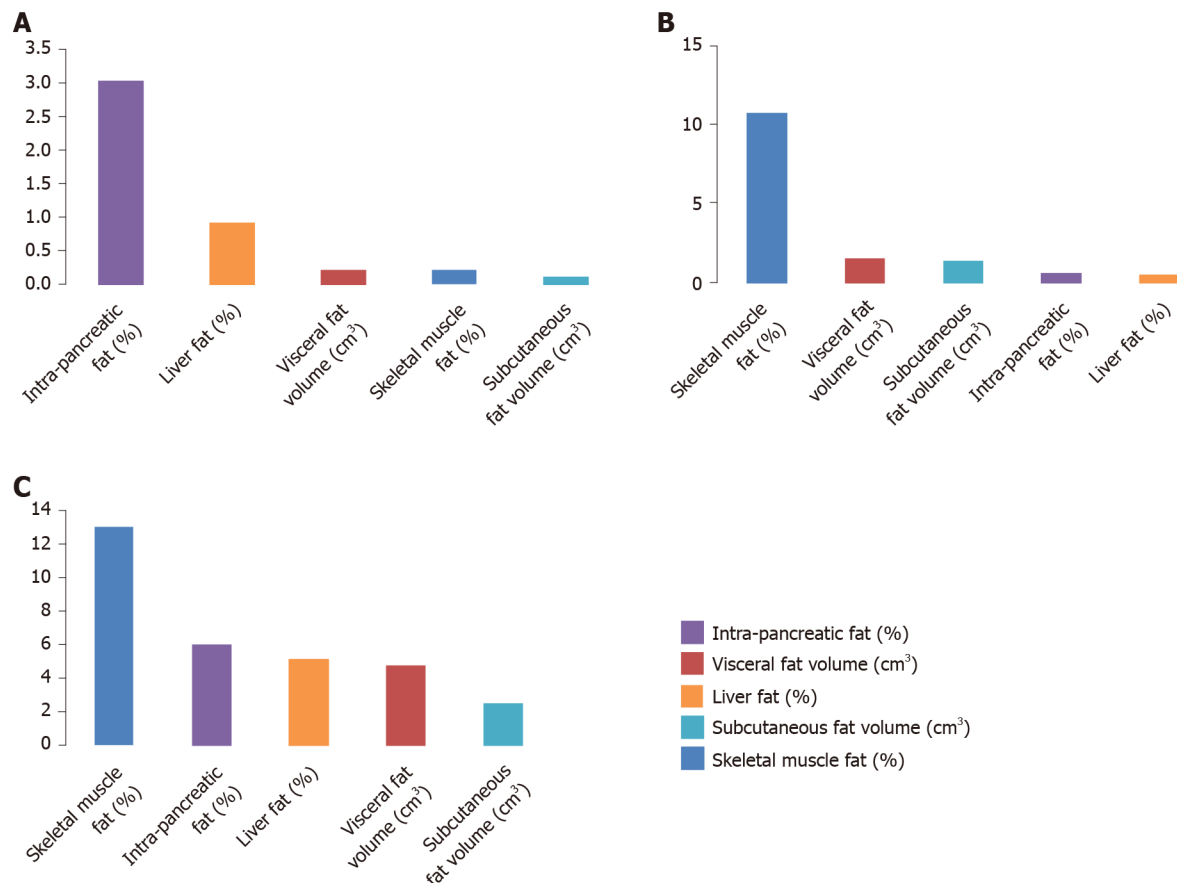




**Figure 1 Contributions of the studied fat phenotypes to the variance in circulating levels of pancreatic amylase in the healthy controls, type 2 prediabetes/diabetes mellitus, and new-onset prediabetes/diabetes after acute pancreatitis groups.** Footnote: Data are presented as a percentage of the corresponding abdominal fat phenotype that explains the variance in circulating levels of pancreatic amylase. A: New-onset prediabetes/diabetes after acute pancreatitis; B: Type 2 prediabetes/diabetes mellitus; C: Healthy controls.

adiposity) than those with type 2 diabetes[28-30]. Moreover, a large study from the United Kingdom demonstrated that the prevalence of type 2 diabetes, but not type 1 diabetes, was significantly associated with BMI[29]. NODAP, another type of diabetes that is much less common than type 2 diabetes, is often misdiagnosed as type 2 diabetes[4]. Specifically, a 2017 population-based study of new-onset diabetes demonstrated that 93% cases of new-onset diabetes after pancreatitis were misclassified as type 2 diabetes (and 4% as type 1 diabetes)[4]. In the present study, the NODAP group did not significantly differ from the T2DM group in terms of visceral adiposity, which was significantly higher in the two case groups in comparison with the healthy control group. Although we did not specifically measure insulin sensitivity in the present study, our earlier study showed that individuals with NODAP were characterized by decreased insulin sensitivity compared with healthy controls[31]. The findings of the two studies are complementary as increased visceral adiposity is known to be more strongly linked with decreased insulin sensitivity than subcutaneous adiposity.

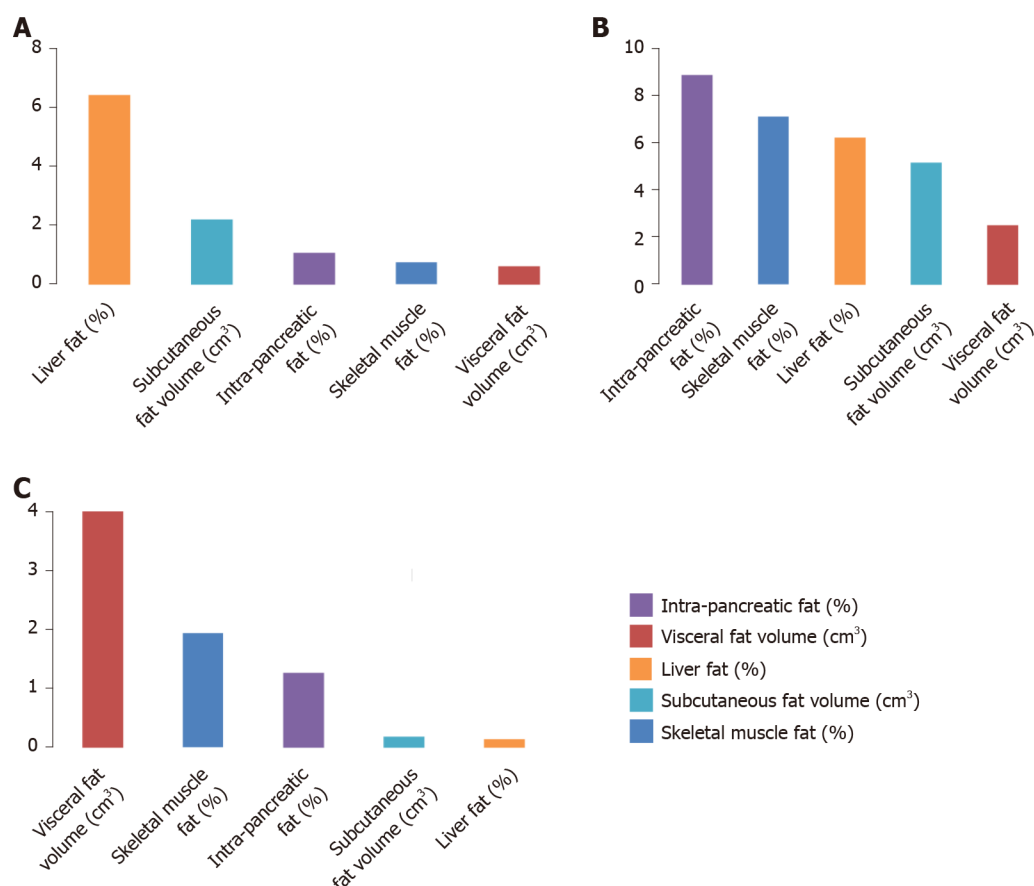
IPFD has recently emerged as another fat phenotype strongly linked with insulin sensitivity[14,31,32]. This is epitomized in the 'twin cycle hypothesis' that posits that type 2 diabetes is caused by excess fat deposition in the pancreas (and liver). During chronic positive caloric balance,  $\beta$ -cells enter a 'survival mode' and fail to function adequately in the pancreas because of the fat-induced metabolic stress[33]. Studies from a primary care-based weight management program in the United Kingdom found that the reduction in fat depositions in the pancreas (and liver) in the first few years after diabetes onset can normalize hepatic insulin responsiveness and possibly



**Figure 2** Contributions of the studied fat phenotypes to the variance in circulating levels of pancreatic lipase in the healthy controls, type 2 prediabetes/diabetes mellitus, and new-onset prediabetes/diabetes after acute pancreatitis groups. Data are presented as a percentage of the corresponding abdominal fat phenotype that explains the variance in circulating levels of pancreatic lipase. A: New-onset prediabetes/diabetes after acute pancreatitis; B: Type 2 prediabetes/diabetes mellitus; C: Healthy controls.

trigger  $\beta$ -cell re-differentiation[34]. Collectively, these changes can lead to normalization of blood glucose levels and reversal of biochemical diabetes status[33-35]. Further, the link between IPFD and insulin traits, specifically in NODAP, was investigated in a 2019 study. It showed that a fasted state index of insulin sensitivity (specifically, Raynaud index) was significantly inversely associated with IPFD in individuals with NODAP and it explained 20% of the variance in IPFD, which was the highest among the nearly 30 body composition variables and insulin traits investigated [31]. The present study bridges the gap in the literature by showing, for the first time, that IPFD is similarly increased in NODAP and T2DM as compared with healthy controls.

The other novel finding in the present study was that, although high IPFD and visceral fat volume characterized both NODAP and T2DM, the two fat phenotypes were significantly inversely associated with circulating levels of pancreatic amylase in the NODAP group only. Further, IPFD contributed the most to the variance in circulating levels of pancreatic amylase ( $R^2 = 15.0\%$ ). By contrast, there was only a small contribution of IPFD to the variance in circulating levels of pancreatic amylase in the healthy controls group ( $R^2 = 3.8\%$ ) and the T2DM group ( $R^2 = 0.7\%$ ). The exact mechanism underlying the above findings is yet to be elucidated but we believe it may relate to a more prominent role of exocrine pancreatic dysfunction in NODAP *vs* T2DM. A 2015 study demonstrated that MRI-derived IPFD was inversely associated with serum lipase activity (pancreatic amylase and other pancreatic enzymes were not studied) in the general population, suggesting that increased IPFD is associated with reduced pancreatic acinar cell mass[36]. A subsequent add-on study of 1458 participants with available fecal elastase measurements showed that MRI-derived IPFD was significantly inversely associated with exocrine pancreatic function (defined based on fecal elastase levels), in both crude analysis and after adjustment for age, sex, and BMI[37]. Interestingly, other fat phenotypes (subcutaneous fat, visceral fat, and liver fat) were not significantly associated with exocrine pancreatic function in that



**Figure 3** Contributions of the studied fat phenotypes to the variance in circulating levels of chymotrypsin in the healthy controls, type 2 prediabetes/diabetes mellitus, and new-onset prediabetes/diabetes after acute pancreatitis groups. Data are presented as a percentage of the corresponding abdominal fat phenotype that explains the variance in circulating levels of chymotrypsin. A: New-onset prediabetes/diabetes after acute pancreatitis; B: Type 2 prediabetes/diabetes mellitus; C: Healthy controls.

study. Another study of fecal elastase and MRI-derived IPFD included 56 individuals without diabetes or pancreatitis and showed that individuals with excess IPFD had a significantly higher frequency of exocrine pancreatic dysfunction than controls[38].

Amylase is the main enzyme responsible for the hydrolysis of carbohydrates[39] and, hence, its observed involvement in the pathogenesis of NODAP may have practical implications for the dietary management of this disorder. In most human populations, starch is the primary source of carbohydrates[40-42]. A 2012 study found that individuals with low amylase activity had higher postprandial plasma glucose concentrations after starch ingestion than individuals with high amylase activity[43]. A 2016 metabolomic study showed that utilization of glucose in the body for energy was attenuated in individuals with low serum amylase who have an energy dependence on fats rather than carbohydrates[44]. Based on the above findings, it is likely that individuals with NODAP have a different glycemic response in comparison with individuals with T2DM. Glycemic load reflects the quantity and quality of carbohydrates in the diet[40,45]. Because individuals with NODAP may not be fully adapted to a diet rich in carbohydrates, they may benefit from the determination of glycemic load for foods that are high in starch. The differential association between amylase and fat phenotypes in NODAP *vs* T2DM may also influence response to weight-loss dietary interventions and this needs to be taken into account in the design of future randomized controlled trials[41].

Several limitations have to be acknowledged. First, genetic factors were not analyzed in the present study. Some genes (*e.g.*, the  $\alpha$ -amylase gene cluster) are highly relevant to the present research. The  $\alpha$ -amylase cluster comprises 2 pancreatic amylase genes (*AMY2A* and *AMY2B*), 3 salivary amylase genes (*AMY1A*, *AMY1B*, and *AMY1C*), and a related pseudogene[46]. Several studies showed that genetic factors (such as copy number variants in amylase genes) may account for as much as 11% of the population variance in body fat[47]. The present study opens up a potential avenue for future research into genetic factors that predispose to IPFD and visceral fat deposition.

Second, levels of amylase, lipase, and chymotrypsin in the gastrointestinal tract were not measured. Hence, it is unknown whether their circulating and intraluminal levels correlate in our study population. However, a 2020 systematic review identified circulating levels of amylase and lipase as biomarkers of diabetes[19]. Third, we did not analyze lifestyle factors (*e.g.*, diet, physical activity)[48,49]. It is well established that obesogenic risk factors are positively associated with ectopic fat deposition in several organs[2,50]. However, there is no evidence that these factors would differentially affect ectopic fat deposition in the liver, pancreas, and skeletal muscle. Third, the study sample size was rather limited. However, this was a pilot study that will inform the design and sample size calculation of future studies. Last, given the cross-sectional nature of the study, inference of causality between IPFD and circulating levels of pancreatic enzymes cannot be drawn. To the best of our knowledge, no longitudinal study has explored the association between IPFD (or the other phenotypes investigated in the present study) and circulating levels of pancreatic enzymes. Their temporal relationship is warranted to be on the research agenda.

## CONCLUSION

IPFD and visceral fat were significantly increased in individuals with NODAP and T2DM (in comparison with healthy controls). However, only individuals with NODAP were characterized by significant inverse associations between the two abdominal fat phenotypes and circulating levels of pancreatic amylase. Pancreatic amylase may have implications for the pathogenesis and management of NODAP and, hence, the role of this pancreatic enzyme in NODAP warrants purposely designed investigations in the future.

## ARTICLE HIGHLIGHTS

### Research background

Abdominal adipose tissue distribution is an important factor in the pathogenesis of diabetes in general and new-onset diabetes after acute pancreatitis in particular.

### Research motivation

The role of pancreatic enzymes in the pathogenesis of new-onset diabetes after acute pancreatitis is unknown.

### Research objectives

The objective was to compare head-to-head abdominal adipose tissue distribution in new-onset prediabetes or diabetes after acute pancreatitis (NODAP), type 2 prediabetes or diabetes, and healthy controls.

### Research methods

The design was a case-control study. Intra-pancreatic fat, liver fat, skeletal muscle fat, visceral fat, and subcutaneous fat were quantified in a blinded fashion with the use of magnetic resonance imaging. Circulating levels of pancreatic amylase, pancreatic lipase, and chymotrypsin were determined.

### Research results

The intra-pancreatic fat percentage was  $9.4 \pm 1.8\%$ ,  $9.8 \pm 1.1\%$ , and  $7.8 \pm 1.9\%$  in NODAP, type 2 prediabetes or diabetes, and healthy controls, respectively ( $P < 0.001$ ). The visceral fat volume was  $2205 \pm 1098 \text{ cm}^3$ ,  $2622 \pm 1172 \text{ cm}^3$ , and  $1209 \pm 808 \text{ cm}^3$  in NODAP, type 2 prediabetes or diabetes, and healthy controls, respectively ( $P < 0.001$ ). The other fat phenotypes did not differ between the groups. The amount of intra-pancreatic fat and visceral fat was significantly associated with circulating levels of pancreatic amylase in NODAP (but not type 2 prediabetes or diabetes or healthy controls).

### Research conclusions

Excess intra-pancreatic fat deposition is a key factor in the pathogenesis of new-onset diabetes after acute pancreatitis. There is a significant inverse relationship between circulating levels of pancreatic amylase and intra-pancreatic fat.



## Research perspectives

Human studies on the role of pancreatic amylase in new-onset diabetes after acute pancreatitis are warranted.

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## Retrospective Cohort Study

# Effect of type 2 diabetic mellitus in the prognosis of acute-on-chronic liver failure patients in China

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**Author contributions:** Lai RM and Chen TB contributed equally to this work; Lai RM, Chen TB, and Zheng Q conceived and designed the study; Chen TB analyzed the data; Hu YH and Wu G collected clinical data of the patients; Lai RM and Zheng Q wrote the manuscript; all authors read and approved the final version of the manuscript.

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### Institutional review board

**statement:** This study was approved by the Institutional Review Board of Fujian Medical University.

### Informed consent statement:

Patients were not required to give informed consent to the study because the analysis used anonymous data that were

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## Abstract

### BACKGROUND

Acute-on-chronic liver failure (ACLF) patients have a high short-term mortality rate, and the severity evaluation of ACLF is necessary for prognostication. Therefore, it was meaningful to evaluate the association between type 2 diabetic mellitus (DM) and ACLF and further explore the feasibility of using DM as a prognostic indicator in ACLF patients. The association between type 2 DM and the prognosis of patients with severe liver disease remains unclear.

### AIM

To examine the effect of type 2 DM on the prognosis of patients with ACLF.

### METHODS

Clinical data from 222 ACLF patients were collected and analyzed. The patients were categorized into two groups depending on whether they had DM or not, and the clinical data of ACLF patients were measured within 48 h after admission. Complications of ACLF were documented during treatment, such as hepatic encephalopathy, hepatorenal syndrome, acute upper gastrointestinal hemorrhage, and spontaneous peritonitis (SBP). Values of laboratory parameters, complication rates, and hospital mortality rates were compared between two groups.

### RESULTS

Among 222 ACLF patients, 38 cases were categorized into DM groups, the mean age was 56.32 years and 73.68% were male. The prognosis of ACLF patients was



obtained after each patient agreed to treatment by written consent.

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significantly correlated with DM in univariate [hazard ratio (HR) = 2.4, 95% confidence interval (CI) = 1.5-3.7,  $P < 0.001$ ] and multivariable analysis (HR = 3.17, 95% CI = 1.82-5.523,  $P < 0.001$ ). The incident of SBP (34.21% *vs* 13.59%,  $P = 0.038$ ) and other infections like lung, urinary, blood, and cholecyst (44.74% *vs* 28.26%,  $P = 0.046$ ) were higher in DM patients than non-DM counterparts. In addition, the ACLF patients with DM tended to have a high mortality rate ( $P < 0.001$ ). Cumulative survival time was also significantly shorter in the ACLF patients with DM than non-DM.

## CONCLUSION

A significant association between DM and the prognosis of ACLF patients was found in China. The ACLF patients with DM had higher incidence of hospital mortality and infection than those without DM.

**Key Words:** Acute-on-chronic liver failure; Diabetic mellitus; Prognosis; Infection; Type 2 diabetic mellitus

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**Core Tip:** This study evaluated the association between type 2 diabetic mellitus (DM) and the prognosis of acute-on-chronic liver failure (ACLF) patients. The 222 ACLF patients were categorized into two groups depending on whether they had DM or not. Values of laboratory parameters, complication rates, and hospital mortality rates were compared between two groups. We observed a significant association between DM and the prognosis of ACLF patients in the study. The ACLF patients with DM had higher incidence of hospital mortality and infection than those without DM.

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## INTRODUCTION

Liver failure is usually manifested as coagulation dysfunction, jaundice, ascites, and hepatic encephalopathy (HE). Acute-on-chronic liver failure (ACLF) is a clinical type of liver failure, and the term was firstly raised in order to describe a condition with two insults operating on the liver simultaneously[1]. ACLF has been featured as a clinical syndrome in which an acute injury factor in a patient with chronic liver disease caused rapid deterioration of liver function, resulting in one or more organ failure. Causes of ACLF could be attributed to hepatitis B virus (HBV), autoimmune hepatitis, severe nonalcoholic fatty liver disease (NAFLD), and other sources. According to Asian Pacific Association for the Study of the Liver (APASL) ACLF Research Consortium consensus, which was published in 2014 and subsequently updated in 2019, ACLF was defined as acute hepatic insult manifested as jaundice and coagulopathy, complicated within 4 wk by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease/cirrhosis, and associated with a high 28-d mortality[1,2]. ACLF diagnostic criteria is uniform in the world, and the Asia-Pacific consensus emphasizes the emergence of liver failure based on chronic liver disease, mainly focusing on the performance of liver failure in order to get early diagnosis and intervention of disease[3]. It was found that ACLF patients had a high short-term mortality rate ( $> 15\%$  at 28 d)[4] and the severity evaluation of ACLF was necessary for prognostication. Hence well-established prognostic indicators are extremely important, which are helpful for early intervention and reversibility of ACLF as well as improving survival rate[5,6].

Diabetic mellitus (DM) is one of the leading causes of morbidity and mortality across the globe[7]. Previous studies showed that DM was associated with higher incidence of encephalopathy, spontaneous bacterial peritonitis, and hepatocellular

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carcinoma in chronic liver disease[8-10]. With the improvement of people's living standards, the prevalence of fatty liver disease and DM has increased significantly in China. NAFLD is the most common chronic liver disease, and the risk of progressed liver fibrosis in NAFLD patients is significantly associated with DM[11,12]. The prevalence of DM has increased significantly in recent decades, results in the coexistence of DM and chronic liver disease being common[13]. DM, NAFLD, and obesity are manifestations of symptoms known as metabolic syndrome, and these symptoms are all related to each other. A recent study showed that morbid obesity is an important risk factor for the development of ACLF in liver cirrhosis patients[14]. However, there are very few studies that have evaluated the association between DM and ACLF. Therefore, it may be meaningful to investigate the association between DM and ACLF and further explore the feasibility of using DM as a prognostic indicator in ACLF patients.

