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EDITORIAL

Pathophysiology of severe gallstone pancreatitis: A new paradigm

Masatoshi Isogai

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Abstract

Severe gallstone pancreatitis (GSP) refractory to maximum conservative therapy has wide clinical variations, and its pathophysiology remains controversial. This Editorial aimed to investigate the pathophysiology of severe disease based on Opie's theories of obstruction, the common channel, and duodenal reflux and describe its types. Severe GSP might be a hybrid disease with pathology polarized between acute cholangitis with mild pancreatitis (biliary type) and necrotizing pancreatitis uncomplicated with biliary tract disease (pancreatic type), in which hepatobiliary and pancreatic lesion severity is inversely related to the presence or absence of impacted ampullary stones. Severe GSP is caused by stones that are persistently impacted at the ampulla with biliopancreatic obstruction (biliary type), and probably, stones that are either temporarily lodged at the duodenal orifice or passed into the duodenum, thereby permitting reflux of bile or possible duodenal contents into the pancreas (pancreas type). When the status of the stones and the presence or absence of impacted ampullary stones with biliopancreatic obstruction are determined, the clinical course and outcome can be predicted. Gallstones represent the main cause of acute pancreatitis globally, and clinicians are expected to encounter GSP more often. Awareness of the etiology and pathogenesis of severe disease is mandatory.

Key Words: Gallstone pancreatitis; Biliary pancreatitis; Gallstone hepatitis; Acute cholangitis; Necrotizing pancreatitis; Pathophysiology

Core Tip: Gallstones represent the main cause of acute pancreatitis globally, and awareness of the etiology and pathogenesis of severe disease is mandatory. Based on the present study aimed to clarify the pathophysiology of severe disease from clinicopathological and historical points of view, severe gallstone pancreatitis may be a hybrid disease with pathology polarized between acute cholangitis and necrotizing pancreatitis in which the severity of hepatobiliary and pancreatic lesions is inversely related to the presence or absence of impacted ampullary stones with biliopancreatic obstruction. When the status of the stones is determined, the clinical course and outcome can be predicted.

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INTRODUCTION

In 1901, Opie[1] first directed attention to the occurrence of pancreatitis caused by gallstone blockage of the pancreatic duct. Generally, biliopancreatic obstruction during gallstone migration into or passage through the ampulla of Vater triggers gallstone pancreatitis (GSP)[2-4]. Stones with a diameter up to 5 mm are important predisposing factors for this disease[5], and it has been reported that the risk of patients with at least one gallstone < 5 mm in diameter is increased more than four-folds[6]. GSP is a rare disease. One study reported that 89 (3.4%) of 2583 residents of Rochester, Minnesota, with gallstones diagnosed between 1950 and 1970 developed acute pancreatitis[7]. Another study reported that 191 (3.4%) of 5663 patients who received surgical treatment for gallstones at Ogaki Municipal Hospital, Japan, between 1976 and 2003 were diagnosed with GSP[8]. Lowenfels *et al*[9] noted that the risk of biliary pancreatitis development in patients with asymptomatic gallstones is not likely > 2% during a 20- or 30-year period. Patients with GSP have highly elevated serum transaminase and pancreatic enzyme levels, and the former could reflect microscopic acute inflammatory hepatocyte necrosis caused by sudden blockage of the ampulla of Vater by the migrating bile duct stones[10]. Thus, elevated liver enzyme levels during acute attacks indicate acute bile duct obstruction by impacted stones and are useful for predicting the gallstone etiology with acute pancreatitis[11-13]. Alanine transaminase (ALT) is the most clinically useful parameter for diagnosing GSP[14]. A prospective study conducted by Anderson *et al*[15] demonstrated that higher the ALT level, the higher the chance of a biliary cause, and that the combination of positive abdominal ultrasound (US) results for gallstones and elevated ALT levels almost completely confirms the diagnosis of GSP. Therefore, the diagnosis of GSP is established by a history of abdominal pain and increases in serum pancreatic and liver enzyme levels. Small stones impacted at the ampulla of Vater cause an increase in biliary pressure, which produces dilatation of the bile duct and, in some cases, subsequent relief of the obstruction[16]. Because of the passage of stones, most patients with GSP have mild disease with hyperamylasemia alone or interstitial edematous pancreatitis (IP), and exhibit rapid objective improvement with rapid decreases in serum hepatic and pancreatic enzyme levels.

Severe GSP refractory to maximum conservative therapy has wide clinical variations, and its pathophysiology remains controversial. In particular, there is no consensus on the events that follow persistent ampullary stone impaction with biliopancreatic obstruction and its effect on the course of GSP[17]. Furthermore, the pathogenesis of necrotizing pancreatitis (NP), which leads to early and late single or multiple organ failure[18], remains controversial. Investigations of humans must rely on findings from autopsies or laparotomies performed early during the rapid course of the disease. In 1901, Opie[1,19] proposed the obstruction and common channel theories to explain the etiology of gallstone-induced IP and NP, respectively. In 1909, Opie and Meakins[20] reported reflux of the duodenal contents as the cause of NP based on the autopsy findings of a patient without gallstones. In 1993, Isogai *et al*[21] identified two categories of severe GSP, biliary and pancreatic, based on a retrospective and observational study of 73 patients with severe GSP who underwent emergency laparotomies between 1976 and 1989, which were common practices at the time. The present study aimed to review a series of autopsy findings on which Opie based his proposed theories of obstruction, the common channel, and duodenal reflux, to describe the two types of severe GSP, and to clarify the pathophysiology of severe disease from clinicopathological and historical points of view.

THEORIES ON THE PATHOGENESIS OF SEVERE GSP

Obstruction theory

In 1901, based on the autopsy findings of a 47-year-old man with a history of jaundice lasting approximately 3 wk accompanied by abdominal pain and fever, Opie[1] proposed pancreatic duct obstruction as the cause of IP with fat necrosis (FN). The patient suddenly experienced severe vomiting accompanied by intense abdominal pain, followed by a gradual increase in abdominal distention. On the third day after admission, he experienced slight jaundice and was irrational at times, and his temperature increased to a maximum of 104°F. He underwent emergency surgery based on the diagnosis of suppurative pancreatitis, but he died 4 h after the procedure. Autopsy revealed a large abscess and FN foci in the lesser peritoneal cavity. The hepatic, cystic, and common bile ducts were dilated. The gallbladder was distended and contained more than 100 calculi with diameters varying from 0.5-1 cm. In the terminal common bile duct, a calculus with

a 7-mm diameter that was lodged approximately 1.5 cm from the duodenal orifice, where the bile and main pancreatic ducts ran parallel to each other, that was separated by a thin interposed septum was observed. The pancreas was enlarged and the interstitial tissue was hemorrhagic; however, the glandular tissue was mostly well-preserved. Histologically, many acini were widely dilated, their cells were flat, and the lumen was distended and contained secretion products. Opie speculated that the stone might have compressed and occluded the main pancreatic duct, and that the obstructed secretion of pancreatic juice might have been forced backward into the pancreatic parenchyma and surrounding tissue, thus making contact with the fat cells and resulting in IP and localized pancreatic FN.

Common channel theory

In 1901, although the patient's sex, age, and clinical course were missing, Opie[19] proposed a continuous closed channel as the mechanism of NP based on autopsy findings. An autopsy revealed a moderate amount of serosanguineous fluid in the peritoneal cavity with disseminated FN. The pancreas was gangrenous. The duodenal papilla was prominent. A small calculus with a 3-mm diameter was lodged at the duodenal apex, thereby occluding the orifice. Careful examination revealed that the impacted stone was so small that the orifices of the common bile and pancreatic ducts were not obstructed, and both ducts were converted into a continuous closed channel 10 mm in length. The pancreatic duct, which passes through the pancreatic head, resembled the common bile duct because it was stained with bile. Opie[19] speculated that bile reflux into the pancreas could repeatedly occur when any slight pressure differences in the bile and pancreatic ducts were overcome by gallbladder contraction; therefore, pancreatic enzymes were activated, producing NP.

Duodenal reflux theory

In 1909, based on the autopsy findings of a 55-year-old man with no gallstones, Opie and Meakins[20] suggested reflux of duodenal contents into the pancreas as the cause of NP. The patient reported sudden and severe epigastric pain followed by vomiting. Two days later, he experienced shock and died 6 h after admission to the hospital. During the autopsy, almost the entire gland of the body and tail and the greater part of the pancreatic head were necrotic. There were several characteristic findings. First, the seat of the pancreatic necrosis was localized to the portion drained by the duct of Santorini and the posterior and lower parts of the pancreatic head, which are in contact with the Wirsung duct, had a normal appearance. Second, the duct of Santorini, which is usually a small accessory duct, was much larger than the Wirsung duct, and ran through the entire length of the pancreas, which was the chief outlet of the gland. Third, the orifice of the duct of Santorini was relatively wide, thus permitting the use of a probe with a diameter of approximately 2 mm. They speculated that duodenal contents with enterokinase might reflux into the pancreatic duct through the patulous orifice of the duct of Santorini, which is unable to prevent regurgitation when the pressure within the duodenum is increased by vomiting, thereby producing NP. Considering the duodenal reflux theory in relation to GSP, it is possible that the evacuation of impacted ampullary stones damages the sphincter, which permits the reflux of duodenal contents into the pancreas[22], thereby causing NP.

TWO CATEGORIES OF SEVERE GSP

Isogai *et al*[21] identified two categories of severe GSP. One is the biliary type, which comprises life-threatening acute cholangitis and minimal pancreatic lesions or IP caused by the persistent impact of stones at the ampulla of Vater with biliopancreatic obstruction. The other is the pancreatic type, which comprises NP, but is uncomplicated by acute biliary tract disease.

Severe biliary-type GSP

Based on Opie's[1] obstruction theory, and the hepatic histopathological changes that occur with acute bile duct obstruction caused by impacted bile duct stones[10,23], the pathogenesis of severe biliary-type GSP may be hypothesized as follows: (1)Pancreatic duct obstruction causes minimal pancreatic lesions or IP; (2) Persistent obstruction of the bile duct leads to acute cholangitis; and (3) The severity of pathological changes in the liver and biliary tract is more significant than that in the pancreas.

Severe pancreatic-type GSP

Based on Opie's[19,20] common channel and duodenal reflux theories, the hypothesis regarding the pathogenesis of severe pancreatic-type GSP is as follows: (1) The reflux of bile or possible duodenal contents into the pancreas causes NP through the activation of pancreatic enzymes; (2) All stones that settle in the narrow duodenal orifice and allow bile reflux into the pancreas are expelled into the duodenum early during the course of disease; (3) Sphincter Oddi incompetence caused by the evacuation of impacted larger stones at the ampullary site permits reflux of duodenal contents into the pancreas, leading to NP; and (4) Virtually all stones responsible for NP pass into the duodenum and are lost or have been evacuated into the duodenum, providing no evidence of their former impaction, with acute hepatobiliary inflammation resolving rapidly.

In 2005, Isogai *et al*[8] conducted another clinical study to investigate the relationship between pancreatitis severity and biliary pathology of 183 consecutive patients with GSP during 1976 to 2002. Of the 183 study patients, 95 (52%) had severe disease and underwent emergency laparotomy (86 patients) or endoscopic retrograde cholangiopancreatography (ERCP; 9 patients) during acute attacks. Of the 95 patients with severe disease, 43 (45%), 23 (24%), and 29 (31%) had impacted ampullary stones, mobile bile duct stones without impaction, and gallbladder stones alone, respectively. Of the 43 patients with impacted ampullary stones, 14 (33%) and 6 (14%) had the Charcot triad and Reynolds pentad, respectively.

Of the 23 patients with floating bile duct stones, 9 (40%) had purulent bile in the bile duct. All 43 patients with impacted ampullary stones and all 23 patients except one with floating bile duct stones had minimal or mild pancreatitis, without the features of severe pancreatitis defined according to the Atlanta classification[24,25]. Of the 95 patients with severe disease, 16 (17%) had NP, which was defined either as a macroscopically dark pancreas or as non-enhanced pancreatic parenchyma on contrast-enhanced CT. None of the 16 patients with NP had stones impacted at the ampulla, and all 16 patients, except one with mobile bile duct stones, retained uniformly small stones in the gallbladder alone; any one of those stones might have caused fatal diversion of bile or duodenal contents into the pancreatic duct, providing no evidence of their former impaction. Of the 95 patients with severe disease, 8 (8%) died. Of these eight fatalities, four patients without NP had impacted ampullary stones (two of those four patients died of acute obstructive cholangitis), and four patients with NP died of multiple organ failure[8]. The afore-mentioned study results supported the hypothesis of the pathogenesis of biliary and pancreatic types of severe disease.

PATHOGENESIS OF SEVERE BILIARY-TYPE GSP

The autopsy findings of the 47-year-old patient on which Opie[1] based the proposed obstruction theory showed that many pancreatic acini were widely dilated; their cells were flat and the lumen was distended and contained products of secretion. Intrapancreatic duct pressure was increased by pancreatic duct obstruction. The main pancreatitis-inducing factors include fatty acids, alcohol, bile acids, and physical pressure[26]. Increased pressure in the pancreatic duct is thought to be a key factor in the development of GSP[27]. Swain *et al*[27] showed that higher and prolonged elevation of the pancreatic duct pressure caused by pancreatic duct ligation in mice activated the calcium-permeable ion channel Piezo 1 in the pancreatic acinar cell, thus causing prolonged elevation of intracellular calcium levels, mitochondrial depolarization, and intracellular trypsin activation, ultimately leading to cell death. However, the clinical picture of the majority of patients with biliopancreatic obstruction caused by stones impacted at the ampulla is more often dominated by life-threatening acute cholangitis and septicemia than by acute pancreatitis[8,21,22,28]. Experimental and clinical investigations have questioned the relationship between the duration of pancreatic obstruction and severity of pancreatitis. In 1901, Opie[1] noted that pancreatic duct obstruction in humans and pancreatic duct ligation in animals do not cause hemorrhage or hemorrhagic inflammation. In 1964, McCutcheon[29] reported that pancreatic duct obstruction had little association with the etiology of pancreatitis, demonstrating that no patient with recurrent pancreatitis who underwent pancreatic duct ligation developed pancreatitis during the immediate postoperative period. In 1997, Arendt *et al*[30] used a rabbit model mimicking gallstone impaction in the human choledochoduodenal junction with biliopancreatic obstruction, and reported that pancreatic duct obstruction without a patent duct of Santorini produced pancreatic edema without acinar necrosis, whereas a patent duct of Santorini prevented the development of pancreatic edema caused by pancreatic duct obstruction. In 2006, Acosta *et al*[31] conducted a prospective randomized clinical trial involving patients with GSP and persistent ampullary obstruction but uncomplicated severe cholangitis and compared the efficacy of ERCP with or without endoscopic sphincterotomy (ES) between the study group (initial conservative treatment and ERCP with or without ES within 48 h if obstruction persisted for 24 h or more) and control group (conservative treatment with or without selective ERCP with or without ES after 48 h in the case of associated persistent jaundice or cholangitis). The study results showed that pancreatic phlegmon consisting of a pancreatic inflammatory mass, peripancreatic fluid, and FN was the most common pancreatic pathology, and that pancreatic phlegmon was identified only in control group patients with persistent obstruction for more than 48 h. The term "pancreatic phlegmon" does not appear in the Atlanta classification of acute pancreatitis[24,25], but it is likely consistent with IP.

Acosta *et al*[17] noted that stones > 3 mm in diameter completely filled the ampulla and prevented pancreatic reflux. However, whether pancreatic duct obstruction without bile reflux causes NP in humans is unknown[32]. The author believes that the stones impacted at the ampulla with biliopancreatic obstruction in the absence of bile reflux probably resulted in clinically minimal pancreatic lesions or IP with or without FN because of the absence of activation of the proteolytic pancreatic proenzymes. Regarding FN caused by pancreatic duct obstruction, the pathogenesis is explained as follows: When the pancreatic duct is obstructed, the pancreatic juice containing fat-splitting enzyme lipase, one of the few pancreatic enzymes that does not need to be activated[33], is forced back into the pancreatic parenchyma, thus penetrating the surrounding tissue and making contact with the fat cells, and the fat is split by the fat-splitting enzyme into fatty acid and glycerin. The insoluble fatty acids subsequently unite with calcium salts and develop into FN, whereas soluble glycerin is absorbed and carried away[1].

In contrast, bacterial cholangiovenous reflux develops if bile duct obstruction persists, thus leading to acute cholangitis. To understand the pathogenesis of acute cholangitis, it is necessary to understand the pathological changes that occur in the liver when the bile duct is obstructed by the impacted stones. As previously noted, patients with GSP have elevated serum transaminase levels. Hepatic histopathological changes in patients with GSP have been reported to be acute inflammatory hepatocyte necrosis and acute cholangitis caused by sudden blockage of the ampulla of Vater because of impacted bile duct stones[10]. These acute hepatic injuries are consistent with those identified in patients with severe biliary colic, marked elevation of serum transaminase levels similar to those with hepatitis, and only a mild increase in serum bilirubin, which, in 1991, Isogai *et al*[23] referred to "gallstone hepatitis" as a new clinical entity. This "medical" enzyme pattern in patients with gallstone hepatitis and GSP who have "surgical" pain is paradoxical and counterintuitive[34]. Mohamed *et al*[35] performed a systematic review and meta-analysis and concluded that marked or extreme transaminase elevations and minimal increases in alkaline phosphatase and bilirubin should prompt clinicians to evaluate and manage biliary obstruction. Culture results of common bile duct aspirate during emergency surgery were positive for 72% of patients with gallstone hepatitis, with *Escherichia coli* being the most frequent pathogen, followed by

Klebsiella pneumoniae and *Streptococcus faecalis*[23]. A rapid increase in the bile duct pressure[13] and mechanical insufficiency of lymph circulation caused by a combination of bile stasis and inflammation[36] have been reported to cause liver cell necrosis. However, the mechanism of hepatocyte necrosis in gallstone hepatitis remains unclear.

The hepatocytes have tight junctional complexes that form a seal between the lumen of the bile canaliculus and hepatic intercellular space, which play the role of a canaliculi-sinusoidal barrier[37], and discontinuities in the junctional meshwork, which provide a direct pathway between the lumen of the bile canaliculus and intercellular space[38]. Elevation of liver enzyme levels is a serological reflection of microscopic hepatocyte necrosis, indicating disruption to the barrier. Bacterial cholangiovenous reflux occurs through damaged tight junctional complexes and also directly through disrupted liver cells[39] if biliopancreatic obstruction persists and the pressure in the bile canaliculus further increases, leading to acute cholangitis.

Conventionally, the cause of death of the 47-year-old patient whose autopsy findings were the basis for the obstruction theory proposed by Opie[1] was considered to be severe pancreatitis; however, this probably was not the actual cause. The patient likely died of acute obstructive cholangitis, which is a distinct clinical syndrome of overwhelming sepsis characterized by complete bile duct obstruction proposed by Reynolds and Dargan[40] in 1959. The patient had mental confusion and possible shock in addition to Charcot's triad, abdominal pain, fever, and jaundice, on the day of surgery, and died during a fulminate postoperative course. Opie[1] did not mention the cause of his death, which might have been unavoidable because, at that time, the clinical concept of acute obstructive cholangitis did not exist; furthermore, even the terms "acute cholangitis" and "Charcot's triad" did not appear in Opie's[1,19,20] original articles. However, Opie[1] seemed to have considered a possible pathology other than pancreatic lesions that determined the disease severity, noting that individuals such as the autopsy patient, who were usually in fairly good health and perhaps, had a history of gallstone attacks suddenly experienced epigastric pain, accompanied by vomiting and followed by collapse and death, usually within 48 h. During autopsy, gallstones were found lodged in the common bile duct near its orifice, which might have caused a fatal attack. Opie's[1] clinical scenario seems typical of acute obstructive cholangitis in patients with GSP with stones persistently impacted at the ampulla of Vater and biliopancreatic obstruction. This severe biliary-type GSP is not widely appreciated by general clinicians, possibly because either longer or permanent stone impaction may lead to severe pancreatitis. In 2023, Zhang *et al*[41] defined obstructive severe acute biliary pancreatitis as persistent single or multiple organ failure (> 48 h) according to the revised Atlanta Classification[25], with the cause of severe pancreatitis being biliary obstruction confirmed by imaging, which may be consistent with severe biliary-type GSP.

As mentioned previously, the real danger for biliary type patients is acute cholangitis that is refractory to supportive therapy and life-threatening. The clinical diagnosis of acute cholangitis has long been based on the clinical findings of Charcot's triad. However, because distinction of the inflammatory response caused by acute pancreatitis from that caused by acute cholangitis may be difficult, reliance on Charcot's triad is insufficient[42]. No standard criteria for the diagnosis of acute cholangitis are available when all of the components of Charcot's triad were not present. However, in 2007, the first diagnostic and severity assessment criteria for acute cholangitis were presented, and a definite diagnosis was made if clinical manifestation, objective laboratory data including liver function test results, and imaging findings that support the evidence of inflammation and biliary obstruction were observed[43]. In 2013, the diagnostic criteria for acute cholangitis were revised and termed the Tokyo Guideline 2013, and the thresholds of numerous variables were accurately and reliably set as follows: Body temperature: > 38°C; white blood cell count: < 4 or > 10 × 1000/μL; C-reactive protein level: ≥ 1 mg/dL; jaundice (total bilirubin): ≥ 2 mg/dL; and liver function levels, such as alkaline phosphatase, γ-glutamyltransferase, aspartate aminotransferase, and ALT, > 1.5-times the upper limit of the normal values[44].

PATHOGENESIS OF SEVERE PANCREATIC-TYPE GSP

Regarding the reflux of bile as the cause of NP, animal models have shown that protease activation is highly dependent on calcium release[45], with bile acids inducing calcium-releasing signals and contributing to pancreatic cell damage[46]. One factor that disputes the common channel theory is the pressure within the pancreatic duct that is higher than that observed in the bile duct that does not allow for retrograde flow from the biliary system to the pancreas[47]. Experimental studies have suggested that after the entry of pancreatic juice into the biliary tree, the following a series of events occurs, thus producing NP: Activation of trypsinogen; reduction of mucin density by trypsin; enhancement of injurious actions and reduction of viscosity by mucin-free bile salts; and a diffusion of dangerous bile-trypsin mixture into the pancreas during a time when intra-abdominal pressure increases[48,49]. Clinically, however, questions regarding the evidence of bile reflux into the pancreatic duct and the presence of impacted stones have prevented wide acceptance of the common channel theory. In 2020, Isogai *et al*[50] reported a case of NP in which bile refluxed into the pancreas was histologically demonstrated in biopsy specimens of the pancreas collected during emergency surgery for GSP (Figure 1), thereby proving Opie's long-speculated common channel theory. However, regarding the case studied by Isogai *et al*[50], intraoperative cholangiography showed no bile duct stones. Moreover, impacted stones are seldom found and remain a mystery.

With the introduction of endoscopic US to investigate the bile duct, biliary sludge and biliary microlithiasis have become widely recognized as causes of acute pancreatitis[51]. Żorniak *et al*[51] proposed consensus definitions of biliary sludge, microlithiasis, and stones as hyperechoic material without acoustic shadowing, calculi ≤ 5 mm with acoustic shadowing, and calculi > 5 mm in the biliary tract and gallbladder, respectively; they showed no difference in the severity of pancreatitis between sludge-induced, microlithiasis-induced, and gallstone-induced pancreatitis. Recently, Hofstrand *et al*[52] reported a case of NP during the postpartum period that presented with clinical symptoms characteristic of severe pancreatitis, without bile duct stones, with gallbladder sludge on contrast-enhanced CT and magnetic resonance cholangiopancreatography, and with highly elevated liver and pancreatic enzymes in the blood. They concluded that the

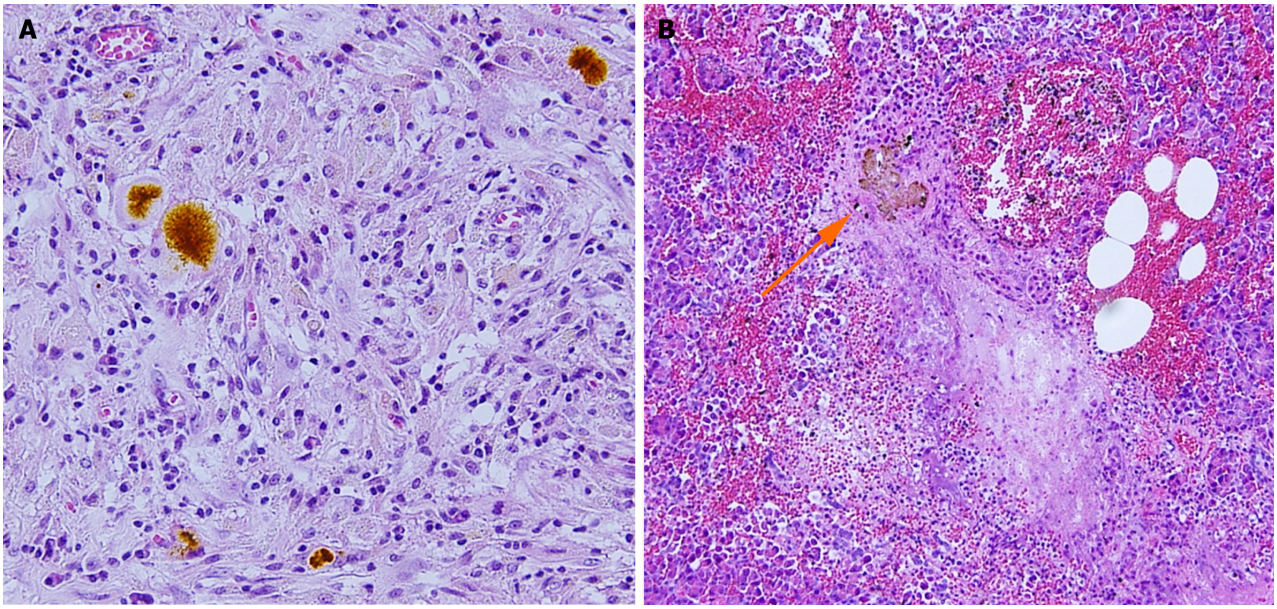

Figure 1 Histological evidence of bile reflux into the pancreas in a patient with gallstone necrotizing pancreatitis. A: Bile pigments among the inflammatory granulomatous tissue; B: Concretion of bile pigment-like material (arrow), periductal necrosis with neutrophil infiltration, and bleeding. Citation: Isogai M, Kaneoka Y, Iwata Y. Histological evidence of bile reflux in necrotizing pancreatitis: A case report. *Med Case Rep Study Protoc* 2020; 1: e0016. Copyright ©The Author 2020. Published by Wolters Kluwer Health Inc[50].

most likely etiology of NP was microlithiasis, which is difficult to detect in the bile duct using imaging techniques. After dissecting the biliary pancreatic junction of autopsy specimens, Kelly[53] reported that the mean diameter of the duodenal orifice was 2.5 mm and the mean length of the ampulla of Vater was 5 mm. It is likely that very small stones of such size, 3 mm in Opie's[19] case, that are lodged at the duodenal orifice and convert the bile and pancreatic ducts into a common channel were either missed, even when surgery or traditional imaging studies were performed within 48 h of admission before sufficient time was allowed for stone passage[53], or expelled and evacuated after short-term occlusion of the duodenal orifice, thereby providing no evidence of their former impaction. Hernández and Lerch[54] noted that migrating stones induce functional stenosis at the sphincter of Oddi and a common channel can arise.