## MATERIALS AND METHODS

### Research population

In this retrospective single-center study, all patients diagnosed with liver failure who were hospitalized at the Department of Hepatology Research Institute of the First Affiliated Hospital, Fujian Medical University, China from July 2013 to July 2020 were recruited in this study. ACLF was defined by APASL definition in 2019, an acute hepatic insult manifesting as jaundice [serum bilirubin  $\geq 5$  mg/dL (85 mmol/L) and coagulopathy (international normalized ratio (INR)  $\geq 1.5$  or prothrombin activity  $< 40\%$ )] complicated within 4 wk by clinical ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease/cirrhosis and was associated with a high 28-d mortality[1]. The diagnosed complications of ACLF patients also were in accord with this definition. Type 2 DM was defined using any of the following criteria: (1) The World Health Organization 2013 criteria; and (2) documented history of diabetes. All ACLF patients were included as follow criteria: (1) patients aged 18 years and 85 years; (2) ACLF diagnostic criteria accord with APASL definition in 2019; and (3) sufficient data for the study. Patients were excluded if they had the following conditions: (1) *de novo* tumors; (2) liver transplantation or liver operation; (3) current use of hormone medication; and (4) other fatal disease, or gestation. After exclusion, a total of 222 patients with basis for chronic liver disease were recruited, including 190 HBV infection patients, 17 alcoholic liver disease patients, 15 other disease patients, among which 94 deaths were observed. This prospective study was approved by the ethics committee at the First Affiliated Hospital of Fujian Medical University, China.

### Clinical and laboratory parameters

All parameters were collected from the electronic medical record system. The demographic parameters included age, gender, and body mass index (BMI). The clinical laboratory parameters collected within 48 h after admission, included prothrombin time (PT), INR, total bilirubin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, blood urea nitrogen (BUN), serum creatinine, albumin (ALB), fasting blood-glucose (FBG), total cholesterol (TCHO), triglyceride,  $K^+$ ,  $Na^+$ , leukocyte count (white blood cell), erythrocyte count [red blood cell (RBC)], hemoglobin, platelet count (PLT), alpha fetoprotein, and ammonia. The BMI was calculated as weight (kg)/height<sup>2</sup> (m<sup>2</sup>). This study used the West Haven criteria for grading HE. This grading system differentiated four grades of clinically manifest HE[15]. Ascites can be divided into 3 grades based on the volume of fluid by ultrasound examination[16]. The Child-Turcotte-Pugh (CTP) score, Model for End-Stage Liver Disease (MELD) score, and MELD with serum sodium (MELD-Na) score were calculated using the latest published criteria[5,17,18]. All prognostic scores were calculated according to all parameters at 48 h of admission.

### Statistical analysis

Statistical analyses were performed using SPSS 24.0 (Armonk, NY, United States). The normally distributed continuous variables are presented as mean  $\pm$  SD, which were further evaluated by Student's *t*-test between those with and without DM, whereas variables showing skewed distributions were evaluated by the Mann-Whitney *U* test, which are presented as median (interquartile range). Categorical variables were described using frequencies and proportions, and Pearson's chi-squared test was used to compare categorical variables.

The Cox proportional hazard regression analysis was used to estimate the hazard ratio (HR) for the association between DM and all-cause mortality. Participants were followed up from date of hospital admission to date of death or date of discharge. Proportional hazard assumption was tested using Kaplan-Meier survival curves method. A multivariable Cox model was chosen by considering all variables with  $P < 0.1$  in univariate analysis, meanwhile DM was considered as the main variable in statistical analysis model. Further, the nomogram and forest plot were built in terms of results of Cox regression analyses. A two sided  $P$  value less than 0.05 were considered significant.

## RESULTS

### ***Demographic and clinical characteristics***

The baseline demographics characteristics and clinical laboratory parameters of the patients are shown in [Table 1](#), and HBV was the most common etiology of ACLF in both groups (68.42% and 75.54%, respectively). The study included 38 DM patients, the mean age was 56.32 years, and 73.68% were male. As expected, BMI and fasting glucose were higher in DMs than their non-DM counterparts. In contrast, the level of ALB was higher in non-DM ( $P = 0.045$ ). The scoring systems included CTP, MELD, and MELD-Na, and there were no statistically significant differences between the two groups. The mortality rate in DM patients was significantly higher than non-DM patients (65.79% *vs* 37.5%,  $P = 0.001$ ).

### ***The incidence of complications between DM and non-DM patients with ACLF***

The incidence of common complications in ACLF patients with or without DM is shown in [Table 2](#), and ascites was the most frequent complication in both groups (89.47% and 75.54%, respectively), most of whom was mild. Compared to non-DM participants, DMs had a higher incidence of HE complication ( $P = 0.026$ ). There was no statistical difference in other clinical complications including ascites, hepatorenal syndrome (HRS), and acute upper gastrointestinal bleeding (AUGIB) between the two groups. Spontaneous peritonitis (SBP) was the common infection complication of ACLF patients, and the incidence of SBP was higher in DMs than their non-DM counterparts ( $P = 0.038$ ). Other infections, including lung infections, urinary infections, blood infections, and cholecyst infections, were statistically different ( $P = 0.046$ ), though the total incidence rates were low. Furthermore, lung infections were the main infection (34.21% and 23.37%, respectively).

### ***Independent risk factor to predict mortality in ACLF patients***

As shown in [Table 3](#), univariate and multivariable analysis were used to evaluate the predictors of prognosis for ACLF patients. Among demographic risk factors, gender and BMI were associated with the prognosis of ACLF patients in univariate analysis. With regard to clinical laboratory parameters, PT, INR, Na<sup>+</sup>, FBG, BUN, PLT, and RBC were significantly associated with the prognosis of ACLF patients in univariate analysis, moreover, INR, FBG, and BUN also had significant association in multivariable analysis. However, TCHO was not significantly associated with the prognosis of ACLF patients in univariate analysis, while it was significantly in multivariable analysis. Among scoring systems reflecting severity of liver dysfunction, CTP, MELD, and MELD-Na were associated with the prognosis of ACLF patients in univariate analysis, while they had no significant association in multivariable analysis. All four risk factors identified in the univariate analysis (DM, HRS, AUGIB, and SBP) remained significant in the multivariable analysis. Further, nomogram and forest plots that incorporate parameters previously shown to be associated with the prognosis of ACLF patients in the multivariable analysis, including gender, DM, INR, BUN, TCHO, HRS, AUGIB, and SBP, were then constructed and are presented in [Figures 1 and 2](#). As shown by nomogram plot in [Figure 1](#), the total points accumulated by the various variables correspond to the predicted probability of survival for individual patient, and the predicted probability of 1- or 2-mo survival ranged from 0.1 to 0.9. The forest plot in [Figure 2](#) illustrated the intuitive correlation between each risk variable, and the prognosis of ACLF and DM was the most important risk factor (HR = 3.257) influencing the prognosis of ACLF patients.

### ***DM was independent risk factor to predict mortality in ACLF patients***

Survival curves were used to describe the survival status of DM and non-DM patients

**Table 1 Demographic characteristics and clinical features of the patients between diabetic mellitus and non-diabetic mellitus**

	DM (yes) (n = 38)	DM (no) (n = 184)	P value
Age (yr)	56.32 ± 14.23	49.16 ± 12.84	0.002
Gender, n (%)			0.309
Male	28 (73.68)	149 (80.98)	
Female	10 (26.32)	35 (19.02)	
Cause of disease, n (%)			0.201
Hepatitis B virus	26 (68.42)	139 (75.54)	
Hepatitis B virus + other	5 (13.16)	20 (10.87)	
Alcohol	2 (5.26)	15 (8.15)	
Others	5 (13.16)	10 (5.44)	
WBC (10 <sup>9</sup> /L)	6.17 ± 4.03	7.35 ± 3.58	0.07
RBC (10 <sup>12</sup> /L)	3.68 ± 0.87	3.94 ± 0.84	0.084
Hb (g/L)	117.21 ± 24.71	121.95 ± 23.13	0.257
PLT (10 <sup>9</sup> /L)	100.34 ± 42.20	118.79 ± 59.09	0.069
PT (s)	23.01 ± 5.38	24.45 ± 6.95	0.229
INR	1.97 ± 0.45	2.10 ± 0.59	0.229
ALT (U/L)	396.08 ± 448.56	560.36 ± 693.06	0.163
AST (U/L)	365.95 ± 391.18	419.99 ± 513.42	0.541
γ-GGT (U/L)	174.16 ± 305.61	137.57 ± 127.33	0.231
TBIL (μmol/L)	320.71 ± 141.31	309.56 ± 134.00	0.644
ALB (g/L)	29.25 ± 4.51	30.73 ± 4.03	0.045
Scr (μmol/L)	56.37 ± 22.00	63.45 ± 27.28	0.134
BUN (mmol/L)	3.94 ± 2.65	4.25 ± 2.98	0.56
TCHO (mmol/L)	2.67 ± 0.81	2.65 ± 1.05	0.919
TG (mmol/L)	1.45 ± 0.67	1.26 ± 0.70	0.124
Na <sup>+</sup> (mmol/L)	136.98 ± 3.97	136.86 ± 4.43	0.878
K <sup>+</sup> (mmol/L)	3.90 ± 0.46	4.08 ± 0.58	0.072
AMON (μmol/L)	64.40 ± 40.39	71.60 ± 41.85	0.332
AFP (ng/mL)	118.63 ± 202.80	128.19 ± 192.02	0.784
BMI (kg/m <sup>2</sup> )	24.99 ± 3.32	22.78 ± 3.03	< 0.001
FBG (mmol/L)	5.34 ± 1.87	3.83 ± 1.07	< 0.001
Scoring systems			
CTP	10.79 ± 1.49	10.40 ± 1.35	0.115
MELD	19.38 ± 4.52	20.74 ± 5.06	0.128
MELD-Na	20.89 ± 5.00	22.27 ± 6.84	0.239
Death, n (%)	25 (65.79)	69 (37.5)	0.001

γ-GGT: Gamma-glutamyl transpeptidase; AFP: Alpha fetal protein; ALB: Albumin; ALT: Alanine aminotransferase; AMON: Ammonia; AST: Aspartate aminotransferase; BMI: Body mass index; BUN: Blood urea nitrogen; CTP: Child-Turcotte-Pugh; DM: Diabetic mellitus; FBG: Fasting blood-glucose; HB: Hemoglobin; INR: International normalized ratio; K<sup>+</sup>: Kalium; MELD: Model for End-Stage Liver Disease; MELD-Na: Model for End-Stage Liver Disease with serum sodium; Na<sup>+</sup>: Natriumion; PLT: Platelet count; PT: Prothrombin time; Scr: Serum creatinine; TBIL: Total bilirubin; TCHO: Total cholesterol; TG: Triglyceride; RBC: Red blood cell; WBC: White blood cell.

with ACLF. **Figure 3** showed that the cumulative survival rate of ACLF patients was significantly distinguished between DM and non-DM ( $P = 0.00019$ ). The survival time



**Table 2** The incidence of complications between diabetic mellitus and non-diabetic mellitus patients with acute-on-chronic liver failure

	DM (yes) (n = 38)	DM (no) (n = 184)	P value
Ascites, n (%)	34 (89.47)	139 (75.54)	0.271
Mild	21 (55.26)	80 (43.48)	
Moderate	5 (13.16)	26 (14.13)	
Severe	8 (21.05)	33 (17.93)	
HE, n (%)	4 (10.53)	5 (2.72)	0.026
Grade (1-2)	4 (10.53)	3 (1.63)	
Grade (3-4)	0 (0)	2 (1.09)	
HRS, n (%)	4 (10.53)	12 (6.52)	0.385
AUGIB, n (%)	3 (7.89)	4 (2.17)	0.066
SBP, n (%)	13 (34.21)	25 (13.59)	0.038
Other infections, n (%)	17 (44.74)	52 (28.26)	0.046
Lung	13 (34.21)	43 (23.37)	
Urinary	1 (2.63)	5 (2.72)	
Blood	2 (5.26)	2 (1.09)	
Cholecyst	1 (2.63)	2 (1.09)	

Ascites mild: The depth of abdomen was < 3 cm; Ascites moderate: The depth of the middle of the abdomen was 3-10 cm; Ascites severe: The depth of the middle of the abdomen was > 10 cm; HE: Hepatic encephalopathy; HE grade 1: Patients showed a lack of attention and some subtle personality changes; HE grade 2: The most intriguing finding was disorientation for time combined; HE grade 3: Patients were stuporous but responded to stimuli; HE grade 4: Patients were in coma. AUGIB: Acute upper gastrointestinal bleeding; AUGIH: Acute upper gastrointestinal hemorrhage; HRS: Hepatorenal syndrome; SBP: Spontaneous peritonitis.

of ACLF patients with non-DM was longer than that in the patients with DM. With the follow-up time increased, the survival time of patients with DM decreased significantly. Therefore, DM was associated with a higher death risk in ACLF patients.

## DISCUSSION

ACLF was characterized by acute decompensation of chronic liver disease, and the course of ACLF was complex and associated with high short-term mortality, complicated with multiple organ failure[19]. The effective prognostic predictors of ACLF would help in identifying assessment of patients at specific time points either requiring early organ support treatment or urgent liver transplantation for ACLF patients, thereafter, doctors may provide a rational therapy for the patients in time[20, 21]. Previous study demonstrated that patients who survived for the first 3 mo usually had a good prognosis[22]. Therefore, the exploration of ACLF related prognostic indicators was helpful to reduce the mortality by predicting the outcome of patients and providing early clinical intervention.

It has been reported that there is a strong association between DM and liver disease. During a 12-year follow-up cohort study, compared to non-DM patients with chronic hepatitis B (CHB), DM patients with CHB developed more frequently to cirrhosis and cirrhosis decompensation[23]. Similarity, it has been shown that the presence of DM was an independent predictor of mortality in CHB patients[24]. Therefore, it is necessary to pay attention to the impact of DM on the clinical outcomes with ACLF. In this study, it was found that DM was as an independent prognostic factor for ACLF patients, and the incidence of infection and mortality were higher in DMs than that in non-DM patients. In addition to DM, gender, INR, BUN, TCHO, HRS, AUGIB, SBP, and FBG were independent risk factors for the prognosis of ACLF patients in the multivariate analysis.

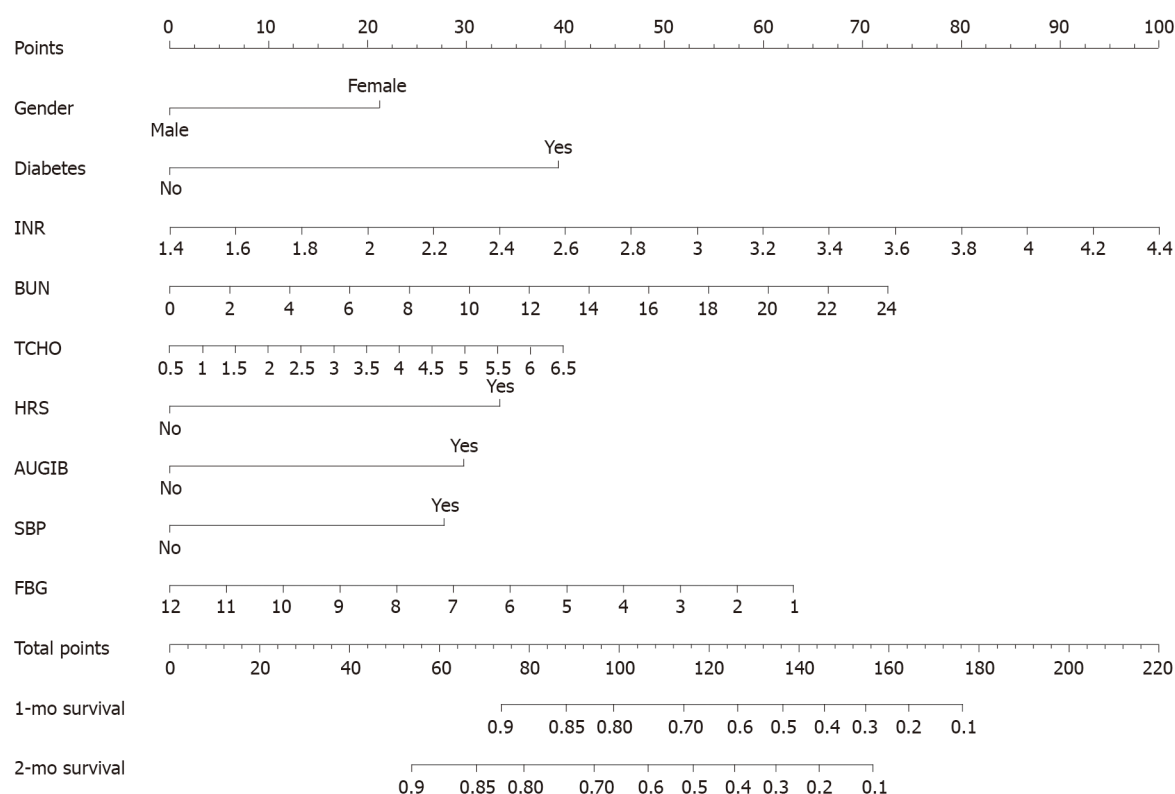
Previous studies had shown that nomogram enabled more-accurate individualized prediction of survival than MELD, MELD-Na, CTP, sequential organ failure assessment, or CLIF-C scores for HBV patients and demonstrated superior net benefits

**Table 3 Factors associated with the survival time of acute-on-chronic liver failure patients based on Cox proportional hazards regression**

	Univariate		Multivariate	
	HR (95%CI)	P value	HR (95%CI)	P value
Gender	0.58 (0.37-0.91)	0.019	0.535 (0.332-0.861)	0.01
Age (yr)	1 (0.99-1)	0.23		
WBC (10 <sup>9</sup> /L)	1 (0.99-1.1)	0.12		
RBC (10 <sup>12</sup> /L)	0.73 (0.57-0.92)	0.009		
Hb (g/L)	0.99 (0.98-1)	0.01		
PLT (10 <sup>9</sup> /L)	0.99 (0.99-1)	0.021		
PT (s)	1.1 (1-1.1)	< 0.001		
INR	1.9 (1.4-2.5)	< 0.001	2.725 (1.949-3.811)	< 0.001
ALT (U/L)	1 (1-1)	0.53		
AST (U/L)	1 (1-1)	0.8		
γ-GGT (U/L)	1 (1-1)	0.93		
TBIL (μmol/L)	1 (1-1)	0.77		
ALB (g/L)	0.95 (0.9-1)	0.079		
Scr (μmol/L)	1 (1-1)	0.15		
BUN (mmol/L)	1.1 (1-1.2)	0.021	1.095 (1.025-1.170)	0.0069
TCHO (mmol/L)	1 (0.85-1.3)	0.76	1.220 (1.006-1.170)	0.0436
TG (mmol/L)	0.78 (0.55-1.1)	0.18		
Na <sup>+</sup> (mmol/L)	0.94 (0.9-0.98)	0.005		
K <sup>+</sup> (mmol/L)	0.83 (0.58-1.2)	0.34		
AMON (μmol/L)	1 (0.99-1)	0.64		
AFP (ng/ml)	1 (1-1)	0.075		
BMI (kg/m <sup>2</sup> )	0.99 (0.93-1.1)	0.7		
FBG (mmol/L)	0.58 (0.39-0.87)	0.009	0.842 (0.719-0.986)	0.0325
CTP	1.7 (1-2.9)	0.042		
MELD	1.1 (1-1.1)	0.02		
MELD-Na	1 (1-1.1)	0.002		
DM	2.4 (1.5-3.7)	< 0.001	3.17 (1.82-5.523)	< 0.001
Ascites	1.2 (1-1.5)	0.036		
HE	1.3 (0.65-2.5)	0.48		
HRS	3.3 (1.9-5.8)	< 0.001	2.71 (1.513-4.857)	0.001
AUGIB	2.6 (1.1-5.9)	0.027	2.444 (1.037-5.76)	0.041
SBP	2.5 (1.6-3.9)	< 0.001	2.262 (1.438-3.558)	< 0.001
Other infections	1.2 (0.82-1.9)	0.32		

γ-GGT: Gamma-glutamyl transpeptidase; AFP: Alpha fetal protein; ALB: Albumin; ALT: Alanine aminotransferase; AMMON: Ammonia; AST: Aspartate aminotransferase; AUGIB: Acute upper gastrointestinal bleeding; AUGIH: Acute upper gastrointestinal hemorrhage; BMI: Body mass index; BUN: Blood urea nitrogen; CI: Confidence interval; CTP: Child-Turcotte-Pugh; DM: Diabetic mellitus; FBG: Fasting blood glucose; HB: Hemoglobin; HE: Hepatic encephalopathy; HR: Hazard ratio; HRS: Hepatorenal syndrome; INR: International normalized ratio; Na<sup>+</sup>: Natriumion; K<sup>+</sup>: Kalium; MELD: Model for End-Stage Liver Disease; MELD-Na: Model for End-Stage Liver Disease with serum sodium; PLT: Platelet count; PT: Prothrombin time; RBC: Red blood cell; SBP: Spontaneous peritonitis; Scr: Serum creatinine; TBIL: Total bilirubin; TCHO: Total cholesterol; TG: Triglyceride; WBC: White blood cell.

over other score models[25,26]. Therefore, an effective prognostic nomogram for ACLF patients was established, as shown in **Figure 1**; the nomogram of our study could

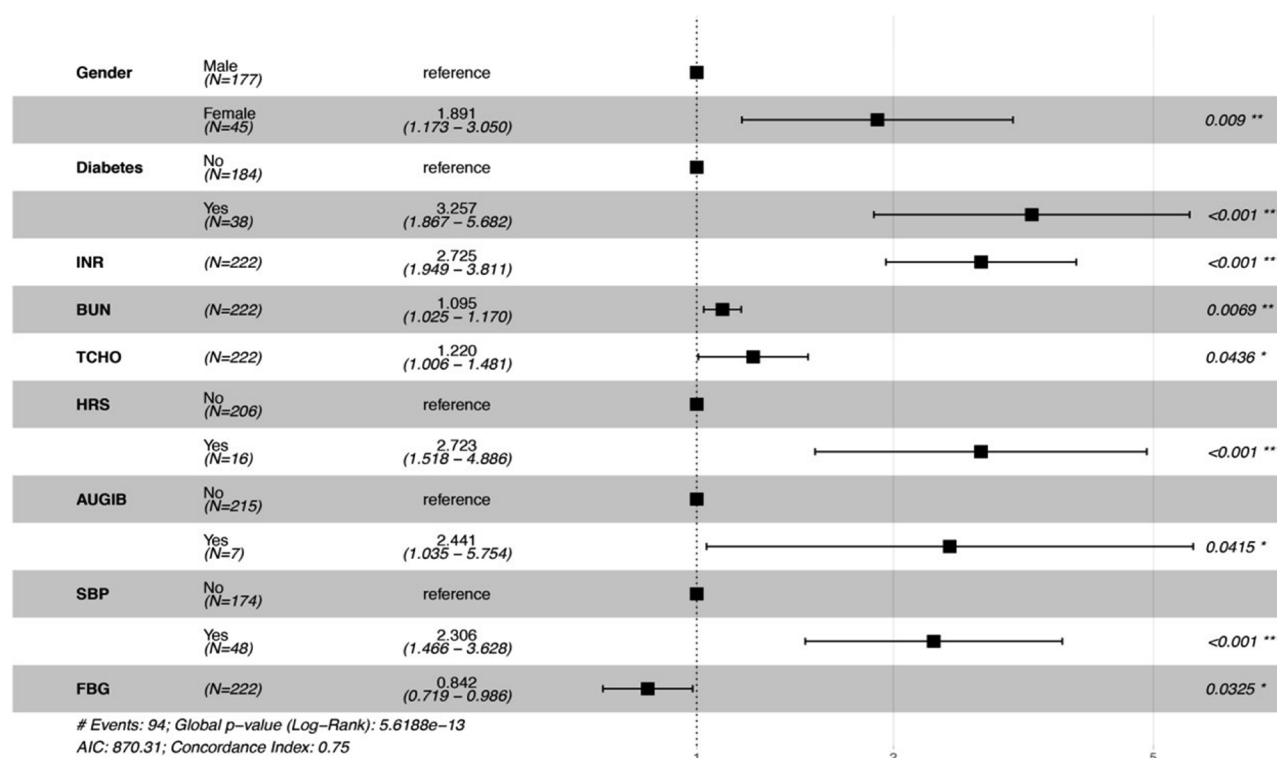


**Figure 1** Acute-on-chronic liver failure patients' survival nomogram predicted the probability of 1- and 2-mo survival. AUGIH: Acute upper gastrointestinal hemorrhage; BUN: Blood urea nitrogen; FBG: Fasting blood-glucose; HRS: Hepatorenal syndrome; INR: International normalized ratio; SBP: Spontaneous peritonitis; TCHO: Total cholesterol.

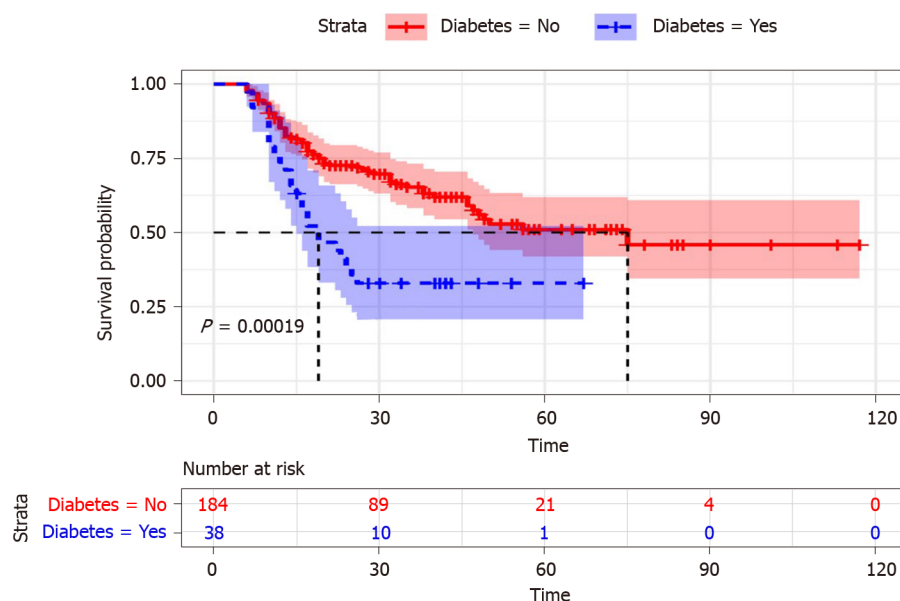
accurately predict 1- and 2-mo survival in patients with ACLF.

Liver plays an important role in glucose homeostasis, and it is the major storage site of glycogen. Massive damage and necrosis of liver cells can cause serious deficiency of liver glycogen synthesis and storage, which are the main features of ACLF disease. Meanwhile, gastrointestinal dysfunction and gastrointestinal mucous edema could lead to impaired nutrient absorption in ACLF patients. Therefore, the mechanism of blood glucose regulation may be abnormal when liver function was severely damaged due to ACLF. In addition, the influence of DM on chronic liver related clinical outcomes had become a focus of research in recent years. It was proved that DM emerged as a risk factor of experiencing liver related death in the chronic liver patients [27]. Furthermore, a meta-analysis showed that DM was independently associated with poor survival in hepatocellular carcinoma patients [28]. Thus, the presence of DM looked as an "at risk" sub-population of liver-related patients. In a conclusion, DM had a tremendous impact on liver disease, which should be fully considered by clinicians. Our study was consistent with previous studies, as shown in Figures 2 and 3, which implied that the level of fast blood glucose and DM were important prognostic indicators for ACLF patients. The cumulative survival time of ACLF patients with non-DM was significantly longer than the patients with DM ( $P = 0.00019$ ). DM was identified as a risk factor in the univariate analysis ( $P = 0.009$ ) and remained significant in the multivariable analysis ( $P = 0.038$ ) in ACLF patients.

DM is a disorder of glucose metabolism; in order to maintain body composition and nitrogen balance, metabolic control and sufficient protein and energy intake are required [29]. ACLF is in a hyper-metabolism state, and upregulated inflammatory response may lead to accelerated proteolysis and increased body energy expenditure, which contributes to negative nitrogen balance [30]. Our study showed that increased level of BUN, which may due to negative nitrogen balance, was associated with the prognosis of ACLF patients in Cox regression analysis. In ACLF patients, the function of protein synthesis was significantly reduced, and ALB had become an effective indicator for ACLF nutritional surveillance [31,32]. A retrospective study had shown that ALB was associated with prognosis in ACLF patients; the patients with hypoproteinemia tended to have a higher mortality rate [33]. However, in this study, ALB was not associated with the prognosis of liver failure, the result may be interfered by exogenous ALB infusion during treatment. Therefore, ACLF patients with DM should



**Figure 2 Forest plot of prognostic factors for acute-on-chronic liver failure patients based on multivariate Cox regression analysis.** AUGIH: Acute upper gastrointestinal hemorrhage; BUN: Blood urea nitrogen; FBG: Fasting blood-glucose; HRS: Hepatorenal syndrome; INR: International normalized ratio; SBP: Spontaneous peritonitis; TCHO: Total cholesterol.



**Figure 3 Cumulative survival time in acute-on-chronic liver failure patients with diabetic mellitus and non-diabetic mellitus.**

be paid more attention to ensure sufficient protein and energy intake.

Malnutrition, protein consumption, and chronic inflammation could lead to sarcopenia in chronic liver disease, which is related with adverse clinical outcomes and the increased risk of mortality[34,35]. Further research found that sarcopenia was strongly associated with ACLF development and impacted the poor prognosis of ACLF[36]. Previous study revealed that DM was significantly associated with sarcopenia[37]. DM increased the risk of sarcopenia, which may be a risk factor to induce the poor prognosis in ACLF patients. Therefore, it was important to give adequate nutritional support for the patients with liver failure in order to reduce the

incidence of sarcopenia.

DM, BMI, and NALFD as metabolic risk factors were strongly related with the pathogenesis of metabolic syndrome. This research showed that BMI in DM patients were higher than that in non-DM counterparts. Our previous study showed BMI was associated with hospital mortality in ACLF patients[33], however, this study showed that BMI was not related with ACLF, which may do with the lack of subgroup analysis of BMI. At the same time, our study found that ACLF patients with DM had a higher BMI than the patients with non-DM, which may be a risk factor for the high mortality in ACLF patients with DM.

DM was a significant risk factor for progression of the chronic liver disease[38]. In fact, several previous surveys indicated that survival was significantly lower in DM than in non-DM cirrhotic[10,39]. DM patients were accompanied by high occurrence of NAFLD, and the prevalence of NAFLD in DM varied from 40% to 70%[40,41]. A recent study implied that NAFLD emerged as the rapidly growing etiology of chronic liver disease associated with ACLF[42]. DM was a significant risk factor for progression of the chronic liver disease. In fact, several previous surveys indicated that survival was significantly lower in DM than in non-DM cirrhotic. Our research further confirmed this finding that DM could increase mortality rate in ACLF patients. DM is an important metabolic risk factor that leads to increased mortality in ACLF patients (HR = 3.257,  $P = 0.001$ ). For all of the above reasons, DM may increase the incidence of NAFLD and affect the prognosis of ACLF patients.

Presence of DM was also shown to be related with the high incidence of HE in patients with liver cirrhosis[43,44]. DM increased the risk of HE in cirrhosis patients, the previous studies showed that insulin resistance, muscle breakdown, and glutaminase activity may be mechanistic[44,45]. In terms of complications of liver failure, DM patients had a higher incidence of HE complication than non-DM participants ( $P = 0.026$ ); our study produced exactly the same result as above studies. Compared with the patients without DM, it was known that the patients with DM had higher rates for all infections[46]. Previous studies had shown that DM was associated with high incidence of infection in cirrhotic individuals[10]. Our study showed the incidence of infection was higher in ACLF patients with DM than in counterparts without DM ( $P = 0.038$ ). SBP and pneumonia were common infectious complication of ACLF patients; especially SBP was significantly related with the prognosis of ACLF patients in Cox regression analysis. Therefore, it was extremely important to prevent and control infection in patients with ACLF, especially in the patients with concurrent DM. Among all possible infections, SBP and pulmonary infection should be considered in priority.

### Limits of the study

There are several limitations in our research. Firstly, this was not a multicenter study and the number of DM patients with ACLF was relatively small. Therefore, the magnitude of association between DM and the prognosis of ACLF could have been less precise. Secondly, because of the retrospective design of the study, not all relevant variables were obtained continuously; glycosylated hemoglobin (HBA1c) parameter and blood sugar control level were available sporadically in ACLF patients with DM. Therefore, these variables were not taken into account in the statistical analysis. Finally, Because of the drawback of this retrospective research, to assess further the impact of DM on the prognosis in ACLF patients, a larger cohort study should be conducted.

## CONCLUSION

DM and chronic liver disease in terms of prevalence and mortality are on the rise worldwide. Because of the liver's important role in glucose metabolism, the correlation between liver disease and DM was close. Through this study, we found that DM could predict the prognosis of ACLF patients, and the ACLF patients with DM had higher incidence of mortality and infection, especially in abdominal and pulmonary infections. Consequently, the clinician should pay attention to the result that DM was a major predictor of short-term mortality in ACLF.



## ARTICLE HIGHLIGHTS

**Research background**

The acute-on-chronic liver failure (ACLF) patients have a high short-term mortality rate, and the severity evaluation of ACLF is necessary for prognostication. The prevalence of diabetic mellitus (DM) has increased significantly in recent decades, resulting in coexistence of DM and chronic liver disease be common. There were very few studies that have evaluated the association between DM and ACLF. Therefore, the association between type 2 DM and the prognosis of patients with ACLF remained unclear.

**Research motivation**

Previous studies suggested that DM increased higher incidences of complications in chronic liver disease, included encephalopathy, spontaneous bacterial peritonitis, and hepatocellular carcinoma, but it was not clear whether DM would similarly affect ACLF patients. We needed further to evaluate the association between DM and the prognosis of ACLF patients in order to explore the feasibility of using DM as a prognostic indicator in ACLF patients.

**Research objectives**

We aimed to examine the effect of DM on the prognosis of patients with ACLF and established a predictive model to predict the risk of mortality for ACLF patients. A well-established prognostic predictive model was extremely important, which was helpful for early intervention of ACLF patients as well as improved survival rate.

**Research methods**

Clinical data from 222 ACLF patients were retrospectively collected and analyzed between July 2013 and July 2020 from the Department of Hepatology Research Institute of the First Affiliated Hospital, Fujian Medical University. The patients were categorized into two groups depending on whether they had DM or not, and complications of ACLF were documented during treatment. Values of laboratory parameters, complication rates, and hospital mortality rates were compared between two groups. The Cox proportional hazard regression analysis was used to estimate hazard ratio (HR) for the association between DM and all-cause mortality. DM was independent risk factor to predict mortality in ACLF patients. Further, the nomogram and forest plot were built in terms of results of Cox regression analyses. A survival nomogram model was constructed to predict the probability of 1- and 2-mo survival for ACLF patients.

**Research results**

The prognosis of ACLF patients was significantly correlated with DM in univariate ( $HR = 2.4$ ,  $P < 0.001$ ) and multivariable analysis ( $HR = 3.17$ ,  $P < 0.001$ ). The ACLF patients with DM tended to have a high mortality rate ( $P < 0.001$ ). Survival curves showed that the cumulative survival rate of ACLF patients was significantly distinguished between DM and non-DM ( $P = 0.00019$ ). The survival time of ACLF patients with non-DM was longer than the patients with DM. As a good prognostic indicator, DM was helpful to predict the outcome of ACLF patients. The incidence of infection was higher in DM patients than non-DM counterparts ( $P = 0.038$ ). Among all possible infections, spontaneous peritonitis and pulmonary infection should be considered a priority for ACLF patients. ACLF patients' survival nomogram could predict the probability of 1- and 2-mo survival, which would be helpful to provide early clinical intervention. Because of the retrospective design of the study, we needed to assess further the impact of DM on the prognosis in ACLF patients by a prospective cohort study.

**Research conclusions**

Our study found a significant association between DM and the prognosis of ACLF patients in China. The survival time of ACLF patients with non-DM was longer than that of patients with DM. The ACLF patients with DM had higher incidence of hospital mortality and infection than those without DM. DM was an independent risk factor affecting the prognosis of ACLF patients.

**Research perspectives**

This was not a multicenter study and the number of DM patients with ACLF was

relatively small. Therefore, a multi-center prospective cohort study would evaluate further the impact of DM on the prognosis in ACLF patients. Meanwhile, a noninvasive model should be established based on an extensive clinical database to predict effectively the survival time of ACLF patients.

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## Observational Study

# Preliminary prospective study of real-time post-gastrectomy glycemic fluctuations during dumping symptoms using continuous glucose monitoring

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## Abstract

### BACKGROUND

Although dumping symptoms constitute the most common post-gastrectomy syndromes impairing patient quality of life, the causes, including blood sugar fluctuations, are difficult to elucidate due to limitations in examining dumping symptoms as they occur.

### AIM

To investigate relationships between glucose fluctuations and the occurrence of dumping symptoms in patients undergoing gastrectomy for gastric cancer.

### METHODS

Patients receiving distal gastrectomy with Billroth-I (DG-BI) or Roux-en-Y reconstruction (DG-RY) and total gastrectomy with RY (TG-RY) for gastric cancer (March 2018-January 2020) were prospectively enrolled. Interstitial tissue glycemic profiles were measured every 15 min, up to 14 d, by continuous glucose monitoring. Dumping episodes were recorded on 5 patient-selected days by diary. Within 3 h postprandially, dumping-associated glycemic changes were defined as a dumping profile, those without symptoms as a control profile. These profiles were compared.

### RESULTS

Thirty patients were enrolled (10 DG-BI, 10 DG-RY, 10 TG-RY). The 47 early dumping profiles of DG-BI showed immediately sharp rises after a meal, which 47



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**Data sharing statement:** Technical appendix, statistical code and dataset available from the corresponding author at [souya.nunobe@jfc.or.jp](mailto:souya.nunobe@jfc.or.jp). Participants gave informed consent for data sharing. No additional data are available.

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control profiles did not ( $P < 0.05$ ). Curves of the 15 late dumping profiles of DG-BI were similar to those of early dumping profiles, with lower glycemic levels. DG-RY and TG-RY late dumping profiles (7 and 13, respectively) showed rapid glycemic decreases from a high glycemic state postprandially to hypoglycemia, with a steeper drop in TG-RY than in DG-RY.

## CONCLUSION

Postprandial glycemic changes suggest dumping symptoms after standard gastrectomy for gastric cancer. Furthermore, glycemic profiles during dumping may differ depending on reconstruction methods after gastrectomy.

**Key Words:** Gastric cancer; Gastrectomy; Billroth-I reconstruction; Roux-en-Y reconstruction; Dumping syndrome; Continuous glucose monitoring

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**Core Tip:** Glucose variability at dumping onset was investigated using continuous glucose monitoring and subject diaries after standard gastrectomy for gastric cancer. Postprandial glycemic changes suggest both early and late dumping symptoms. Glycemic profiles during dumping may differ depending on reconstruction methods after gastrectomy, considering the similar glucose fluctuation curves with both early and late dumping after distal gastrectomy with Billroth I reconstruction and rapidly decreasing glucose profiles with late dumping after distal and total gastrectomy, both with Roux-en-Y reconstruction.

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## INTRODUCTION

Globally, gastric cancer (GC) is among the most life-threatening malignancies[1,2]. Gastric resection with lymph node dissection is still the only curative treatment option for resectable GC, though it is noteworthy that early lesions can be resected endoscopically[3]. However, gastrectomy can result in various gastrointestinal symptoms known as post-gastrectomy syndrome, which is characterized by functional deficits and disorders due to loss of some or all of the stomach, often giving rise to clinical issues reflecting deterioration of quality of life for patients[4,5].

Dumping symptoms constitute the most common post-gastrectomy syndrome adversely affecting quality of life[6-8]. According to the time of onset, dumping syndrome is classified into early and late symptoms[9,10] but cannot always be clearly separated into these two categories. Therefore, while some patients develop either early or late dumping symptoms, others may have both. The mechanisms underlying late dumping symptoms especially are thought to involve hypoglycemia in response to hyperinsulinemia after carbohydrate ingestion[10,11]. However, blood glucose changes appearing while patients experience dumping symptoms are still not fully understood because a method allowing blood glucose to be measured easily and continuously has been lacking.

However, the continuous glucose monitoring (CGM) system developed for the management of diabetes allows interstitial glucose levels, which are closely related to blood glucose levels, to be tracked continuously[12,13]. Therefore, details of the 24 h glycemic profile, which includes both postprandial and nocturnal trends not measurable with a simple conventional glucometer, can be obtained using CGM. This also means that CGM has the potential to provide essential information about the glucose profiles of patients suffering from dumping symptoms after gastrectomy.

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Herein, we designed a prospective exploratory pilot study to investigate relationships between glucose fluctuations and the occurrence of dumping symptoms in patients who had undergone gastrectomy for GC, with various reconstructions. To our knowledge, this is the first examination of real-time glucose variability during the onset of dumping symptoms.

## MATERIALS AND METHODS

### Patients

During the period from March 2018 to January 2020, patients who underwent distal or total gastrectomy for GC at the Department of Gastroenterological Surgery, Cancer Institute Hospital, Tokyo, Japan, were prospectively enrolled in this study. The inclusion criteria were as follows: Diagnosed as having pathological stage I or II gastric adenocarcinoma, underwent R0 resection, age 20 to 75 years, 3 mo to 3 years after the operation and Eastern Cooperative Oncology Group Performance Status score 0 or 1. Patients with simultaneous resection of other organs (other than cholecystectomy or splenectomy), diabetes under treatment, receiving adjuvant chemotherapy, and/or taking supplements including enteral nutrition were excluded. Pathological stages were determined according to the 14<sup>th</sup> edition of the Japanese classification of gastric carcinoma[14]. This study was approved by the institutional review board of the Cancer Institute Hospital (No. 2017-1110). All participants signed a written informed consent for the present study. All protocols are carried out in accordance with relevant guidelines.

### Continuous glucose monitoring

FreeStyle Libre Pro (Abbot Diabetes Care Inc., Alameda, CA, United States), a CGM device, was used to continuously measure glucose concentrations. The sensor attached to the posterior surface of the patient's upper arm continuously measured and recorded the glucose concentration in the subcutaneous tissue interstitial fluid every 15 min for up to 14 d. Measurement results automatically saved on the sensor were transferred wirelessly to the reader and then analyzed using FreeStyle Libre Pro Software *via* the reader.

### Assessment of dumping syndrome

Dumping symptoms were evaluated using a diary recording diet and symptoms. Diary entries were made every 15 min and listed the 15 typical symptoms related to dumping, as previously reported[15,16]. The patient filled in the times of starting and completing meals, whether symptoms appeared, allowing the times and corresponding symptoms to be checked. Considering that patient dietary records after gastrectomy often document three or more meals, snacks described by the patient as being about the same amount as an ordinary meal were regarded as meals and were recorded as such in the diary. The diary entries were made for 5 patient-selected days within the 14 d period with the sensor attached.

### Definition of dumping and control

Our strategy for defining the dumping and control profiles is presented in Figure 1. Dumping syndrome is defined as the development of one or more of the 15 symptoms listed in the diary within 3 h of eating a meal. Furthermore, symptoms that occurred within 1 h after the start of a meal were regarded as early dumping and those occurring within 1 h to 3 h after starting a meal were regarded as late dumping, as previously reported[10,17]. To avoid effects of the intakes of other foods, we excluded cases in which another meal was consumed from two hours before to three hours after the baseline meal. The total number of symptoms was described as N-symp. In addition, within 3 h after the start of a meal, glycemic changes associated with early or late dumping symptoms were defined as a dumping profile, while those with no symptoms were defined as a control profile. The control profiles consisted of up to one series per patient per day. The total number with each profile was designated the N-profile. If multiple symptoms appeared simultaneously, the symptoms were counted accordingly, and the profile was only counted once.