DiMagno *et al*[55] classified the pancreatic and bile duct entries as a common channel, an interposed septum, or separate opening categories; furthermore, they noted that a well-defined ampulla as a common channel that formed an ampullary structure and a long common channel > 3 mm without an ampullary structure are likely to have bile reflux into the pancreatic duct. However, common channels that allow bile reflux through impacted stones at the duodenal orifice are not universally present in patients with gallstones. McCutcheon[56] favored the reflux of duodenal contents rather than bile as a rational and simple explanation for the activation of pancreatic enzymes and subsequent pathological changes. Experimentally, he demonstrated that duodenopancreatic reflux occurred readily at physiological pressure if the papillary mucosal folds of the pancreatic duct that prevent reflux were damaged[29]. Clinically, the damage caused by the passage of stones possibly leads to insufficiency of the sphincter of Oddi, that is, loss of resistance to flow between the duodenum and pancreatic duct[57], and permits reflux of the duodenal contents into the pancreas, causing NP[22]. A case of NP for which intraoperative cholangioscopy was performed during emergency surgery and showed no stones in the bile duct but an injured and gaping sphincter has been reported elsewhere[8]. After surgery, stool samples were screened for gallstones, and a stone that was approximately 4 mm in diameter and grossly similar to that in the gallbladder was found[8], indicating possible reflux of the duodenal contents into the pancreas as the cause of NP. However, it is difficult to histologically prove that reflux is the cause of NP because the duodenal contents have no pigment to indicate their presence, and duodenal reflux has not been scientifically or clinically well-supported[28].

Thus, if the reflux of bile or duodenal content is the cause of NP, then the offending calculus will be expelled early during the disease course or pass into the duodenum and become lost. Genetic control has been explored as a possible determinant of pancreatitis severity[58]; however, the exact event responsible for progressive pancreatic inflammation after stone passage into the duodenum may be multifactorial and remains to be determined[22].

PATHOPHYSIOLOGY AND MANAGEMENT OF SEVERE GSP

Severe GSP may be a hybrid disease[59], with pathology polarized between acute cholangitis and NP, each of which has a distinct etiology and is inversely related to the presence or absence of impacted ampullary stones with biliopancreatic obstruction. When the status of the stones and the presence or absence of biliopancreatic obstruction without bile reflux are determined, the clinical course and outcome can be predicted.

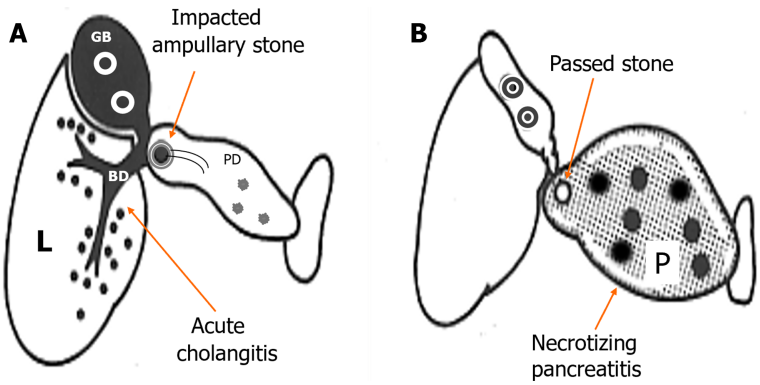

Figure 2 Subdivision of severe gallstone pancreatitis into gallstone cholangiopancreatitis and gallstone necrotizing pancreatitis. A: Gallstone cholangiopancreatitis with persistent ampullary stone impaction and ascending acute cholangitis complicated with minimal or mild pancreatic inflammation caused by biliopancreatic obstruction. B: Gallstone necrotizing pancreatitis caused by the reflux of bile or possible duodenal contents into the pancreas, not complicated by acute biliary tract disease caused by the passage of stones. L: Liver; BD: Bile duct; GB: Gallbladder; PD: Pancreatic duct; P: Pancreas. Citation: Isogai M. Proposal of the term "gallstone cholangiopancreatitis" to specify gallstone pancreatitis that needs urgent endoscopic retrograde cholangiopancreatography. *World J Gastrointest Endosc* 2021; 13: 451-459. Copyright ©The Author(s) 2021. Published by Baishideng Publishing Group Inc[74].

Severe biliary-type GSP should not be misdiagnosed as severe pancreas-type GSP. If an impacted ampullary stone is detected using imaging modalities including a high-quality evaluation of the biliary system with endoscopic US[60] or magnetic resonance cholangiopancreatography[61], an accurate diagnosis of acute cholangitis can be made, or ongoing biliary obstruction can be strongly predicted by serum total bilirubin > 4mg/dL[62,63], then ERCP with stone removal through the duodenal papilla is indicated[63-65], for severe biliary-type GSP.

When an impacted ampullary stone with biliopancreatic obstruction is ruled out, contrast-enhanced CT is a safe and accurate method of identifying NP[66]. Kovalska *et al*[67] noted that during the early stages of NP, the pathological changes were impaired microcirculation caused by intravascular microthrombosis with endothelial desquamation and sludge. Thus, after the first week of disease, a non-enhancing area of the pancreatic parenchyma on contrast-enhanced CT should be considered pancreatic necrosis[25]. The CT severity index[68], when calculated after the first week of disease, has been reported to show the highest diagnostic and predictive accuracies[18]. Song *et al*[69] reported the usefulness of contrast-enhanced CT features at the time of admission, such as the presence of peripancreatic fluid and heterogeneous pancreatic parenchyma enhancement, for predicting the progression to NP in patients initially diagnosed with IP. Generally, however, it is difficult to determine the final severity of pancreatitis early during the disease course of patients with predicted severe pancreatic-type GSP, and maximum intensive care treatment is mandatory. A recent multicenter randomized controlled trial showed that urgent ERCP with ES within 24 h after presentation did not reduce major complications or mortality of patients with predicted severe GSP and without cholangitis[70].

FUTURE PERSPECTIVES

Conventionally, clinicians have focused less attention on the hepatobiliary diseases with GSP that may be unavoidable because the terminology of GSP refers to "pancreatitis" alone, without "hepatitis." This can be explained by the lower availability of liver enzyme testing during the early 1960s, when Howard and Ehrlich[71] proposed the term "GSP "as a distinct clinical entity in 1962. In 1955, Wroblewski and LaDue[72] first reported increases in serum glutamic-oxaloacetic transaminase levels associated with liver disease that occurred as a result of the release of enzymes from damaged liver tissue. To provide a better understanding of the nature of GSP (*i.e.*, gallstone-induced acute hepatitis and pancreatitis[73]) and direct the clinician's attention to hepato-biliary-pancreatic lesions that occur in both the liver and pancreas, in 2021, Isogai[74] devised the term "gallstone hepatopancreatitis" in place of GSP. At the same time, the terms "gallstone cholangiopancreatitis" and "gallstone NP" were proposed for the biliary and pancreatic types of severe disease, respectively, with the former emphasizing that acute cholangitis is not a comorbid disease; instead, it is an essential condition that outweighs pancreatic lesions (Figure 2)[74].

Gallstones represent the main cause of acute pancreatitis globally[75,76], with a contributory rate twice that of alcohol [75]. Thus, clinicians are expected to encounter GSP more often, and awareness of the etiology and pathogenesis of severe disease is mandatory.

CONCLUSION

Severe GSP may be a hybrid disease with pathology polarized between acute cholangitis and NP in which the severity of hepatobiliary and pancreatic lesions is inversely related to the presence or absence of impacted ampullary stones with biliopancreatic obstruction. When the status of the stones is determined, the clinical course and outcome can be predicted.

FOOTNOTES

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EDITORIAL

Trauma to the solid abdominal organs: The missed dark box of colonoscopy

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Abstract

Colonoscopy is an integral part of the lower bowel care and is generally considered a potentially safe diagnostic and therapeutic procedure performed as a daycare outpatient procedure. Colonoscopy is associated with different complications that are not limited to adverse events related to the bowel preparation solutions used, the sedatives used, but to the procedure related as well including bleeding and perforation. Injuries to the extra-luminal abdominal organs during colonoscopy are uncommon, however, serious complications related to the procedure have been reported infrequently in the literature. Life threatening injuries to the spleen, liver, pancreas, mesentery, and urinary bladder have been reported as early as in mid-1970s. These injuries should not be overlooked by clinicians and endoscopists. Steadily increasing abdominal pain, abdominal distension, and hemodynamic instability in absence of rectal bleeding should raise the possibility of severe organ injury. Splenic and hepatic injury following colonoscopy are usually serious and may be life threatening. Although conservative management may help, yet they usually need interventional radiology or surgical intervention. Acute pancreatitis following colonoscopy is usually mild and is mostly managed conservatively. The mechanism of abdominal organ injuries during colonoscopy is not fully understood, however many risk factors have been identified, which can be classified as- organ related, procedure related, and local abdominal factors. Difficult colonoscopy and prior intra-abdominal adhesions are probably the most relevant risk factors for these injuries. Left lateral position, avoidance of looping and excessive force during the procedure would probably reduce the risk of such injuries.

Key Words: Colonoscopy; Pancreatitis; Trauma; Complications; Adhesions

Core Tip: Although colonoscopy is seen as a potentially safe procedure performed as a daycare practice, yet it is associated with a wide range of complications. Emerging evidence in the literature documents injuries to abdominal organs beyond the bowel wall. The most commonly affected organ is the spleen, followed by the pancreas and infrequently the liver, mesentery and urinary bladder. Despite its low frequency, some cases are serious especially the rupture of visceral organs like spleen. In such cases, conservative management may not always work and interventional radiologic procedures and/or surgery may be required.

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INTRODUCTION

Colonoscopy is potentially a safe diagnostic and therapeutic procedure performed daily as an outpatient care service. Colonoscopy refers to the examination of colon and terminal ileum using flexible colonoscopes. The introduction of colonoscopy was a paradigm shift in the diagnosis and treatment of lower gastrointestinal (GI) diseases. It is indicated for evaluation of lower GI symptoms including chronic diarrhea, constipation, bleeding per rectum, tenesmus and abdominal pain among others. It is also an integral part of monitoring activity and response to treatment in inflammatory bowel diseases (IBD), follow up of patients with diverticular disease and mucosal tissue acquisition for different purposes. Furthermore, it is now a cornerstone not only in screening but also in the treatment of early stages of colorectal cancer (CRC). In fact, with the advancements achieved in colonoscopy, CRC is now classified as a preventable disease through screening of the high-risk group of the patients and application of different therapeutic interventions. The therapeutic potential extends from simple hot and cold snare polypectomy to advanced endoscopic mucosal resection, and endoscopic submucosal dissection for superficial bowel neoplasia and stenting for both benign and malignant lesions [1-3].

The operator should maintain an acceptable level of competency to clearly identify the indications for the procedure, technical ease with avoidance of looping and excessive force application while advancing the scope, and high experience in handling high risk patients. In addition, early identification and treatment of procedure related complications is very crucial[2].

The common adverse events related to this procedure vary and include abdominal pain, distension, perforation, bleeding, and adverse events related to the bowel preparation solutions used to clean the colon before the endoscopic examination. Furthermore, adverse events due to the used sedation medications have been described also[3].

Severe acute abdominal pain occurring post-colonoscopy usually directs the attention towards bowel perforation, however, extra-luminal adverse events related to colonoscopy presenting with acute abdominal pain are associated with a wide range of manifestations, and are increasingly reported in the literature[3-6].

Post-colonoscopy abdominal pain is a common manifestation, usually benign, and mostly related to the gaseous distension and tractions induced by the procedure[5,6]. However, continuous and steadily increasing pain should alarm the endoscopists and clinicians to the development of complications. Furthermore, development of symptoms not related to the procedure, *e.g.*, nausea, vomiting, marked and progressive abdominal distension, hypotension and hemodynamic instability should raise the possibility of serious complications especially post-procedural perforation, bleeding and injury to other organs. The overall rate of colonoscopy related serious complications is 2.8/1000 procedure[3] and mortality is estimated to be 0.23 to 0.91 per 10000 among FIT-positive and negative participants undergoing screening colonoscopy in a recently published Dutch study respectively[7].

Injury to organs beyond the colonic wall induced by colonoscopy is uncommon. These injuries are sometimes serious and life-threatening, and that is why a high index of suspicion should be raised upon evaluating patients' post-procedural state. There are no accurate estimates to overall organ injuries induced by colonoscopy, however, many reports are evolving in the literature figuring out these injuries. The first report for organ injury by colonoscopy was reported as early as mid-1970s[8], and thereafter many case reports of different organ injuries were published.

We analyzed many articles across the literature through search of the major databases including PubMed, Embase, and Cochrane. The retrieved articles are mainly case reports, case series and reviews while the observational studies were infrequently found.

SPLENIC INJURY DURING COLONOSCOPY

Although splenic injury after colonoscopy is a rare complication, yet spleen is the most frequently injured abdominal organ during colonoscopy. There is no accurate estimate for the incidence of post colonoscopy splenic injury. However, a study from a single institution focusing on all colonoscopies done between 1980 and 2008 reported the incidence of splenic injury to be 0.2 per 10000 procedures[9]. Another study documented a rate of 0.72 per 10000 procedures[10]. An intermediate incidence was recently figured out by Laanani *et al*[11], with figures of 0.20-0.34 per 10000 procedures.

It was first documented by Wherry and Zehner[12], and is a serious complication that can lead to life threatening conditions. Catastrophic complete splenic rupture was reported among 22% of cases, while the majority of cases (63%) experienced other types of splenic injuries such as, spleen laceration, sub-capsular hematoma, and spleen avulsion[13].

Diagnosis of splenic injury may be challenging as post-colonoscopy abdominal discomfort and pain is common and mostly due to trapped air in colon. However, in splenic injury, symptoms develop within 24 h most commonly as persistent abdominal pain without radiographic evidence of perforation, peritoneal irritation, and a drop in hematocrit and hemodynamic instability, depending on the severity of the injury. Contrast enhanced computed tomography (CT) scan is the gold standard method for diagnosis of splenic injuries because it can detect the presence of haemoperitoneum, describe the extent of injury and exclude other organ injuries[14].

Treatment varies according to the type and severity of splenic injury and the associated hemodynamic instability, if any. In hemodynamically stable patients with closed sub-capsular hematoma and no intra-peritoneal blood conservative treatment should be tried. This includes close monitoring, intravenous fluids, bed rest, serial hemoglobin and hematocrit monitoring, and serial imaging. In hemodynamically unstable patients with active bleeding and associated peritonitis, selective splenic artery embolization is an option, however, splenectomy is usually the definitive management[14,15].

HEPATIC INJURY DURING COLONOSCOPY

Regarding liver, the first report of liver injury following colonoscopy was reported as early as 1979 by Ellis *et al*[16], who reported combined liver, splenic, and mesenteric injury in a 33-year-old lady with active IBD. Combined hepatic and splenic lacerations due to rupture of tight vascular adhesions between transverse colon and both the spleen and liver was reported 8 years later by Levine *et al*[17]. Combined hepatic and splenic injuries during colonoscopy are rather common than isolated hepatic injury that is extremely rare. Noreña *et al*[18], reported a large subcapsular hepatic hematoma, right lobe laceration and hemoperitoneum 4 d after a routine colonoscopy. One more, lone hepatic injury was reported by Jammal *et al*[19], who described a subcapsular hepatic hematoma 6 h after colonoscopy.

In these cases (Table 1), most patients experienced persistent right side abdominal pain, peritoneal irritation, drop in hematocrit and hemodynamic instability, depending on the degree of hepatic injury. Although abdominal ultrasound has been used to diagnose large sized hepatic hematoma, yet contrast CT scan is the gold standard method for confirming diagnosis, assessing extent, and diagnosing associated other organ injuries. Management options for hepatic injury depend on hemodynamic stability and extent of injury including conservative approach, percutaneous drainage, angiographic embolization, or exploratory laparotomy[17-21].

PANCREATIC INJURY DURING COLONOSCOPY

Regarding pancreas, there are no accurate estimates for pancreatic injuries induced by colonoscopy. However, the frequency of pancreatic injury following colonoscopy seems low. The literature presents many published cases (Table 2), figuring out this serious complication. Probably the first reported pancreatic injury in relation to colonoscopy reported in English literature was described by Thomas and Mitre[22]. Thereafter, many cases were reported. The reported pancreas related adverse events comprised acute pancreatitis (AP)[5,6,23], pancreatitis with hemorrhage related to tail of pancreas [21] and pancreatic duct leak with development of colo-pancreatic fistula[22]. Diagnosis should be suspected with the development of the symptoms after colonoscopy usually by 2-6 h and these include epigastric pain, nausea, vomiting, haemodynamic instability associated with pancreatic enzymes' elevation[24]. However, the presentation may be delayed to many hours.

Diagnosis usually combines clinical manifestations of epigastric pain and vomiting, with elevated pancreatic enzymes [22-24], and CT scan (Figure 1) not only confirms the diagnosis but also assesses the severity of the injury.

The risk factors for pancreatic injuries include direct mechanical trauma to the pancreas due to procedural difficulties particularly around the splenic flexure, cautery induced transmural colonic burns, over-insufflation of colon, direct abdominal pressure exerted to facilitate endoscope advancement[5,22-24] and prior abdominal surgeries with pre-existing adhesions[24].

Treatment varies based on severity and etiology. For uncomplicated AP, conservative treatment with fluids, analgesics with or without prophylactic antibiotics is usually sufficient[5,22]. The duration of conservative treatment is usually few days[5,24], and may be prolonged to 1-2 wk in complicated cases[5]. Complicated cases may require further interventions, *e.g.*, endoscopic retrograde cholangiopancreatography[24].

Table 1 Case reports of hepatic injuries during colonoscopy

Ref.	Type of injury	Age, sex, and risk factors	Presentation	Treatment	Comments
Ellis *et al* [16], 1979	Combined liver and spleen	33-year-old female, her manifestations and colonoscopy findings were suggestive of IBD	Left upper quadrant followed by hemodynamic instability	Surgical	The spleen ruptured, laceration to left liver lobe, injury to mesocolon. Ruptured? IBD related adhesions were seen
Noreña *et al*[18], 2013	Lone liver injury	73-year-old male, undergoing a screening colonoscopy	Severe right upper abdominal pain radiating to lumbar region with abdominal distension and rigidity	Laparoscopic evaluation and drainage were done with conservative management	A subcapsular hepatic hematoma and a right hepatic; lobe laceration as well as a large hemoperitoneum
Jammal *et al*[19], 2013	Lone liver injury	30-year-old woman undergoing screening colonoscopy for polyps	Right upper quadrant pain radiating to the right scapula	Conservative	Abdominal ultrasonography and CT achieved diagnosis of a subcapsular hematoma
Hussain *et al*[20], 2020	Combined liver and spleen	A 71-year-old woman with many medical co-morbidities had an EGD; and colonoscopy for nonspecific abdominal pain	Brought to emergency room in code blue hours after having colonoscopy	Emergency laparotomy with splenectomy and hepatorrhaphy	Adhesion of omentum to bilateral abdominal wall and pelvis. The patient passed away dye to brain hypoxic events

IBD: Inflammatory bowel diseases; EGD: Esophagogastroduodenoscopy; CT: Computed tomography.

Table 2 Case reports of pancreatic injuries during colonoscopy

Ref.	Type of injury	Age, sex, and risk factors	Presentations	Treatment	Comments
Sidiqi and Gong[5], 2019	Acute pancreatitis	53-yr-old female	Epigastric pain with nausea and vomiting	Conservative	Probably direct trauma to tail of the pancreas by movement of the endoscope
Limb *et al*[6], 2016	Acute pancreatitis	69-yr-old female, multiple abdominal surgeries, and previous episode of acute pancreatitis	Epigastric pain	Conservative	Crohn's disease; controlled under mesalazine therapy
Thomas and Mitre[22], 1994	Acute pancreatitis	A 25-yr-old male, technical difficulties with passage of the scope beyond the splenic flexure after manipulations, position change and external abdominal pressure	Mid-epigastric pain with nausea and vomiting	Conservative	Alpha loop formation. The pancreatic inflammation was limited to the tail (close proximity to splenic flexure)
Khashram and Frizelle [24], 2011	Hemorrhage around tail of pancreas	Trauma of insufflation transmitted to the pancreas	Epigastric pain	Conservative	
Ahmed *et al* [32], 2019	Pancreatic duct leak	62-yr-old female, prior left nephrectomy	Worsening left sided abdominal pain, nausea and vomiting	ERCP with PD stenting	A collection involving pancreatic tail and splenic flexure (possible following adhesions of the prior surgery) was seen in the CT

ERCP: Endoscopic retrograde cholangiopancreatography; PD: Pancreaticoduodenectomy; CT: Computed tomography.

OTHER ABDOMINAL ORGAN INJURIES DURING COLONOSCOPY

Apart from acute kidney injury due to bowel preparation solutions[25], direct physical injuries to the kidney(s) or supra-renal gland(s) by colonoscopy was not reported.

Other uncommon abdominal organ injuries during colonoscopy that have been reported involve the mesentery with a tear[26], and urinary bladder with multiple perforations[27]. Small bowel injury has been reported also-these injuries are mostly perforations and encountered in ileum following electrocautery within colon[28] or pneumatic distension[29], or proximal jejunum due to rupture of jejunal diverticulum in elderly people[30]. These uncommon injuries and perforations are related to previous abdominal surgeries with presence of adhesions[27,29,31], weak bowel wall due to age[26], and excessive air insufflation during colonoscopy[28,31]. It may be a single[30] or multiple perforations[27,31].