### Statistical analysis

The patient background characteristics, surgical details and postoperative findings were collected from our database and information contained in electronic medical

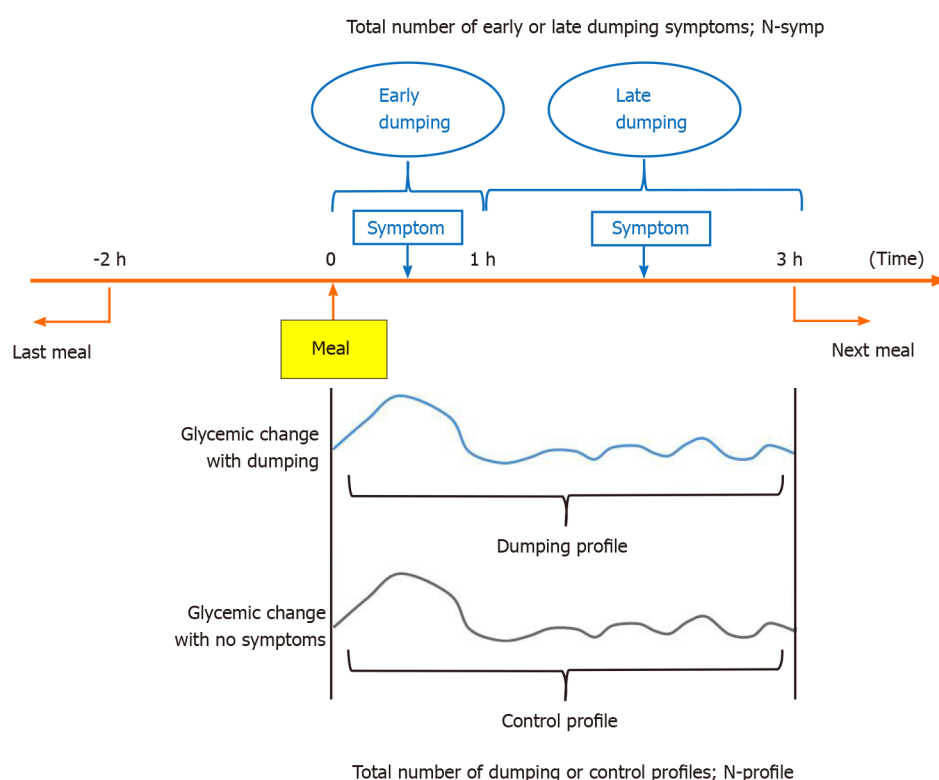


Figure 1 How dumping and control profiles were defined.

records. Based on the glucose concentration values measured every 15 min by CGM and the details of the dumping symptoms described in the diary, dumping and control profiles were compared. All missing values in the data obtained employing CGM were replaced by linear interpolation as single imputation method[18]. All continuous variables were expressed as median values. Although we used *P* value of Mann-Whitney *U* test and the  $\chi^2$  test in addition to basic statistics, statistical analysis is solely exploratory and descriptive without any formal general linear models. The statistical methods of this study were reviewed by Naoki Ishizuka, a biostatistician. A *P* value less than 0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed with JMP Pro 13 (SAS Institute Japan Ltd, Japan) for Windows.

## RESULTS

### Patient characteristics

Patient background data are presented in Table 1. In total, 30 patients were enrolled prospectively: 10 patients each underwent distal gastrectomy with Billroth I reconstruction (DG-BI), distal gastrectomy with Roux-en-Y reconstruction (DG-RY) and total gastrectomy with Roux-en-Y reconstruction (TG-RY). The DG-BI group had a significantly shorter period since surgery than the other two surgical groups (*P* = 0.01). Surgical approach, degree of lymph node dissection and pathological stage differed significantly among the three groups. There were no significant differences in nutritional status or HbA1c levels among the three groups.

### Details of dumping symptoms

The details of early and late dumping symptoms are shown in Tables 2 and 3. In all patients, early dumping symptoms consisted mainly of abdominal symptoms such as borborygmi, bloating and abdominal pain (Table 2). The rates of borborygmi in TG-RY, bloating in DG-BI and drowsiness in DG-RY were significantly higher than those associated with other procedures. On the other hand, late dumping exhibited a wide range of symptoms in all three patient groups, and hypoglycemic symptoms such as cold sweat and drowsiness were relatively common among the features of late dumping syndrome (Table 3).

### Dumping and control profiles

Dumping and control profiles obtained by CGM and the diary results are shown in Figure 2. During the 5 d of diary recording, the respective N-profiles of early dumping were 47, 25 and 54, those of late dumping were 15, 7 and 13, in the order of DG-BI, DG-RY and TG-RY.

The early dumping profiles in DG-BI showed a sharp and immediate rise when starting a meal and then dropped, with significant increases up to 60 min postprandially as compared with the control group ( $P < 0.05$ , Figure 2A). The curves of late dumping profiles in DG-BI were similar to those of early dumping profiles, with generally lower glucose levels (Figure 2A). The early dumping profiles in TG-RY increased significantly from the start of a meal up to 60 min postprandially as compared to the control profiles ( $P < 0.05$ , Figure 2C).

When late dumping developed in DG-RY and TG-RY (Figure 2B and 2C), the dumping profiles in the former showed a sharp decrease from the peak glycemic value at 75 min after starting a meal. A similar but more rapid drop was observed in the dumping profiles in TG-RY from the hyperglycemic state at 45 min after starting a meal. Most notably, glucose levels in TG-RY ultimately decreased to 69 mg/dL at 90 min postprandially.

## DISCUSSION

Dumping syndrome was first reported by Mix in 1922 as a serious complication of gastrectomy[19]. Several mechanisms have been speculated to underlie the development of dumping symptoms[9,10,20]. In early dumping, because of the rapid flow of hyperosmolar food into the jejunum, the plasma fluid rapidly moves into the intestinal lumen and the plasma volume decreases. Furthermore, the release of gastrointestinal hormones, including vasoactive agents, incretins and glucose modulators, is also increased[21]. Consequently, vasomotor symptoms such as palpitations, weakness and faintness and gastrointestinal symptoms such as abdominal pain, bloating and borborygmi develop[10]. On the other hand, hypoglycemia in response to hyperinsulinemia after carbohydrate ingestion has been said to cause hypoglycemic symptoms including the drowsiness and cold sweat characteristic of late dumping[9,10,20]. In fact, early and late dumping symptoms similar to those previously reported were also observed in the present study. However, the causes of the onset of dumping symptoms have not yet been sufficiently elucidated because, as mentioned above, it is difficult to measure changes in plasma volume, various hormones and blood glucose levels when a dumping symptom is actually occurring.

To our knowledge, this is the first exploratory study of real-time changes in glucose profiles when a dumping symptom actually occurs after gastrectomy using CGM and daily recording of diet and symptoms. Although a few studies have demonstrated continuous glucose profiles using CGM after GC surgery, with most simply measuring the glucose fluctuations, the occurrence of dumping symptoms over time has not previously been examined[22-24]. Results of the present study indicated glucose fluctuations to be involved in the onset of late dumping as well as early dumping symptoms after standard gastrectomy for GC.

The early dumping profiles in DG-BI showed rises immediately after the start of meals and subsequently dropped in contrast to the control profiles. Postprandial hyperglycemia is usually observed in patients with impaired glucose tolerance, which is representative of diabetes. However, it was recently found that healthy subjects without diabetes show a similar phenomenon called a blood glucose spike[25]. Although the main mechanisms have been considered to differ between early and late dumping symptoms, a series of similar mechanisms might underlie the development of both early and late dumping symptoms, considering that similar curves of glycemic changes were observed in both symptomatic groups. Mine *et al*[8], who demonstrated a strong correlation between early and late dumping, suggested that a faster or greater flow of food into the small intestine may be the cause of both of early and late dumping symptoms.

On the other hand, in the late dumping profiles in DG-RY and TG-RY, a marked decrease in glucose levels was observed from a high glycemic state around 1 h after starting a meal to a hypoglycemic level around 2 h postprandially. Although hypoglycemia has been regarded as a cause of late dumping symptoms as described above, many hypoglycemic episodes are reportedly asymptomatic, and the appearance of hypoglycemia-like symptoms does not consistently correlate with biochemically verified hypoglycemia after gastrectomy with RY reconstruction for obese patients[26,

**Table 1 Patient demographic and clinical features**

	DG-BI, <i>n</i> = 10	DG-RY, <i>n</i> = 10	TG-RY, <i>n</i> = 10	<i>P</i> value
Sex, <i>n</i> (%)				0.30
Male	3 (30)	6 (60)	6 (60)	
Female	7 (70)	4 (40)	4 (40)	
Age, yr (IQR)	60 (48-70)	63 (55-68)	62 (46-70)	0.97
Period from operation, mo (IQR)	7.1 (6.7-19.2)	23.5 (19.5-26.6)	20.3 (14.9-26.1)	0.01
Body mass index, kg/m <sup>2</sup> (IQR)	19.0 (16.1-23.1)	21.3 (18.4-23.5)	20.5 (19.4-23.1)	0.32
Serum total protein, g/dL (IQR)	7.1 (6.8-7.4)	7.0 (6.7-7.3)	6.8 (6.5-7.3)	0.57
Serum prealbumin, mg/dL (IQR)	23.4 (18.6-28.7)	23.0 (21.8-29.3)	22.0 (18.4-26.5)	0.62
Serum albumin, g/dL (IQR)	4.3 (4.2-4.6)	4.2 (4.0-4.5)	4.1 (4.1-4.3)	0.45
Serum hemoglobin, g/dL (IQR)	12.9 (12.2-14.3)	13.6 (12.2-15.0)	12.1 (11.3-13.2)	0.21
Blood glucose level, mg/dL (IQR)	96 (95-99)	96 (89-114)	93 (88-100)	0.73
HbA1c, % (IQR)	5.7 (5.3-5.9)	5.7 (5.6-5.9)	5.6 (5.5-5.8)	0.73
Approach				< 0.01
Open	0 (0)	2 (20)	6 (60)	
Laparoscopic	10 (100)	8 (80)	4 (40)	
Lymph node dissection, <i>n</i> (%)				< 0.01
D1+	10 (100)	5 (50)	2 (20)	
D2	0 (0)	5 (50)	8 (80)	
pStage, <i>n</i> (%)				< 0.01
I	10 (100)	9 (90)	3 (30)	
II	0 (0)	1 (10)	7 (70)	

DG-BI: Distal gastrectomy with Billroth I reconstruction; DG-RY: Distal gastrectomy with Roux-en-Y reconstruction; HbA1c: Hemoglobin A1c; IQR: Interquartile range; TG-RY: Total gastrectomy with Roux-en-Y reconstruction.

**Table 2 Details of early dumping symptoms in all patients, *n* (%)**

	Early dumping symptoms				<i>P</i> value
	All, N-symp = 185	DG-BI, N-symp = 80	DG-RY, N-symp = 35	TG-RY, N-symp = 70	
Borborygmi	49 (26.5)	14 (17.5)	9 (25.7)	26 (37.1)	0.02
Bloating	47 (25.4)	28 (35.0)	4 (11.4)	15 (21.4)	0.01
Abdominal pain	21 (11.4)	9 (11.3)	2 (5.7)	10 (14.3)	0.42
Drowsiness	21 (11.4)	9 (11.3)	8 (22.9)	4 (5.7)	0.03
Palpitation	15 (8.1)	3 (3.8)	2 (5.7)	10 (14.3)	0.05
Weakness	11 (5.9)	6 (7.5)	3 (8.6)	2 (2.9)	0.37
Others	21 (11.4)	11 (13.8)	7 (20.0)	3 (4.3)	0.03

DG-BI: Distal gastrectomy with Billroth I reconstruction; DG-RY: Distal gastrectomy with Roux-en-Y reconstruction; N-symp: Total number of dumping symptoms; TG-RY: Total gastrectomy with Roux-en-Y reconstruction.

27]. Therefore, the results of the present study raise the possibility that the development of late dumping after RY reconstruction may be related not to hypoglycemia but rather to rapid drops in glucose profiles following meals. A possible mechanism would be that postprandial hyperglycemia, resulting from increased carbohydrate absorption from the upper jejunum, further promotes the secretion of



**Table 3** Details of late dumping symptoms in all patients, *n* (%)

	Late dumping symptoms				<i>P</i> value
	All, N-symp = 62	DG-BI, N-symp = 28	DG-RY, N-symp = 12	TG-RY, N-symp = 22	
Cold sweat	9 (14.5)	3 (10.7)	2 (16.7)	4 (18.2)	0.73
Drowsiness	8 (12.9)	3 (10.7)	1 (8.3)	4 (18.2)	0.64
Diarrhea	8 (12.9)	5 (17.9)	3 (25.0)	0 (0)	0.06
Weakness	6 (9.7)	3 (10.7)	0 (0)	3 (13.6)	0.42
Bloating	6 (9.7)	3 (10.7)	1 (8.3)	2 (9.1)	0.96
Abdominal pain	6 (9.7)	1 (3.6)	3 (25.0)	2 (9.1)	0.10
Borborygmi	5 (8.1)	3 (10.7)	2 (16.7)	0 (0)	0.18
Palpitations	4 (6.5)	1 (3.6)	0 (0)	3 (13.6)	0.21
Tremulousness	4 (6.5)	2 (7.1)	0 (0)	2 (9.1)	0.57
Others	6 (9.7)	4 (14.3)	0 (0)	2 (9.1)	0.37

DG-BI: Distal gastrectomy with Billroth I reconstruction; DG-RY: Distal gastrectomy with Roux-en-Y reconstruction; N-symp: Total number of dumping symptoms; TG-RY: Total gastrectomy with Roux-en-Y reconstruction.

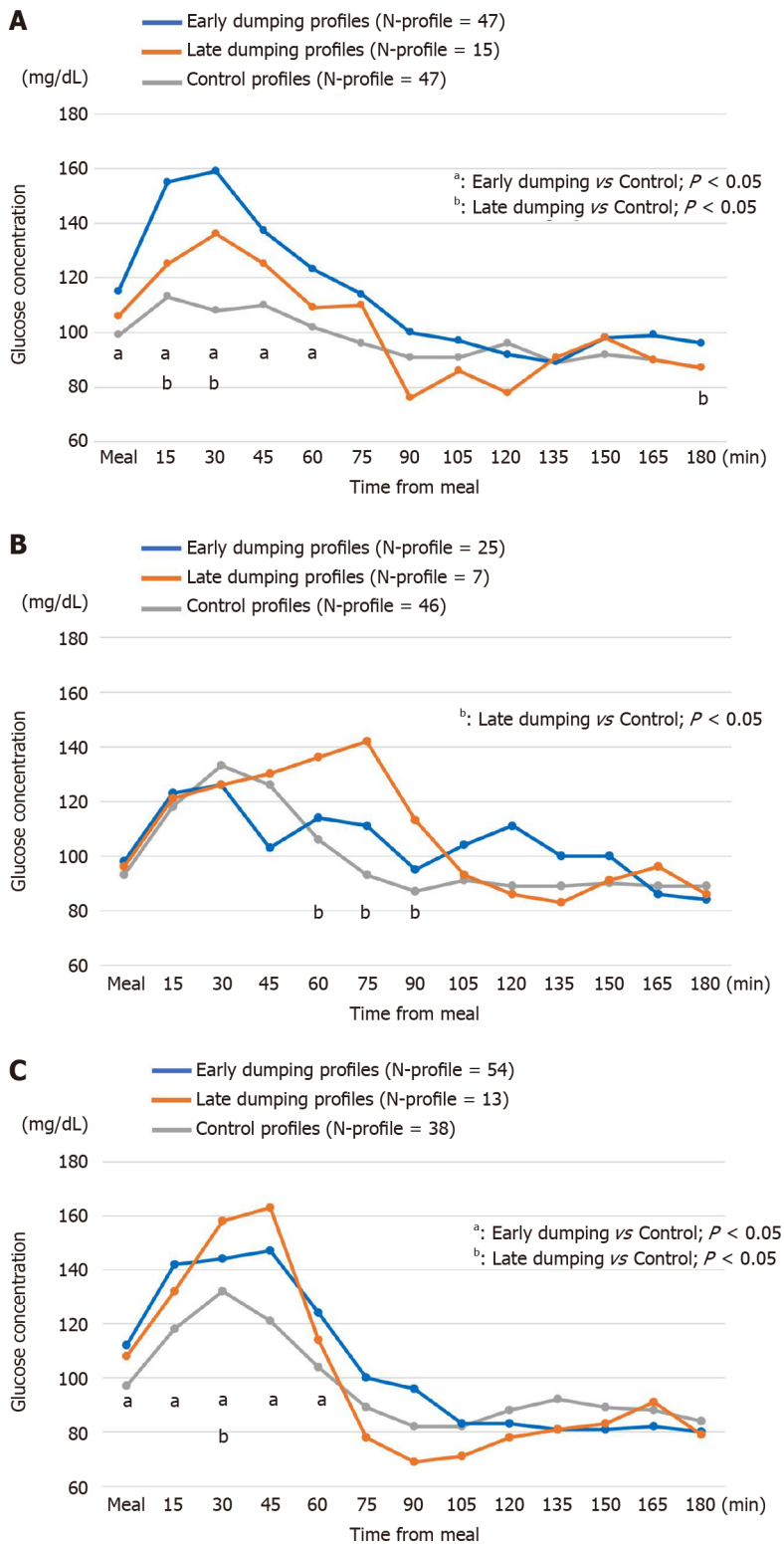
incretins such as GLP-1, one of the gastrointestinal hormones, resulting in hyperinsulinemia and subsequently a rapid decrease in glucose levels[28].

Although a similar glycemic variability was observed in the late dumping groups that had undergone DG-RY and TG-RY, the curve of glucose profiles was steeper in the TG-RY than in the DG-RY dumping group. Due to the lack of storage capacity, that is the absence of part or all of the stomach, there might be a faster flow of food into the jejunum in TG-RY than in DG-RY, resulting in a more rapid glucose level change and higher number of dumping symptoms. In contrast, some of the observed variation in glycemic profiles might have been due to differences in the size of the remnant stomach in DG-RY, resulting in a lack of statistical significance. In addition, considering that the control profiles in DG-RY and TG-RY were more remarkable than those in DG-BI, RY reconstruction, a non-physiological reconstruction method in which food does not pass through the duodenum, may impact glucose fluctuations after a meal more adversely than the other procedures.

The present study has potential limitations. First, although prospective, this was a preliminary study with a small sample size conducted at a single institution. It is possible that statistical differences could not be demonstrated due to the small number of events. In addition, variation in the frequency of dumping among patients might have produced statistical bias. Further accumulation of cases, allowing a study with a larger sample size, is needed. Second, postoperative periods were not similar among the three groups. Although we aimed herein to enroll patients in the mid to long term after surgery, differences in postoperative periods may have influenced the onset of dumping symptoms and glycemic change. Third, dietary details were not documented in this study. Caloric intake has a significant effect on blood glucose levels, and the manner in which a meal is consumed may affect the onset of dumping symptoms. Finally, although glucose profiles and dumping symptoms were sequentially investigated, other factors possibly involved in the onset of dumping were not examined. The evaluation of changes in hemodynamics and various hormones, which are considered to be factors causing dumping, is another topic for future research.

## CONCLUSION

Postprandial rapid glycemic changes appear to be involved in the onset of early and late dumping symptoms after standard gastrectomy for GC. Given the similar glucose fluctuation curves with early and late dumping in DG-BI and the rapid decrease in glucose profiles with late dumping in DG-RY and TG-RY, the glycemic profiles associated with dumping symptoms may differ depending on the reconstruction methods employed after gastrectomy.



**Figure 2** Glycemic changes with dumping symptoms (dumping profiles) and without symptoms (control profiles) after gastrectomy. A: Dumping and control profiles after distal gastrectomy with Billroth I reconstruction; B: Dumping and control profiles after distal gastrectomy with Roux-en-Y reconstruction; C: Dumping and control profiles after total gastrectomy with Roux-en-Y reconstruction.

## ARTICLE HIGHLIGHTS

### Research background

Dumping symptoms constitute the most common post-gastrectomy syndrome adversely affecting quality of life. However, the causes of dumping symptoms, including blood glucose changes, remain poorly understood due to limitations in examining dumping symptoms as they occur.

### Research motivation

The continuous glucose monitoring (CGM) system, which continuously measures interstitial glucose levels to reflect blood glucose levels, was developed for the management of diabetes. CGM also has the potential to provide long awaited essential information about the glucose profiles of patients suffering from dumping symptoms after gastrectomy.

### Research objectives

We designed a prospective pilot study to investigate relationships between glucose fluctuations and the occurrence of dumping symptoms in patients undergoing gastrectomy for gastric cancer (GC). Our results may contribute to devising future treatments for dumping syndrome.

### Research methods

During the period from March 2018 to January 2020, GC patients who underwent distal gastrectomy with Billroth I reconstruction (DG-BI), distal gastrectomy with Roux-en-Y reconstruction (DG-RY) or total gastrectomy with Roux-en-Y reconstruction (TG-RY) were prospectively enrolled in this study. Based on the glucose concentration values measured every 15 min by CGM and the details of the dumping symptoms (early dumping within 1 h postprandially, late dumping within 1 h to 3 h postprandially) described in diaries, patients with dumping-associated glycemic changes (dumping profiles) were compared to those without symptoms (control profiles). This is the first examination of real-time glucose variability during the onset of dumping symptoms using CGM.

### Research results

Thirty patients were enrolled (10 DG-BI, 10 DG-RY, 10 TG-RY). The early dumping profiles of DG-BI (47 profiles) showed a sharp and immediate rise after a meal, with significant increases up to 60 min postprandially as compared with the control group (47 profiles) ( $P < 0.05$ ). The curves of late dumping profiles in DG-BI were similar to those of early dumping profiles, with generally lower glucose levels. DG-RY and TG-RY late dumping profiles (7 and 13, respectively) showed rapid glycemic decreases from a high glycemic state postprandially to hypoglycemia, with the drop being steeper in TG-RY than in DG-RY.

### Research conclusions

Postprandial rapid glycemic changes appear to be involved in the onset of early and late dumping symptoms after standard gastrectomy for GC. In addition, the glycemic profiles associated with dumping symptoms may differ depending on the reconstruction methods employed after gastrectomy, considering the similar glucose fluctuation curves with both early and late dumping after DG-BI and rapidly decreasing glucose profiles with late dumping after DG-RY and TG-RY.

### Research perspectives

We will conduct a prospective interventional study with the aim of developing new treatments ameliorating dumping symptoms associated with GC surgery.

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## Prospective Study

# Real-world treatment patterns and disease control over one year in patients with inflammatory bowel disease in Brazil

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#### Institutional review board

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The clinical trial is registered with ClinicalTrials.gov using identifier NCT02822235. Details can be found at <https://clinicaltrials.gov/ct2/show/NCT02822235>.

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## Abstract

### BACKGROUND

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBDs) with a remission-relapsing presentation and symptomatic exacerbations that have detrimental impacts on patient quality of life and are associated with a high cost burden, especially in patients with moderate-to-severe disease. The Real-world Data of Moderate-to-Severe Inflammatory Bowel Disease in Brazil (RISE BR) study was a noninterventional study designed to evaluate disease control, treatment patterns, disease burden and health-related quality of life in patients with moderate-to-severe active IBD. We report findings from the prospective follow-up phase of the RISE BR study in patients with active UC or CD.