MECHANISM OF ABDOMINAL ORGAN INJURY DURING COLONOSCOPY

The mechanism of intra-abdominal organ injures during colonoscopy is not fully understood. However, many risk factors

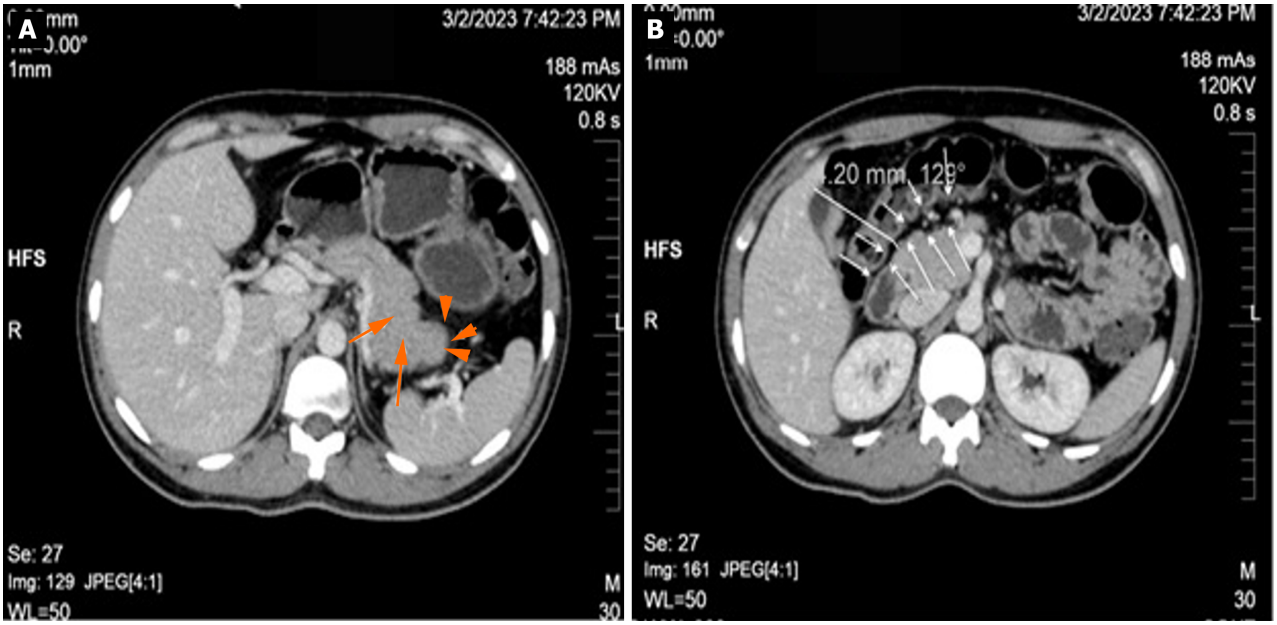

Figure 1 Computed tomography scan of the abdomen with contrast of a 34-year-old male patient who underwent a colonoscopy for 2-months history of bleeding per rectum and significant involuntary weight loss and was found to have severe pan-ulcerative colitis and eventually developed acute pancreatitis hours after colonoscopy. The patient was admitted for 3-d and treated conservatively and experienced uneventful course. A: The pancreatic body and tail are homogenously enlarged (long arrows) with post-contrast enhancement, and faint peri-pancreatic fat stranding (short arrow); B: Shows the close proximity of the pancreas and the colon with distance of 4.2 mm (arrows). The close distance (4.2 mm) between the pancreas and colon wall may potentiate our assumption that direct mechanical trauma exerted to the colonic wall and transmitted to the pancreatic tissue is the precipitating cause of acute pancreatitis in the current case.

have been identified and it can be categorized into organ related, procedure related, and local abdominal factors. Organomegaly either due to infiltrative, hematologic, or intrinsic diseases predispose the organ to trauma during colonoscopy; this was evident in many injuries involving spleen[14]. Previous episodes of inflammation also increase the likelihood of organ affection and was described in many cases of post colonoscopy pancreatitis[6]. Furthermore, the procedure of colonoscopy itself plays a pivotal role in occurrence of such injuries, where looping or hooking to straighten the left colon [23], marked inflation with air[24,29,32,33], use of electrical current[28], supine position, external abdominal pressure have been proposed as possible precipitants of colonoscopy related injuries. Local abdominal factors have been verified as potential precipitants for colonoscopy related injuries. The close proximity of splenic flexure to spleen and the tail of pancreas (Figure 1) explain why injuries are more frequently reported in this anatomical site (pancreatitis and splenic injuries). Intra-abdominal adhesions due to prior abdominal surgeries have been focused as the most important predisposing factor for colonoscopy related injuries[16,17] reported to the spleen, pancreas, liver, mesentery, and urinary bladder due to the transmitted traction exerted through the adhesion bands from the colon to these organs, *e.g.*, spleen, liver, and urinary bladder or to the ligaments fixing these organs in their anatomical positions, *e.g.*, rupture of spleno-colic ligament due to manipulations in the splenic flexure predispose to extensive splenic injuries[8]. The underlying bowel diseases especially IBD[16], bowel polyposis, diverticulae[30] were described as possible predisposing factors for these injuries[14], especially in elderly[30].

PREVENTION OF ABDOMINAL ORGAN INJURY DURING COLONOSCOPY

There are no published guidelines to guard against the abdominal organ injuries during colonoscopy. However, certain precautions and preventive strategies can be extrapolated from the colonoscopy practice guidelines and from the case reports focusing on these injuries. The prevention strategies should focus not only on preventing organ injuries but also should prevent the delay in diagnosis of such potentially fatal injuries.

Preventive measures include good colonoscopy technique[8], to avoid loop formation and to avoid the use of excessive force[34]; and it is probably that emerging endoscopic technologies and artificial intelligence will lead to a reduced risk of these organ injuries.

The left lateral position of the patient may reduce the risk of splenic injury after colonoscopy. It is postulated that if the patient assumed a supine position, the forces exerted on the spleen due to gravity and traction during colonoscopy oppose each other. This factor will increase the chance of splenic capsular avulsion, especially if there are other predisposing factors, such as previous abdominal surgery. The adhesions in supine position will be tightened while in left lateral position the spleen will fall beside the colon and any fibrotic band will be loose. Consequently, it is recommended that, patients belonging to the high-risk group should be placed in the left lateral position, and the supine position needs to be avoided during colonoscopy[34]. Manual abdominal counter-pressure applied correctly and safely during

colonoscopy can reduce the risk of tears during the procedure[26].

Abdominal organ injuries may remain asymptomatic post-colonoscopy for a variable period of time that extends from hours to days and hence patients should be informed about the possible complications and the leading manifestations and they should be instructed to access the ER for suggestive complaints especially abdominal pain, distension, and hemodynamic instability[35]. High risk patients should be monitored before discharge and clinicians should raise the high level of suspicion when evaluating those patients[34].

CONCLUSION

In conclusion, colonoscopy related abdominal organ injuries are uncommon, however serious, that should not be overlooked by clinicians and endoscopists. Severe abdominal pain, distension, hypotension and hemodynamic instability in absence or rectal bleeding should raise the possibility of severe organ injury. Splenic and hepatic injury following colonoscopy is serious and may be life threatening and usually needs interventional radiology or surgical intervention. AP following colonoscopy is usually mild and usually managed conservatively. Other abdominal organs are less frequently injured during colonoscopy. Left lateral position, avoidance of looping and excessive force during the procedure would probably reduce the risk of such injuries.

FOOTNOTES

Author contributions: Emara MH, Mazid U, Malik DF, and Mahros AM developed the concept; Emara MH, Elshaer YA, and Elkerdawy MA searched the literature; Emara MH, Mahros AM, and Malik DF analyzed the retrieved literature; Emara MH, Mazid U, Elshaer YA, and Elkerdawy MA prepared the tables and figure; Emara MH and Mazid U managed the case; Emara MH, Malik DF, and Mahros AM drafted the manuscript; all authors agreed and approved the final manuscript.

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From prediction to prevention: Machine learning revolutionizes hepatocellular carcinoma recurrence monitoring

Mariana Michelle Ramírez-Mejía, Nahum Méndez-Sánchez

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Abstract

In this editorial, we comment on the article by Zhang *et al* entitled Development of a machine learning-based model for predicting the risk of early postoperative recurrence of hepatocellular carcinoma. Hepatocellular carcinoma (HCC), which is characterized by high incidence and mortality rates, remains a major global health challenge primarily due to the critical issue of postoperative recurrence. Early recurrence, defined as recurrence that occurs within 2 years posttreatment, is linked to the hidden spread of the primary tumor and significantly impacts patient survival. Traditional predictive factors, including both patient- and treatment-related factors, have limited predictive ability with respect to HCC recurrence. The integration of machine learning algorithms is fueled by the exponential growth of computational power and has revolutionized HCC research. The study by Zhang *et al* demonstrated the use of a groundbreaking preoperative prediction model for early postoperative HCC recurrence. Challenges persist, including sample size constraints, issues with handling data, and the need for further validation and interpretability. This study emphasizes the need for collaborative efforts, multicenter studies and comparative analyses to validate and refine the model. Overcoming these challenges and exploring innovative approaches, such as multi-omics integration, will enhance personalized oncology care. This study marks a significant stride toward precise, efficient, and personalized oncology practices, thus offering hope for improved patient outcomes in the field of HCC treatment.

Key Words: Hepatocellular carcinoma; Early recurrence; Machine learning; XGBoost model; Predictive precision medicine; Clinical utility; Personalized interventions

Core Tip: Machine learning is an important approach for personalized oncology care, as it paves the way for precise and individualized postoperative strategies, thereby enhancing patient outcomes in the field of hepatocellular carcinoma treatment. Ongoing collaboration, larger sample sizes, and multicenter studies are crucial for validating and refining this innovative predictive model, thus ensuring its applicability and reliability in diverse clinical settings.

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INTRODUCTION

In this editorial, we comment on the article by Zhang *et al*[1] entitled Development of a machine learning-based model for predicting the risk of early postoperative recurrence of hepatocellular carcinoma. Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and is considered a major global health challenge due to its high incidence and mortality rates[2,3]. Despite advances in medical and surgical interventions, recurrence remains a critical problem affecting the long-term survival of patients with HCC[4,5]. Recurrence of HCC within the 2 years posttreatment is categorized as early recurrence. Early recurrence typically occurs due to the hidden spread of the primary tumor within the liver, and its incidence is correlated with the tumor's size and extent. On the other hand, recurrence after 2 years posttreatment is categorized as late recurrence. Late recurrence is associated with *de novo* HCC, indicating the development of new cancerous growth independent of the original tumor[6]. Several predictive factors associated with recurrence have been recognized. Factors contributing to early recurrence include patient-related aspects such as age, the presence of underlying health conditions, liver function, viral load, the presence and activity of hepatitis, metabolic dysfunction-associated fatty liver disease, alcoholic liver disease and other etiologies, and the existence and activity of liver cirrhosis. Additionally, treatment-related factors, including the type of treatment employed, surgical margins, and specifics of the resection, also play a crucial role in predicting early recurrence[7]. The intricate nature of liver cancer, coupled with the diverse factors influencing recurrence, makes it challenging to provide an accurate prognosis[8,9]. Due to the constantly evolving landscape of HCC research, the quest for methods for predicting early recurrence has undergone a remarkable transformation in recent decades. Initially, researchers focused on deciphering the morphological characteristics of tumors as a basis for predictions[10]. Factors such as vascular invasion, tumor multiplicity and large tumor size have emerged as fundamental, although somewhat rudimentary, indicators that establish the basis for understanding the complexities of HCC recurrence[11,12]. Nevertheless, the paradigm shifted with the arrival of molecular analysis. Elevated alpha-fetoprotein (AFP) levels emerged as one of the first markers used for HCC prediction, offering insight into the intricate molecular landscape of this aggressive cancer[13,14]. Despite these advances, the multifaceted nature of HCC recurrence requires more nuanced and sophisticated approaches. Researchers and clinicians alike recognize the limitations of relying solely on morphologic and molecular analyses[15]. The quest for increasing predictive accuracy has led the scientific community to explore uncharted territory and harness the transformative power of technology, especially in the fields of imaging, genetics and computational sciences[16].

The evolution of technology has emerged as a pivotal catalyst, propelling HCC research into an era of unprecedented possibilities. Cutting-edge imaging techniques, coupled with advancements in genetic profiling, provide researchers with a comprehensive understanding of the tumor microenvironment[17-20]. These insights, combined with the computational progress of modern data analysis, paved the way for a new generation of predictive models. These models transcended the limitations of traditional analyses, offering a more nuanced and accurate glimpse into the future course of HCC[21-24].

MACHINE LEARNING IN HCC RESEARCH

The exponential growth of computational power has heralded a new era in HCC research, where machine learning algorithms have emerged as valuable tools in handling vast datasets and deciphering complex patterns[25,26]. This convergence of computational capabilities and healthcare needs represents a significant paradigm shift, transforming the landscape of HCC research. The integration of machine learning into the study of HCC offers a multitude of benefits and promises to address long-standing challenges in this field[27,28].

In a retrospective study, Zhang *et al*[1], harnessed the potential of supervised machine learning to develop a state-of-the-art preoperative prediction model for early postoperative HCC recurrence. Leveraging readily available clinical and imaging data, the team built six different risk prediction models, using ensemble learning, linear and neural network models, each meticulously designed to identify patients at high risk of recurrence. The study methodology consisted of analyzing the demographic and clinical data of 371 patients with HCC, excluding cases with incomplete data or previous

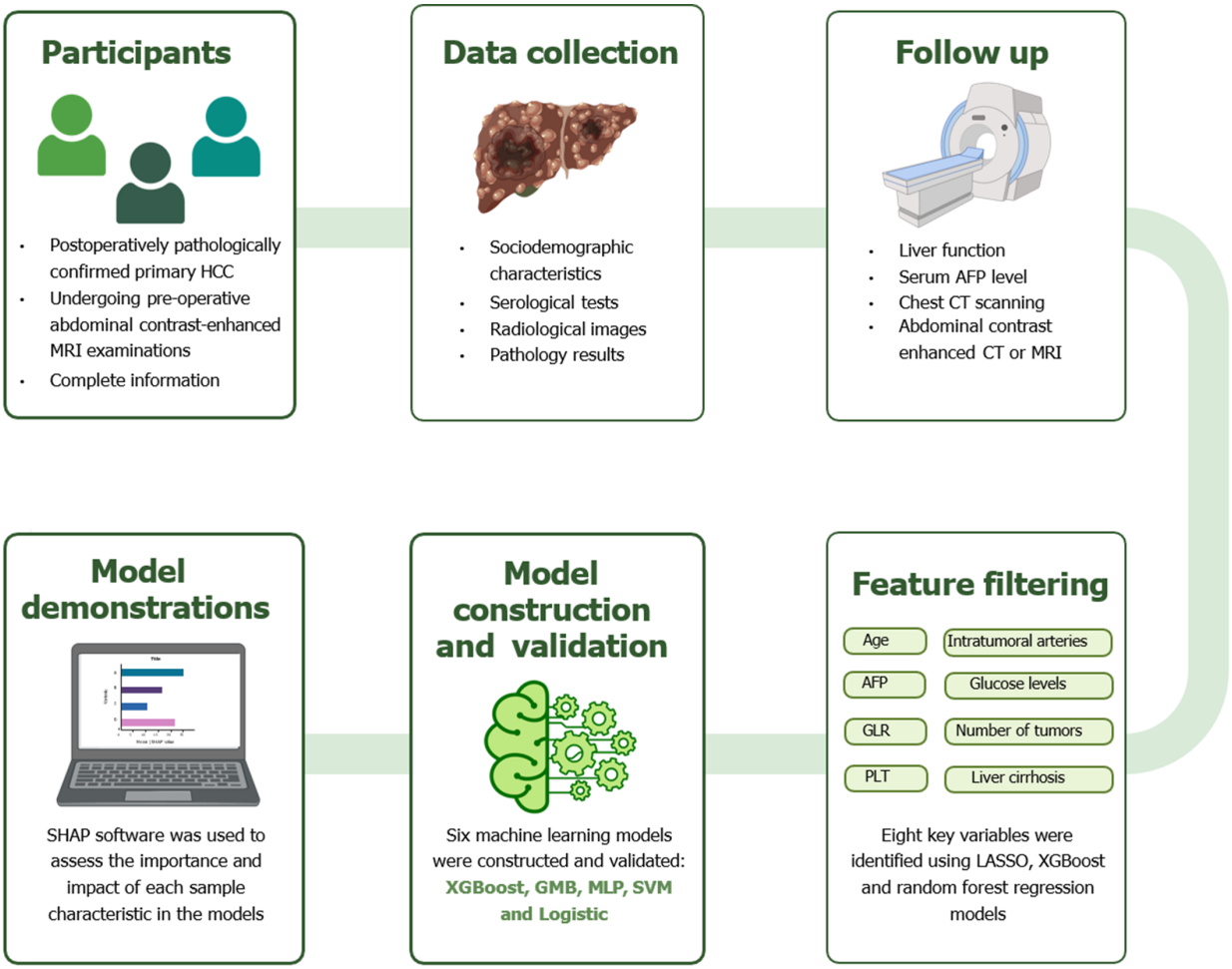

Figure 1 Summary of the study process. AFP: Alpha-fetoprotein; CT: Contrasted tomography; GLR: γ-glutamyl transferase-to-lymphocyte ratio; GMB: Complement NB; HCC: Hepatocellular carcinoma; MLP: Multilayer perceptron; MRI: Magnetic resonance imaging; PLT: Blood platelet; SVM: Support vector machine.

neoadjuvant treatments. Using machine learning algorithms, the researchers identified eight key variables to predict early HCC recurrence: Age, intra-tumoral arteries, AFP, blood glucose, number of tumors, glucose-to-lymphocyte ratio (GLR), liver cirrhosis, and platelet count. These variables formed the basis for the construction of six different prediction models, of which the XGBoost model proved to be the most robust (Figure 1). The XGBoost model, outperformed its peers, showing unmatched performance on several metrics. In the training dataset, the model achieved an impressive area under the receiver operating characteristic curve (AUROC) of 0.993, proof of its accuracy. Even in the validation and test data sets, the XGBoost model maintained its excellence, with AUROC values of 0.734 and 0.706, respectively. Calibration curve analysis underscored the reliability of the model, confirming its alignment with real-world results. Furthermore, decision curve analysis highlighted the clinical utility of the XGBoost model, highlighting its potential to guide surgical strategies and usher in an era of individualized postoperative medicine. By employing the SHAP (SHapley Additive exPlanations) package, the study provided a detailed interpretation of the model results, unraveling the intricate relationships between variables. Preoperative glycemia emerged as a key factor, in line with previous research highlighting its role in HCC progression. The predictive power of the model was further demonstrated using an online calculator, designed to assist physicians in their daily practice. This user-friendly tool represents a major breakthrough, as it ensures the seamless integration of predictive analytics into clinical decision making.

The relevance of this study goes far beyond conventional medical research. Machine learning algorithms, used to decipher the intricate web of preoperative variables, have ushered in an era where predictive precision medicine reigns supreme. The identification of these eight key variables is a pivotal moment, providing physicians with unprecedented insight into the intricate factors that determine early postoperative recurrence.

Several research projects have been conducted to explore the use of machine learning in predicting HCC recurrence. Kucukkaya *et al*[29] developed a predictive model based on the analysis of pre-treatment magnetic resonance imaging using the VGG16 and XGBoost machine learning models. This model aimed to predict recurrence in six different time intervals, ranging from 1 year to 6 years, and demonstrated performance with AUROC values between 0.71 and 0.85. In another study, Zeng *et al*[25] compared the performance of random survival forest (RSF) models with Cox proportional hazard (CPH) models in predicting early recurrence using clinical features of the participants. In training and internal and external validation cohorts, the C-index of the RSF model was 0.725, 0.762, and 0.747, respectively. Although both studies highlighted the utility of machine learning, neither included the analysis of clinical and imaging variables, assuming a linear interaction of predictors for HCC recurrence. In this context, Zhang *et al*[1] proposed a solution in their

study, addressing the need to include clinical and imaging variables in the analysis. Their approach seeks to overcome the limitation of assuming linear interactions among predictors of HCC recurrence.

CHALLENGES AND FUTURE DIRECTIONS

This represents a significant advancement in early postoperative HCC recurrence prediction. Future research should focus on overcoming challenges related to sample size, data handling, validation, and interpretability. The authors acknowledge these limitations, emphasizing the need for future research endeavors to validate and refine the model further. These findings call for additional research, urging the scientific community to collaborate, expand sample sizes, and conduct multicenter studies. Comparative analyses with existing prediction models are crucial for ensuring the reliability and applicability of this innovative approach[30,31].

CONCLUSION

The development of accurate, interpretable, and widely applicable prediction models for early postoperative HCC recurrence represents a significant advance in personalized medicine. Addressing the challenges associated with data quality and model interpretability while exploring innovative approaches, such as multi-omics integration and continuous model refinement, will pave the way for improved patient outcomes and healthcare practices in the field of HCC treatment. Through collaborative efforts, continued research and the use of patient-centered approaches, the field of oncology can continue its journey toward more precise, efficient and personalized oncology care.

FOOTNOTES

Author contributions: Méndez-Sánchez N and Ramírez-Mejía MM contributed to this paper; Méndez-Sánchez N designed the overall concept and outline of the manuscript; Ramírez-Mejía MM contributed to the discussion and design of the manuscript; Méndez-Sánchez N and Ramírez-Mejía MM contributed to the writing and editing of the manuscript, the illustrations, and the review of the literature.

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EDITORIAL

Muscle strength and non-alcoholic fatty liver disease/metabolic-associated fatty liver disease

Xuan-Yu Hao, Kai Zhang, Xing-Yong Huang, Fei Yang, Si-Yu Sun

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Abstract

This editorial comments on an article published in a recent issue of *World Journal of Gastroenterology,* entitled "Association of low muscle strength with metabolic dysfunction-associated fatty liver disease: A nationwide study". We focused on the association between muscle strength and the incidence of non-alcoholic fatty liver disease (NAFLD) and metabolic-associated fatty liver disease (MAFLD), as well as the mechanisms underlying the correlation and related clinical applications. NAFLD, which is now redefined as MAFLD, is one of the most common chronic liver diseases globally with an increasing prevalence and is characterized by malnutrition, which may contribute to decreased muscle strength. Reduction of muscle strength reportedly has a pathogenesis similar to that of NAFLD/MAFLD, including insulin resistance, inflammation, sedentary behavior, as well as insufficient vitamin D. Multiple studies have focused on the relationship between sarcopenia or muscle strength and NAFLD. However, studies investigating the relationship between muscle strength and MAFLD are limited. Owing to the shortage of specific medications for NAFLD/MAFLD treatment, early detection is essential. Furthermore, the relationship between muscle strength and NAFLD/MAFLD suggests that improvements in muscle strength may have an impact on disease prevention and may provide novel insights into treatments including dietary therapy, as well as tailored physical activity.

Key Words: Muscle strength; Non-alcoholic fatty liver disease; Metabolic-associated fatty liver disease; Sarcopenia; Insulin resistance; Inflammation

Core Tip: The relationship between muscle strength and the incidence of non-alcoholic fatty liver disease (NAFLD) and metabolic-associated fatty liver disease (MAFLD), as well as the mechanisms underlying the correlation and related clinical applications were discussed. Muscle strength may play an imperative role in the incidence and development of NAFLD/MAFLD and interventions to improve muscle strength in the management of NAFLD/MAFLD may provide novel insights into the treatment of these diseases.

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INTRODUCTION

As an imperative component of human health and a crucial predictor of physical function, muscle strength has drawn great interest in the areas of disease prediction and treatment[1,2]. Non-alcoholic fatty liver disease (NAFLD), recently renamed metabolic-associated fatty liver disease (MAFLD), with liver biopsy as the gold standard for diagnosis, is a common chronic liver disease, and its prevalence is still increasing, representing a global healthcare burden[3,4]. NAFLD/ MAFLD is characterized by malnutrition, which may contribute to decreased muscle mass, strength, and sarcopenia[5,6]. NAFLD encompasses a broad disease spectrum ranging from non-alcoholic fatty liver to cirrhosis, which is a representative cause of sarcopenia due to protein and energy metabolic disorders[7,8].

Sarcopenia is now defined as low muscle strength combined with low muscle quantity or quality with or without poor physical performance after revision by the European Working Group on Sarcopenia in Older People 2[9]. However, the measurements for diagnosis of sarcopenia differs according to clinical practice and research. In addition, some studies have utilized the previous definition of sarcopenia, which focused only on decreased muscle mass and not on low muscle strength. Multiple studies have focused on the association between sarcopenia or muscle strength and NAFLD; however, studies on the relationship between muscle strength and MAFLD are rare. As the primary parameter for sarcopenia in the updated definition, decreased muscle strength has also been suggested as a better predictor of mortality and poor disease outcomes than has muscle mass in previous studies[9-11]. Thus, further exploration of the association between low muscle strength and NAFLD/MAFLD is necessary. Muscle strength is usually measured using grip strength, which is noninvasive, low-cost, uncomplicated, and has been validated reliably in research and clinical practice[12]. Lower limb strength can also be measured when grip strength is not available[13].

MUSCLE STRENGTH AND NAFLD/MAFLD

In a recent issue of *World Journal of Gastroenterology*, Lee *et al*[14] published an absorbing article titled "Association of low muscle strength with metabolic dysfunction-associated fatty liver disease: A nationwide study". This study verified a significant relationship between muscle strength and MAFLD in the general Korean population. The present cross-sectional study examined 31649 participants aged ≥ 19 years who took part in the Korea National Health and Nutrition Examination Survey between 2015 and 2018. Of the enrolled participants, 29.3% had MAFLD diagnosed using the hepatic steatosis index and the presence of metabolic risk factors. The muscle strength of the participants was defined by relative handgrip strength and divided into quartiles. Multivariate logistic regression analysis revealed that the prevalence of MAFLD was higher in the lower muscle strength quartiles. The lowest quartile exhibited a 3.12-fold increased risk of MAFLD compared to that in the highest quartile. A significantly elevated odds ratio for MAFLD was also observed in the lower muscle strength quartiles in a dose-dependent manner. These associations persisted across all subgroups, including age, obesity, and diabetes mellitus. Patients with MAFLD in the highest quartile also had increased risks of severe liver fibrosis compared to those in the other quartiles. The study indicated that decreased muscle strength was related to a dose-dependent higher risk of MAFLD as well as to a high probability of liver fibrosis in participants with MAFLD.

Studies that have explored the association between muscle strength and NAFLD are presented in Table 1. The relationship between NAFLD and low muscle strength was demonstrated in a cross-sectional study by Gan *et al*[15]. The occurrence of NAFLD was higher in the sarcopenic state, and this risk was further elevated if obesity was present. The results also showed that low muscle strength, measured using weight-adjusted handgrip strength, was positively, independently, and significantly associated with NAFLD. An association between low muscle strength and increased incidence of NAFLD has been detected in the Korean population[16-19]. Similar findings have been validated in the Chinese population and HIV-infected men in Italy, as well as in the male patients diagnosed with type 2 diabetes mellitus [20-23]. Further, low muscle strength was related to a higher incidence of severe NAFLD in a prospective study using data derived from the UK Biobank[24]. These results indicated that lower muscle strength was significantly associated with an increased incidence of severe NAFLD. In another study by Kang *et al*[25], decreased muscle strength was

Table 1 Studies fucus on the relationship between muscle strength and non-alcoholic fatty liver disease

Ref.	Study design	Subjects	Sample size and gender [female; *n* (%)]	Mean age (yr)	Strength position/measures	Outcome
Gan *et al*[15], 2020	Cross-sectional study	General adults in China	3536 (71.3)	51.72 for participants without NAFLD/55.2 for participants with NAFLD	Handgrip strength (kg)/electronic hand dynamometer	Incidence of NAFLD
Lee *et al*[16], 2018	Cross-sectional study	General adults in Korea	8001 (55.5)	49.9	Handgrip strength (kg)/digital grip strength dynamometer	Incidence of NAFLD
Kim *et al*[17], 2019	Cross-sectional study	Men aged ≥ 50 yr and postmenopausal women in Korea	4103 (53.8)	61.9 for men without NAFLD/59.5 for men with NAFLD/61.8 for women without NAFLD/62 for women with NAFLD	Handgrip strength (kg)/digital grip strength dynamometer	Incidence of NAFLD
Cho *et al*[18], 2021	Cross-sectional study	Middle-aged adults in Korea	5272 (68.2)	57.1	Handgrip strength (kg)/digital grip strength dynamometer	Incidence of NAFLD
Lee *et al*[19], 2022	Cross-sectional study	General adults in Korea	19852 (62.5)	45.8 for men/48.3 for women	Handgrip strength (kg)/digital grip strength dynamometer	Incidence of NAFLD
Debroy *et al*[20], 2019	Cross-sectional study	Adults living with HIV and receiving treatment in Italy	169 (0)	56.8	Handgrip strength (kg)/handheld dynamometer	Incidence of NAFLD
Meng *et al*[21], 2016	Cross-sectional study	General adults in China	20957(49)	41.2	Handgrip strength (kg)/electronic hand-grip dynamometer	Incidence of NAFLD
Bulur *et al*[22], 2023	Cross-sectional study	Middle-aged male patients diagnosed with type 2 diabetes mellitus	145 (0)	55.2	hand muscle strength (kg)/hydraulic dynamometer	Incidence of NAFLD
Wang *et al*[23], 2021	Cross-sectional study	Senior hospital staff in China	578 (84.1)	72.9 for men without NAFLD/68.9 for men with NAFLD/62.9 for women without NAFLD/67.5 for women with NAFLD	Handgrip strength (kg)/hydraulic hand dynamometer	Incidence of NAFLD
Petermann-Rocha *et al*[24], 2022	prospective study	General adults in Scotland, England and Wales	333295 (55)	56.6	Handgrip strength (kg)/hydraulic hand dynamometer	Incidence of severe NAFLD
Kang *et al*[25], 2020	Cross-sectional study	General adults in Korea	13502 (57.6)	45.6	Handgrip strength (kg)/digital grip strength dynamometer	Incidence and severity of NAFLD
Park *et al*[26], 2020	Cross-sectional study	General adults in the USA	3922 (58.1)	45 for men/46.9 for women	Handgrip strength (kg)/digital grip strength dynamometer	Incidence and severity of NAFLD
Zhao *et al*[27], 2023	Cross-sectional study	General adults in the USA	8888 (50.43)	46.07	Handgrip strength (kg)/digital grip strength dynamometer	Incidence and severity of NAFLD
Kim *et al*[28], 2023	Cross-sectional study	General adults with NAFLD in the USA	4655 (46.9)	48	Handgrip strength (kg)/digital grip strength dynamometer	All-cause and cause-specific mortality in NAFLD
Charatcharoenwitthaya *et al*[29], 2022	Observational study	General adults with NAFLD in the Thailand	7083 (69.4)	49.3	Handgrip strength (kg)/digital dynamometer	All-cause mortality in NAFLD

NAFLD: Non-alcoholic fatty liver disease.

independently related to the incidence and progression of NAFLD in a Korean population. Additionally, grip strength was inversely associated with NAFLD severity in a United States population[26,27]. Furthermore, higher muscle strength was independently related to lower all-cause and cardiovascular mortality after full adjustment in patients with NAFLD in a United States population[28]. Muscle strength was independently associated with long-term all-cause mortality in participants with NAFLD in a study conducted in Thailand[29]. However, few studies have compared the predictive value of muscle strength in NAFLD and MAFLD.

POTENTIAL MECHANISM FOR THE RELATIONSHIP BETWEEN MUSCLE STRENGTH AND NAFLD/MAFLD

The potential mechanisms that may explain the relationship between muscle strength, NAFLD/MAFLD, and cirrhosis have yet to be explored. Insulin resistance may play a crucial role in the inverse relationship between muscle strength and NAFLD/MAFLD. Skeletal muscle is the primary target organ of insulin-stimulated glucose disposal; therefore, a reduction in muscle strength may exacerbate insulin resistance, which is a crucial pathogenic component of NAFLD/MAFLD, through hepatic fat accumulation, inflammation, and energy metabolism alteration[30-32]. Insulin resistance in turn aggravates proteolysis and itself leads to muscle depletion[33]. Several inflammatory mechanisms related to NAFLD, such as cirrhosis, have been identified[34,35]. As a characteristic of NAFLD, dysfunction of adipocyte lipolysis is suggested to lead to ectopic fat accumulation in hepatic parenchyma as well as skeletal muscle[36,37]. Higher levels of inflammatory markers, including C-reactive protein, interleukin (IL)-6, and tumor necrosis factor-α, observed in populations with lower muscle strength indicated that decreased muscle strength may be associated with NAFLD/MAFLD and cirrhosis based on inflammation[38]. Elevated levels of proinflammatory cytokines may result in muscle degradation by reducing the muscle response to insulin and insulin-like growth factor-1[39]. Alterations in myokines (such as irisin, IL-6, myostatin, and adiponectin) secreted by skeletal muscle may be involved in NAFLD/MAFLD development through their influence on hepatic fat accumulation[40]. A previous study found that decreased muscle strength was related to lower vitamin D levels and that vitamin D supplementation contributed to muscle strength preservation[41,42]. Epidemiological data also support that vitamin D is associated with the incidence of NAFLD and that the vitamin D/vitamin D receptor axis is involved in the modulation of inflammatory and metabolic pathways related to MAFLD[43,44]. Decreased muscle strength is related to physical disability and functional decline as well as sedentary behavior, which is related to the development of NAFLD/MAFLD[45-47].

CLINICAL IMPLICATION

Recovery from decreased muscle strength may decrease complications and improve survival in individuals with fatty liver disease[10,48]. Because sarcopenia is related to nutritional status, dietary therapy based on optimal nutritional intake has been suggested for its treatment in fatty liver disease and cirrhosis[49]. Supplementation with branched-chain amino acids and adequate energy in conjunction with a protein-enriched dietary intake has been recommended for individuals with liver cirrhosis[50]. As a non-pharmacological and innovative strategy, proper physical activity can be effective in recovering lost muscle strength. A previous study illustrated that progressive resistance training could increase muscle strength and improve general performance, including functional exercise capacity, mental health, and body composition, in patients with cirrhosis[51]. Furthermore, implementation of tailored physical activity may improve outcomes in patients awaiting liver transplantation[52]. A recent meta-analysis indicated that combining protein supplementation with resistance training is advisable for optimizing muscle strength[53]. The findings of a cross-sectional study suggested that adherence to an anti-inflammatory nutrient pattern, characterized by a high intake of polyunsaturated fat, monounsaturated fat, copper, vitamin E, and omega-3 fatty acids was associated with reduced odds of low muscle strength, which indicated that anti-inflammatory pattern might be a therapeutic approach for decreased muscle strength[54]. Despite the beneficial impact of physical activity on muscle strength, specific exercise training guidelines for individuals with fatty liver disease and cirrhosis are lacking in terms of the amount, intensity, and forms of exercise in clinical practice[55,56]. Electrical stimulation has also emerged as a popular modality for enhancing muscle strength among athletes and fitness enthusiasts; however, the lack of standardized protocols pertaining to its specific implementation remains a challenge[57].

The correlation between muscle strength and NAFLD/MAFLD might provide a fresh perspective for treatment owing to the lack of specific medications[58]. Although there are no specific pharmacotherapeutic interventions for reduced muscle strength to date, multiple efforts have already been made, including a variety of clinical trials that focused on pharmacological interventions for sarcopenia[59,60]. Several drugs have been investigated for their ability to augment muscle strength based on different molecular targets. Bimagrumab, a fully human monoclonal antibody targeting the MSTN-ActRII pathway, was found to be useful for increasing muscle strength in patients with sarcopenia in phase 2 clinical trials[61]. However, in a clinical trial performed by Rooks *et al*[62], bimagrumab demonstrated no positive effect on muscle strength. Medications targeting the renin–angiotensin system, such as inhibitors of angiotensin-converting enzyme, have also been found to influence the decline in muscle strength[63]. The efficacy of dipeptidyl peptidase-4 inhibitors for enhancing muscle strength in geriatric patients with type 2 diabetes mellitus was investigated in a recent retrospective cohort study[64]. Furthermore, several studies have demonstrated the potential benefits of testosterone for improving muscle strength. The most recent Clinical Practice Guideline by the Endocrine Society suggested that

testosterone treatment can enhance muscle strength in men with hypogonadism[65]. A meta-analysis also found that intramuscular testosterone replacement therapy is effective in improving muscle strength in middle-aged and older men [66]. In addition, owing to their beneficial effects on muscle strength, exercise mimetics, which can induce energy expenditure without changes in activity, have been recognized as a potential therapeutic strategy[67].

CONCLUSION

In summary, muscle strength has an imperative function in the incidence of NAFLD/MAFLD and may serve as a potential predictor for early diagnosis, as well as a better means of evaluating NAFLD/MAFLD. Interventions based on muscle strength may provide novel insights into the treatment of NAFLD/MAFLD.

FOOTNOTES

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MINIREVIEWS

Colon and rectal cancer: An emergent public health problem

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Abstract

Colorectal cancer ranks third globally, with a high mortality rate. In the United States, and different countries in Europe, organized population screenings exist and include people between 50 and 74 years of age. These screenings have allowed an early diagnosis and consequently an improvement in health indicators. Colon and rectal cancer (CRC) is a disease of particular interest due to the high global burden associated with it and the role attributed to prevention and early diagnosis in reducing morbidity and mortality. This study is a review of CRC pathology and includes the most recent scientific evidence regarding this pathology, as well as a diagnosis of the epidemiological situation of CRC. Finally, the recommendation from a public health perspective will be discussed in detail taking into account the context and the most current recommendations.

Key Words: Colon and rectal neoplasia; Colon and rectal tumor; Mortality; Morbidity

Core Tip: Colon and rectal cancers (CRC) are important public health problems. Epidemiological studies, morbidity and mortality indicators demonstrate the high burden of colorectal cancer on individuals and society. To face this issue, several measures are urgently need. Improved therapies and precision medicine for patients with CRC are required. Prevention is fundamental through dietary and lifestyle measures, along with early diagnosis.

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INTRODUCTION

Colon and rectal cancer (CRC) is one of the most incident cancer in the western world with 1.8 million cases in 2018, being more frequent after the age of 50. By 2022, it is estimated that around 2 million cases of CRC will have arisen worldwide, constituting the second most common cause of cancer mortality worldwide with 880000 fatalities[1]. Incidence rates have been increasing globally, with the highest rates being reached in high-income countries and rates lower in low-income countries. This difference in the incidence of CRC seems to result from environmental and mainly nutritional factors. The determinants associated with CRC are identified and included, age, male sex, inflammatory bowel disease, diet, and physical exercise, among others[1,2]. It is well-established that a healthy lifestyle decreased the likelihood of developing CRC[3]. This comprehensive review discussed CRC pathology, and its associated determinants, diagnosis methods, treatment, and prognosis.

CRC

CRC pathology results from the accumulation of multiple genetic and epigenetic alterations in the previously healthy colon and rectal epithelium, leading to the progression of colon and rectal adenomas to carcinoma (Figure 1)[1]. Approximately half of the patients with CRC will develop metastases in the course of the disease and the majority of metastatic CRC (mCRC) are incurable[3]. About 90% of CRC are adenocarcinomas, *i.e.*, they are malignant tumors that derive from the glandular epithelium of the colon and rectum. Despite adenocarcinoma, other types of CRC include colorectal lymphoma, squamous cell carcinoma, leiomyosarcomas, and melanomas[3]. According to the literature, about 65% of CRC cases develop sporadically, without any family history or predisposition to hereditary genetic mutations, occurring through somatic genomic and epigenetic alterations[4,5]. The remaining cases have a familial association. From these a few percentage (representing only 5% of cases) are hereditary cancer syndromes, and other unknown genomic alterations [2]. CRC is considered a heterogeneous disease from a molecular perspective. One of the main molecular pathways that are altered is chromosomal instability, occurring in 85% of cases of sporadic CRC, and characterized by changes in structure, number, loss of heterozygosity in the tumor suppressor gene, gain or loss of chromosome segments, and rearrangements, which results in variations in the number of copies of the gene[6]. These changes are often associated with mutations in specific oncogenes or tumor suppressor genes, such as tumor suppressor adenomatous polyposis coli (APC), kirsten rat sarcoma virus, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit-a, b-raf proto-oncogene (BRAF), SMAD family member 4, and p53 that regulate cell proliferation and deoxyribonucleic acid (DNA) cell cycle, playing an important role in initiation and progression pathways. Another important mechanism for the development of CRC is microsatellite instability caused by the dysfunction of DNA mismatch repair (MMR) genes during DNA recombination, DNA replication, and DNA damage. Other important pathways for the development of CRC include the CpG island methylator phenotype pathway[6]. However, in most cases, the process of CRC development begins with the transformation of previously healthy colon and rectal epithelial cells under the influence of spontaneous mutations, environmental factors, and genetic or epigenetic alterations[7,8]. These cells expand to form aberrant cells, crypt foci, and early adenomas, driven by mutations that cause hyperproliferation, such as APC mutations, or other signaling pathways with the release of cytokines and tumor microenvironment growth factors[5]. These factors lead to the growth of these adenomas and their transformation into malignant tumors in a progressively slow process that usually lasts for 10-20 years[5]. The presence of other mutations will amplify the process of CRC cells and facilitate the metastasis of these tumors to distant organs and tissues, a process called tumor progression[9].

Causes and risk factors

Epidemiological studies show that the male sex and increasing age show a strong association with CRC incidence (Figure 2)[2]. Both environmental and hereditary factors also play an important role in the development of CRC[10]. The risk of developing CRC is influenced by several acquired risk factors, including environmental exposures and medical conditions where many of which have an associated genetic load (Figure 2)[2]. Data for some risk factors (*e.g.*, male sex, smoking and processed meat consumption) are well-established in the literature, other factors (supplements, drugs) are not supported by experimental studies.

The risk factors described for CRC are based almost exclusively on data from observational studies and therefore some caution is required concerning their interpretation[11]. Acquired risk factors for the development of CRC include dietary factors, lifestyle factors, side effects of medical interventions, and pre-existing medical conditions[12]. Dietary factors that potentially increase the risk of CRC include low intake of fruits, vegetables, or fiber, high consumption of red meat or saturated fat, and high exposure to caffeine or alcohol. Of these factors, the association between reduced intake of fruits, vegetables, and fiber-rich foods has been questioned due to contradictory results from large observational studies and randomized clinical trials[13]. The association of high consumption of red meat or saturated fat with increased risk of CRC is strongly supported by research, but only by observational data[14-17]. The association between CRC and smoking and physical exercise has also been supported by observational data, but these studies are also of moderate quality. Medical interventions that appear to increase the risk of CRC include pelvic irradiation, cholecystectomy, and ureter-

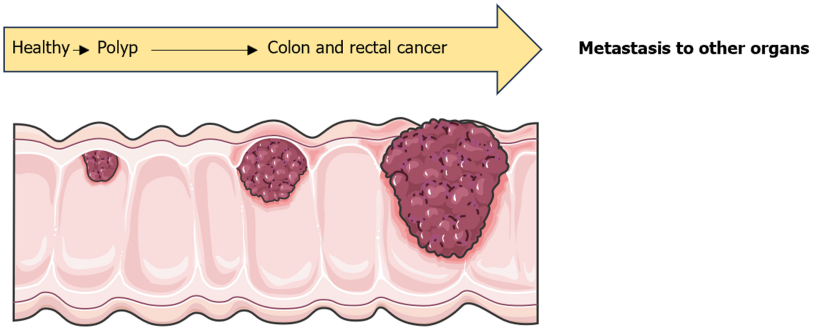

Figure 1 Progression of colon and rectal cancer pathology.

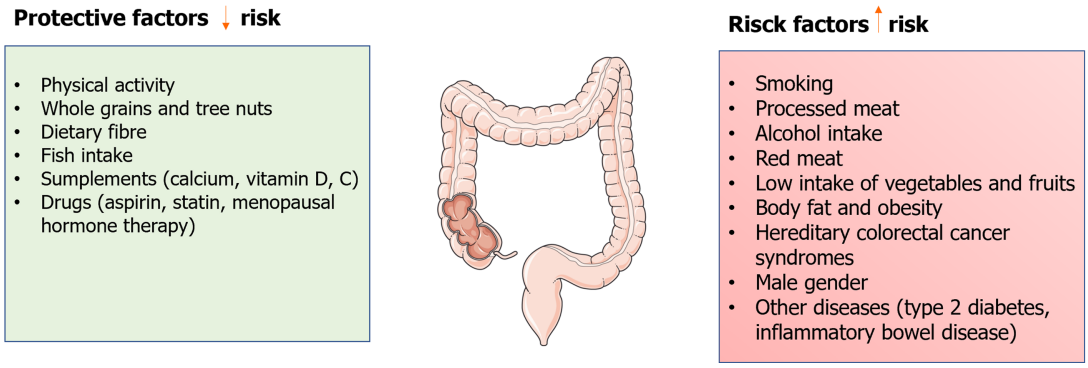

Figure 2 List of modifiable and non-modifiable determinants for colon and rectal cancer.

ocolic anastomosis after major surgery of the urinary and intestinal systems. These risk factors are supported only by observational data from small studies, so their validity is also not well established with the necessary rigor. Finally, medical conditions that are associated with an increased risk of CRC include Barrett's esophagus, human immunodeficiency virus infection, acromegaly, and inflammatory bowel disease. The association between CRC and inflammatory bowel disease, the most prevalent being Crohn's disease and ulcerative colitis, is well established and forms the basis for colonoscopy surveillance recommendations widely adopted by national and international medical organizations at earlier ages concerning population-based screening ages. The association of CRC with other associated medical conditions is only supported by very limited and controversial observational data. Epidemiological studies and scientific research demonstrate a strong influence of diet and physical exercise not only on the risk of developing CRC but also on its impact on CRC. In this regard, the American Cancer Society has reinforced the role of diet and physical activity as important determinants in CRC prevention[17]. Regarding vitamin supplements that include calcium and vitamin D in reducing CRC[15], results have been controversial and their use did not demonstrate a significant reduction in the risk of developing CRC in healthy subjects[16]. In physically active patients, the risk of developing CRC is reduced by about 15% compared to people who do not practice any type of physical exercise. Another important aspect is that physical exercise has a highly beneficial impact (*i.e.*, tertiary prevention) even in patients already diagnosed with CRC[17]. A recent study has shown that patients with CRC who regularly engage in moderate-intensity physical exercise (60-75 min/d of moderate-intensity exercise) have a better prognosis and a lower mortality rate than people who do not exercise. The physically active group demonstrated a lower rate of disease recurrence, being the group with the highest survival compared to the inactive group. Thus, the American Cancer Society recommends physical activity to all cancer patients, however, it has not defined the intensity and duration of physical exercise to be practiced to enjoy its beneficial effects on CRC[10]. Still in this context of tertiary prevention, a clinical trial conducted in 2018 showed that having a healthy life, with a body mass index considered normal, being physically active, and eating a diversified diet rich in vegetables and fruits in physically active individuals diagnosed with CRC in stage III is associated with greater survival compared with controls (non-physically individuals) that are not physically active and with a diet with deficiencies in vegetables and fruit[17].

Prevention

The above-mentioned determinants are important to take into account at the level of primary, secondary, and tertiary prevention of CRC. Since CRC survival outcomes are closely related to the stage of cancer at diagnosis, CRC is one of the only types of cancer in which screening is considered a key preventive measure (secondary and in some cases even primary). In addition to adopting a healthy lifestyle and exercising, the most effective method of preventing CRC and reducing CRC-associated mortality in the population is screening individuals with an associated average risk. Most European countries, including Portugal, Canada, specific regions of North and South America, countries on the Asian

continent, and Oceania have started population screening programs as a strategy. Population-based screening aims to show the disease during its development period among the average-risk population, allowing interventions at an early stage with a consequent decrease in mortality. Thus, screening is particularly appropriate in CRC, as this cancer consists of a gradual development of the adenoma-carcinoma sequence[8]. Although the time taken for an early adenoma to progress to an established CRC is still unknown, current evidence suggests that it is no less than ten years, offering a window of opportunity for early diagnosis and treatment. In addition, CRC can be prevented by removing adenomas, and the earlier CRC is detected, the less likely the patient risks dying. Treatment outcomes are therefore positively impacted by interventions along the adenoma-carcinoma pathway. Other effective strategies include identifying and monitoring high-risk populations, including individuals with inflammatory bowel disease, families with inherited CRC syndrome, individuals whose family history suggests a genetic predisposition to CRC but have no detectable genetic markers, and individuals whose phenotypic appearance indicates high risk. As tertiary prevention in CRC, it is worth mentioning the practice of physical exercise that, as previously mentioned, improves the prognosis in patients with CRC.

Diagnosis

The United States Preventive Services Task Force strongly recommends the use of endoscopy in the diagnosis of CRC, in addition to fecal tests and computed tomography (CT)[18]. The recommendation of endoscopy as means of complete diagnosis and preferred therapy is based on the fact that polyps in the pre-malignant stage can thus not only be detected but also removed, which reduces the incidence of CRC[19]. New sequencing techniques have allowed a detailed characterization of tumors through the use of predictive biomarkers. However, its application in clinical practice is very difficult, and specific recommendations with guidelines are important to support therapeutic decisions[20]. The most used means in the diagnosis of CRC are fecal tests and colonoscopy, and other means are still under investigation. For staging, it is essential to perform a CT scan of the chest, abdomen, and pelvis before surgical resection of the CRC to diagnose possible metastases[21]. Performing CT allows detection and assessment of the extent of metastases, which may require a change in treatment strategy, for example, the need to perform CT instead of surgery first or simultaneous resection of the primary tumor and metastases[21].

Stages

The pathological stage represents the most important prognostic factor in patients with CRC[22]. The tumor-nodes-metastasis classification system, as defined by the American Joint Committee on Cancer, is the most commonly used staging system and is based on the depth of intestinal wall invasion, the extent of regional lymph node involvement, and presence of distant sites of the disease[23]. The depth of tumor invasion defines the T stage and varies from *in situ* (Tis), T1 (a tumor that invades the subserosa), T2 (a tumor that invades the muscular layer), T3 (a tumor that invades the muscularis propria up to the subserosa) and T4 (invasion of the serosa or adjacent structures). As the depth of tumor invasion increases, the risk of nodal and distant spread also increases. Pathological review of surrounding lymph nodes defines three categories: N: N0 (no lymph nodes involved), N1 (1-3 lymph nodes involved), and N2 (more than 3 lymph nodes involved). Current guidelines recommend identifying 12 or more lymph nodes in the resected sample, as testing with fewer regional lymph nodes has been associated with worse outcomes in patients with negative lymph node disease and with positive lymph nodes[24]. Examination with fewer lymph nodes may reflect a less complete operative procedure or inadequate inspection of the pathology specimen, leading to tumor "under-staging" and subsequent omission of beneficial adjuvant therapy[23]. Table 1 summarizes the staging of CRC.

Treatment

The different modalities available for the treatment of CRC include surgery, CT, and radiotherapy. Surgery is the main form of treatment for CRC, and CT is used as an adjuvant treatment. Approximately 92% of patients with colon cancer and 84% with rectal cancer undergo surgery as a first therapeutic option, the majority with curative intent. Approximately 20% of patients with CRC have advanced disease at diagnosis, while 30%-40% of patients undergoing curative surgery eventually relapse locally or at distance[25]. The metastatic pattern is influenced either by the histological subtype or primary tumor localization. Indeed, colon cancer patients develop a higher rate of abdominal metastases (*e.g.*, liver), while rectal cancer more often metastasizes to extra-abdominal sites including the lungs. The more common histology of adenocarcinoma metastasizes to the liver, while mucinous and signet-ring histology more frequently has peritoneal metastases[26]. The advancement of surgical techniques has made it possible for patients with mCRC to have a curative nature. Surgery should be performed laparoscopically whenever possible. However, certain lesions are not amenable to a minimally invasive approach due to several factors, such as a large tumor mass, or the fact that the tumor is locally advanced. The laparoscopic procedure should achieve the same goals as the open approach procedure (laparotomy) and when this is not possible, conversion to a laparotomy approach is recommended. There are several studies, including a meta-analysis that demonstrate oncologic non-inferiority and better short-term outcomes with laparoscopy compared to the open surgical approach when performed by experienced surgeons[21].

After surgical resection, patients with stage II disease may benefit from adjuvant CT in case of the presence of major risk factors for relapses, such as an inadequate lymph node sampling < 12 or pT4 stage including perforation. Other minor risk factors for stage II risk assessment are the presence of high-grade tumors, vascular, lymphatic, and perineural invasion, tumor presentation with obstruction, and high preoperative carcinoembryonic antigen (CEA) levels. The main agents used in CT are 5-fluorouracil (5-FU) or oral capecitabine (fluoropyrimidines) for 6 months. The addition of oxaliplatin to 5-FU or capecitabine (FOLFOX or XELOX) may be evaluated in presence of major risk factors for relapse and younger age. Moreover, deficient MMR (dMMR)/microsatellite instability (MSI) testing must be performed to identify a small subset of stage II patients with a very low risk of recurrence and in whom the benefits of fluorop-

Table 1 Tumor-nodes-metastasis staging classification for colon and rectal cancer

Stage	Tumor	Regional nodules	Metastases
Stage 0	Tis	N0	M0
Stage I	T1, T2	N0	M0
Stage II	T3, T4	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage III	Any T	N1, N2	M0
Stage IIIA	T1, T2	N1	M0
	T1	N2a	M0
Stage IIIB	T3, T4a	N1	M0
	T2, T3	N2a	M0
	T1, T2	N2b	M0
Stage IIIC	T4a	N2a	M0
	T3, T4a	N2b	M0
	T4b	N1, N2	M0
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

yrimidines have not been demonstrated[27].