### AIM

To describe the 12-mo disease evolution and treatment patterns among patients with active moderate-to-severe IBD in Brazil.

### METHODS

This was a prospective, noninterventional study of adult patients with active Crohn's disease (CD: Harvey-Bradshaw Index  $\geq 8$ , CD Activity Index  $\geq 220$ ), inadequate CD control (*i.e.*, calprotectin  $> 200 \mu\text{g/g}$  or colonoscopy previous results), or active ulcerative colitis (UC: Partial Mayo score  $\geq 5$ ). Enrollment occurred in 14 centers from October 2016 to February 2017. The proportion of active IBD patients after 9-12 mo of follow-up, Kaplan-Meier estimates of the time to mild or no activity and a summary of treatment initiation, discontinuation and dose changes were examined.

### RESULTS

The study included 118 CD and 36 UC patients, with mean  $\pm$  SD ages of  $43.3 \pm 12.6$  and  $44.9 \pm 16.5$  years, respectively. The most frequent drug classes at index were biologics for CD (62.7%) and 5-aminosalicylate derivatives for UC patients

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(91.7%). During follow-up, 65.3% of CD and 86.1% of UC patients initiated a new treatment at least once. Discontinuations/dose changes occurred in 68.1% of CD patients [median 2.0 (IQR: 2-5)] and 94.3% of UC patients [median 4.0 (IQR: 3-7)]. On average, CD and UC patients had  $4.4 \pm 2.6$  and  $5.0 \pm 3.3$  outpatient visits, respectively. The median time to first mild or no activity was 319 (IQR: 239-358) d for CD and 320 (IQR: 288-358) d for UC patients. At 9-12 mo, 22.0% of CD and 20.0% of UC patients had active disease.

## CONCLUSION

Although a marked proportion of active IBD patients achieved disease control within one year, the considerable time to achieve this outcome represents an unmet medical need of the current standard of care in a Brazilian real-world setting.

**Key Words:** Crohn's disease; Ulcerative colitis; Inflammatory bowel diseases; Prospective study

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**Core Tip:** This was a prospective, noninterventional study of 118 adult patients with active Crohn's disease (CD) and 36 with active ulcerative colitis (UC). The aim was to describe the 12-mo disease evolution and treatment patterns of active moderate-to-severe inflammatory bowel disease (IBD) patients in Brazil. The median time to first mild or no activity was 319 (IQR: 239-358) d for CD and 320 (IQR: 288-358) d for UC patients. A marked proportion of active IBD patients achieved disease control within one year, which is considered a long time to achieve the goal of reducing inflammation.

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## INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBDs) with a remission-relapsing presentation and symptomatic exacerbations that have a detrimental impact on patient quality of life and are associated with a high cost burden, especially in the case of moderate to severe disease[1,2]. The pharmacological treatment of IBD aims to achieve and maintain long-term remission and heal the gut mucosa, thus delaying the progression of disease and associated complications as well as the need for surgery[3]. For CD patients with moderate-to-severe disease activity, guidelines usually recommend the use of antitumor necrosis factor- $\alpha$  (anti-TNF $\alpha$ ) either as a monotherapy or combined with an immunosuppressant to induce remission for steroid-refractory disease or steroid-intolerant patients[4]. With regard to moderate-to-severe active UC, an immunosuppressant and/or a biologic drug are recommended[5,6]. Systemic 5-aminosalicylate (5-ASA) agents are also indicated for maintenance treatment of UC[1]. Corticosteroids are recommended for short-term use ( $\leq 3$  mo) during relapses of both CD and UC and are not recommended as maintenance therapy[1,4,6].

In clinical practice, physicians often try several options and treatment sequences for IBD management, both in monotherapy or combined regimens[3]. Primary nonresponse, secondary loss of response and development of adverse reactions to anti-TNF $\alpha$  drugs may occur and lead to discontinuation, with the subsequent prescription of a second advanced therapy with a different mechanism of action (*e.g.*, anti-integrins

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or JAK inhibitors)[7-11]. However, some studies have reported that up to half of IBD patients who discontinue their first-line biologic (infliximab or adalimumab) do not restart or switch to another therapy[3,12]. In Brazil, vedolizumab, ustekinumab and tofacitinib are available at private health services, the latter being approved only for UC treatment since 2018. Moreover, the VARSITY study recently demonstrated that the gut-selective anti-integrin vedolizumab was superior to the anti-TNF $\alpha$  adalimumab in achieving clinical remission in patients with moderate-to-severe active UC after one year[13].

Real-world data on IBD management could unveil unmet medical needs and treatment gaps[3]. This is of utmost relevance in developing countries such as Brazil, where the prevalence of IBD is increasing but access to biologic treatment may be restricted, and information on IBD treatment, in general, and associated outcomes is scarce[14,15]. The Real-world Data of Moderate-to-Severe Inflammatory Bowel Disease in Brazil (RISE BR) study was a noninterventional study designed to evaluate disease control, treatment patterns, burden of disease and health-related quality of life in patients with a previous diagnosis of moderate-to-severe active IBD[16,17]. Here, we report findings from the prospective follow-up phase of the RISE BR study in patients with active UC or CD and aim to describe the real-world treatment patterns and outcomes of IBD-related therapies during a 12-mo follow-up period.

## MATERIALS AND METHODS

### Study design

The RISE BR study (ClinicalTrials.gov Identifier: NCT02822235) was a national, multicenter, noninterventional study designed to evaluate disease control, treatment patterns, burden of disease and health-related quality of life among IBD patients in Brazil. It involved a cross-sectional evaluation of IBD patients (index date) with a retrospective data collection component and a 12-mo prospective follow-up period of IBD patients who had active disease (at the index date). The cross-sectional evaluation was conducted at routine IBD outpatient visits between October 2016 and February 2017, and patients were consecutively recruited from 14 study sites (nine clinics from the public health system and five private clinics) covering Brazil's most populated regions. In this article, we present data from the follow-up period. All participants provided written informed consent prior to the study procedures. The study was approved by the local ethics committees and was conducted according to the principles of the Helsinki Declaration.

### Study population

At the index date, patients were included if they were aged 18 years or older and had a confirmed diagnosis of moderate-to-severe CD or UC at least 6 mo prior to index. Patients were excluded if they presented indeterminate/unclassified colitis, mental incapacity, unwillingness, or language barriers precluding adequate understanding or cooperation with the study, were hospitalized, participated in a clinical trial within the last 3 years, or received off-label treatment with vedolizumab. All patients presenting active disease at the index date were invited to participate in the prospective follow-up phase of the study. Active CD was defined as a Harvey-Bradshaw Index (HBI)  $\geq 8$  points or a Crohn's Disease Activity Index (CDAI)  $\geq 220$  points or fecal calprotectin levels  $> 200 \mu\text{g/g}$  or colonoscopy results in the previous year suggestive of inadequate control (as per investigator criteria)[18]. Active UC was defined as a partial Mayo (pMayo) score  $\geq 5$  points.

### Prospective follow-up period and study variables

The prospective observational period was defined as a 12-mo follow-up since the index date. Data were collected from patients' medical records by staff personnel of the participating site. Sociodemographic characteristics (age, sex, educational level, and professional status), weight and body mass index (BMI), smoking habits, and family history of IBD were collected at the study appointment. Other clinical variables included the time since first IBD diagnosis, extension/severity and location of CD or UC (Montreal classification), corticosteroid dependency/intolerance status, and presence of extraintestinal manifestations. In addition, IBD treatment patterns and changes during the follow-up period (at the index date and the following medical appointments) were collected.

Grade E (Poor): 0

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CD activity during follow-up was evaluated with the HBI[18] and/or CDAI[19], depending on local practice. Moderate-to-severe active CD was defined as patients with an HBI  $\geq 8$  or a CDAI  $\geq 220$ , while CD disease control (*i.e.*, mild or no disease activity) was defined as an HBI  $< 8$  or a CDAI  $< 220$ [18-20]. UC disease activity during follow-up was assessed with the 9-item pMayo score; moderate-to-severe active UC was defined as pMayo  $\geq 5$ , and UC disease control (*i.e.*, mild or no UC activity) was defined as pMayo  $< 5$ [21]. Given the noninterventional design of the study, there were no predefined time points of IBD activity assessment, and measures were collected in the course of typical disease treatment.

### Statistical analysis

Descriptive statistics [mean, median, standard deviation (SD) and minimum-maximum] were used to analyze sociodemographic, clinical, and treatment-related variables. Student's *t*-test for independent samples or the Mann-Whitney test were used to compare CD and UC patients regarding quantitative variables. Fisher's exact test and the chi-square test were used for qualitative variables.

Overall, 407 patients (264 CD and 143 UC) were enrolled in the cross-sectional assessment. All patients with active IBD at the cross-sectional appointment were eligible for the prospective phase of the study, including 118 CD patients and 36 UC patients. These sample sizes allowed the use of a desirable central limit theorem in outcome inference, even accounting for sample losses [11/118 (9.3%) in CD; 2/36 (5.6%) in UC] after 12 mo of follow-up.

The time to disease control was calculated using the Kaplan-Meier approach, defined as the time (in days) from index date until the date of the first documentation of disease control [*i.e.*, achieved disease control, mild or no active disease (defined as event)]. Patients who did not achieve disease control, died (without the event), discontinued the study or were lost to follow-up had their time censored at death or at the date of the last visit.

The proportion of patients with moderate-to-severe active IBD at 12 mo was calculated by IBD type at the last visit between 9 and 12 mo of follow-up.

Treatment changes were defined as drug discontinuations or dose changes (*i.e.*, changes in frequency and/or dosage administered) during follow-up for ongoing medications or initiated at the index date and any new medication initiated after the index date. Patients who did not have any visits during follow-up or patients who had no ongoing treatments were considered missing in the survival analysis.

For each IBD type, bivariate analyses were conducted to evaluate sociodemographic, clinical and treatment-related variables between subjects who achieved disease control during the 12-mo follow-up period *vs* those who did not. Chi-square or Fisher's exact tests were used to compare categorical variables, *t*-tests were used for independent samples, and the nonparametric Mann-Whitney test was used to compare quantitative variables. The incidence rate of treatment changes by person-years was also determined.

All statistical tests were two-tailed, with a significance level of 0.05, using SAS® (version 9.4, SAS Institute Inc., Cary).

## RESULTS

### Patient disposition and characteristics

Of 407 subjects (75.4%,  $n = 307$ , from public clinics) evaluated at the index date, 37.8% ( $n = 118$  CD and 36 UC) were included in the prospective study phase (Figure 1). The majority of included patients were from public clinics: 74.6% ( $n = 88$ ) CD and 72.2% ( $n = 26$ ) UC patients. During the 12-mo follow-up, 11 (9.3%) CD patients and 2 (5.6%) UC patients discontinued the study, and one CD patient died. Overall, the mean  $\pm$  SD follow-up times were  $340.4 \pm 59.2$  d and  $339.2 \pm 64.8$  d for CD and UC patients, respectively. A total of 107 CD patients and 34 UC patients completed the study and had a medical appointment between months 9 and 12, of which 82 CD patients and 25 UC patients had at least one evaluation of disease activity during this time window.

Most patients were female (58.5% and 78.8% of CD and UC patients, respectively), and the mean  $\pm$  SD age at baseline was  $43.3 \pm 12.6$  years for CD patients and  $44.9 \pm 16.5$  years for UC patients (Table 1). A total of 45 (38.1%) CD patients and 22 (61.1%) UC patients had their first diagnosis of moderate-to-severe IBD within the past 5 years. The median CD disease duration was similar between patients receiving biologics and those not receiving biologics at the index date [11.0 (IQR: 0.5-11.0) *vs* 11.5 (IQR: 0.5-29.1) years]. UC patients on biologics at index had a shorter disease duration than



**Table 1 Sociodemographic and disease characteristics of patients at the index date**

	CD patients (n = 118)	UC patients (n = 36)
Age (yr), mean $\pm$ SD	43.3 $\pm$ 12.6	44.9 $\pm$ 16.5
Female, n (%)	69 (58.5)	26 (72.2)
Employed, n (%)	36 (36.7)	5 (16.1)
Attended higher education, n (%)	32 (34.4)	5 (23.8)
Body mass index (kg/m <sup>2</sup> ), mean $\pm$ SD	24.9 $\pm$ 4.7	24.9 $\pm$ 5.1
Current smokers, n (%) <sup>1</sup>	7 (5.9)	0 (0.0)
Family history of IBD, n (%)	15 (12.7)	2 (5.6)
IBD duration (yr), median (IQR)	11.0 (0.5-11.0)	6.5 (0.5-29.1)
Biologic group	11.0 (5.0-16.0)	6.0 (3.0-13.0)
Nonbiologic group	11.5 (4.5-18.5)	10.0 (4.0-17.0)
5-ASA derivatives group	9.0 (4.0-13.0)	5.5 (4.0-14.0)
Non-5-ASA derivatives group	11.5 (5.0-17.5)	13.0 (4.0-15.0)
Corticosteroid status, n (%) <sup>2</sup>		
Steroid-dependent	17 (14.4)	10 (27.8)
Steroid-refractory disease	5 (4.2)	3 (8.3)
Not applicable (no previous use)	33 (28.0)	3 (8.3)
Unknown	37 (31.4)	15 (41.7)
Location of CD, n (%)		
L1 ileal	26 (22.0)	-
L2 colonic	21 (17.8)	-
L3 ileocolonic	71 (60.2)	-
CD Behavior, n (%)		
B1 nonstricturing/penetrating	31 (26.3)	-
B2 stricturing	52 (44.1)	-
B3 penetrating	35 (29.7)	-
Symptomatic perianal disease, n (%)	37 (31.6)	-
HBI result (available for 101 CD patients), median (IQR)	5.0 (1.0-9.0)	-
CDAI result (available for 43 CD patients), median (IQR)	200.0 (114.0-274.0)	-
Fecal calprotectin level > 200 $\mu$ g/g during previous year, n (%)	40 (33.9)	-
Colonoscopy with CD activity <sup>3</sup> during previous year, n (%)	69 (58.5)	-
Extent of UC inflammation - Montreal classification, n (%)		
E1 ulcerative proctitis	-	10 (27.8)
E2 left-sided UC	-	8 (22.2)
E3 pancolitis	-	18 (50.0)
Partial Mayo score, median (IQR)	-	6.0 (5.0-6.0)
Total Mayo score (available for 13 UC patients)	-	8.0 (7.0-8.0)
Extra intestinal manifestations <sup>4</sup> , n (%)	29 (24.6)	8 (22.2)
Patients with at least one IBD surgery during previous 3 yr, n (%)	32 (27.1)	1 (2.8)
Total colectomy	0 (0.0)	1 (2.8)
Fistulectomy	7 (5.9)	0 (0.0)
Enterostomy	6 (5.1)	0 (0.0)



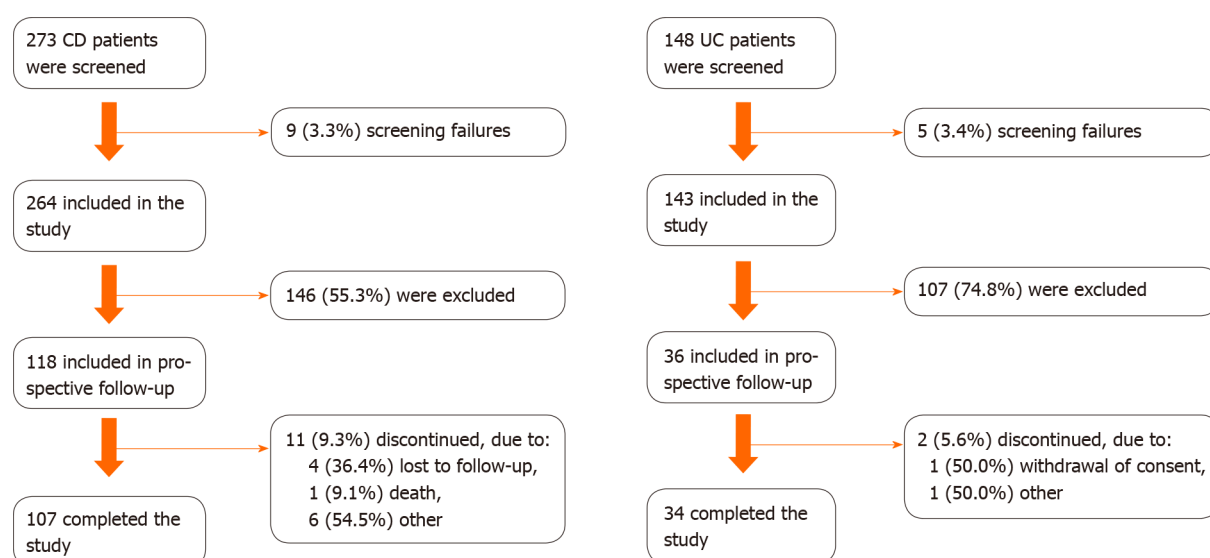
Partial colectomy	5 (4.2)	0 (0.0)
Closure of enterostomy	5 (4.2)	0 (0.0)
Drainage of anorectal abscess	3 (2.5)	0 (0.0)
Jejunostomy/Ileostomy	1 (0.8)	0 (0.0)
Other	15 (12.7)	1 (2.8)
Patients with at least one IBD-related hospitalizations during previous 3 yr, n (%)	51 (43.1)	11 (30.6)

<sup>1</sup>Smoking at Day 1 and has smoked 100 cigarettes in his/her lifetime.

<sup>2</sup>Steroid-dependent disease - defined as: (1) Being unable to reduce steroids below the equivalent of prednisolone at 10 mg/day within 3 mo of starting steroids, without recurrent disease; or (2) having a relapse within 3 mo of stopping glucocorticoids. Steroid-refractory disease - defined as having active disease despite prednisolone of up to 0.75 mg/kg/d over a period of 4 wk.

<sup>3</sup>As per investigator criteria.

<sup>4</sup>During the entire study period (3 retrospective years and 1 year of prospective follow-up), extraintestinal manifestations included arthralgia, arthritis, sacroiliitis, ankylosing spondylitis, erythema nodosum, pyoderma gangrenosum, pustular dermatitis, psoriasis, uveitis, sclerosing cholangitis, cholelithiasis, nephrolithiasis, autoimmune disease, hypertension, and dyslipidemia. SD: Standard deviation; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; HBI: Harvey-Bradshaw index; CDAI: Crohn's disease activity index; pMayo: Partial Mayo score; 5-ASA: 5-aminosalicylate; IQR: Interquartile.



**Figure 1 Patient disposition for Crohn's disease patients and ulcerative colitis patients.** UC: Ulcerative colitis; CD: Crohn's disease.

patients not receiving biologics (6.0 years *vs* 10.0 years).

At the index date, 46 (39.0%) CD patients had active disease according to the CDAI or HBI, of whom 24 were identified based on these indexes only, with six presenting severely active CD (HBI > 16 or CDAI > 450). Regarding disease behavior, 52 (44.1%) and 35 (29.7%) CD patients had B2 stricturing and B3 penetrating disease, respectively. In addition, 40 (33.9%) patients had fecal calprotectin levels > 200 µg/g (22 patients identified only by calprotectin levels), and 69 (58.5%) had a colonoscopy results suggestive of disease activity during the previous year (35 patients identified by this criterion only). UC patients with active disease had a median pMayo of 6.0 (IQR: 5.0-6.0) at baseline, four (11.1%) had severe UC based on pMayo > 7, and 50.0% of patients presented with pancolitis.

### **IBD treatment patterns and changes during follow-up**

Biologics were the most frequent drug class that CD patients were receiving at the index date appointment (62.7%), and 5-ASA derivatives (91.7%) were the most common for UC patients; however, it is important to highlight that biologics were not available as a UC treatment strategy in Brazil at the time of the study (Table 2). Approximately 48% of CD patients and 58.3% of UC patients were receiving corticosteroids at the index date. Half (50.0%) of UC patients had received at least one corticosteroid for a period of 3 mo or longer. At the start of the 12-mo follow-up period, 58.5% of CD

**Table 2 Treatment patterns and medical appointments during the follow-up period (Crohn's disease and ulcerative colitis patients)**

	CD patients (n = 118)	UC patients (n = 36)
IBD treatment at index date appointment, n (%) <sup>1</sup>	111 (94.1)	35 (97.2)
5-ASA derivatives	36 (30.5)	33 (91.7)
Corticosteroids	56 (47.5)	21 (58.3)
Immunosuppressants	65 (55.1)	21 (58.3)
Biologics	74 (62.7)	14 (38.9)
Patients who received at least one corticosteroid for $\geq 3$ mo, n (%) <sup>2</sup>	31 (26.3)	18 (50.0)
Treatments at the start of 12-mo follow-up (index date), n (%)		
IBD treatment initiated or maintained	112 (94.9)	35 (97.2)
Biologic therapy		
Maintained or initiated at index date	75 (63.6)	14 (38.9)
Maintained	69 (58.5)	11 (30.6)
Initiated new	6 (5.1)	3 (8.3)
Naïve to biologics <sup>3</sup>	1 (0.8)	1 (2.8)
Discontinued at index date	0 (0.0)	0 (0.0)
Immunosuppressants		
Maintained or initiated at index date	73 (61.9)	19 (52.8)
Maintained	71 (60.2)	14 (38.9)
Initiated new	2 (1.7)	5 (13.9)
Discontinued at index date	1 (0.8)	0 (0.0)
5-ASA compounds		
Maintained or initiated at index date	19 (16.1)	27 (75.0)
Maintained	17 (14.4)	20 (55.6)
Initiated new	2 (1.7)	5 (13.9)
Naïve to 5-ASA compounds	2 (1.7)	5 (13.9)
Discontinued at index date	1 (0.8)	0 (0.0)
Corticosteroids		
Maintained or initiated at index date	16 (13.6)	15 (41.7)
Maintained	9 (7.6)	6 (16.7)
Initiated new	6 (5.1)	8 (22.2)
Naïve to corticosteroids <sup>3</sup>	2 (1.7)	1 (2.8)
Discontinued at index date	2 (1.7)	4 (11.1)
Any antibiotic initiated at index date	12 (10.2)	5 (13.9)
Treatment changes during 12-mo follow-up period		
New treatment initiated during follow-up	77 (65.3)	31 (86.1)
Discontinuation or dose change during follow-up <sup>2</sup>	77 (68.1)	33 (94.3)
Median (IQR) number of treatment changes by patient	2 (2-5)	4 (3-7)
Biologic therapy		
Initiated	45 (38.1)	6 (16.7)
Naïve to biologics <sup>4</sup>	13 (11.0)	1 (2.8)
Discontinued	23 (19.5)	5 (13.9)
Dose change	14 (11.9)	4 (11.1)

Immunosuppressants		
Initiated	24 (20.3)	14 (38.9)
Naïve to immunosuppressants <sup>4</sup>	4 (3.4)	2 (5.6)
Discontinued	20 (16.9)	11 (9.3)
Dose change	7 (5.9)	7 (19.4)
5-ASA derivatives		
Initiated	10 (8.5)	21 (58.3)
Naïve to 5-ASA compounds <sup>4</sup>	1 (0.8)	2 (5.6)
Discontinued	7 (5.9)	8 (22.2)
Dose change	4 (3.4)	14 (38.8)
Corticosteroids		
Initiated	24 (20.3)	16 (44.4)
Naïve to corticosteroids <sup>4</sup>	5 (4.2)	2 (5.6)
Discontinued	23 (19.5)	17 (47.2)
Any antibiotic initiated after index date	23 (19.5)	5 (13.9)

<sup>1</sup>Inflammatory bowel disease (IBD) treatment patients were receiving at the time of enrollment, irrespective of the physician's decision to continue or discontinue it at the end of the medical appointment.

<sup>2</sup>Percentages calculated from 113 Crohn's disease and 35 ulcerative colitis patients, since two patients had no IBD medication and four had no medical appointments during follow-up.

<sup>3</sup>Assuming the information from the previous 3 year.

<sup>4</sup>Assuming the information from the previous 3 years and index date. Values are *n* (%), and percentages calculated within the total of the study population in each group, except if otherwise mentioned. SD: standard deviation; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; 5-ASA: 5-aminosalicylate.

patients maintained their current biologic therapy, while 5.1% initiated a new biologic. Regarding UC patients, 30.6% maintained their biologic therapy, while 8.3% initiated a new biologic treatment. Current 5-ASA agents were maintained by 14.4% of CD patients, while 55.6% of UC patients maintained the same therapy.

During follow-up, 65.3% of CD and 86.1% of UC patients switched to a new treatment at least once. Discontinuation or dose changes (excluding antibiotics) of IBD-related treatments occurred in 68.1% of CD and 94.3% of UC patients.

The incidence rates of drug discontinuations and dose changes were 9.2 and 6.8 changes/person-years for CD and UC patients, respectively (data not shown). During follow-up, the median number of treatment changes among CD patients was 2.0 (IQR 2-5), with 26 (33.8%) patients having more than four changes. Regarding drug classes, 19.5% of CD patients discontinued biologic therapy, 16.9% discontinued immunosuppressants, and 5.9% discontinued 5-ASA derivatives.

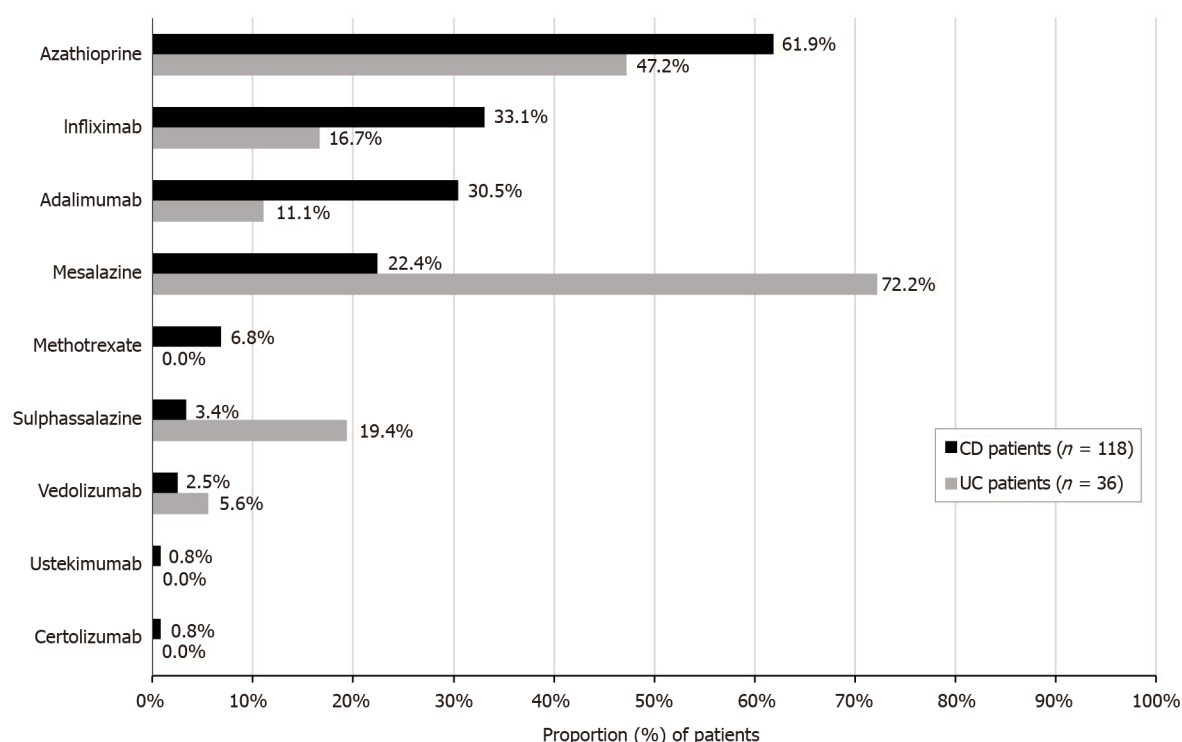
In UC patients, the median number of treatment changes was 4.0 (IQR 3-7), with 18 (54.5%) patients having more than four treatment changes. Regarding drug classes, 22.2% of UC patients discontinued 5-ASA derivatives, 13.9% discontinued biologics, and 9.3% discontinued immunosuppressants.

Azathioprine was the most frequently maintained or newly initiated agent in CD patients (61.9%), while mesalazine was the agent most maintained or newly initiated treatment by UC patients (72.2%), whether as monotherapy or combination therapy (Figure 2).

### **Treatment patterns at the medical appointments during follow-up**

The vast majority (96.6%) of CD patients had at least one outpatient visit during the follow-up period, with a mean  $\pm$  SD number of  $4.4 \pm 2.6$  visits per patient and a total of 518 visits. Treatment changes were observed in 25.3% (*n* = 131) of CD outpatient visits, and disease activity was assessed by the HBI and/or CDAI scores in 12.6% (*n* = 51) and 6.4% (*n* = 26) of visits, respectively.

Biologics were the most frequent treatments used during follow-up (76.8% in the first quarter of the follow-up), followed by immunosuppressants (65.2%) and 5-ASA compounds (16.1%). The use of corticosteroids started to decrease from the third quarter of follow-up (Figure 3).



**Figure 2** Proportion (%) of Crohn's disease and ulcerative colitis patients who maintained or initiated new inflammatory bowel disease drugs at the index date. UC: Ulcerative colitis; CD: Crohn's disease.

All UC patients had at least one visit during the follow-up period, with a mean  $\pm$  SD number of  $5.0 \pm 3.3$  visits per patient and a total of 179 visits. Treatment changes were observed among 40.2% ( $n = 72$ ) of UC outpatient visits, and the assessment of UC activity with the Mayo score was performed in 11.2% ( $n = 16$ ) of visits. The most prescribed agent during the first quarter of follow-up was 5-ASAs (83.3%), followed by immunosuppressants (58.3%) and biologics (38.9%). Corticosteroid use decreased from 50.0% in the first trimester to 26.5% in the last trimester of follow-up (Figure 3).

### Time to IBD disease control during follow-up

During the 12-mo follow-up period, 86 (72.9%) CD patients achieved disease control. The median time to first disease control for CD from the index date was 319 d (IQR 239-358) [95%CI: 295-330], *i.e.*, approximately 10.6 mo (Figure 4A). There were five switches from "mild or no disease activity" to "moderate or severe disease" among the patients who had the event (control disease,  $n = 86$ ).

Overall, 25 (69.4%) UC patients achieved disease control at least once, with 30.6% ( $n = 11$ ) of patients remaining with "moderate or severe" disease at the end of the 12-mo period (censored observations). The median time for achieving the first episode of disease control from the index date was 320 d (IQR: 288-358) [95%CI: 302-358], *i.e.*, approximately 10.7 mo (Figure 4B), and there were two changes from "mild or no disease activity" to "moderate or severe disease".

Of the 107 patients who had a final evaluation during the remaining 90 d of the follow-up period, 23 (21.5%) showed moderate or severe disease activity. The proportions of CD and UC patients with active disease at the last follow-up visit were 22.0% ( $n = 15/82$ , 95%CI: 13.6%-32.5%) and 20.0% ( $n = 5/25$ , 95%CI: 6.8%-40.7%), respectively.

CD patients with active disease at the end of follow-up had a higher mean BMI at index than patients with 'mild or no activity' ( $27.5 \pm 3.7$  vs  $24.2 \pm 4.4$ ;  $P = 0.007$ ) (Table 3). Higher education was also more frequent among CD patients with mild or no disease activity at the end of follow-up than among patients with moderate-to-severe activity (42.6% vs 12.5%;  $P = 0.029$ ).

Despite a higher proportion of CD patients with symptomatic perianal disease among patients with moderate to severe disease activity, the results were not statistically significant (55.6% vs 30.2% among patients with mild or no disease activity;  $P = 0.066$ ).

**Table 3 Sociodemographic, clinical and treatment characteristics of ulcerative colitis and Crohn's disease patients at the index date according to the final disease activity assessment**

Characteristics at index date	CD patients (n = 82)			UC patients (n = 25)		
	Moderate-to-severe activity (n = 18)	No or mild activity (n = 64)	P value	Moderate-to-severe activity (n = 5)	No or mild activity (n = 20)	P value
Age (yr), mean $\pm$ SD	42.0 $\pm$ 13.0	43.1 $\pm$ 12.7	0.732 <sup>3</sup>	38.0 $\pm$ 14.4	46.9 $\pm$ 17.5	0.310 <sup>4</sup>
Female, n (%)	12 (66.7)	39 (60.9)	0.786 <sup>5</sup>	4 (80.0)	15 (75.0)	> 0.999 <sup>5</sup>
Employed, n (%)	4 (23.5)	22 (45.8)	0.328 <sup>6</sup>	1 (20.0)	2 (10.5)	0.977 <sup>5</sup>
Attended higher education, n (%)	2 (12.5)	20 (42.6)	0.029 <sup>6</sup>	1 (20.0)	2 (18.2)	0.462 <sup>5</sup>
Body Mass Index (kg/m <sup>2</sup> ), mean $\pm$ SD	27.5 $\pm$ 3.7	24.2 $\pm$ 4.4	0.007 <sup>4</sup>	25.4 $\pm$ 7.5	24.8 $\pm$ 5.5	0.838 <sup>3</sup>
Current smokers, n (%) <sup>1</sup>	2 (12.5)	4 (7.0)	0.607 <sup>5</sup>	0 (0.0)	0 (0.0)	NA
IBD duration (yr), median (IQR)	6.0 (1-27)	13.0 (0-45)	0.0433 <sup>3</sup>	5 (1-23)	6.5 (1-23)	0.7032 <sup>4</sup>
Time since moderate to severe IBD (yr), median (IQR)	5.5 (1-25)	7.0 (0-26)	0.8839 <sup>3</sup>	2 (0-18)	3.5 (1-16)	0.8101 <sup>3</sup>
Extra intestinal manifestation, n (%)	3 (50.0)	17 (51.5)	0.946 <sup>6</sup>	1 (33.3)	4 (40.0)	NA
HBI result, median (IQR)	9.0 (7-13)	4.0 (1-8)	NA	-	-	-
CDAI result, median (IQR)	200.0 (114-311)	224.0 (113-281)	NA	-	-	-
Previous calprotectin level > 200 $\mu$ g/g, n (%)	3 (16.7)	24 (37.5)	0.097 <sup>6</sup>	-	-	-
Previous colonoscopy with CD activity, n (%)	12 (66.7)	33 (51.6)	0.256 <sup>6</sup>	-	-	-
Ileocolonic disease, n (%)	13 (72.2)	36 (56.3)	0.282 <sup>5</sup>	-	-	-
Stricture or penetrating CD behavior, n (%)	11 (61.1)	48 (75.0)	0.247 <sup>6</sup>	-	-	-
Symptomatic perianal disease, n (%)	10 (55.6)	19 (30.2)	0.066 <sup>6</sup>	-	-	-
Partial Mayo score, median (IQR)	-	-	-	7.0 (5-8)	6.0 (5-6)	NA
Left-sided or pancolitis UC, n (%)	-	-	-	3 (60.0)	15 (75.0)	-
Current IBD treatment at baseline <sup>2</sup> , n (%)	15 (83.3)	61 (95.3)	0.233 <sup>5</sup>	5 (100.0)	19 (95.0)	0.871 <sup>5</sup>
Treatment ongoing or initiated at index, n (%)	16 (88.9)	62 (96.9)	0.416 <sup>5</sup>	5 (100.0)	20 (100.0)	NA
5-ASA compounds	2 (12.5)	10 (16.1)	3 (60.0)	-	16 (80.0)	-
Biologic therapy	13 (81.3)	41 (66.1)	3 (60.0)	-	4 (20.0)	-
Corticosteroids	3 (18.8)	9 (14.5)	3 (60.0)	-	9 (45.0)	-
Immunosuppressants	5 (31.3)	44 (71.0)	3 (60.0)	-	10 (50.0)	-

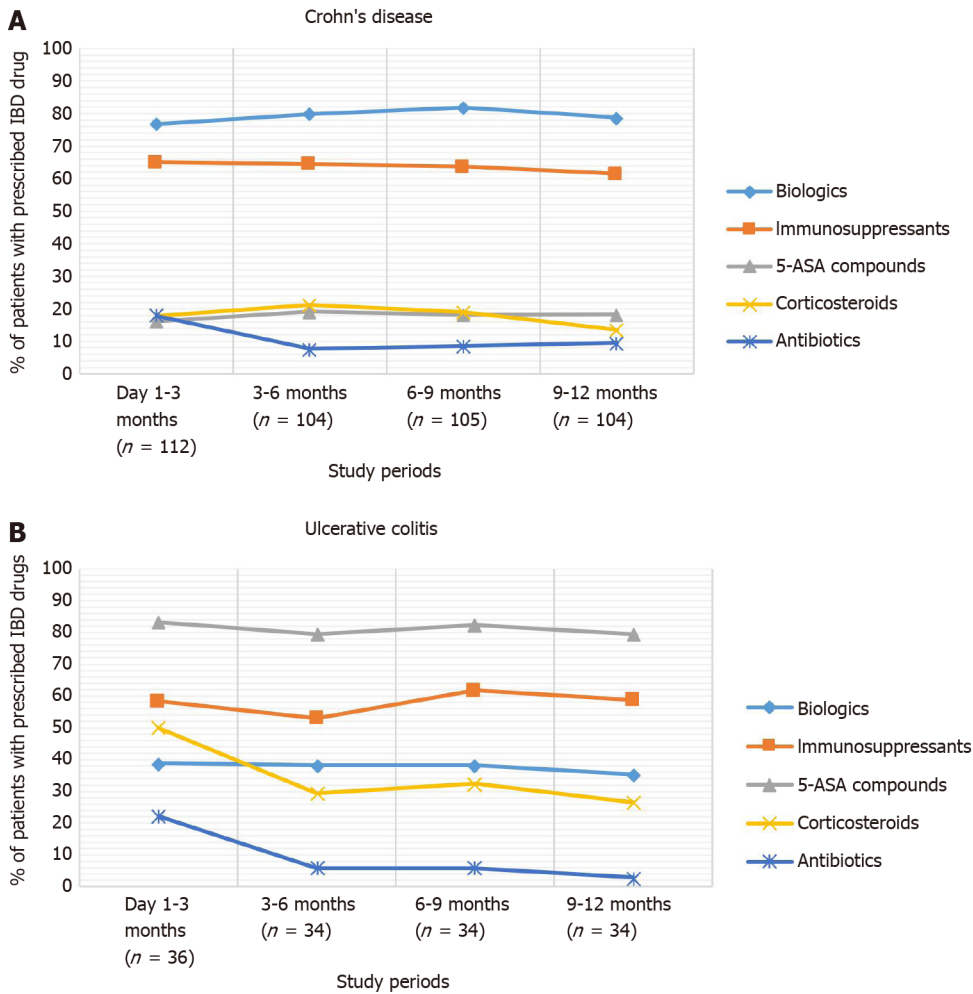
<sup>1</sup>Smoking at Day 1 and has smoked 100 cigarettes in his/her lifetime.<sup>2</sup>Ongoing or discontinued at baseline.<sup>3</sup>P values from the Mann-Whitney test.<sup>4</sup>t-test.<sup>5</sup>Fisher's exact test.<sup>6</sup>Chi-square test. SD: Standard deviation; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; HBI: Harvey-Bradshaw index; CDAI: Crohn's disease activity index; pMayo: Partial Mayo score; 5-ASA: 5-aminosalicylate; NA: Not applicable.

No statistically significant differences were observed when comparing sociodemographic, clinical and treatment characteristics between UC patients with *vs* without disease activity at the end of follow-up.

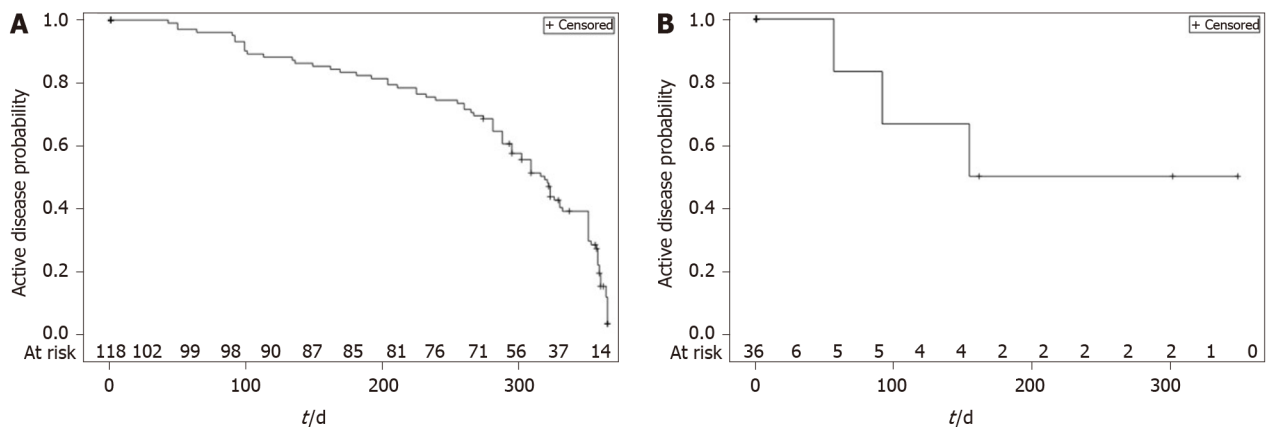
## DISCUSSION

This study aimed to provide a real-world perspective on the management of moderate-to-severe active IBD patients in Brazil, with a focus on treatment patterns





**Figure 3** Proportion of patients by prescribed inflammatory bowel disease treatment classes during the prospective phase and grouped by trimester for Crohn's disease (A) and ulcerative colitis patients (B) with at least one treatment. 5-ASA: 5-aminosalicylate.



**Figure 4** Probability of staying in active disease over 12 mo in Crohn's disease (A) and ulcerative colitis (B) patients.

and outcomes.

We found that more than two-thirds of IBD patients (73% and 69% for CD and UC patients) with active disease at the index date achieved disease control at least once during the 12-mo period. In addition, at the end of follow-up, approximately 20% of IBD patients had moderate-to-severe activity.

On average, almost 11 mo were required for half of UC and CD patients to achieve disease control. Considering that this is an observational study with no predefined visits, some patients did not undergo the 12-mo visit. Ideally, the control of disease

activity should be achieved within 4-6 mo[22,23]. One possible explanation for this long period could be attributed to the difficulty in the public setting in Brazil to access fecal biomarkers (such as calprotectin) or routinely perform colonoscopy, which are essential assessments to evaluate the response to treatment[2,6,24]. These barriers can delay the assessment of treatment response and thus the optimization of or change in medications.

The lack of public access to serum and anti-drug antibody level testing (unless solicited and justified) could also contribute to this delay. Evidence shows the usefulness of these tests in the decision to proactively or reactively change or optimize biologic therapies[25,26]. During the follow-up period, a mean of 4.4 medical appointments were required for CD patients and 5.0 for UC patients. We observed that clinical scores to assess disease activity were not collected at the majority of appointments (the HBI was assessed only in 12.6%, the CDAI in 6.4% and the Mayo score in 11.2% of outpatient visits).

Since the assessment of symptoms is part of several disease activity scores and relevant for both patients and physicians[27-30], these data suggest that assessment of IBD activity can be improved.

For the purpose of this study, we grouped “no activity” and “mildly active” disease in the same category. The ultimate treatment goal in IBD consists of achieving mucosal healing; however, the definition for this outcome can vary. Some studies in UC consider an endoscopic response as a Mayo endoscopic score  $\leq 1$ [6].

Biological agents were the most frequent treatment among CD patients, while 5-ASA compounds were largely the most common therapy among UC patients. Despite 5-ASA derivatives not being recommended for routine use in CD patients for induction or maintenance therapy, 14.4% of CD patients received this treatment strategy. This highlights the need for continuing education of gastroenterologists in Brazil. In addition, the lack of other therapies in some regions of the country may have led to the prescription of salicylic derivatives as the only CD treatment option. In a systematic literature review conducted in 2019, the lack of evidence for conventional treatment effectiveness, especially for remission maintenance, mucosal healing and fecal calprotectin, was highlighted[31].

We observed that the majority (65.5%) of CD patients started a new therapy after the study appointment, and the most common new treatment was biologics. New treatments were found for 86.1% of UC patients, with 5-ASA compounds being the most frequent. The more recent treatment guidelines, which are also followed in Brazil, recommend 5-ASA compounds in UC treatment irrespective of disease activity, while CD patients with moderate-to-severe disease activity would benefit more from the early use of biologics and immunosuppressants[2,6]. However, we cannot exclude differences in accessibility to new and more effective drugs, as clinics from the public health system in Brazil have no reimbursement when prescribing biologics to UC patients. Furthermore, the use of immunosuppressants and biologics increased among UC patients during follow-up, suggesting that 5-ASA compounds may not be enough for achieving disease control in many patients.