In the case of patients with CRC with lymph node involvement (stage III), they should undergo adjuvant CT, to reduce mortality. Combination CT with fluoropyrimidines and oxaliplatin is the standard of treatment. The duration of treatment is different depending on the risk of relapse. In low-risk patients (T1-3 N1) options are XELOX for 3 months or FOLFOX for 6 months. Differently, high-risk patients (T4 and/or N2) should receive 6 months of therapy either with FOLFOX or XELOX[27].

Radiotherapy is not considered effective in the treatment of colon cancer. In the case of rectal cancer, radiotherapy and CT are indicated for T3 and T4 tumors with lymph node involvement. In the case of locally advanced rectal cancer, the use of radiotherapy as a neoadjuvant therapy has been shown to decrease local recurrence rates by approximately 50%-60% compared to surgical treatment performed alone. In terms of CT for the localized and locally-advanced stages, the most used in the treatment of rectal carcinoma are 5-FU, leucovorin, capecitabine, and oxaliplatin[26]. The usual treatment regimen for stage II and III rectal cancer is based on total neoadjuvant CT (based on oxaliplatin and capecitabine or 5-FU) followed by concomitant 5-FU or capecitabine-based chemoradiotherapy, followed by surgical resection[28].

In stage IV both colon and rectal carcinoma, the main objective of therapy is usually to improve quality of life, with treatment being directed at symptoms, such as pain. Palliative surgery is performed to avoid complications such as intestinal obstruction or perforation and bleed and is often preceded by CT to control metastases. Palliative radiotherapy is indicated only for rectal cancer, while CT is the main treatment for mCRC. The use of more targeted therapies has drastically modified the treatment of mCRC, having changed the natural history of the disease. For nearly a decade, patients with mCRC have been among the cancer patients most benefited from targeted therapies such as monoclonal antibodies against vascular endothelial growth factor (VEGF) (*e.g.*, bevacizumab, aflibercept, and ramucirumab) and epidermal growth factor receptors (EGFR) (*e.g.*, cetuximab and panitumumab). In general, the first and second-line treatment in mCRC is made by a CT backbone, for example, FOLFOX (5-fluorouracil and oxaliplatin), FOLFIRI (5-fluorouracil and irinotecan) or FOLFOXIRI (5-fluorouracil, oxaliplatin, and irinotecan). Together with either an anti-VEGF agent or anti-EGFR antibody, which have been demonstrated to improve clinical outcomes when combined with CT. Many factors contribute to the choice of the treatment strategy. First of all, the patient's comorbidities, age, and general clinical conditions have to be considered. Secondly, the determination of the RAS and BRAF status on tumor biopsy is mandatory to guide the best treatment decision. Moreover, dMMR/MSI testing must be performed as part of the initial molecular work-up as well to select patients for immune checkpoint inhibition. In addition, sidedness (left or right primary tumor) drives the choice of the most suitable monoclonal antibody[29] (Figure 3). Some of the difficulties in the development of these drugs have been the resistance mechanisms developed by the cells, which are often only possible to be identified in clinical trials[30].

Prognosis

The stage at diagnosis is a determining factor in patient survival. Since most relapses occur within the first four years

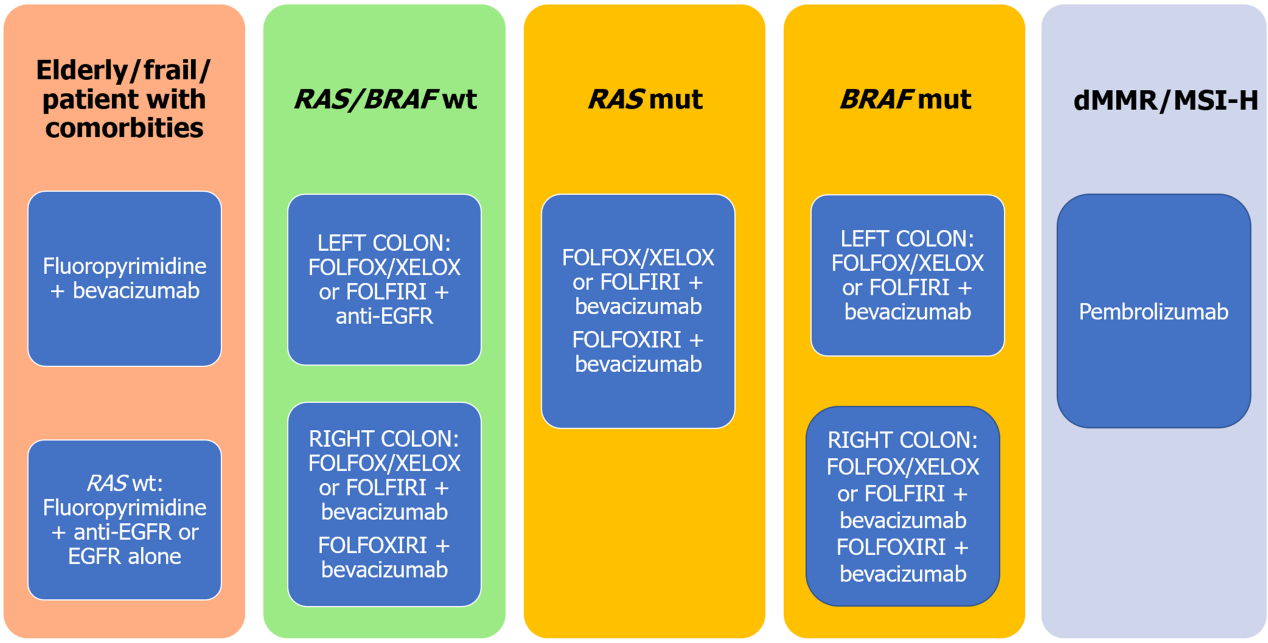

Figure 3 First-line treatment for metastatic colon and rectal cancer patients. anti-EGFR: Anti-epidermal growth factor receptor agent; dMMR/MSI-H: Deficient mismatch repair/microsatellite instability; FOLFOX: 5-fluorouracil + oxaliplatin; FOLFOXIRI: 5-fluorouracil + oxaliplatin + irinotecan; FOLFIRI: 5-fluorouracil + irinotecan; mut: Mutant; XELOX: Capecitabine + oxaliplatin; wt: Wild-type; BRAF: B-raf proto-oncogene.

after initial diagnosis, five-year survival is a commonly used indicator of cure. For stage I the 5-year survival rate is greater than 90%, decreasing to 70%-85% in stage II, to 25%-80% in stage III, and to less than 10% in IV[29]. In resettable tumors, factors that increase the risk of recurrence after surgery are poorly differentiated histology, lymphatic and venous invasion, tumor invasion through the intestinal wall with pericolic fat reaching, intestinal perforation or obstruction, as well as levels of elevated CAE[31]. Thus, the preoperative measurement of CEA is extremely relevant, as its high levels are associated with a higher risk of cancer recurrence[31]. The two molecular markers most implicated in the prognosis of patients with colorectal cancer rank are microsatellite instability and specific chromosomal deletions, such as allelic loss on chromosome 18q of tumor cells. Microsatellite instability is associated with a better prognosis than microsatellite stability. Deletion of chromosome 18q is associated with a worse prognosis[32].

CONCLUSION

CRC is an emerging public health problem with an increasing trend in its incidence and mortality worldwide[33]. Globally, CRC accounts for approximately 10% of all diagnosed cancers and associated deaths annually[34]. The determinants of CRC are older age, male sex, family history of CRC, tobacco, overweight, alcohol, processed meats, and physical inactivity, among others[2,10]. Most cancers arise from the polyps that give rise to CRC after about 10-15 years. The diagnosis can be made in several ways, including clinical presentation, endoscopy, CT, and fecal test[2]. The fecal test is a non-invasive means of diagnosis, some studies demonstrate that its use contributes not only to the reduction of CRC mortality but also to the reduction of the incidence[35]. Surgical treatment is intended to be curative and constitutes the gold standard of treatment. Prevention is fundamental and can be done at several levels, especially primary, secondary, and tertiary. From a public health perspective, primary prevention plays a key role in the management of this scourge, and measures such as smoking cessation, a healthy diet, and regular physical exercise can prevent the onset of CRC cancer[10]. The recommendations are based on the practice of daily physical exercise, for at least 30 min, the consumption of healthy foods, and a diversified diet (milk, fresh fruits, nuts, vegetables, foods with calcium, and foods rich in fiber) [10]. As secondary prevention, screening is recommended. Ideally, in the future, it may be possible to perform a colonoscopy in population-based screening, but more studies are needed to prove its superiority compared to fecal tests [36]. Clinical trials comparing the fecal blood test *vs* colonoscopy strategies are fundamental to compare the incidence and mortality associated with the two strategies in population-based screening diagnosis. Program logistics should be simplified and we should recognize a visit to the primary health care of the target population as a unique opportunity to carry out screening if it is lacking. It is also necessary to reinforce the invitations made for screening to users who do not adhere to remind them of the importance of screening.

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MINIREVIEWS

Recent advances in age-related metabolic dysfunction-associated steatotic liver disease

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Abstract

Metabolic dysfunction-associated steatotic liver disease (MASLD) affects approximately 25% of the world's population and has become a leading cause of chronic liver disease. In recent years, an increasing amount of data suggests that MASLD is associated with aging. As the population ages, age-related MASLD will become a major global health problem. Targeting an aging will become a new approach to the treatment of MASLD. This paper reviews the current studies on the role of aging-related factors and therapeutic targets in MASLD, including: Oxidative stress, autophagy, mitochondrial homeostasis, bile acid metabolism homeostasis, and dysbiosis. The aim is to identify effective therapeutic targets for age-related MASLD and its progression.

Key Words: Metabolic dysfunction-associated steatotic liver disease; Aging; Mitochondrial homeostasis; Bile acid homeostasis; Dysbiosis

Core Tip: Aging is closely associated with metabolic dysfunction-associated steatotic liver disease (MASLD). We will focus on the main features of age-related MASLD, the mechanisms by which age-related factors, such as oxidative stress, autophagy, mitochondrial homeostasis, bile acid metabolism and bacterial dysbiosis induce MASLD, as well as the study of related therapeutic targets.

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INTRODUCTION

Aging is broadly defined as the time-dependent decline in function that affects most organisms. López-Otín *et al*[1], in conjunction with recent research findings, has revised the concept of "aging hallmarks" by incorporating an additional 12 aging features, including macroautophagy deficiencies, chronic inflammation, and dysbiosis. Owing to advancements in living standards, economic growth, and healthcare infrastructure, population aging has emerged as the primary demographic phenomenon globally. Currently, the proportion of elderly people over the age of 60 worldwide is about one ninth, with projections indicating that by the year 2050, this ratio will escalate to one-fifth[2]. Human aging is characterized by molecular, structural, and functional alterations in various organ systems, including the liver. Within the intensifying milieu of aging, age-associated liver dysfunction has become a compelling clinical challenge that demands urgent attention and resolution.

Cellular damage, if left unattended or irreparable, can lead to cellular apoptosis or senescence, which is the fundamental cellular process employed by the organism in its defense against cancer. Similarly, following exposure to damage and stress signals, aging can irreversibly arrest the G0/G1 phase of the cell cycle. This phenomenon restricts the proliferative potential of damaged cells[3], ultimately leading to changes in the microenvironment and tissue homeostasis. Upon entering a state of cellular senescence, cells undergo morphological transformations characterized by permanent cell cycle arrest, displaying distinctive traits such as altered secretion profiles, macromolecular damage, and metabolic shifts. These alterations include an upregulated expression of senescence-associated β-galactosidase, acquisition of the senescence-associated secretory phenotype (SASP), elevated levels of P16INK4a (P16) and P21Cip1/Waf1 (P21), and increased levels of reactive oxygen species (ROS)[4]. In particular, the senescence of hepatic cells can potentially contribute to intracellular lipid accumulation, fibrosis, and inflammation, while concurrently secreting age-associated inflammatory mediators.

In 2023, the global hepatology community renamed nonalcoholic fatty liver disease (NAFLD) as metabolic dysfunction-associated steatotic liver disease (MASLD). MASLD is the most common chronic liver disease worldwide. Patients presenting hepatic steatosis and at least one of five cardiometabolic risk factors are diagnosed with MASLD. Generally, the initial hepatic steatosis observed in MASLD is considered reversible. However, persistent metabolic disturbances can lead to aberrant liver cell metabolism, resulting in the accumulation of lipids, such as fatty acids, cholesterol, and other lipid metabolites. This, in turn, induces the onset of MASLD. Excessive lipid accumulation in the liver can lead to lipotoxicity, mitochondrial dysfunction, increased ROS levels, and inflammation. These events signify the transition from MASLD to metabolic dysfunction-associated steatohepatitis (MASH) or the progression to more severe stages[5]. Younossi *et al*[6] conducted an extensive analysis involving 8515431 samples from 22 countries and demonstrated that the global prevalence of MASLD was 25.24%. The highest rates of prevalence were identified in the Middle East and South America, with Africa exhibiting the lowest prevalence. Notably, MASLD exhibited strong associations with the components of metabolic syndrome, including obesity, diabetes, and dyslipidemia. The median age of the MASLD population in the United States in 2015 was 50 years old. Researchers predict that it will increase to 55 years old by 2030. This indicates that with an increase in population aging, the incidence of MASLD-related liver diseases and mortality rates will increase in the United States[7].

RELATIONSHIP BETWEEN AGING AND MASLD

The aging phenotype exhibits remarkable stability, displaying resistance to mitotic stimuli and apoptosis[8]. In healthy cells, senescence may be triggered primarily by factors such as oxidative stress, mitochondrial imbalance, and chronic inflammation either independently or synergistically. The progressive accumulation of senescent cells can elicit bystander effects, ultimately leading to organ aging and functional impairments[4]. Age dependence is the main risk factor for chronic liver diseases, including MASLD and MASH. The accumulation of aging cells drives liver steatosis, and liver cell aging is closely related to the progression of MASLD[9]. The intricate mechanisms governing the pathogenesis of MASLD are not completely clear. Elevated hepatic lipid accumulation is a significant risk factor for MASLD, with hepatic cell senescence playing a contributory role in the development of hepatic steatosis. This can be best described by the "two-hit" hypothesis. "First hit" denotes the initial occurrence of excessive hepatic lipid accumulation due to alterations in lipid metabolism, which leads to the development of nonalcoholic fatty liver (NAFL). The "second hit" entails the induction of hepatic cell damage, including oxidative stress, inflammatory cytokine release, and mitochondrial dysfunction, building upon the backdrop of hepatic lipotoxicity. This second hit can precipitate the progression of NAFL towards MASH and, in more severe cases, fibrosis, cirrhosis, hepatocellular carcinoma, and even death[10]. In recent years, the "multiple-hit" hypothesis has emerged as a more elaborate concept for elucidating the pathogenesis of MASLD. This theory underscores the pivotal roles played by gut microbiota (GM), insulin resistance, and adipose tissue-derived adipokines. These factors, through their interconnected relationships along the gut–liver axis, interact synergistically and causally, contributing to

the progression of MASLD[11].

Oxidative stress

In response to oxidative stress, organisms generate substances, including ROS or free radicals, that can inflict damage on cellular constituents such as the cell membrane, proteins, and DNA. This damage can ultimately lead to cellular senescence or even apoptosis. The acceleration of oxidative stress due to hepatocyte aging reportedly augments lipid accumulation in the liver, suggesting the pivotal role of oxidative stress in the etiology and progression of MASLD. Oxidative stress initiates DNA damage, as well as instigates autophagy and the secretion of SASP. These processes are concomitant with the activation of the p53-p21 and p16-Rb pathways, leading to premature cellular senescence. Concurrently, oxidative stress exacerbates disruptions in lipid metabolism, promotes inflammatory responses, and induces hepatocyte damage[4]. During the normal aging process of the liver, hepatic stellate cells help drive macrophage differentiation towards the M1 phenotype, exerting anti-tumor effects. Conversely, a study has found that p53-knockout hepatic stellate cells promote macrophage differentiation towards the pro-tumor M2 phenotype, thereby inducing the progression of liver fibrosis, cirrhosis, and even HCC[12].

MASLD is characterized by increased levels of senescent cells compared with the control group. Additionally, patients with MASLD demonstrate significantly higher telomere shortening, along with increased cell cycle arrest, which coincides with an increased expression of p21[13]. Reportedly, Krüppel-like factor 16 (KLF16) can bind to the promoter of peroxisome proliferator-activated receptor alpha (PPARα), thereby activating it. Upregulation of KLF16 expression expedites fatty acid β-oxidation and mitigates oxidative stress responses in db/db and high-fat diet (HFD) mice. This, in turn, results in a reduction in hepatic lipid accumulation and an improvement in MASLD[14]. Oxidative stress induced cellular aging can alter the liver microenvironment, leading to disease progression from simple steatosis to inflammation and fibrosis, as well as hepatocellular carcinoma.

Autophagy

In hepatic cells, autophagy is subject to modulation by both individual and organ-level aging processes, with pronounced attenuation of hepatic autophagy evident in the aging state[15]. The process of aging decreases the quantity and efficiency of autophagosomes, subsequently causing a buildup of lipid droplets and resultant liver damage. Autophagy inhibition is posited as a potential risk factor in the progression of age-related MASLD. An animal study has revealed that the administration of plasma from younger mice restores autophagic activity in aged mice, effectively mitigating liver aging, lipid accumulation, and fibrosis[16]. Using Atg7-deficient mice, researchers have substantiated that the autophagic impairment in liver sinusoidal endothelial cells not only accelerates liver inflammation and fibrosis in the early phases of MASLD but also exacerbates hepatic inflammation and fibrosis during the advanced stages[17].

Mice deficient in Omi/HtrA2 exhibit premature aging symptoms and age-related autophagy inhibition, which results in hepatic dysfunction[18]. Therefore, augmenting autophagy may mitigate aging and hepatic steatosis, thus alleviating MASLD. Mice overexpressing Omi/HtrA2 have demonstrated enhanced autophagic activity, diminished hepatic steatosis, and elevated hepatic fatty acid β-oxidation, which ameliorated HFD-induced MASLD along with hepatic inflammation[19]. P62/sequestosome 1 facilitates the phosphorylation of unc-51 Like autophagy activating kinase 1, thereby promoting autophagy activation and triggering NFE2L2/NRF2 activation. This protects mouse liver cells against lipotoxic damage[20]. AMPK activation is a regulator of autophagy and aging-related changes in AMPK activation may impact autophagic processes, which results in decreased formation of autophagosomes and further hastening of the aging process[21]. In hepatic cells, PPARδ activates the autophagy-lysosome pathway *via* the AMPK/mammalian target of rapamycin (mTOR) signaling to induce fatty acid β-oxidation, which reduces hepatic lipid levels[22]. Autophagy changes may be an important target for the treatment of age-related MASLD.

Mitochondrial homeostasis

In mouse liver, increased mitochondrial ROS levels are linked with the aging process. Oxidative stress triggers mutations in the mitochondrial DNA, leading to the accumulation of mitochondrial DNA fragments within the cell nucleus. This accumulation subsequently contributes to mitochondrial dysfunction[23]. It has been shown that the absence of the gene encoding the nonhomologous end-joining enzyme known as DNA ligase IV (DNL4) exacerbates linear mitochondrial DNA (mtDNA) aggregation in the nucleus. Cheng *et al*[24], proposed that linear nuclear mtDNA fragments accelerate aging in yme1-1 mutant cells by affecting nuclear DNA replication, recombination, repair, and transcription. In addition, mice deficient in the antioxidant enzyme superoxide dismutase 1 (SOD1) demonstrate premature aging, along with hepatic damage. Investigations have unveiled that SOD1-deficient (SOD1-/-) mice display shifts in the composition of their GM, including alterations in the ratio of Firmicutes and Bacteroidetes, a significant reduction in lactobacilli, increased hepatic metabolites, and manifestation of a systemic aging phenotype[25]. Moreover, cellular senescence results in mitochondrial dysfunction, which induces respiratory chain disturbances, membrane potential anomalies, and concomitant ROS generation, all of which further induce the development of MASLD[26].

Free fatty acids in the liver are primarily metabolized *via* two pathways: mitochondrial β-oxidation and esterification into triglycerides. The preservation of mitochondrial homeostasis is a pivotal factor in hepatic lipid metabolism. However, excessive free fatty acids burden the process of mitochondrial β-oxidation, subsequently leading to an imbalance in mitochondrial homeostasis. This imbalance further exacerbates the accumulation of lipids within the hepatic cells, ultimately contributing to the development of MASLD[27]. Omi/HtrA2 is a mitochondrial serine protease and a pro-apoptotic factor that plays a pivotal role in maintaining mitochondrial homeostasis[28]. Within the liver, Omi/HtrA2 mediates mitochondrial stability and autophagy, thus contributing to the amelioration of MASLD. Reportedly, the overexpression of HtrA2/Omi in the mouse liver enhances the expression of genes related to mitochondrial fatty acid β-

oxidation, reduces hepatic lipid accumulation, and regulates glucose homeostasis[19]. KLF16 tightly links hepatic lipid metabolism to mitochondrial homeostasis by regulating the transcriptional activity of PPARα. Knockdown of hepatic KLF16 also leads to increased mitochondrial stress and promotes the development of hepatic steatosis and insulin resistance in mice, whereas hepatic-specific PPARα overexpression effectively ameliorates hepatic steatosis induced by KLF16 deficiency and improves mitochondrial imbalance and insulin resistance[14]. The mitochondrial homeostasis is closely related associated with hepatic lipid metabolism and exacerbates the development of MASLD.

Bile acid-mediated metabolism homeostasis

Metabolic irregularities serve as both inducers and outcomes of the aging process and are intricately intertwined in the development of various diseases[29]. Hepatic metabolism declines with aging and is accompanied by a reduction in enzymatic activity, owing to which the elderly are more susceptible to lipid accumulation. Bile acids (Bas), in conjunction with their homologous receptors such as the farnesoid X receptor (FXR) and Takeda G-protein-coupled receptor 5, play integral roles in numerous signaling pathways that are closely linked with MASLD. These pathways encompass BA self-regulation, glucose and lipid metabolism, energy modulation, cell proliferation, detoxification, and immune regulation [30]. BA analogs targeting FXR, TGR5, or both have been shown to effectively mitigate the progression of MASH[31].

BAs regulate metabolism by interacting with nuclear receptors and tightly modulating the diversity and relative abundance of the GM. The GM, along with their metabolic byproducts, may mediate MASLD by inducing endotoxemia, triggering insulin resistance, increasing short-chain fatty acids, elevating endogenous ethanol production, altering choline and BA metabolism, and impacting the host's immune response[32]. Therefore, the disruption of intracellular BA homeostasis may be a pivotal factor influencing the development of MASLD.

Dysbiosis

Aging results in the restructuring of the GM and is characterized by a decreased Firmicutes-to-Bacteroidetes ratio and reduced overall microbial diversity[33]. The lipopolysaccharide (LPS)/toll-like receptor 4 (TLR4) signaling pathway plays a crucial role in mediating the pathological mechanisms of MASLD. Patients with MASLD demonstrate an overgrowth of intestinal bacteria, which disrupts the intestinal barrier function. This altered gut barrier permeability leads to the translocation of LPS that triggers the activation of the LPS/TLR4/nuclear factor-κB (NF-κB) signaling pathway. That mediates the progression of MASLD to MASH[34]. A study has demonstrated that TLR4-deficient mice showed amelioration in insulin resistance and hepatic steatosis induced by HFD[35]. However, another study revealed that TLR5-deficient mice exhibited characteristics of metabolic syndrome, such as obesity, insulin resistance, and hepatic steatosis. Furthermore, transplanting the GM from TLR5-deficient mice into healthy mice exhibited the performance of metabolic syndrome in healthy mice[36].

Reportedly, GM additionally stimulates the generation of endogenous ethanol[37]. Dysbiosis within the gut microbiome can lead to the overgrowth of ethanol-producing bacteria, resulting in an increase in endogenous ethanol levels and subsequent induction of MASH. This has been demonstrated by Yuan *et al*[38], who isolated a high-alcohol-producing strain of Klebsiella pneumoniae (HiAlc Kpn) from the fecal samples of patients with Auto-brewery syndrome(ABS)/MASH and found that orally administering the strain to healthy sterile mice induced hepatic steatosis. Their results revealed that HiAlc-Kpn induced mouse MASLD model, the high-alcohol-producing strain of K. pneumoniae, upon colonization in the mouse gut, induced endogenous ethanol production that subsequently impaired the intestinal mucosal barrier. This resulted in heightened intestinal permeability in mice, which exacerbated inflammation in MASLD. Additionally, the transplantation of GM from younger mice to older mice could reverse age-related changes in the gut, eyes, and brain. Aged mice receiving young donor microbiota had reduced cortical and callosal microglia, reduced expression of inflammatory complement protein C3 in the retina, and reduced circulating concentrations of lipopolysaccharide (LPS)-binding protein (LBP), to levels comparable to young mice[39]. Furthermore, Hoyles *et al*[40] transplanted GM from patients with MASLD into mice maintained on a normal diet and found that the mice developed hepatic steatosis and their gut microbial characteristics realigned to those observed in MASLD.

POTENTIAL THERAPEUTIC TARGETS FOR MASLD

Regarding MASH treatment, this article predominantly focuses on clinical trials assessing agents targeting metabolic pathways, cellular stress responses, and interactions with GM.