A relevant proportion of CD and UC patients were receiving biologic therapy, not only at the time of the study appointment but also during the follow-up period. This finding suggests that despite difficulties in accessing biologic therapies in hospitals in Brazil, especially in UC, physicians prescribe these agents for patients with moderate-to-severe disease activity. At the time of data collection, biologic therapy was available for use but not reimbursed by public or private healthcare systems in Brazil (which would mean medication without cost for patient); however, patients with UC had access to biologics *via* other strategies such as judicial petition and out-of-pocket. Only from March 2020, was infliximab available for patients diagnosed with UC in the public healthcare system; infliximab, vedolizumab and golimumab were only available in the private setting in 2021. We observed a decrease in the use of corticosteroids during follow-up for both CD and UC, suggesting an adequate control of disease overtime. Nevertheless, approximately one-quarter of CD patients and half of UC patients required corticosteroids for a period longer than three months.

One of the strengths of this study was the inclusion of treated IBD patients from both public and private settings, allowing a broad characterization of this population.

Some study limitations should be recognized. The inclusion of prevalent IBD cases, with baseline variability in terms of time since diagnosis and previous treatments, was a barrier to the assessment of the association between treatment options and disease activity. Even though we found no statistically significant association between clinical and treatment variables and presenting disease activity at the end of follow-up, we cannot exclude that treatment at the index date and during follow-up may have contributed to this outcome, as we did not adjust for combined therapy, dosing

information or duration of treatment (due to missing information). Moreover, the disease activity outcome was assessed at one time point only during the prospective phase (end of follow-up or month 12), making it difficult to determine whether it was a sustained control.

The inclusion of patients who had a previous event of moderate-to-severe IBD (irrespective of disease activity at index date) may affect the generalization of results for the whole IBD population in Brazil, even though these patients were selected for their higher burden of disease and thus needed closer medical follow-up. Disease activity was not assessed with the proposed scores in all outpatient medical appointments. Nevertheless, concerning disease activity, one limitation was the subjective assessment of colonoscopy, which was based on investigator criteria, which also impacted the selection of UC patients. The prospective design aimed to reduce missing data, but due to the observational character of the study, we did not proactively interfere with the usual practices for IBD activity evaluation. Finally, the sample size affected the group comparison regarding the main baseline and follow-up variables, especially for the UC patients. Furthermore, due to the small sample, one should be cautious when extrapolating the findings to a nationwide population. Additional studies are required to further understand the management of IBD in Brazil and understand the gaps and barriers in this setting.

## CONCLUSION

In conclusion, to our knowledge, this was the first study aiming to characterize IBD evolution in a real-world setting in Brazil. Although a marked proportion of patients with active IBD achieved disease control within one year, the prolonged time to achieve this outcome suggests an area of unmet need with the current standard of care.

## ARTICLE HIGHLIGHTS

### Research background

The Real-world Data of Moderate-to-Severe Inflammatory Bowel Disease in Brazil (RISE BR) study was a noninterventional study designed to evaluate disease control, treatment patterns, disease burden and health-related quality of life in patients with moderate-to-severe active inflammatory bowel diseases (IBD).

### Research motivation

Real-world data on IBD management could unveil unmet medical needs and treatment gaps. This is of utmost relevance in developing countries such as Brazil, where the prevalence of IBD is increasing, but access to biologic treatment may be restricted, and information on IBD treatment, in general, and associated outcomes is scarce.

### Research objectives

The aim was to describe the 12-mo disease evolution and treatment patterns among patients with active moderate-to-severe IBD in Brazil.

### Research methods

We report findings from the prospective follow-up phase of the RISE BR study in patients with active ulcerative colitis (UC) or Crohn's disease (CD). This was a prospective, noninterventional study of adult patients with active CD, inadequate CD control or active UC. The proportion of active IBD patients after 9-12 mo of follow-up, the time to mild or no activity and a summary of treatment initiation, discontinuation and dose changes were evaluated.

### Research results

The study included 118 CD and 36 UC patients. The most frequent drug classes at index were biologics for CD (62.7%) and 5-aminosalicylate derivatives for UC patients (91.7%). During follow-up, 65.3% of CD and 86.1% of UC patients initiated a new treatment at least once. Considering the prospective follow-up period, discontinuations/dose changes occurred in 68.1% of CD patients [median 2.0 (IQR: 2-5)] and

94.3% of UC patients [median 4.0 (IQR: 3-7)]. On average, CD and UC patients had  $4.4 \pm 2.6$  and  $5.0 \pm 3.3$  outpatient visits, respectively. The median time to first mild or no activity was 319 (IQR: 239-358) d for CD and 320 (IQR: 288-358) d for UC patients. At 9-12 mo, 22.0% of CD and 20.0% of UC patients had active disease.

### Research conclusions

Although a marked proportion of active IBD patients achieved disease control within one year, the considerable time to achieve this outcome represents an unmet medical need of the current standard of care in a Brazilian real-world setting.

### Research perspectives

This was the first study aiming to characterize IBD evolution in a real-world setting in Brazil. Additional studies are required to further understand the management of IBD in Brazil and understand the gaps and barriers in this setting.

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## Local ablation of pancreatic tumors: State of the art and future perspectives

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### Abstract

#### BACKGROUND

Currently, the technologies most commonly used to treat locally advanced pancreatic cancer are radiofrequency ablation (RFA), microwave ablation, and irreversible (IRE) or reversible electroporation combined with low doses of chemotherapeutic drugs.

#### AIM

To report an overview and updates on ablative techniques in pancreatic cancer.

#### METHODS

Several electronic databases were searched. The search covered the years from January 2000 to January 2021. Moreover, the reference lists of the found papers were analysed for papers not indexed in the electronic databases. All titles and abstracts were analysed.

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## RESULTS

We found 30 studies (14 studies for RFA, 3 for microwave therapy, 10 for IRE, and 3 for electrochemotherapy), comprising 1047 patients, which were analysed further. Two randomized trials were found for IRE. Percutaneous and laparotomy approaches were performed. In the assessed patients, the median maximal diameter of the lesions was in the range of 2.8 to 4.5 cm. All series included patients unfit for surgical treatment, but Martin *et al* assessed a subgroup of patients with borderline resectable tumours who underwent resection with margin attenuation with IRE. Most studies administered chemotherapy prior to ablative therapies. However, several studies suggest that the key determinant of improved survival is attributable to ablative treatment alone. Nevertheless, the authors suggested chemotherapy before local therapies for several reasons. This strategy may not only downstage a subgroup of patients to curative-intent surgery but also support to recognize patients with biologically unfavourable tumours who would likely not benefit from ablation treatments. Ablation therapies seem safe based on the 1047 patients assessed in this review. The mortality rate ranged from 1.8% to 2%. However, despite the low mortality, the reported rates of severe post procedural complications ranged from 0%-42%. Most reported complications have been self-limiting and manageable. Median overall survival varied between 6.0 and 33 mo. Regarding the technical success rate, assessed papers reported an estimated rate in the range of 85% to 100%. However, the authors reported early recurrence after treatment. A distinct consideration should be made on whether local treatments induce an immune response in the ablated area. Preclinical and clinical studies have shown that RFA is a promising mechanism for inducing antigen-presenting cell infiltration and enhancing the systemic antitumour T-cell immune response and tumour regression.

## CONCLUSION

In the management of patients with pancreatic cancer, the possibility of a multimodal approach should be considered, and conceptually, the combination of RFA with immunotherapy represents a novel angle of attack against this tumour.

**Key Words:** Pancreatic cancer; Ablation treatment; Radiofrequency ablation; Microwave ablation; Irreversible; Electrochemotherapy

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**Core Tip:** In the current state of knowledge, the most commonly used technologies in locally advanced pancreatic cancer are radiofrequency ablation, microwave ablation, and irreversible or reversible electroporation combined with low doses of chemotherapeutic drugs. Our purpose is to report an updated overview of these techniques, highlighting the advantages and limitations of each technology.

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## INTRODUCTION

Worldwide, an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020[1]. Pancreatic cancer accounts for almost as many deaths (466000) as cases (496000) because of its poor prognosis, and it is the seventh leading cause of cancer death in both sexes. Both incidence and mortality rates have been stable or slightly increased in many countries, likely reflecting the increasing prevalence of obesity, diabetes, and alcohol consumption, although improvements in diagnostic and cancer registration practices may also be factors in some countries.

Given that the rates of this disease are rather stable relative to the declining rates of breast cancer, it has been projected in a study of 28 European countries that pancreatic cancer will surpass breast cancer as the third leading cause of cancer death by 2025[1].

The only curative treatment is surgery; however, many patients have locally advanced or metastatic disease at diagnosis, and systemic chemotherapy is usually the main treatment[2-6]. The median survival of patients with metastatic disease treated with FOLFIRINOX therapy is only 3 mo[2,5]. FOLFIRINOX or modified FOLFIRINOX and gemcitabine/albumin-bound nab-paclitaxel remain the first-line treatment regimens, and for patients with *BRCA1/2* and *PALB2* mutations, FOLFIRINOX or modified FOLFIRINOX and gemcitabine/cisplatin are a second option[2,3]. Despite the recent introduction of novel chemotherapeutic schemes, these treatments still correlate with inadequate survival and significant systemic complications. Additionally, only one-third of patients are responsive to chemotherapy[6,7].

Local ablation treatment is considered in some centres for patients with persistent locally advanced disease after chemotherapy. Although randomized trials to establish the role of ablation treatments in addition to chemotherapy alone are absent and there are no concluded trials that have compared various ablative modalities[8], patients with persistent locally advanced disease who are in good clinical condition (World Health Organization performance status 0-1) and Response Evaluation Criteria in Solid Tumours (RECIST) stable disease after 2-4 mo of chemotherapy can be treated by local ablation therapies. Moreover, there is a growing interest in these techniques related to the fact that they can encourage a systemic antitumour response. Therefore, it is proposed to combine ablative treatments with immunotherapy to improve disease control[8]. Nonetheless, ablative treatments should be employed in pancreatic cancers that show a local growth pattern without systemic involvement and should be chosen as consolidative treatments in a multimodal approach. The superior technique between the two remains unknown; therefore, the choice to employ one or the other should be reserved for a multidisciplinary team, considering the patients' comorbidities, the tumour characteristics and, particularly, the response to medical therapies[9-19].

In the current state of knowledge, the most commonly used technologies in locally advanced pancreatic cancer (LAPC) are radiofrequency ablation (RFA), microwave ablation (MWA), and irreversible electroporation (IRE) or reversible electroporation combined with low-dose chemotherapeutic drugs (ECT).

We report an overview and an update of these procedures, highlighting the advantages and limitations of each technology.

## MATERIALS AND METHODS

This study is an autonomous study with no protocol or registration number.

### Search criteria

The following electronic databases were used for search: PubMed (United States National Library of Medicine, <http://www.ncbi.nlm.nih.gov/pubmed>), Scopus (Elsevier, <http://www.scopus.com/>), Web of Science (Thomson Reuters, <http://apps.webofknowledge.com/>), and Google Scholar (<https://scholar.google.it/>). The following search criteria were used: "Pancreatic Cancer" AND "Ablative Therapies"; "Pancreatic Cancer" AND "RFA"; "Pancreatic Cancer" AND "MWA"; "Pancreatic Cancer" AND "IRE"; "Pancreatic Cancer" AND "ECT".

The search covered the years from January 2000 to January 2021. Reference lists of the found papers were analysed for papers not indexed in the electronic databases. The included papers were required to be clinical studies (*e.g.*, retrospective analyses, case series, and prospective cohort studies) evaluating the safety and efficacy of ablative therapies in pancreatic adenocarcinoma. Articles published in the English language from January 2000 to January 2021 were included. The exclusion criteria were: Different topics, unavailability of full text, insufficient data, and case reports, reviews, or letters to editors.

## RESULTS

The search strategy resulted in 30 studies [14 studies for RFA, 3 for MWA, 10 for IRE, and 3 for electrochemotherapy (ECT)] (Figure 1), including 1047 patients, which were



**Figure 1** Included and excluded studies in systematic review.

further assessed. We found two randomized trials for IRE. Percutaneous and laparotomy approaches were performed. In the assessed patients, the median maximal diameter of the lesions was in the range of 2.8-4.5 cm, including patients unfit for surgical treatment. Additionally, Martin *et al*[19] evaluated patients with borderline resectable tumours who underwent resection with margin attenuation with IRE. Most series administered chemotherapy prior to IRE. The specific types of drugs varied between series, but gemcitabine- or 5-FU-based regimens were common.

In **Table 1**, we report the sample size, overall survival (OS), major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with RFA according to the studies assessed.

In **Table 2**, we report the sample size, OS, major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with MWA according to the studies assessed.

In **Table 3**, we report the sample size, OS, major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with IRE according to the studies assessed.

In **Table 4**, we report the sample size, OS, major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with ECT according to the studies assessed.

## DISCUSSION

### ***Ablation techniques-physical principles***

RFA and MWA are hyper-thermic techniques that utilize energy to heat the lesions to at least 60°C[16].

RFA produces necrosis due to thermocoagulation. With this treatment, the area of active tissue heating is limited to a few millimetres near the electrode[16]. Consequently, the efficacy is closely correlated to the lesion size, and the maximum result is obtained for targets less than 3.5 cm[16]. Additionally, some tissue features, such as electrical conductivity, thermal conductivity, dielectric permittivity, and blood perfusion rate, have an effect on the efficacy of the RFA procedure. In particular, RFA treatment should be avoided when the target is near large vessels because of the heat sink effect[19]. However, the bipolar system of RFA can reduce the heat sink effect and lower pancreatic injury[10].

MWA uses the dielectric effect, which occurs when an imperfect dielectric material is subjected to an alternating electromagnetic field, to generate a larger area of active heating (up to 2 cm close to the antenna), allowing more homogeneous necrosis in the target zone compared to RFA[16]. Additionally, MWA has several supposed improvements with respect to RFA: The target can be greater given that it generates a larger area of necrosis; the treatment time is quicker; and it is less influenced by the defence of the neighbouring tissues, which is due to vaporization and charring, so the heat-sink effect impacts the efficacy of MWA[16].

In contrast to RFA and MWA, IRE and ECT are non-thermal techniques that cause ablation, changing cell membrane permeability through an induced electric field



**Table 1 Sample size, overall survival, major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with radiofrequency ablation**

Ref.	Sample size	Overall survival	Major complication rate	Minor complication rate	Mortality rate
D'Onofrio <i>et al</i> [28], 2016	51	-	-	-	-
D'Onofrio <i>et al</i> [29], 2017	18	Median, 185 d (range, 62-398 d)	0%	0%	0%
Giardino <i>et al</i> [30], 2013	168	34.0 mo	3.70%	17.70%	1.80%
Hadjicostas <i>et al</i> [31], 2006	4	6 mo	0%	25%	0%
Kallis <i>et al</i> [33], 2015	23	226 d (range, 140-526 d)	0%	4.30%	0%
Song <i>et al</i> [37], 2016	6	NR	0%	33.30%	0%
Spiliotis <i>et al</i> [38], 2007	25	33 mo	0%	-	0%
Varshney <i>et al</i> [39], 2006	3	-	0%	66.70%	0%
Zou <i>et al</i> [41], 2010	32	17.5 mo	3.10%	0%	0%
Giardino <i>et al</i> [44], 2017	10	NR	30%	0%	0%
D'Onofrio <i>et al</i> [46], 2020	35	310 (65-718) d	0%	0%	0%
Wang <i>et al</i> [48], 2020	11	12 mo	0%	0%	0%
He <i>et al</i> [50], 2020	18	1-yr 40.5%; 2-yr 27.0%	22.20%	50%	0%
Fegrachi <i>et al</i> [52], 2019	17	9 mo (range, 5-11 mo)	6%	24%	0%

NR: Not reported.

**Table 2 Sample size, overall survival, major complication rate, minor complication rates, and mortality rate in pancreatic cancer treated with microwave ablation**

Ref.	Sample size	Overall survival	Major complication rate	Minor complication rate	Mortality rate
Carrafiello <i>et al</i> [59], 2013	10	80% at 1 yr	10%	20%	0%
Ierardi <i>et al</i> [60], 2018	5	-	0%	20%	0%
Vogl <i>et al</i> [61], 2017	20	-	0%	9.10%	0%

(electroporation). IRE is considered a direct ablation tool since electroporation is used in an irreversible manner[17-26]. The use of short high-voltage electric current fields (up to 3000 V and 50 A for milliseconds) cause irreversible permeabilization of the lipid bilayer, disruption of cellular homeostasis, and stimulation of apoptotic pathways, causing death of neoplastic cells[9-17,26]. Taking into account its mechanism of action, IRE can protect surrounding structures, such as vessels, and it is a central element if the tumour encases the peripancreatic vessels in which the employment of RFA could be unsafe[22]. ECT conceives of the electroporation of cells and the associated administration of low doses of non-permanent or poorly permanent chemotherapeutic agents[11,13-27]. The application of an electrical field to a cell causes a transient and reversible orientation of its polar membrane molecules, with increased permeability[11-13]. This transient permeability allows the cell to receive a higher dose of chemotherapeutic drugs than would occur otherwise, increasing the cytotoxic effects of the agents. This local potentiation of chemotherapy allows reducing the doses of the drugs, lowering the side effects and increasing the chemotherapy efficacy [11-13].

### Clinical study

**RFA:** RFA is the typical treatment worldwide for the treatment of LAPC if further benefit from chemotherapy is expected. Several studies have evaluated the role of RFA in metastatic pancreatic cancer[9]. To the best of our knowledge, 14 studies assessed the safety and efficacy of RFA in pancreatic cancer[28-52]. In several studies, the use of RFA was limited to patients with locally advanced cancer and/or metastatic cancer. Only in cases where the patients were unfit for surgery was RFA used in resectable

**Table 3 Sample size, overall survival, major complication rate, minor complication rate, and mortality rate in pancreatic cancer treated with irreversible electroporation**

Ref.	Sample size	Overall survival	Major complication rate	Minor complication rate	Mortality rate
Vroomen <i>et al</i> [14], 2017	25	-	8%	20%	0%
Martin <i>et al</i> [19], 2015	200	24.9 mo (range, 4.9–85 mo)	18.50%	50,5%	2%
Martin <i>et al</i> [20], 2013	54	20 mo	24%	55,5%	2%
Lambert <i>et al</i> [23], 2016	21	10.2 mo	23.80%	-	0%
Yan <i>et al</i> [24], 2016	25	-	36%	16%	0%
Scheffer <i>et al</i> [26], 2017	25	11 mo	40%	40%	0%
Ruarus <i>et al</i> [65], 2020	50	11.6 mo (no induction chemotherapy or gemcitabine-based induction chemotherapy) and 14.9 mo (FOLFIRINOX)	42%	28%	2%
van Veldhuisen <i>et al</i> [66], 2020	30	17.0 (range, 5-35 mo)	20%	23%	0%
Narayanan <i>et al</i> [67], 2017	50	27 mo	20%	-	0%
Liu <i>et al</i> [68], 2019	54	16.2 and 20.3 mo in the IRE and IRE + chemo groups	7.40%	81%	0%

IRE: Irreversible electroporation.

**Table 4 Sample size, overall survival, major complication rates, minor complication rate, and mortality rate in pancreatic cancer treated with Electrochemotherapy**

Ref.	Sample size	Overall survival	Major complication rate	Minor complication rate	Mortality rate
Granata <i>et al</i> [11], 2015	13	-	0%	23%	0%
Granata <i>et al</i> [12], 2017	19	-	-	-	-
Granata <i>et al</i> [71], 2020	25	In fixed geometry, treated patients 6 mo (range, 1-74 mo); in variable geometry treated patients 12 mo (range, 2-50 mo)	0%	23%	0%

cancer[25-52]. Recently, RFA has been used as an upfront option at the time of diagnosis[9], justified based on immunological antitumoral stimulation[43,44].

The results of the assessed studies in terms of OS, major and minor complication rates, and mortality rates are reported in Table 1.

In the treatment of pancreatic adenocarcinoma, RFA has been mainly applied during laparotomy or with an endoscopic approach. The percutaneous approach has rarely been described in the literature, and worldwide experience is still limited[46]. However, the percutaneous approach should be favoured to avoid the invasiveness of the intraoperative approach. If percutaneous RFA is feasible, it could avoid unnecessary laparotomy, thus reducing the risk of surgical complications as well as the time and costs of the treatment[46]. Moreover, while surgery involves an impaired immune response, enhanced immune system stimulation and immune response against the tumour have been described in a percutaneously treated patient[43,47].

Nonrandomized studies showed a promising OS up to 25.6 mo after RFA for LAPC [53]. However, no randomized controlled trials have been performed, so the true effectiveness of RFA combined with systemic chemotherapy regimens remains unknown. Several studies[9] reported an excellent outcome with a median OS of 30 mo for patients subjected to RFA and a median OS of 25.6 mo in the patients subjected to primary treatments plus RFA plus further systemic treatments. Few studies have assessed the efficacy of RFA compared to other treatments[50]. He *et al*[50] compared

the efficacy of IRE with RFA in patients with LAPC, showing that IRE after induction chemotherapy is superior to RFA after induction chemotherapy for treating LAPC, while these two therapies have comparable efficacy for tumours that were larger than 4 cm. However, the study was not a randomized controlled trial but a retrospective study.

Morbidity rates range from 14% to 28% and seem to depend on RFA temperature settings, preventive duodenal cooling, and safety margins from vital structures[52]. Since pancreatic tissue is sensitive to heat and rich in blood vessels and since the anatomical position is close to arteries and bile ducts, the application of thermotherapy techniques carries a high risk. However, as RFA application becomes increasingly mature, the incidence of postoperative complications has decreased significantly[52]. Complications related to RFA included pancreatic fistulae, portal vein thrombosis, gastrointestinal bleeding, and acute pancreatitis. The rates of RFA-related mortality ranged from 0% to 19%[51]. The RFA-related complications that resulted in patient deaths included gastrointestinal bleeding and sepsis. The rates of overall complications ranged from 10% to 43%[51]. The types of complications reported varied widely and included pneumonia, peritoneal cavity abscess, acute renal failure, transient ascites, hepatic insufficiency, pseudomembrane colitis, hemoperitoneum, abdominal fluid collection, gastric bypass fistula, gastric ulcer, and choledocholithiasis.