Drugs targeting metabolism

FXR agonists: FXR plays a pivotal role in regulating lipid metabolism, BA homeostasis, and glucose equilibrium. The dysregulation of FXR function has been implicated in the pathogenesis of MASLD, cholestasis, and chronic inflammatory disorders that affect the liver and gastrointestinal tract. Despite a multitude of clinical trials targeting FXR for the management of MASLD/MASH, to date, only obeticholic acid has received approval for the treatment of primary biliary cholangitis. MET409[41] is another agent that has a unique non-BA composition and sustained pharmacokinetic/pharmacodynamic properties. A study has assessed the efficacy of oral MET409 administered once daily over 12 wk in patients with MASH. By the end of the 12-week treatment cycle, MET409 remarkably reduced hepatic fat levels, with an average reduction of 55% (80 mg) and 38% (50 mg) compared with the 6% reduction observed with placebo ($P < 0.001$). Vonafexor (EYP001a) is a second-generation synthetic, non steroidal, non-bile salt, orally active carboxylic acid FXR agonist currently in development. In patients with MASH and suspected fibrosis, vonafexor has demonstrated efficacy in reducing hepatic fat content and improving liver enzymes. Specifically, 50.0% and 39.3% of patients treated with VONA-

100 mg and VONA-200 mg once daily, respectively, demonstrated a reduction in hepatic fat levels of > 30%, whereas only 12.5% of patients treated with placebo demonstrated this effect[42].

PPARs agonists: PPARs represent a subgroup of nuclear transcription factors activated by ligands and are categorically classified within the nuclear receptor superfamily. Post activation, PPARs form heterodimers with retinoid X receptors (RXRs). The resultant PPARγ–RXR heterodimer binds to peroxisome proliferator response elements that are situated upstream of target gene promoters, consequently modulating the transcription of these specific target genes. The PPARs comprise PPARα, PPARβ/δ, and PPARγ that function as sensitive receptors for fatty acids and their derivatives, exerting crucial roles in lipid metabolism[43]. Notably, they can effectively mitigate hepatic steatosis, inflammation, and fibrosis in preclinical models of MASLD, thus underscoring their potential as promising targets for MASLD treatment. Selective agonists targeting PPARα and PPARγ have already demonstrated clinical efficacy, while clinical trials are currently assessing PPARγ agonists such as pioglitazone; dual PPARα/δ agonists such as chiglitazar, saroglitazar magnesium, and elafibranor; and pan-PPAR agonists such as lanifibranor. The activation of intestinal PPARα signaling plays a role in upregulating the expression of fatty acid-binding protein 1, thus facilitating intestinal fatty acid uptake and potentially contributing to the progression of MASH[44]. Elafibranor can improve steatosis, mitigate inflammation, and attenuate fibrosis in rodent models of MASLD/MASH[45]. Moreover, human liver *in vitro* models have unveiled that PPAR agonists can effectively diminish the increase in lipid levels, quell the secretion of inflammatory chemokines, and modulate the expression of pro-fibrotic genes *via* diverse mechanisms[46].

Glucagon-like peptide-1 agonists: The discovery of glucagon-like peptide-1 (GLP-1) represents a pivotal milestone in the field of biology, as the molecule significantly affects the regulation of blood glucose levels and the management of body weight. Cotadutide, an agonist of GLP-1R/GcgR, improves MASH and liver fibrosis by regulating mitochondrial function and lipid biosynthesis. Notably, in C6BL29/J mice exposed to an Amylin liver MASH diet for 57 wk, cotadutide displayed greater efficacy in treating MASH than cotadutide combined with obeticholic acid[47]. Reportedly, the dual agonist GLP-1-Fc-FGF21 D1 exhibits notable and sustained hypoglycemic effects in diabetic mouse models. Moreover, in an HFD-induced ob/ob mouse model, GLP-1-Fc-FGF21 D1 has demonstrated robust anti-MASH properties *via* significant enhancements in liver function, alterations in serum and hepatic lipid profiles, and reduction in the MASLD activity score. Remarkably, its therapeutic efficacy surpasses that of singular FGF21 or GLP-1 analogs[48].

Thyroid hormone receptor-beta agonist: Activation of hepatic thyroid hormone receptor-beta (THR-β) reduces systemic lipid levels, enhances BA synthesis, and promotes lipid oxidation. Resmetirom (MGL-3196) is a liver-targeted and selective THR-β agonist. Studies have demonstrated that administering resmetirom (MGL-3196) to mice with diet-induced fibrotic DIO-MASH can lead to substantial reductions in liver weight, hepatic steatosis, plasma ALT levels, and hepatic and plasma cholesterol levels, as well as a decrease in blood glucose levels. Moreover, the treatment remarkably improved the MASLD activity score, with no discernible impact on body weight[49]. Compared with the placebo group, the resmetirom group was more effective in mitigating hepatic steatosis and ameliorating liver enzyme levels as well as inflammatory markers, resulting in a pronounced improvement in MASH as evidenced by liver biopsy assessments[50]. Furthermore, both 80 and 100 mg resmetirom result in favorable tolerability profiles, devoid of any severe or serious adverse events. The Phase 3 MASH clinical trial (NCT03900429) is now underway and is poised to enroll 900 volunteers with liver biopsy MASH with fibrosis grades I-III in a multinational, double-blind, randomized, placebo-controlled study to address the progression of MASH, cirrhosis, and/or hepatic decompensation (Figure 1).

Other agents: Sodium-dependent glucose co-transporter 2 (SGLT2) inhibitors are a novel class of antidiabetic drugs that inhibit glucose reabsorption in the proximal renal tubules, and have been shown to be effective in reducing hepatic fat content and AST/ALT levels and ameliorating hepatic fibrosis in several studies. Specifically, empagliflozin prevents MASLD progression in ApoE (-/-) mice by inducing autophagy *via* increased AMPK phosphorylation, reduced mTOR activity, and elevated LC3B expression. Furthermore, it mitigates endoplasmic reticulum stress and inhibits hepatocyte apoptosis[51]. Dapagliflozin, another SGLT2 inhibitor, has been found to activate AMPK and reduce mTOR phosphorylation in Zucker diabetic fatty rats. This effect has additionally been replicated in LO2 cells and HepG2 cells stimulated with palmitic acid. Consequently, the activation of AMPK promotes fatty acid oxidation and induces autophagy, ultimately improving hepatic steatosis[52]. Reportedly, fructose is catalyzed by ketohexokinase to produce fructose-1-phosphate, a metabolite that is primarily metabolized within the liver. Inhibition of fructose metabolism using ketohexokinase inhibitors can mitigate hepatic injury and fibrosis in both murine models and human subjects[53]. High fructose intake induces de novo lipid biosynthesis in the liver. This process does not depend on ATP citrate lyase (ACLY) but rather on the intestinal microflora which metabolizes fructose to acetate and converts the latter to acetyl coenzyme A (acetyl-CoA). Altered intestinal permeability, gut dysbiosis, and increased fructose intake exacerbate hepatic lipid accumulation and contribute to the development of MASLD in elderly patients[54].

Cell stress and cellular senescence

ROS inhibition: Excessive oxidative stress culminates in hepatocyte senescence, thereby instigating the accrual of hepatic fat[55]. Furthermore, hepatic lipotoxicity triggers oxidative stress and the release of inflammatory cytokines, thereby fostering the progression of MASH and, in more severe cases, fibrosis. Senolytics are a class of selective drugs that kill aging cells and can be used for the targeted intervention of cellular senescence. Quercetin, as an example, can elevate hepatic levels of SOD, catalase, and glutathione, concurrently decreasing interleukin (IL)-1β, IL-6, tumor necrosis factor-α, and hepatic lipid accumulation in db/db mice. Furthermore, it can activate the FXR1/TGR5 signaling pathway, thereby contributing to the amelioration of MASLD[56]. In addition, quercetin demonstrates the ability to regulate GM dysbiosis and attenuate endotoxemia-induced upregulation of the TLR-4 pathway. This results in the inhibition of inflammasome

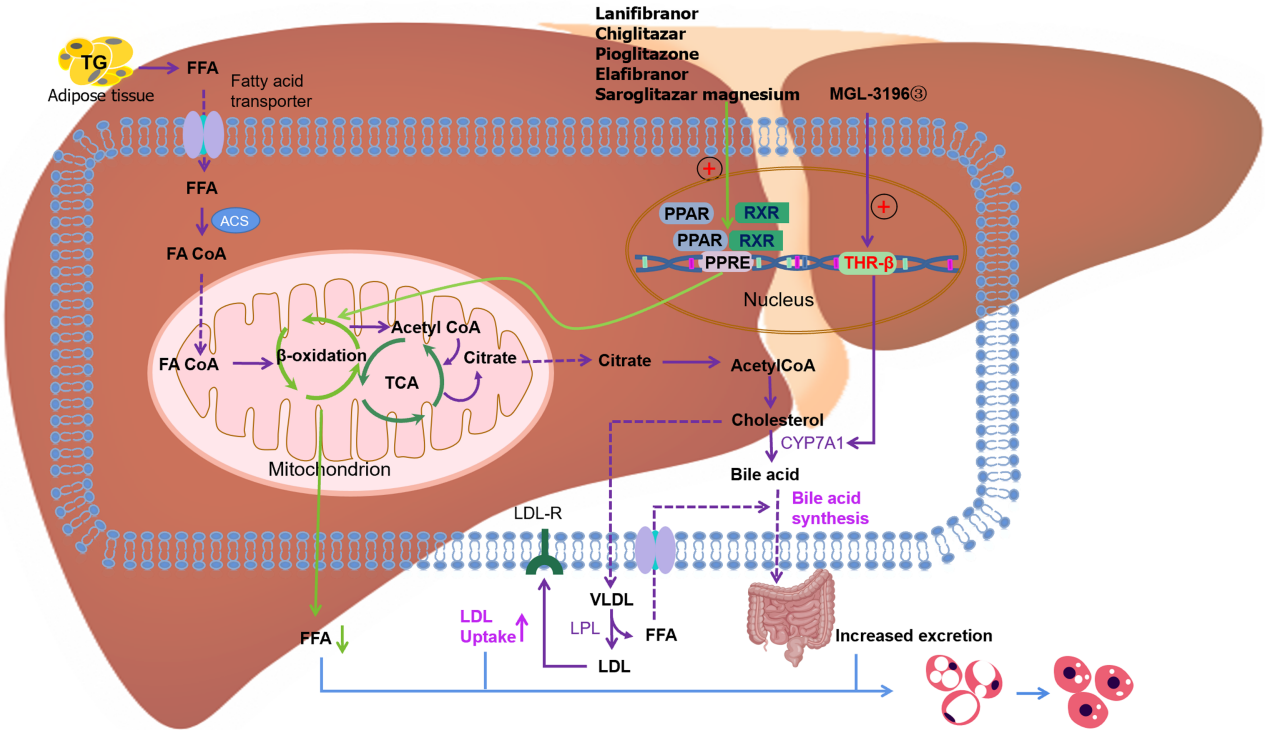

Figure 1 Signaling pathways of proliferator-activated receptor alpha and thyroid hormone receptor-beta and drugs targeting these pathways. PPAR: Proliferator-activated receptor; TG: Triglyceride; FFA: Free fatty acid; ACS: Acyl coenzyme A synthetase; FA CoA: Fatty acyl coenzyme A; TCA: Tricarboxylic acid cycle; RXR: Retinoid X receptor; PPRE: PPAR reaction element; THR: Thyroid hormone receptor-beta; LDL: Low-density lipoprotein; LDL-R: Low-density lipoprotein receptor; VLDL: Very low-density lipoprotein.

activation and stress pathway activation, which reinstates the equilibrium in lipid metabolism gene expression[57]. An ongoing clinical trial (NCT05506488) assessing a combination of dasatinib and quercetin for the clearance of senescent cells offers a potential avenue for addressing MASLD-associated fibrosis. N-acetylcysteine (NAC), an antioxidant with the capacity to reduce ROS levels and induce cellular apoptosis, can significantly reduce obesity, dyslipidemia, hepatic dysfunction, and GM dysbiosis induced by HFD in murine models. However, it is noteworthy that in these mice, using antibiotics for GM depletion resulted in a resurgence of hepatic steatosis and liver injury[58]. Another study has found that supplementing NAC to mice with diet-induced obesity and non-alcoholic steatohepatitis increases the CD4+ T cell population within liver tumor cells. Additionally, it elevates the levels of immunotherapeutic agents M30 and aOX40, thus effectively inhibiting the progression of liver tumors[59].

Myeloperoxidase inhibitor: Recent studies have revealed that patients with MASLD have elevated levels of plasma myeloperoxidase (MPO) and increased hepatic MPO expression compared with healthy controls. Notably, this elevation is more pronounced in patients with MASH. Studies have demonstrated that treatment with the MPO inhibitor AZM198 results in significantly reduced MASH-induced liver damage and fibrosis as well as decreased serum ALT levels and amelioration of hepatic steatosis[60]. Specifically in the elderly population, MASLD was associated with notable alterations in GM abundance, which resulted in compromised gut barrier function and heightened susceptibility to intestinal inflammation and subsequent systemic inflammatory responses. The administration of AZM198 improved the Firmicutes-to-Bacteroidetes ratio and regulated GM composition. Reportedly, TXNIP-deficient mice demonstrated decreased expression of inflammatory factors, reduced LPS levels, improved liver health, and restored intestinal barrier function. Notably, TXNIP is significantly upregulated in the intestinal mucosa of MASH mice. Moreover, studies have demonstrated that inhibiting the activation of the TXNIP-NLRP3 axis can effectively reduce MPO activity and oxidative stress, leading to the restoration of intestinal barrier function in the context of MASH. MPO Inhibitors reduce liver lipid accumulation, inflammation, and fibrosis and ameliorate the development of MASH.

PTUPB inhibitor: PTUPB, a dual inhibitor of soluble epoxide hydrolase and cyclooxygenase-2, exerts its effects by suppressing the PI3K/AKT/mTOR pathway *via* the modulation of Sirt3[61]. This leads to increased autophagy and decreased hepatocyte senescence, thus inducing the progression of MASLD. Reportedly, PTUPB can mitigate liver injury in HFD-induced MASLD murine models by inducing collagen deposition and lipid accrual; reducing hepatic triglyceride levels; and suppressing the expression of liver aging-associated molecules, such as p16, p53, and p21. Aspirin, an oral irreversible inhibitor of the cyclooxygenases COX-1 and COX-2, prevents the development of MASLD and MASH-related HCC[62].

Other agents: Lubiprostone functions by selectively stimulating type 2 chloride channels located on the apical cell membrane of gastrointestinal epithelial cells, leading to increased intestinal permeability. Research findings have

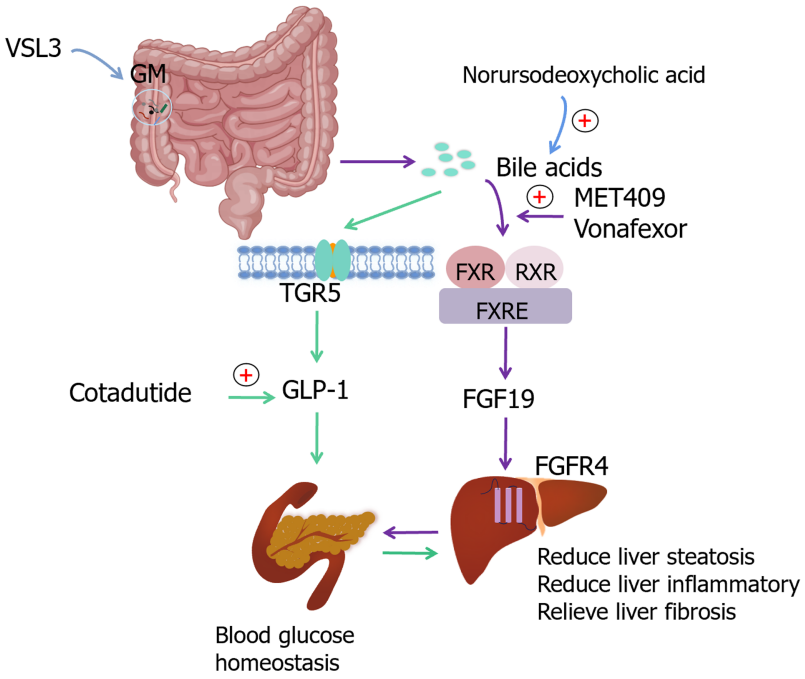

Figure 2 Targets related to lipids, bile acids, glucose homeostasis and intestinal microbiota in age-related metabolic dysfunction-associated steatotic liver disease. GM: Gut microbiota; FXR: Farnesoid X receptor; RXR: Retinoid X receptor; FXRE: FXR reaction element; FGF19: Fibroblast growth factor 19; FGFR4: Fibroblast growth factor receptor 4; TGR5: Takeda G protein–coupled receptor 5; GLP-1: Glucagon-like peptide-1.

indicated that in patients with constipation and MASLD, lubiprostone reduces liver enzyme levels and exhibits favorable tolerability[63]. Heat shock protein (HSP) 47 is a collagen-specific molecular chaperone residing within the endoplasmic reticulum and plays a pivotal role in ensuring the correct folding, assembly, and extracellular secretion of collagen proteins within the extracellular matrix (ECM). The anomalous accumulation of collagen proteins within the ECM disrupts its structural integrity, thereby precipitating fibrotic processes[64]. These findings suggest that targeting HSP47 is of paramount importance for the treatment of liver fibrosis[65].

Drugs targeting the gut–liver axis

Probiotics: The GM assumes a crucial role in MASLD. Dysbiosis of the GM, attributed to oxidative stress, lifestyle choices, and excessive antibiotic use, leads to impaired intestinal permeability. An increased permeability facilitates the induction of pro-inflammatory cytokines and interferon-mediated factors through the activation of pattern recognition receptors by microbiota, bacterial byproducts, and LPS, thereby significantly contributing to the pathogenesis of MASLD [66]. Aging is concomitant with the deterioration of multiple physiological functions and the exacerbation of inflammatory processes. Age-related alterations in the gastrointestinal tract contribute to an elevated incidence of gastrointestinal inflammatory disorders. Aging is associated with increased production of ROS, which leads to lipid accumulation, DNA damage, and concomitant cellular functional impairments. Furthermore, modifications in GM composition result in the endogenous production of ethanol, which consequently compromises the integrity of the intestinal barrier and incites ROS accumulation in hepatic stellate cells and Kupffer cells[37]. Studies have shown that probiotics can restore homeostasis in the GM and mitigate oxidative stress[67]. Specifically, members of the Lactobacillus genus have demonstrated the ability to modulate the expression of inflammatory cytokines, including but not limited to IL-6, IL-1β, IL-1α, IL-12, and interferon-γ, both in serum and colonic tissues. This immunomodulatory effect is attributed to their capacity to inhibit NF-κB activation *via* the G protein-coupled receptor 109A pathway, thereby fostering improvements in immune function among aging mice[68]. In animal models of chronic liver injury, probiotics have demonstrated a protective role against hepatic steatosis and liver inflammation by modulating and potentially restoring the GM. Studies evaluating patients with MASH/MASLD have revealed that the probiotic formulation VSL3 displays inherent anti-inflammatory properties and insulin-sensitizing effects, indicating its potential for the treatment of liver fibrosis[69]. Evidence suggests that GM can ameliorate an array of biomarkers associated with inflammation, blood glucose regulation, insulin resistance, lipid anomalies, obesity, and hepatic impairment, including reductions in liver enzymes, hepatic steatosis, and fibrosis. Probiotics exert their influence on the immune system, potentially improving MASLD outcomes, either by fortifying the intestinal barrier or preventing the formation of hepatotoxic metabolites[70] (Figure 2).

Weight loss surgery: Invasive weight loss surgeries may be associated with reduced tolerance among patients but demonstrate notable efficacy, particularly in severely obese individuals. A study assessing patients with severe obesity and MASH who underwent bariatric surgery revealed that 5 years post-surgery, 84% of the patients experienced resolution of inflammation, 70% demonstrated improvements in fibrosis, and 56% exhibited regression of liver fibrosis [71]. Bariatric surgery further modifies the ileal milieu, thereby inducing alterations in the GM or its metabolic products,

ultimately ameliorating MASLD.

CONCLUSION

The escalating trend of population aging underscores the increasing importance of investigating age-related diseases. Although research on MASLD has proliferated in recent years, the intricate mechanisms underlying the condition remain only partially elucidated. The factors precipitating aging exhibit reciprocal interactions with the pathogenesis and progression of MASLD. Oxidative stress and dysbiosis of the GM, for instance, can trigger both the aging process and inducing MASLD, which is frequently encountered in the elderly population. It is conceivable that such convergent targets, including oxidative stress and GM, may hold promise in formulating strategies for the management of age-related MASLD.

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FOOTNOTES

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MINIREVIEWS

Current landscape of preoperative neoadjuvant therapies for initial resectable colorectal cancer liver metastasis

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Abstract

Colorectal cancer liver metastasis (CRLM) presents a clinical challenge, and optimizing treatment strategies is crucial for improving patient outcomes. Surgical resection, a key element in achieving prolonged survival, is often linked to a heightened risk of recurrence. Acknowledging the potential benefits of preoperative neoadjuvant chemotherapy in managing resectable liver metastases, this approach has gained attention for its role in tumor downsizing, assessing biological behavior, and reducing the risk of postoperative recurrence. However, the use of neoadjuvant chemotherapy in initially resectable CRLM sparks ongoing debates. The balance between tumor reduction and the risk of hepatic injury, coupled with concerns about delaying surgery, necessitates a nuanced approach. This article explores recent research insights and draws upon the practical experiences at our center to address critical issues regarding considerations for initially resectable cases. Examining the criteria for patient selection and the judicious choice of neoadjuvant regimens are pivotal areas of discussion. Striking the right balance between maximizing treatment efficacy and minimizing adverse effects is imperative. The dynamic landscape of precision medicine is also reflected in the evolving role of gene testing, such as RAS/BRAF and PIK3CA, in tailoring neoadjuvant regimens. Furthermore, the review emphasizes the need for a multidisciplinary approach to navigate the comp-lexities of CRLM. Integrating technical expertise and biological insights is crucial in refining neoadjuvant strategies. The management of progression following neoadjuvant chemotherapy requires a tailored approach, acknowledging the diverse biological behaviors that may emerge. In conclusion, this review aims to provide a comprehensive perspective on the considerations, challenges, and advancements in the use of neoadjuvant chemotherapy for initially resectable CRLM. By combining evidence-

based insights with practical experiences, we aspire to contribute to the ongoing discourse on refining treatment paradigms for improved outcomes in patients with CRLM.

Key Words: Neoadjuvant therapy; Colorectal cancer liver metastasis; Multidisciplinary teams; Chemotherapeutic regimens; Resectability criteria

Core Tip: Optimizing treatment for colorectal cancer liver metastasis (CRLM) is essential. This review explores the dynamic landscape of preoperative neoadjuvant chemotherapy for initially resectable CRLM, addressing debates, criteria for patient selection, and the role of gene testing. Emphasizing a multidisciplinary approach, it navigates complexities in managing progression post-chemotherapy, contributing to ongoing discussions on refining treatment paradigms for improved outcomes.

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INTRODUCTION

Colorectal cancer liver metastasis (CRLM), identified in approximately 25% of cases at the primary diagnosis, significantly contributes to both the incidence and mortality rates of colorectal cancer (CRC)[1]. The prognosis for CRC patients with liver metastasis remains challenging, with a 5-year survival rate of less than 10%[2]. Although the outcome of metastatic CRC varies, the surgical removal of isolated CRC liver metastases holds the potential for curative effects. Recent studies have shown an upward trend in survival rates following hepatic resection for metastatic CRC, indicating improved prognoses[3]. Notably, for initially resectable CRLM patients, perioperative chemotherapy combined with surgery has been explored, emphasizing outcomes such as progression-free survival (PFS) and overall survival (OS)[4].

Despite surgery being the cornerstone for treating initially resectable CRLM, a considerable number of patients experience recurrence within a year post-surgery, highlighting the limitations of surgery alone[5]. This prompts the consideration of alternative treatment modalities, including direct surgery followed by adjuvant chemotherapy and neoadjuvant therapy followed by surgery and postoperative adjuvant chemotherapy.

The implementation of neoadjuvant chemotherapy as part of the treatment strategy offers distinct advantages and poses challenges. On the positive side, it enables the early control of microscopic metastatic lesions, facilitates the assessment of tumor response to chemotherapy, and provides patients with a "biological waiting window" to potentially avoid surgery in cases of early progression[6-9]. However, neoadjuvant chemotherapy introduces disadvantages such as the risk of chemotherapy-induced liver damage, including sinusoidal injury from oxaliplatin and irinotecan-induced steatohepatitis, thereby elevating the risk of surgical complications and mortality[10,11]. Furthermore, the disappearance of imaging lesions post-chemotherapy presents a challenge in determining the extent of surgical resection[12,13].

Thus, neoadjuvant chemotherapy acts as a double-edged sword, emphasizing the paramount importance of clinically identifying patients suitable for neoadjuvant chemotherapy *vs* direct surgery. The primary challenge faced by neoadjuvant treatment lies in the difficulty of patient selection, stemming from the vague definition of resectability and limited understanding of risk factors for recurrence in metastatic CRC, including biological behavior and inadequate assessment.

REFINEMENT OF TERMINOLOGY IN THE CONTEXT OF RESECTABILITY CRITERIA FOR CRLM

In clinical practice, the central question of "resectability" has traditionally focused on technical aspects. The criteria for deeming a case resectable have centered on the ability to achieve R0 resection for all liver lesions while ensuring an adequate residual liver volume[14]. However, this technical definition introduces inherent subjectivity, influenced by factors such as varying clinical volumes across different centers and the diverse technical prowess of hepatobiliary surgeons. Moreover, not all technically resectable liver metastases translate into meaningful postoperative benefits, with up to 80% of patients experiencing recurrence within three years post-resection[15-18]. This underscores the critical need for a more nuanced understanding and strategic planning of perioperative treatments to effectively mitigate the often-devastating consequences of postoperative recurrence.

Recognizing the limitations of a purely technical definition, the European Society for Medical Oncology (ESMO) guidelines advocate for a comprehensive evaluation of initially resectable CRLM that integrates both "surgical technical standards" and "oncological prognostic factors"[19]. The surgical technical standards are further categorized into "easily

resectable" and "difficult to resect," while oncological prognostic information is classified into "excellent," "good," and "poor" prognostic factors[19,20]. This dual-dimensional approach acknowledges the inherent complexity of assessing resectability, recognizing that successful surgery extends beyond technical feasibility to crucial oncological factors that significantly influence prognosis.

In 1999, Fong *et al*[21] introduced the Clinical risk score (CRS) to assess the risk of postoperative recurrence in CRLM patients[21]. This scoring system incorporates five clinical indicators, providing a valuable tool for identifying patients at high risk of recurrence[21]. However, despite its widespread use, there is a current gap in the literature regarding whether neoadjuvant treatment can improve outcomes for high-risk patients identified by CRS[22,23].

Addressing the biological dimension, Guinney *et al*[24] made a significant stride in understanding the molecular landscape of CRC with the introduction of Consensus molecular subtypes (CMS) in 2015[24]. This classification, encompassing four molecular subtypes, offers a more nuanced view of the disease[24]. However, the application of CMS in identifying beneficiaries of neoadjuvant treatment remains unexplored to date[25-28]. Bridging this gap in research is imperative for a comprehensive understanding of the molecular intricacies influencing treatment responses in CRC.

In recent years, advancements in prognostic assessment systems have been prompted by our evolving understanding of the molecular biology of tumors and treatment paradigms for CRLM. Notably, Brudvik *et al*[29] introduced the modified clinical score (m-CS) in 2017, an enhanced system incorporating RAS gene status, size of liver metastases, and lymph node status of the primary tumor[29]. While representing progress in simplification compared to previous scores, the m-CS lacks granularity in weighing diverse high-risk factors and fails to account for chemotherapy sensitivity. Wang *et al*'s study[30] underscores the significance of neoadjuvant chemotherapy insensitivity, CRS > 2 points, and KRAS mutations as independent prognostic indicators, providing valuable insights for refining prognostic tools[30]. Utilizing a score-based prediction model, their findings present a concise yet effective means of predicting long-term survival postoperatively.

Moreover, a new frontier in prognostic assessment emerged in 2018 with the introduction of the tumor burden score (TBS) by Sasaki *et al*[31] Utilizing tumor size and number, TBS categorizes patients into low, intermediate, and high-risk groups[31]. External validation by Tsilimigras *et al*[32] showcased TBS's superior discriminatory ability compared to the widely used CRS[32]. The subsequent integration of genetic and morphological factors in the Genetic and morphological evaluation (GAME) score by Margonis *et al*[33] represents a notable step forward[33]. Though GAME scores outperformed CRS in external validation by Wang *et al*[34], a potential limitation lies in the exclusion of chemotherapy as an evaluation parameter. Nevertheless, this innovative integration of genetic and morphological factors in the GAME score enhances its prognostic utility in CRLM, urging further exploration of its practical implications.

The comprehensive evaluation of relapse risk (CERR) score emerges as a robust prognostic system, integrating the refined TBS (mTBS) model. Building upon TBS, the mTBS introduces parameters accounting for unilateral or bilateral metastases, addressing inherent limitations in TBS calculations. Chen *et al*[35] crafted the CERR score, considering factors such as KRAS/NRAS/BRAF mutation status, primary tumor lymph node involvement, presence of extrahepatic disease, and elevated carcinoembryonic antigen or carbohydrate antigen-199 Levels[35]. These elements, combined with mTBS, provide a CERR. Stratifying patients into high-risk (CERR score ≥ 4), intermediate-risk (2 ≤ CERR score ≤ 3), and low-risk groups (CERR score < 2), the CERR score exhibits superior discriminatory ability over CRS and GAME scoring systems [35]. This nuanced approach enhances our understanding of tumor biological behavior, aligning with both clinical and molecular perspectives. However, while the CERR score represents a comprehensive tool, its mathematical complexity and abstract metrics pose challenges for widespread clinical application.

Recent years have witnessed significant strides in the integration of artificial intelligence (AI) in medical practice, presenting a promising avenue for predicting patient prognosis following CRLM resection. Chakedis *et al*[36] pioneered a machine learning-based model that, through Bootstrap resampling and multifactorial logistic regression analysis, demonstrated remarkable accuracy in predicting recurrence risk[36]. Compared to the conventional CRS, the model exhibited a substantial increase in discriminative ability, showcasing its potential to predict postoperative recurrence. However, it is crucial to acknowledge the inherent limitations of machine learning, including the risk of model overfitting and the black-box nature of the model. These factors hinder its seamless integration into clinical practice, demanding careful consideration of its application.

In conclusion, the guidance of Multidisciplinary Teams (MDT) remains pivotal for stratified management based on patient-specific and tumor-specific factors. Adhering to diagnostic and therapeutic guidelines, respecting individual patient features, and aligning with established principles are crucial for optimizing patient care strategies and maximizing survival benefits. This comprehensive and personalized approach, incorporating the latest advancements in preoperative neoadjuvant therapies for initially resectable CRLM, underscores our commitment to achieving the highest standards in patient care. Future research should delve into refining prognostic models, addressing their limitations, and exploring innovative applications of AI in enhancing precision medicine for CRC patients. Refer to Table 1 for a detailed overview of biological behavior assessment systems, their advantages, and limitations.

THE CRUCIAL ROLE OF MDT IN CUSTOMIZING INDIVIDUALIZED APPROACHES FOR PATIENTS WITH INITIALLY RESECTABLE CRLM

The engagement of MDT in tailoring individualized approaches for patients with initially resectable CRLM stands as a cornerstone in contemporary oncology[37]. Placing patient-centered care at the forefront, this approach involves the collaboration of a diverse team of qualified medical professionals. Ideally, the MDT should encompass experts in colorectal surgery, gastrointestinal surgery, hepatic surgery, medical oncology, radiation oncology, interventional

Table 1 Overview of biological behavior assessment systems for colorectal cancer liver metastasis

Evaluation System	Components included	Advantages	Limitations	Ref.
CRS	Five clinical indicators	Widely used; identifies high-risk patients	Limited evidence on improving outcomes for high-risk CRS patients	[21-23]
CMS	Molecular classification into 4 subtypes	Offers nuanced view of disease	Application in identifying neoadjuvant treatment beneficiaries remains unexplored	[24]
m-CS	RAS gene status, size of liver metastases, lymph node status of the primary tumor	Enhanced system compared to previous scores	Lacks granularity in weighing diverse high-risk factors and does not account for chemotherapy sensitivity	[29, 30]
TBS	Categorizes patients into low, intermediate, and high-risk groups based on tumor size and number	Superior discriminatory ability	Limited by excluding chemotherapy as an evaluation parameter	[31]
GAME	Genetic and morphological factors	Outperformed CRS in external validation	Potential limitation in excluding chemotherapy as an evaluation parameter	[33, 34]
CERR	Integrates mTBS with additional parameters	CERR	Mathematical complexity and abstract metrics pose challenges for widespread clinical application	[35]
AI model	Machine learning-based model predicting recurrence risks	Remarkable accuracy in predicting recurrence risk	Inherent limitations include model overfitting and the black-box nature, hindering seamless integration into clinical practice	[36]

CRS: Clinical risk score; CMS: Consensus molecular subtypes; m-CS: Modified clinical score; mTBS: Refined tumor burden score; GAME: Genetic and morphological evaluation; CERR: Comprehensive evaluation of relapse risk; AI: Artificial intelligence.

radiology, radiology, ultrasound imaging, pathology, and other pertinent specialties. This collaborative and comprehensive team ensures a holistic evaluation of each patient, allowing for a well-rounded and specialized treatment plan that considers the intricacies of CRLM from various medical perspectives[38].

The management of CRLM has progressively incorporated MDTs for comprehensive patient care[39]. Extensive evidence supports the impact of MDT processes on patient survival, demonstrating improved OS under MDT care[40, 41]. Notably, a Chinese study reported that MDT discussions contributed to prolonged OS in patients with advanced gastrointestinal cancers, including CRC[42]. Additionally, preoperative MDT assessment has shown associations with enhanced survival in patients with locally advanced colon cancer[43].

The significance of MDTs in managing CRLM has been underscored by its substantial impact on patient outcomes and treatment strategies. A retrospective study by Milana *et al*[44] demonstrated the effects of MDT on CRC patients with liver metastases, highlighting the potential benefits of a multidisciplinary approach in tailoring individualized treatment strategies[44]. The study identified patients undergoing liver resection or simultaneous resection for primary CRC and liver metastases with curative intent, emphasizing the role of MDT in optimizing patient care and outcomes[44]. Additionally, a review illustrated the advantages of a multidisciplinary team approach, particularly in treating patients with CRLM. The review emphasized the significance of MDTs in developing tailored treatment plans and optimizing patient management, stressing the importance of a collaborative and comprehensive approach to address the complexities of CRLM[45]. Moreover, Cheng *et al*[46] highlighted the importance of a multidisciplinary treatment plan in achieving successful resection of colorectal liver metastases and intrahepatic cholangiocarcinoma, further emphasizing the role of MDTs in guiding complex treatment decisions[46].

In conclusion, the engagement of MDTs in customizing individualized approaches for patients with initially resectable CRLM is pivotal for optimizing patient care, treatment strategies, and outcomes. The collaborative nature of MDTs allows for comprehensive assessments, personalized treatment plans, and the integration of diverse expertise, ultimately contributing to improved patient management and prognosis.

INDICATIONS FOR NEOADJUVANT CHEMOTHERAPY IN CRLM

In recent years, the management of CRLM has witnessed significant advancements, prompting a nuanced consideration of neoadjuvant chemotherapy. The National Comprehensive Cancer Network guidelines, starting from the 2009 edition, suggest that patients with resectable CRLM and fewer adverse prognostic factors may benefit more from direct surgery. Conversely, those with borderline resectability might find neoadjuvant chemotherapy more suitable[28,47,48]. Notably, for patients who have not undergone chemotherapy or have been chemotherapy-free for the past 12 months, the clinical benefits of neoadjuvant chemotherapy may be more significant[49,50].

The 2009 European expert consensus proposes specific recommendations, advocating neoadjuvant chemotherapy followed by surgical resection for CRLM patients with a CRS of ≥ 2[51]. The 2012 edition of the ESMO guidelines refines these recommendations[52]. For initially resectable CRLM with a single lesion and a diameter < 2 cm, direct surgery is recommended, while other scenarios favor preoperative neoadjuvant mFOLFOX6 chemotherapy[52].

The 2016 ESMO guidelines introduce a comprehensive approach, considering both surgical complexity and tumor biology. Patients with a poor prognosis or challenging resections are recommended for preoperative neoadjuvant chemotherapy, while those with technically feasible and favorable prognosis CRLM are advised to undergo direct surgery[19]. The 2023 ESMO guidelines highlight careful consideration for patients with small metastatic lesions (10-15 mm), which may disappear after systemic treatment[53]. In such cases, neoadjuvant chemotherapy, if indicated, should not exceed two months. Alternatively, under MDT discussion, early surgery or other local treatment methods for small lesions, such as percutaneous ablative therapy, may be considered[53].

In summary, for initially R0 resectable or locally treatable CRLM, adopting the ESMO guidelines in clinical practice is recommended. While the ESMO guidelines do not provide specific criteria for classifying prognosis as good, moderate, or poor, the CRS is generally regarded as the gold standard. Patients with scores 0-2 are considered to have a 'good prognosis,' whereas those with a score of 3 or above are classified as having a 'poor prognosis.' The more adverse prognostic factors in CRLM, the more neoadjuvant chemotherapy is recommended.

SELECTION OF NEOADJUVANT CHEMOTHERAPEUTIC REGIMENS FOR CRLM

In the landscape of neoadjuvant chemotherapy for initially resectable CRLM, the gold standard, as established by randomized controlled trials, remains the FOLFOX regimen, a cornerstone reaffirmed by the EPOC study[54,55]. FOLFOX's prominence persists as the standard approach, and its equivalency with oxaliplatin in combination with capecitabine (CAPOX) in palliative treatment has seamlessly integrated CAPOX into clinical neoadjuvant chemotherapy practices. However, regimens containing irinotecan are generally cautioned against for neoadjuvant use, unless necessitated by a patient's history of oxaliplatin-based adjuvant chemotherapy and subsequent development of liver metastasis within one year of completing treatment. The complexity of patient selection criteria poses a challenge, impacting the outcomes of clinical studies focused on neoadjuvant treatment for CRLM. Notable among these studies are EORTC-40983, COMET, and NEW EPOC.

The EORTC-40983 trial, initiated in 2008, delved into the perioperative use of the FOLFOX4 regimen (6 cycles preoperatively + 6 cycles postoperatively) for CRLM. Results indicated enhanced PFS with perioperative chemotherapy (median PFS 18.7 *vs* 11.7 months), yet failed to demonstrate conclusive OS benefits [median OS (mOS) 61.3 *vs* 54.3 months, $P > 0.05$]. The study's broad categorization without distinguishing between synchronous and metachronous liver metastases, combined with generally low overall tumor burden, introduces complexities in assessing neoadjuvant benefits[4,56,57]. The COMET trial, a phase II study exploring neoadjuvant chemotherapy and targeted therapy's impact on PFS for initially resectable CRLM, administered 6 cycles of FOLFOX or 4 cycles of XELOX preoperatively[58]. The addition of cetuximab or bevacizumab was contingent on KRAS status. While the study demonstrated a median PFS of 22.5 months for wild-type KRAS and 10.5 months for mutant KRAS, showcasing modest improvement compared to EORTC-40983, its efficacy was influenced by a significant proportion of low-risk patients, with around half having a single liver metastasis, potentially diluting the overall neoadjuvant treatment impact[59]. The NEW EPOC trial aimed to evaluate the effectiveness of adding cetuximab to chemotherapy in the neoadjuvant treatment of KRAS exon 2 wild-type CRLM. The combination group exhibited a higher proportion of synchronous liver metastases (53% *vs* 47%), larger metastatic volumes, and worse prognosis-related indicators (14% poorly differentiated *vs* 10%)[55,60]. However, statistical *P*-values were not provided. While the neoadjuvant treatment effective rate (Complete Response + Partial Response) was higher in both groups than COMET, it lacked statistical significance (70% for the combination group, 62% for chemotherapy alone, $P > 0.05$). Intriguingly, the combination group showed significantly shorter PFS than the chemotherapy-alone group (14.1 *vs* 20.5 months, $P = 0.030$). Long-term follow-up even suggested that neoadjuvant treatment with combined targeted therapy could shorten OS (mOS for the combination group 55.4 months *vs* chemotherapy alone 81 months, Hazard Ratio 1.45, $P = 0.036$). Further stratified analysis indicated that for patients with favorable prognostic factors, neoadjuvant combination targeted therapy did not confer a survival benefit. The unexpected reduction in survival with combined targeted therapy remains unclear, possibly influenced by significant differences in postoperative adjuvant treatment between the two groups[55,60].

In a retrospective analysis, Hao *et al*[61] evaluated 3038 cases of resectable colon cancer with single-organ metastases (either in the liver or lung). They compared patients who underwent neoadjuvant treatment (either preoperative or perioperative) with those who received postoperative adjuvant treatment alone. The neoadjuvant treatment group showed a substantial extension in mOS, reaching 47.24 months, compared to 38.08 months in the postoperative adjuvant treatment alone group[61,62]. Chakrabarti *et al*[63] retrospectively analyzed seven cases of Microsatellite Instability-High (MSI-H) non-metastatic digestive tract tumors treated with neoadjuvant immunotherapy at their center. Among three cases of CRC, two achieved a pathological Complete Response (pCR), showcasing promising results[63]. However, given the small sample size and the absence of M1 samples, the generalizability of these findings awaits confirmation in larger studies. This study raises parallels with a 2022 The New England Journal of Medicine publication reporting a 100% complete clinical response in 12 cases of stage II-III dMMR rectal cancer treated with single-agent programmed cell death 1 (PD-1) (dostarlimab) in the neoadjuvant setting[64]. This suggests that immunotherapy, especially for dMMR patients, might be considered earlier in the treatment timeline. Xiao *et al*[65] conducted an analysis on 10 cases of pMMR/Microsatellite Stable (MSS) CRLM subjected to neoadjuvant treatment involving PD-1, bevacizumab, and chemotherapy. The findings revealed a noteworthy pCR in one case, accompanied by a Disease Control Rate of 100% and an Overall Response Rate (ORR) of 62.5%[65]. Comparing these outcomes with the ORR observed in the COMET study, the addition of PD-1 appears to augment the ORR, closely approaching the results of NEW EPOC[55,58,60]. However, considering the NEW EPOC study demonstrated a 70% ORR with a regimen involving eight agents plus chemotherapy, the distinct

contribution of PD-1 remains uncertain. This observation is reminiscent of a study in 2021, which explored neoadjuvant dual immunotherapy for CRLM[66]. In this study, 23 CRLM patients underwent preoperative treatment with a combination of CTLA-4 and durvalumab, followed by postoperative monotherapy involving four administrations of durvalumab. Among the four patients achieving pCR, two had pMMR tumors. This suggests that, analogous to liver cancer, differentiating between MSI-H or MSS may not be imperative for CRLM, and both subtypes could potentially derive benefits from immunotherapy[66]. Alternatively, there might be unidentified factors influencing the efficacy of immunotherapy in these specific patients.

Several ongoing studies (https://clinicaltrials.gov/) are currently investigating various neoadjuvant treatments for CRLM, encompassing different regimens such as FOLFIRINOX (NCT03487939, NCT05362825), combined immunotherapy (NCT03844750, NCT05359393), post-neoadjuvant local treatment for recurrent liver metastases (NCT05861505), and neoadjuvant treatment combined with VEGFR (NCT00659022, NCT01508000, NCT01646554).

In summary, the ORR for neoadjuvant treatment currently falls within the 50%-70% range, with traditional chemotherapy drugs being the predominant agents. However, there is a notable lack of robust clinical evidence regarding the addition of targeted therapies, and the experience with combining immunotherapeutic agents is even scarcer. The investigation of neoadjuvant treatments for CRLM is advancing by refining patient selection criteria beyond the CRS and incorporating factors such as CMS and genetic status. Moreover, there is a growing emphasis on exploring extensive combinations of drugs, including immunotherapy, targeted therapy, and differentiating between MSS and MSI-H, as well as the exploration of dual immunotherapy.

MANAGEMENT STRATEGIES FOR PROGRESSION IN HEPATIC METASTASES DURING NEOADJUVANT THERAPY

The management of CRLM following neoadjuvant therapy and subsequent progression poses a complex therapeutic challenge. Studies indicate that hepatectomy significantly improves OS in patients with progressive disease (PD) compared to continuing chemotherapy, especially when considering a balanced patient profile[67]. However, in patients undergoing extensive liver resection, survival analysis reveals that PFS and OS are influenced by independent prognostic factors. The acceptance of second-line chemotherapy is not an independent predictor of patient survival. This observation suggests that patients undergoing extensive liver resection, often burdened with a larger tumor load and high CRS, may not derive substantial benefits from second-line chemotherapy. Therefore, timely surgical intervention is advisable for eligible patients to achieve a disease-free state[67].

Comparing patients who achieved complete or partial response to second-line chemotherapy with those undergoing direct surgery, no significant advantage in terms of PFS and OS is evident for effective responders[68]. This implies that the efficacy of second-line chemotherapy has limited impact on the long-term outcomes of initially resectable patients, particularly those who do not achieve objective relief from neoadjuvant therapy. For patients experiencing PD after neoadjuvant treatment, the consideration of liver resection becomes pivotal. Observational studies indicate a significant improvement in OS for PD patients undergoing liver resection, particularly when patient characteristics are meticulously balanced[67]. In the context of patients progressing after first-line chemotherapy and experiencing PD following neoadjuvant treatment, the continuation of chemotherapy may diminish survival rates. Therefore, prompt consideration of surgical options is crucial to avoid unnecessary adverse effects of prolonged chemotherapy.

A detailed analysis of the type of progression during neoadjuvant therapy, including numeric, dimensional, and biological changes, aids in determining the suitability for surgery[69]. Tumor markers hold potential value in assessing treatment response and disease progression. Monitoring protein levels in blood samples, especially during progression, enhances the understanding of the patient's disease biology[70]. When formulating individualized treatment strategies, factors such as the patient's overall condition, treatment sensitivity, and biological characteristics should be considered. Personalized treatment plans may involve surgery, targeted therapy, or more potent chemotherapy regimens. This underscores the importance of a comprehensive approach, considering multiple factors, to optimize treatment strategies when dealing with progression after neoadjuvant therapy for CRLM.

CONCLUSION

With an increasingly comprehensive and precise definition of 'initially resectable', the significance of neoadjuvant therapy has gained recognition. Combining patient-specific factors and the tumor's intrinsic characteristics, a stratified management model has emerged under the guidance of MDT (Figure 1). This model emphasizes surgical intervention as the primary approach, with perioperative systemic therapy serving as auxiliary support. It facilitates more precise treatment decisions for initially resectable CRLM. Neoadjuvant therapy, tailored to individual differences, offers varying intensities of regimens.

For patients with adverse prognostic factors like a high CRS, GAME risk scores, initial treatment insensitivity, or elevated ctDNA levels, exploring two-drug combination targeted therapies or more potent three-drug chemotherapy regimens may optimize survival benefits. The adjustment of specific regimens and efficacy predictions is increasingly guided by gene testing (such as RAS/BRAF, PIK3CA), now recommended in major guidelines. Simultaneously, the promising biomarker ctDNA awaits further validation through advanced-level evidence-based research as a prospective indicator for treatment response. Future research directions should focus on validating and refining these approaches to

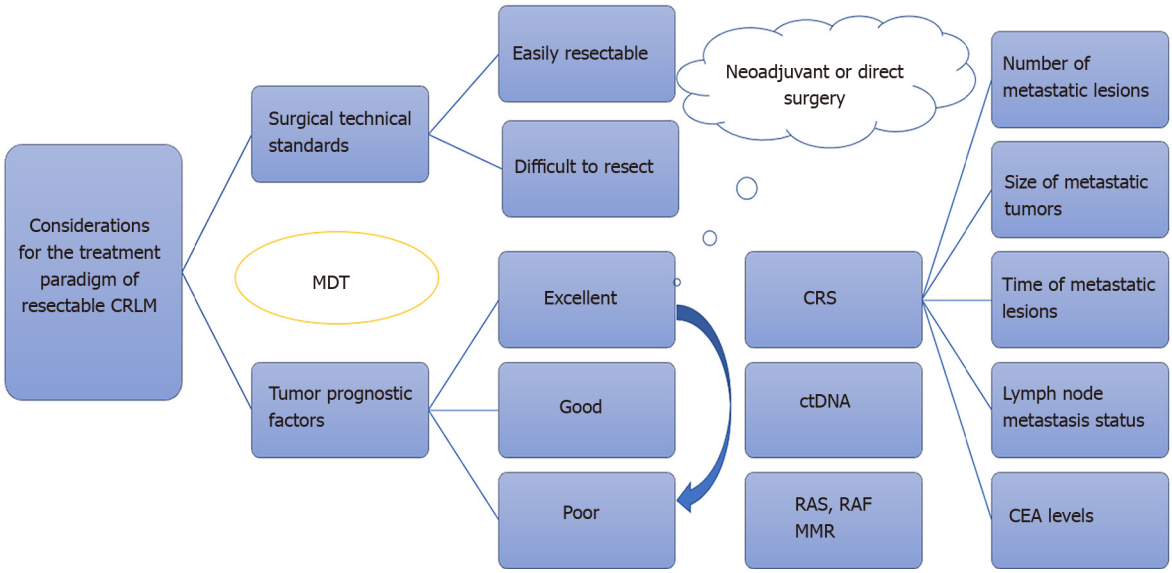

Figure 1 Key Considerations for the paradigm of neoadjuvant or direct surgery in resectable colorectal cancer liver metastasis. The figure outlines factors such as surgical standards, technical considerations, tumor characteristics, prognostic factors, and key markers influencing treatment decisions. MDT: Multidisciplinary team; CRS: Clinical risk score; CEA: Carcinoembryonic antigen; MMR: Mismatch repair; ctDNA: Circulating tumor DNA.

enhance the precision and effectiveness of neoadjuvant therapy in the management of initially resectable CRLM.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Endoscopic features and treatments of gastric cystica profunda

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Abstract

BACKGROUND

Gastric cystica profunda (GCP) represents a rare condition characterized by cystic dilation of gastric glands within the mucosal and/or submucosal layers. GCP is often linked to, or may progress into, early gastric cancer (EGC).

AIM

To provide a comprehensive evaluation of the endoscopic features of GCP while assessing the efficacy of endoscopic treatment, thereby offering guidance for diagnosis and treatment.

METHODS

This retrospective study involved 104 patients with GCP who underwent endoscopic resection. Alongside demographic and clinical data, regular patient follow-ups were conducted to assess local recurrence.