Computed tomography (CT) is the diagnostic tool most often employed to evaluate treatment in terms of efficacy and safety. Although CT is best known for its role in the evaluation of abdominal emergencies, it is also an excellent tool in the evaluation of posttreatment complications[54-58]. However, CT has significant limitations in assessing treatment effectiveness[6]. RFA produces side effects such as interstitial oedema, haemorrhage, carbonization, necrosis, and fibrosis. These are responsible for heterogeneous appearances on imaging. The assessment of the treatment response in terms of dimensional criteria, according to RECIST 1.1[15] criteria, is not applicable because effectiveness is not always correlated to a size decrease[6]. Nevertheless, the assessed studies evaluated short- and long-term RFA efficacy according to dimensional criteria[15]. The assessment time was between 7 and 34 mo considering only dimensional criteria. According to Paiella *et al*[9] for RFA the technique to choose is CT, and the effectiveness is related to a post treatment hypodense zone. However, pancreatic tumours are also hypodense, so a “qualitative evaluation” based on a visual assessment could cause misdiagnosis. A quantitative evaluation founded on functional analysis allows a more objective assessment and a more correct diagnosis[6].

Distinct consideration should be made regarding whether RFA induces an immune response in the ablated area. Preclinical and clinical studies have shown that RFA is a favourable tool to induce antigen-presenting cell infiltration and to enhance the systemic antitumour T-cell immune response and tumour regression. The treatment is followed by a significant inflammatory response with intense T-cell infiltration[43,45].

Therefore, in the management of patients with pancreatic cancer, the possibility of a multimodal approach should be considered, and theoretically, the association of RFA with immunotherapy is a novel strategy against this tumour.

### **Microwave ablation**

The results of the assessed studies in terms of OS, major and minor complication rates, and mortality rates are reported in Table 2.

Carrafiello *et al*[59] evaluated the efficacy of MWA in ten unresectable pancreatic head adenocarcinomas. The mean follow-up was 9.2 mo (range, 3 to 16 mo). The rate of MWA-related morbidity was 30% (3 patients). The authors found pancreatitis in two patients and gastroduodenal artery pseudoaneurysm in one patient. CT was executed up to 15 mo after the procedure. At the first follow-up, the researchers found one case with partial response (PR), eight with stable disease (SD), and one with progressive disease.

Ierardi *et al*[60] evaluated the feasibility and safety of MWA in five head pancreatic locally advanced cancer patients using a new technology with a high power and frequency of 2450 MH. CT was performed after 1, 3, 6, and 12 mo. No major complications were reported with a safe treatment in all patients (100%). Minor complications resolved during hospitalization (median, 4 d)[60].

Vogl *et al*[61] cured 22 lesions: In 17 (77.3%) patients, the tumour was in the pancreatic head and in 5 (22.7%) in the pancreatic tail. The rate of MWA efficacy was 100%. No major complications were reported; however, in two (9.1%) cases, minor complications were found because of severe local pain post-MWA treatment. Only ten patients underwent follow-up magnetic resonance imaging (MRI) examinations (median, 3 mo); local tumour progression was reported in one (10%) case.

Unlike RFA, the percutaneous approach was the most commonly used during MWA treatment, probably explaining the lower complication and death rates. However, the complication rates of MWA varied among the assessed studies. This finding might be due to the heterogeneity and the sample size of the studies assessed. In fact, Carrafiello *et al*[59] treated ten unresectable head pancreatic adenocarcinomas, Ierardi *et al*[60] treated five locally advanced head pancreatic cancers, and Vogl *et al* [61] treated 22 patients: In 17 (77.3%) patients, the tumour was in the pancreatic head and in five (22.7%) in the pancreatic tail.

CT is the diagnostic tool most often employed to evaluate the treatment, for either efficacy and/or safety. However, an assessment centred only on dimensional criteria is inappropriate to evaluate this procedure.

No data on MWA and immune response in the ablated area are described in the literature.

Although MWA is a promising treatment for LAPC, further studies are needed to increase the data about its safety and efficacy as well as the oncological outcome.

### **Irreversible electroporation**

Currently, IRE is applied in stage III LAPC[17,26], even if several studies have reported three cases of IRE in stage IV with liver metastases[62] and the option to employ IRE as a technique to reduce the rate of R1 resections[19,21,63]. According to the reported data[17-26,62,64], IRE works better on target areas not larger than 3-3.5 cm; in addition, IRE should be more suitable than thermal tools when the tumour encapsulates the superior mesenteric artery. However, IRE has the disadvantage of necessitating general anaesthesia.

The results of the assessed studies in terms of OS, major and minor complication rates, and mortality rates are reported in Table 3.

Rombouts *et al*[53] described an IRE-related complication rate of 13%, with a mortality of 2%. The complication rate increases with the percutaneous treatment (29% vs 13%)[53]. Martin *et al*[19], evaluating 200 cured lesions, reported an overall rate of adverse events of 37% and a mortality rate of 2%. The most common complications reported are abdominal pain as a minor complication and pancreatitis, bile leakage, pancreatic leakage, duodenal leakage, duodenal ulcer, pneumothorax, haematoma, and deep vein thrombosis as major complications[6]. Several studies have confirmed the safety profile of IRE, with encouraging survival outcomes. Most studies, however, were retrospective, had limited sample sizes, and had a relatively short follow-up time. The aim of the PANFIRE study was to evaluate the efficacy and safety of percutaneous IRE for LAPC and isolated local recurrence following surgical resection of pancreatic cancer[65]. In this prospective single-arm phase II study, 40 patients with LAPC and 10 with local recurrence after resection were treated. The primary endpoint was the median survival times with primary LAPC (median OS, 17 mo) and with local recurrence (median OS, 16 mo), which exceeded the target median survival times based on chemotherapy alone. These results show a survival benefit compared with the current standard of care[65]. The reported overall complication rate was of 58%, including 21 major adverse events and two deaths within 90 d of the treatment. In addition, 13 (33%) patients were treated upfront with IRE. No survival benefit was demonstrated for patients receiving a 5-FU-based regimen. This finding suggests that the key determinant of improved survival is attributable to IRE treatment alone. Nevertheless, the authors recommend at least four cycles of a 5-FU-based regimen before IRE for several reasons. First, a 5-FU-based regimen enables the identification of patients with aggressive tumour biologic features, allowing the exclusion of those who would not benefit from IRE. Second, a 5-FU-based regimen can result in downstaging, potentially rendering 15%–25% of patients with resectable disease. Last, an upfront 5-FU-based regimen has the potential to result in a longer OS[65]. The combination of systemic chemotherapy and cytoreductive ablation using IRE may prove synergistic for several reasons[66]. Induction chemotherapy may not only downstage a subgroup of lesions to curative-intent surgery but also identify biologically unfavourable tumours with rapid progression that would likely not benefit from ablative treatment [66]. As systemic chemotherapy remains the only treatment for LAPC proven to be beneficial, patients should first receive systemic chemotherapy followed by experimental treatment in the setting of a clinical trial[66-69].

IRE can be implemented successfully as an adjunctive measure for attempting to achieve negative microscopic operative margins in selected patients[21]. This treatment is limited to patients who have generally stable disease at the time of resection. To date, there are few options for effective therapy to facilitate microscopically negative margin resections outside of patient selection and meticulous operative dissection. Accepting that true margin-positive rates are significantly high (> 75%) in

resected pancreatic cancers, intraoperative IRE could accentuate negative-margin dissection of the retroperitoneal margin and its surrounding perivascular soft tissue, primarily the perineural and mesenteric tissue adjacent to critical vascular structures [21].

MRI and CT are the diagnostic tools mostly used to assess IRE.

IRE produces the formation of nanoscale pores within the cell membrane, changing the transmembrane potential and causing cell death. Experimental models showed that diffusion-weighted imaging (DWI) could be used to assess therapeutic effects [14, 70]. Vroomen *et al* [14] evaluated specific imaging parameters with contrast-enhanced (ce) MRI and ce-CT. The authors evaluated pre- and post-treatment, for MRI, the signal intensity (SI) on T2-weighted (W) images, on T1-W images before and after contrast medium, on DWI, and on apparent coefficient of diffusion (ADC) maps and for CT attenuation in the arterial and portal-venous phases. These authors showed that the most significant features to evaluate efficacy and outcome were SI on images with  $b = 800\text{s/mm}^2$  and contrast-enhanced MRI.

Only two studies [19, 67] reported an outstanding median OS of 24.9 and 27 mo, respectively. Consequently, there is a need for a greater number of studies that assess efficacy in terms of oncological outcomes.

### Electrochemotherapy

Today, few studies have evaluated the role of ECT in LAPC [11-13, 71]. In our previous study, we assessed 13 patients with LAPC. In seven (53.8%) patients, the tumour was localized in the head, and in six (46.2%), it was localized in the body tail (Figure 2). The treatment was safe in all patients without major complications. The types of minor complications reported varied widely and included transient ascites, transient pleural effusion, and gastric emptying documented by radiological studies, without clinically significant signs. The mean duration of hospitalization was 12 d. CT and MR were utilized for the follow-up [11]. In an ongoing study, we found that the median OS was 11.5 mo with a range in values of 73 mo. At 1 mo after ECT, 76.0% of patients were in PR, and 20.0% were in SD. Moreover, we found that the use of pre-treatment planning (Figure 3), which optimizes the multiple insertions of single electrodes, increases the local disease control rate (LDCR) and the OS compared with the use of fixed-geometry electrodes (hexagonal or linear). The patients treated with fixed geometry had an LDCR of 46.1%, whereas the group treated with variable geometry (Figure 4) had an LDCR of 66.7%. For the 13 patients treated with fixed geometry, the median OS was 6 mo (range, 1-74 mo), whereas for the 12 patients treated with variable geometry, the median OS was 12 mo (range, 2 to 50 mo) [71].

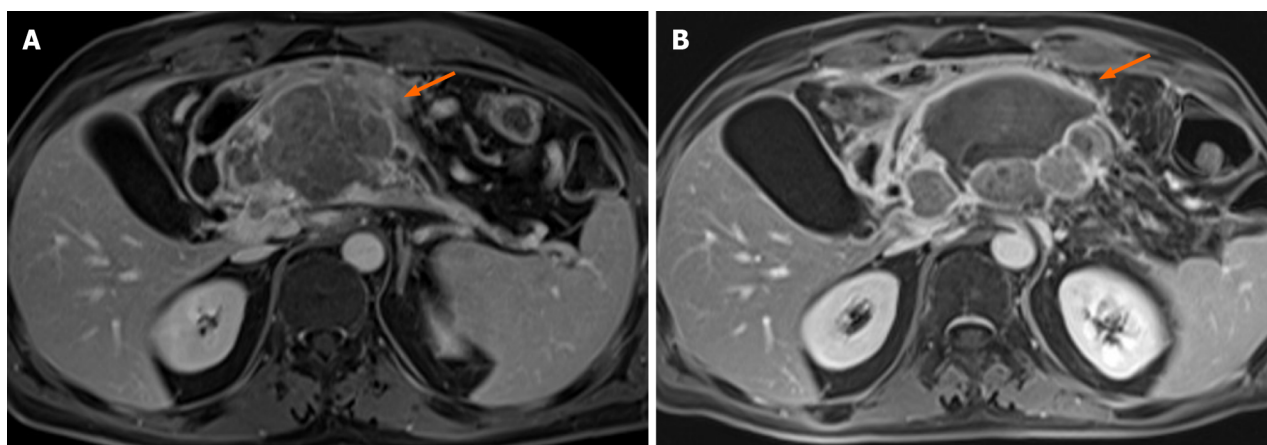
Although ECT is a promising tool for cancer treatment, how to assess tumour treatment response is still a problem. In fact, as highlighted in our preliminary experience, the RECIST 1.1 criterion, using the variation of the largest diameter on both CT and MRI images, does not provide appropriate patient stratification in responders or non-responders [12, 13]. It is clear that when considering therapeutic effects on tumours, imaging observations are sometimes difficult to interpret, so functional imaging should resolve this problem. We evaluated several functional features as follows: for MRI, wash-in slope and wash-out slope by dynamic contrast-enhanced MRI, pseudo-diffusivity, perfusion fraction, and tissue diffusivity by the intravoxel incoherent motion model, ADC by the conventional mono-exponential approach, and the mean of the diffusion coefficient and the mean of diffusional kurtosis by diffusion kurtosis imaging. In addition, for positron emission tomography, maximum standardized uptake value was assessed and for CT, lesion density was evaluated. We found that conventional morphologic criteria were not able to differentiate partial, complete, or incomplete responses after ECT, while changes in functional parameters could be more suitable to assess ECT responses [11, 13].

Today, ECT is recommended during clinical trials in dedicated centres [11-13].

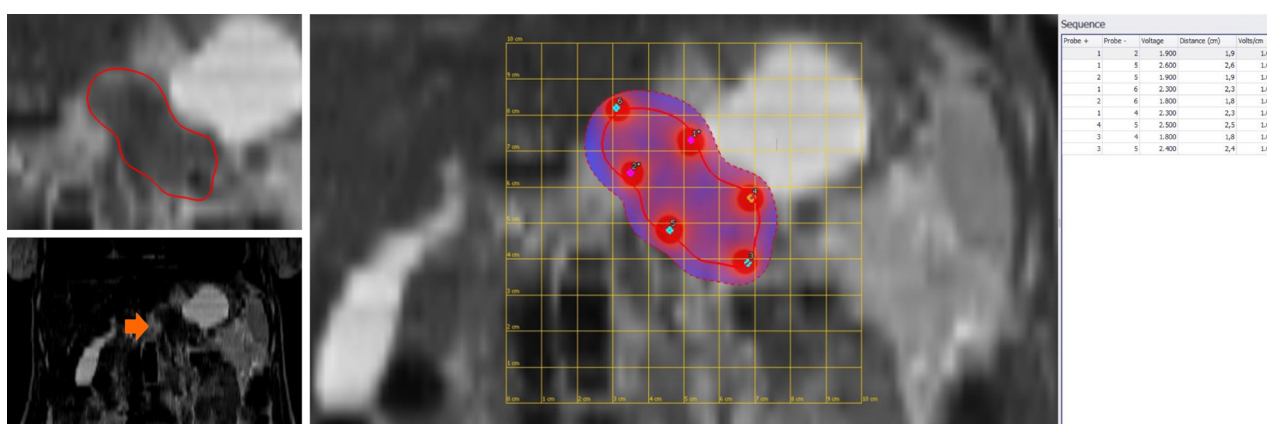
### Comments and future perspectives

Several studies have suggested that the key determinant of improved survival is attributable to ablative treatment alone [72-75]. Nevertheless, the authors recommend at least four cycles of a 5-FU-based regimen before local therapy for several reasons. Induction chemotherapy may not only downstage a subgroup of lesions to curative-intent surgery but also identify biologically unfavourable tumours with rapid progression that would likely suffer from ablation treatment [66, 76-80]. As systemic chemotherapy remains the only treatment for LAPC proven to be beneficial, patients should first receive systemic chemotherapy followed by experimental treatment in the setting of a clinical trial [66].





**Figure 2** A patient with body pancreatic cancer. A: Volume-interpolated breath-hold examination (VIBE) T1-W post contrast sequence during the portal phase in axial plane for pretreatment evaluation of the lesion (arrow); B: VIBE T1-W post contrast sequence during the portal phase in axial plane showing the ablated area (arrow). According to qualitative assessment (significant differences in signal intensity in pre and post treatment sequences), the lesion was in partial response.

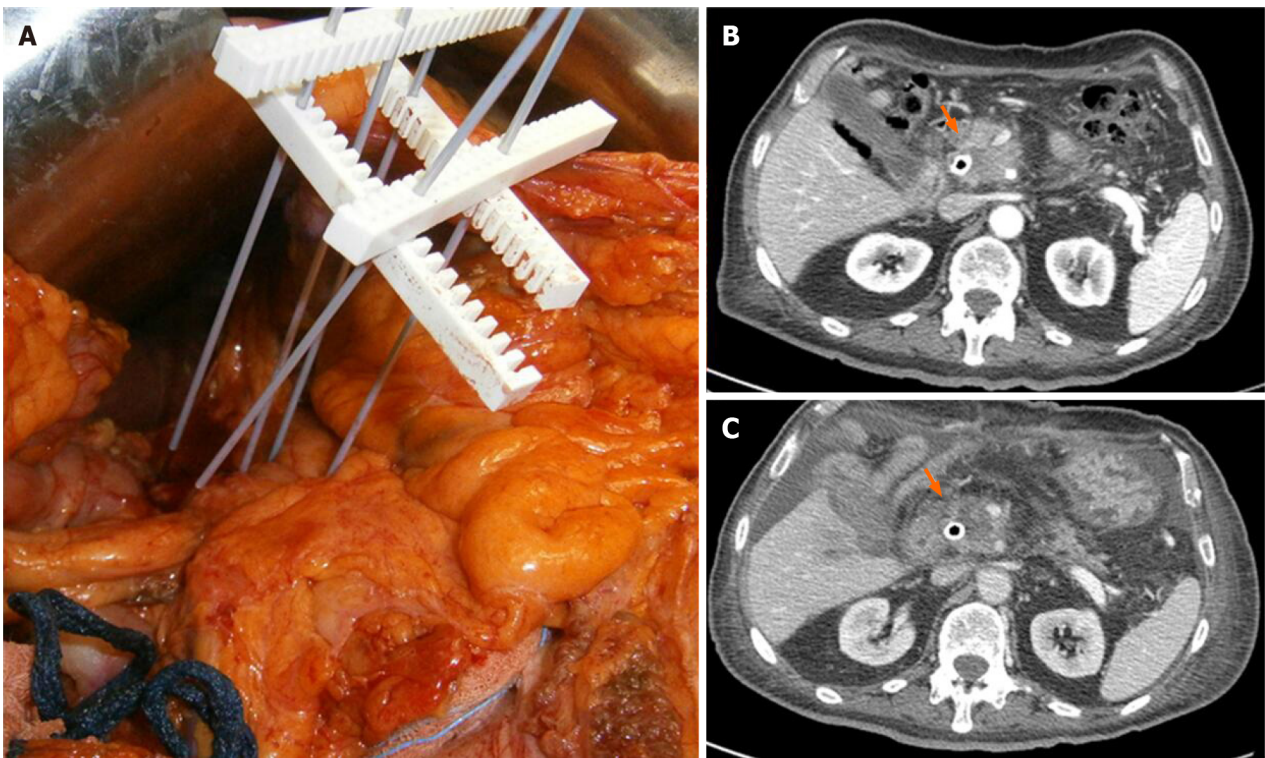


**Figure 3** Preoperative planning for electrochemotherapy treatment with multiple single needles in a variable geometry for locally advanced pancreatic cancer.

Ablation therapies seem to be safe in 1047 patients assessed in this study. The mortality rate ranged from 1.8% to 2%. However, despite the low mortality, the reported rates of severe post procedural complications ranged from 0%–42%. Additionally, for laparotomy, the series reported by Martin *et al*[19] had more severe complications, including procedure-related deaths. The major drawback inherent to all thermal ablation techniques is the fact that these therapies comprise the risk of the heat-sink effect[69]. This issue is particularly important, as the pancreas is an organ with a peculiar position that is closely related to the duodenum, bile duct, and major vessels. This feature turned IRE into an attractive tool for LAPC ablation[69]. Taken together, pathological studies revealed that one-third of patients died of PC as a result of local tumour infiltration, without evidence of metastatic disease. This population appears to be ideal for IRE to increase patient survival and, importantly, quality of life [69]. A registry-based study showed a high rate of complications (42%) post-IRE. An important point demonstrated in this study is the correlation of the learning curve to the rate of complications, which seems to drop after a cumulative experience of a minimal of five IRE cases in PC[69].

Median OS varied between 6.0 and 33 mo. However, these data are very problematic to understand because of the heterogeneity between the series.

Regarding the technical success rate, several studies reported an estimated technical success rate in the range of 85%–100%. However, the authors reported early recurrence after treatment, indicating the limitations of the radiological assessment post-treatment [16]. In addition, none of these studies assessed the relationship between the technical success rate and tumour size.



**Figure 4 Electrochemotherapy with variable geometry and computed tomography pre-treatment and 6 mo post-treatment.** A: Electrochemotherapy treatment with variable geometry; B: Computed tomography pre-treatment; C: Computed tomography 6 mo post-treatment. Computed tomography density showed a reduction as a positive response to treatment.

A distinct consideration should be made on whether local treatments induce an immune response in the ablated area. Preclinical and clinical studies have shown that RFA is an interesting tool to induce antigen-presenting cell infiltration and to enhance the systemic antitumour response. To the best of our knowledge, no data on other local treatments are available; therefore, studies that also evaluate this aspect for the other methods would be interesting.

Therefore, in the management of patients with pancreatic cancer, the possibility of a multimodal approach should be considered, and theoretically, the association of RFA with immunotherapy is a novel strategy against this tumour.

## CONCLUSION

In conclusion, ablation therapies seem effective and safe with low post-treatment mortality. Although complications are mostly self-limiting, severe complications do occur. The technical success rate is high at 85%–100%, but this feature may be an over-estimation. Further efforts are also needed to address patient selection, as well as the use of IRE for “margin accentuation” during surgical resection, so the combination of RFA with immunotherapy represents a novel strategy against this tumour.

## ARTICLE HIGHLIGHTS

### Research background

In the current state of knowledge, the most commonly used technologies in locally advanced pancreatic cancer (LAPC) are radiofrequency ablation (RFA), microwave ablation, and irreversible electroporation (IRE) or reversible electroporation combined with low doses of chemotherapeutic drugs.

### Research motivation

In the management of patients with pancreatic cancer, the possibility of a multimodal approach should be considered.

### Research objectives

The research purpose was to report an overview and an update on ablation techniques, highlighting the advantages and limitations of each technology.

### Research methods

The search covered the years from January 2000 to January 2021 and was performed using data from several electronic databases.

### Research results

Ablation therapies seem effective and safe with low post-treatment mortality. Although complications are mostly self-limiting, severe complications do occur.

### Research conclusions

Overall survival varies widely between different studies, and the additional value of ablation treatments for LAPC needs to be further explored.

### Research perspectives

Further efforts are also needed to address patient selection, as well as the use of IRE for “margin accentuation” during surgical resection, so the combination of RFA with immunotherapy represents a novel angle of attack against this tumour type.

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