RESULTS

Among the 104 patients diagnosed with GCP who underwent endoscopic resection, 12.5% had a history of previous gastric procedures. The primary site predominantly affected was the cardia (38.5%, n = 40). GCP commonly exhibited intraluminal growth (99%), regular presentation (74.0%), and ulcerative mucosa (61.5%). The leading endoscopic feature was the mucosal lesion type (59.6%, n = 62). The average maximum diameter was 20.9 ± 15.3 mm, with mucosal involvement in 60.6% (n = 63). Procedures lasted 73.9 ± 57.5 min, achieving complete resection in 91.3% (n = 95). Recurrence (4.8%) was managed *via* either surgical intervention (n = 1) or through endoscopic resection (n = 4). Final pathology confirmed that 59.6% of GCP cases were associated with EGC. Univariate analysis indicated that elderly males were more susceptible to GCP associated with EGC. Conversely, multivariate analysis identified lesion morphology and endoscopic features as significant risk factors. Survival analysis demonstrated no statistically significant difference in recurrence between GCP

with and without EGC (P = 0.72).

CONCLUSION
The findings suggested that endoscopic resection might serve as an effective and minimally invasive treatment for GCP with or without EGC.

Key Words: Gastric cystica profunda; Early gastric cancer; Endoscopic features; Endoscopic resection; Endoscopy

Core Tip: Gastric cystica profunda (GCP) associated early gastric cancer (EGC) was found to be relatively common. Irregular morphology and mucosal lesion type might be the risk factors for development of EGC in GCP. Endoscopic resection can be recommended as an effective and minimally invasive treatment for GCP with or without EGC.

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INTRODUCTION

Gastric cystica profunda (GCP) represents a rare gastric lesion characterized by hyperplasia of connective tissues within the interstitium of the glands, involving the submucosal layer and occasionally extending to the muscularis propria of the stomach[1]. Initially, GCP was believed to be an inflammatory pseudotumor associated with ischemia, chronic inflammation, and mucosal defects that may arise from surgical procedures, biopsies, or polypectomies[2]. Widespread chronic active or atrophic gastritis is considered a significant contributing factor to the development of GCP[3]. Over recent years, the emergence of advanced endoscopic techniques such as endoscopic ultrasonography (EUS) and endoscopic resection has led to a gradual increase in the detection of non-surgically resected GCP cases.

Patients with GCP may either remain asymptomatic or present with non-specific digestive symptoms, including abdominal pain and belching[4]. Owing to the unremarkable clinical characteristics and nonspecific endoscopic manifestations, most clinicians possess limited understanding of GCP. Furthermore, GCP has been regarded as a potential premalignant lesion[5]; hence, the endoscopic diagnosis and early excision of GCP are deemed crucial[6,7]. In this study, we conducted a retrospective analysis of 104 cases of GCP treated by endoscopic resection at our center from October 2011 to December 2022. Our analysis was based on their clinical manifestations, endoscopic findings, pathological results, and treatments. The primary objectives were to delineate the endoscopic features of GCP associated with early gastric cancer (EGC) and to assess the impact of endoscopic resection on the diagnosis and treatment of GCP with EGC.

MATERIALS AND METHODS

Patients

We conducted a single-center retrospective study involving 104 consecutive patients diagnosed with GCP who underwent endoscopic resection at Zhongshan Hospital, Fudan University (Shanghai, China) between October 2011 and December 2022. Only patients with complete demographic and clinical information, along with available follow-up data, were included in the study. Patients were assessed based on findings from endoscopy, computed tomography (CT) scans, or EUS during the preoperative phase. All patients with suspected GCP following endoscopic examination underwent biopsy for pathological confirmation. Lesion characteristics, endoscopic methods, complications, *en-bloc* resection rate, complete resection rate, and the occurrence of local recurrence were evaluated for all patients. This study was approved by the Ethics Committee of Zhongshan Hospital in accordance with the Declaration of Helsinki (B2021-029), and written consent was obtained from all participating patients.

Lesion classification and pathological examination

In this study, lesions were categorized into four types: Mucosal lesion type, polypoid type, submucosal lesion type, and thickened mucosa with rough wrinkles type (Figure 1A-D). According to the pathological diagnostic criteria for GCP, the presence of cystic structure expansion within the mucosal muscle layer and submucosal layer could confirm the diagnosis [8]. Building upon this criterion, the presence of cancerous changes in the gastric mucosal glands, with the lesion tissue confined to the mucosal and submucosal layers, led to a diagnosis of GCP with EGC (Figure 2). Each case was independently reevaluated by two experienced pathologists in a blinded manner, without access to clinical or endoscopic information.

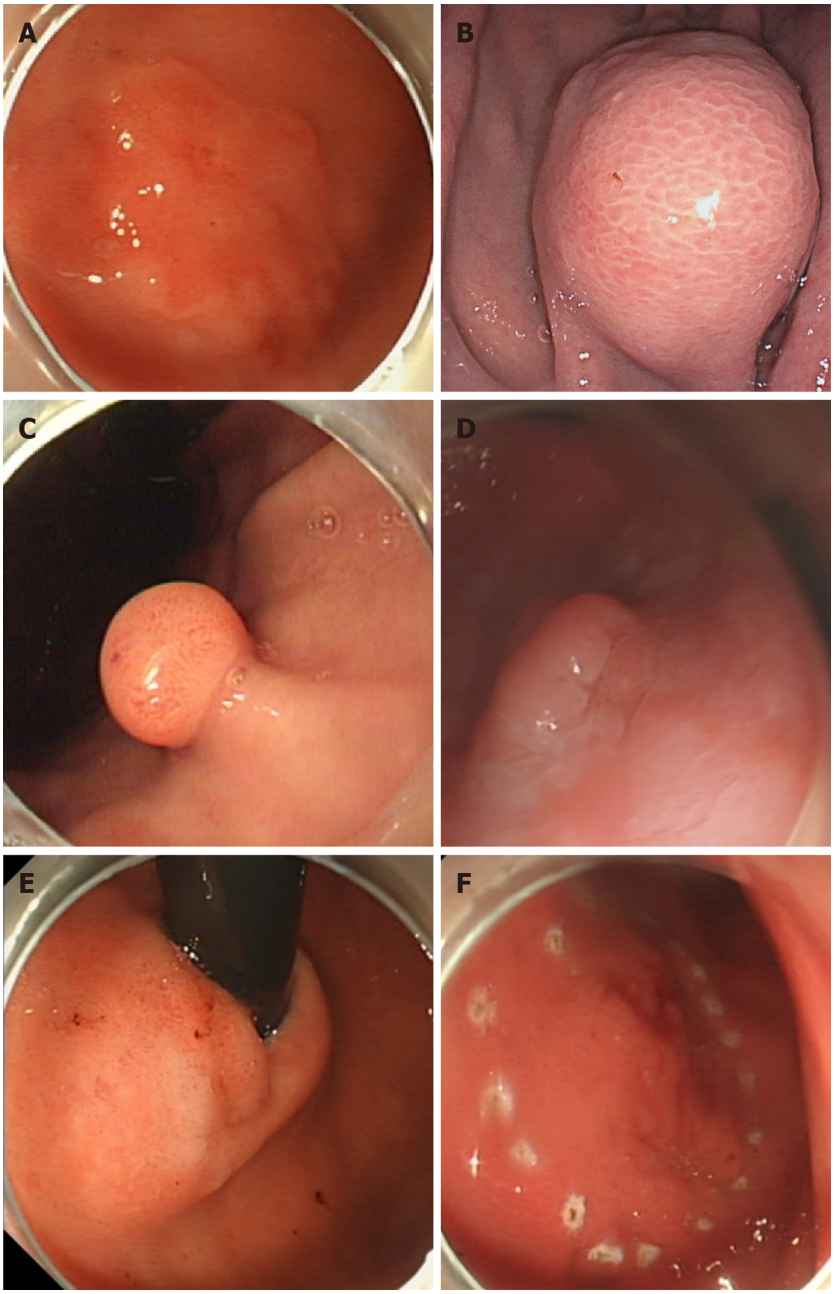

Figure 1 Classification of gastric cystica profunda lesions. A: Mucosal lesion type; B: Submucosal lesion type; C: Polypoid type; D: Thickened mucosa with rough wrinkles type; E and F: Irregular mucosal lesion type in gastric cystica profunda.

Moreover, irregular shapes of GCP primarily encompassed three types: Mucosal lesion type, polypoid type, and submucosal lesion type. The irregular mucosal lesion type manifested as uneven surfaces with raised and depressed areas, often accompanied by surface erosion or ulcers. Irregular polypoid type GCP referred to type III and IV polyps in the Yamada classification[9]. As for the irregularity of the submucosal lesion type, it mainly denoted an irregular shape, presenting as lobulated or branching[10].

Endoscopic resection method and outcome assessments

The choice of endoscopic resection for GCP depended on the appearance during endoscopy. If it appeared as a mucosal lesion, submucosal tumor, or thickened and folded mucosa, then endoscopic submucosal dissection (ESD) would be employed. During ESD, operators cut the mucosa, dissected the submucosal layer, and subsequently removed the tumor after locating the lesions. If it appeared to be polyp-like and raised, then endoscopic mucosal resection (EMR) or electric cutting would be performed.

Following endoscopic resection, a nasogastric tube was inserted to both decompress and monitor potential delayed bleeding from the wound. Additionally, we monitored postoperative symptoms. In cases where patients experienced persistent fever, hematemesis, melena, or pain, emergency endoscopy and CT scans were conducted. Moreover, proton pump inhibitors, antibiotics, and hemocoagulase injections were administered.

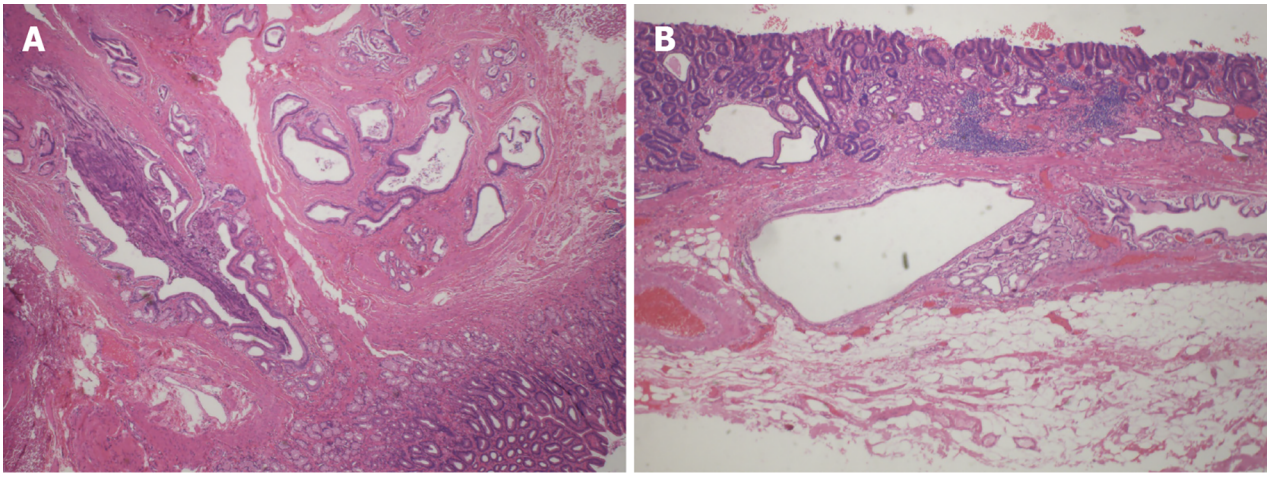

Figure 2 Pathological images of gastric cystica profunda and gastric cystica profunda with early gastric cancer. A: Gastric cystica profunda; B: Gastric cystica profunda with early gastric cancer.

Endoscopic outcome assessments included: (1) The duration of procedure and hospital stay; (2) *en-bloc* resection (the excision of the tumor was performed in one piece without fragmentation) and complete resection (based on *en-bloc* resection, the excision was performed in a manner that ensures the absence of discernible residual tumors upon macroscopic evaluation at the resection site, coupled with negative margins upon pathologic examination); and (3) complications and local recurrence.

Follow-up

Patients underwent regular follow-up for the assessment of wound healing and the detection of local recurrence through endoscopy at 6 months post-resection. In cases where patients experienced relapses, EUS and CT scans were conducted to check for recurrent lesions.

Statistical analysis

Continuous variables were presented as means and SD, while categorical variables were displayed as numbers and percentages. Statistical analysis was performed using SPSS 26.0 and R 4.0.2.

RESULTS

Clinical characteristics of the patients

A total of 104 consecutive patients, including 27 women and 77 men, with a mean age of 63.4 ± 11.0 years, were diagnosed with GCP and underwent endoscopic resection at Zhongshan Hospital, Fudan University in Shanghai, China. Among these patients, 12.5% had a history of prior gastric endoscopic or surgical treatment. The majority of patients were asymptomatic (n = 66, 63.5%), while 28 (26.9%) reported experiencing epigastric discomfort. Additionally, other symptoms such as regurgitation and melena were also observed (Table 1).

Characteristics of lesions

The most commonly involved sites were the cardia (n = 40, 38.5%), followed by the gastric body (n = 35, 33.7%), gastric antrum (n = 21, 20.2%), and gastric fundus (n = 8, 7.7%). Furthermore, 13 patients (12.5%) with GCP had a history of gastric endoscopic or surgical treatment. Among them, three patients had a history of gastrectomy, where GCP occurred specifically at the cardia, particularly at the anastomotic site. Additionally, ten patients with GCP had undergone previous gastric endoscopic procedures, and seven of these GCP cases (70%) were located at the sites of prior gastric endoscopic interventions.

It was observed that 99% of GCP cases manifested an intraluminal growth pattern. In terms of morphology, 74.0% of GCP presented as regular, while 61.5% exhibited an ulcerative mucosa. The most common endoscopic feature was the mucosal lesion type (n = 62, 59.6%), including IIa (n = 29), IIa+IIc (n = 4), and IIc (n = 29), followed by polypoid type (n = 23, 22.1%), submucosal lesion type (n = 17, 16.3%), and thickened mucosa with rough wrinkles type (n = 1, 1.0%). The maximum diameter ranged from 20.9 ± 15.3 mm. The mucosa was the most commonly involved layer (n = 63, 60.6%), followed by the submucosa (n = 40, 38.5%), and muscularis propria (n = 1, 1.0%; Table 1).

We conducted further comparisons of the endoscopic features between the regular (n = 77) and irregular (n = 27) lesions. We found that the irregular lesion group predominantly consisted of mucosal lesion type (n = 17, 63.0%), polypoid type (n = 4, 14.8%), and submucosal lesion type (n = 6, 22.2%; Supplementary Table 1).

Table 1 Demographic information, lesion characteristics, and procedural outcomes of early gastric cancer, *n* (%)

	GCP (*n* = 104)
Demographic information	
Male	77 (74.0)
Age (yr), mean ± SD	63.4 ± 11.0
History of gastric endoscopic or surgical treatment	13 (12.5)
Symptom	
Asymptomatic	66 (63.5)
Epigastric discomfort	28 (26.9)
Regurgitation	8 (7.7)
Melena	2 (1.9)
Lesion characteristics	
Growth pattern	
Intraluminal growth	103 (99.0)
Extraluminal growth	1 (1.0)
Morphology	
Regular	77 (74.0)
Irregular	27 (26.0)
Mucosa	
Smooth	40 (38.5)
Ulcerative	64 (61.5)
Max diameter (mm), mean ± SD	20.9 ± 15.3
Location	
Cardia	40 (38.5)
Gastric fundus	8 (7.7)
Gastric body	35 (33.7)
Gastric antrum	21 (20.2)
Endoscopic features	
Mucosal lesion type	62 (59.6)
IIa	29 (46.8)
IIa + IIc	4 (6.5)
IIc	29 (46.8)
Polypoid type	23 (22.1)
Submucosal lesion type	17 (16.3)
Thickened mucosa with rough wrinkles type	2 (1.9)
Infiltration depth	
Mucosa	63 (60.6)
Submucosa	40 (38.5)
Muscularis propria	1 (1.0)
GCP with EGC	62 (59.6)
Procedural outcomes	
Endoscopic methods	
Electric cutting	7 (6.7)

EMR	11 (10.6)
ESD	80 (76.9)
ESE	6 (5.8)
En-bloc resection	95 (91.3)
Complete resection	95 (91.3)
Suture method	
Unstitched	62 (59.6)
Metal clip	40 (38.5)
Nylon rope	1 (1.0)
Metal clip and nylon rope	1 (1.0)
Surgery time (min), mean ± SD	73.9 ± 57.5
Complications	1 (1.0)
Hospital stay (d), mean ± SD	3.4 ± 2.3
Additional surgery	1 (1.0)
Recurrence	5 (4.8)

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; ESE: Endoscopic submucosal excavation; GCP: Gastric cystica profunda; EGC: Early gastric cancer.

Endoscopic methods and outcomes

Endoscopic resection stands as the primary treatment for GCP. In this study, all 104 patients underwent endoscopic resection, including electric cutting (n = 7, 6.7%), EMR (n = 11, 10.6%), ESD (n = 80, 76.9%), and endoscopic submucosal excavation (n = 6, 5.8%). The suture methods employed included a metal clip (n = 40, 38.5%), nylon rope (n = 1, 1.0%), and a combination of a metal clip and nylon rope (n = 1, 1.0%). The average duration ranged from 73.9 ± 57.5 min. Overall, *en-bloc* resection was performed for 95 GCP cases (91.3%), and complete resection was achieved in 95 cases (91.3%; Table 1). Further analysis revealed no statistical difference in the rates of *en-bloc* and complete resection between irregular and regular GCP groups (Supplementary Table 1).

The average duration of hospital stay was 3.4 ± 2.3 d. One patient (1.0%) experienced delayed wound bleeding and required the use of a nylon rope to stop the bleeding. Another patient (1.0%) underwent additional surgery subsequent to endoscopic resection due to pathologic findings indicating invasion of gastric cancer into the submucosa. Recurrence was observed in five patients (4.8%). Among these cases, only one patient had undergone incomplete resection. Ultimately, one patient received treatment through surgery, while the remaining four underwent endoscopic resection (Table 1). Patients undergoing surgery received a pathological diagnosis of gastric cancer, whereas those undergoing endoscopic resection were all diagnosed with GCP without concomitant EGC.

Comparisons between GCP with EGC and GCP without EGC groups

According to the pathologic examination, 59.6% of patients were found to have concomitant EGC. Moreover, we observed significant differences in six variables (sex, age, morphology, mucosa, location, and endoscopic features) between the groups with GCP and those with GCP accompanied by EGC (Table 2). As mucosa and endoscopic features exhibited a significant correlation, the multivariate logistic regression considered five explanatory variables (sex, age, morphology, location, and endoscopic features). The analysis demonstrated that irregular morphology and mucosal lesion type were significant risk factors for GCP accompanied by EGC ($P < 0.05$; Table 3, Figure 1E and F). The sensitivity analysis depicted the variable importance of risk factors for GCP accompanied by EGC (as shown in Figure 3). Furthermore, survival analysis indicated no statistical difference in recurrence between the groups with GCP accompanied by EGC and those without EGC (P = 0.72; Figure 4).

DISCUSSION

Given the limited literature and reports on GCP, our research might hold significance in raising awareness of GCP as a high-risk factor for EGC. Clinical differentiation from conditions such as hypertrophic gastritis, mesenchymal tumors, gastric cancer, and ectopic pancreas is crucial. Due to GCP's malignant potential, prompt removal through endoscopy or surgery is essential, coupled with regular postoperative follow-up[11]. In this study, we delineated the endoscopic features of GCP and evaluated the impact of endoscopic resection on the diagnosis and treatment of GCP.

Table 2 Demographic information, lesion characteristics, and procedural outcomes of the early gastric cancer without early gastric cancer s and early gastric cancer with early gastric cancer s groups, *n* (%)

	GCP without EGCs (*n* = 42)	GCP with EGCs (*n* = 62)	*P* value
Demographic information			
Male	23 (54.8)	54 (87.1)	< 0.001
Age (yr), mean ± SD	58.5 ± 11.9	66.7 ± 9.1	< 0.001
History of gastric endoscopic or surgical treatment	4 (9.5)	9 (14.5)	0.450
Symptom			0.158
Asymptomatic	23 (54.8)	43 (69.4)	
Epigastric discomfort	15 (35.7)	13 (21.0)	
Regurgitation	4 (9.5)	4 (6.5)	
Melena	0 (0)	2 (3.2)	
Lesion characteristics			
Growth pattern			1.000
Intraluminal growth	42 (100)	61 (98.4)	
Extraluminal growth	0 (0)	1 (1.6)	
Morphology			0.007
Regular	37 (88.1)	40 (64.5)	
Irregular	5 (11.9)	22 (35.5)	
Mucosa			< 0.001
Smooth	27 (64.3)	13 (21.0)	
Ulcerative	15 (35.7)	49 (79.0)	
Max diameter (mm), mean ± SD	18.0 ± 14.4	22.9 ± 15.7	0.110
Location			0.003
Cardia	9 (21.4)	31 (50.0)	
Gastric fundus	7 (16.7)	1 (1.6)	
Gastric body	16 (38.1)	19 (30.6)	
Gastric antrum	10 (23.8)	11 (17.7)	
Endoscopic features			< 0.001
Mucosal lesion type	8 (19.0)	54 (87.1)	
IIa	6 (75.0)	23 (42.6)	
IIa + IIc	0 (0)	4 (7.4)	
IIc	2 (25.0)	27 (50.0)	
Polypoid type	19 (45.2)	4 (6.5)	
Submucosal lesion type	13 (31.0)	4 (6.5)	
Thickened mucosa with rough wrinkles type	2 (4.8)	0 (0)	
Infiltration depth			0.363
Mucosa	24 (57.1)	39 (62.9)	
Submucosa	17 (40.5)	23 (37.1)	
Muscularis propria	1 (2.4)	0 (0)	
Procedural outcomes			
Endoscopic methods			< 0.001
Electric cutting	7 (16.7)	0 (0)	

EMR	11 (26.2)	0 (0)	
ESD	18 (42.9)	62 (100)	
ESE	6 (14.3)	0 (0)	
En-bloc resection	38 (90.5)	57 (91.9)	1.000
Complete resection	38 (90.5)	57 (91.9)	1.000
Suture method			0.011
Unstitched	18 (42.9)	44 (71)	
Metal clip	23 (54.8)	17 (27.4)	
Nylon rope	1 (2.4)	0 (0)	
Metal clip and nylon rope	0 (0)	1 (1.6)	
Surgery time (min), mean ± SD	38.5 ± 38.6	96.6 ± 56.3	< 0.001
Complications	1 (2.4)	0 (0)	0.404
Hospital stay (d), mean ± SD	2.6 ± 1.8	3.9 ± 2.5	0.006
Additional surgery	0 (0)	1 (1.6)	1.000
Recurrence	2 (4.8)	3 (4.8)	1.000

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; ESE: Endoscopic submucosal excavation; GCP: Gastric cystica profunda; EGC: Early gastric cancer.

Table 3 Multivariate logistic regression analysis for gastric cystica profunda with early gastric cancers

Factors	Multivariate analysis		
	OR [95%CI]	β coefficient	*P* value
Location			
Cardia	1		
Non-cardia	0.881 [0.226-3.424]	-0.126	0.853
Sex			
Male	3.323 [0.771-14.764]	1.201	0.104
Female	1		
Morphology			
Regular	1		
Irregular	15.278 [2.965-111.712]	2.726	0.003
Endoscopic features			
Mucosal lesion type	1		
Non-mucosal lesion type	0.029 [0.006-0.108]	-3.531	< 0.001
Age	1.026 [0.968-1.090]	0.025	0.392

OR: Odds ratio.

Out of the five patients with GCP who experienced recurrence, only one had a recurrence at the original resection site. The remaining four recurrences occurred at sites distinct from the original resection site. Additionally, the patient who experienced a recurrence at the original site had multiple lesions and was unable to undergo *en-bloc* resection at that time. Hence, it can be inferred that ESD is effective for lesions necessitating *en-bloc* resection.

GCP is typically regarded as a benign lesion, yet it can serve as a precancerous gastric condition. Given that GCP is commonly associated with gastric adenocarcinoma or EGC, its malignant potential should be underscored. In our study, we noted that 59.6% of GCP cases were linked with EGC. Through multivariate and sensitivity analyses, irregular morphology and mucosal lesion type emerged as significant risk factors for GCP accompanied by EGC. The mucosal lesion type encompassed IIa (mucosal flat elevation), IIa+IIc (mucosal flat elevation with mild depression), and IIc (mild

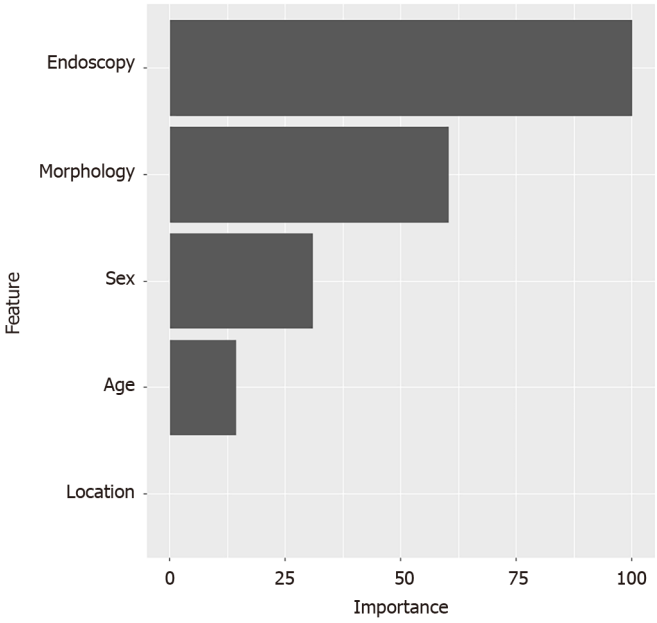

Figure 3 Significance of variable risk factors for gastric cystica profunda with early gastric cancer.

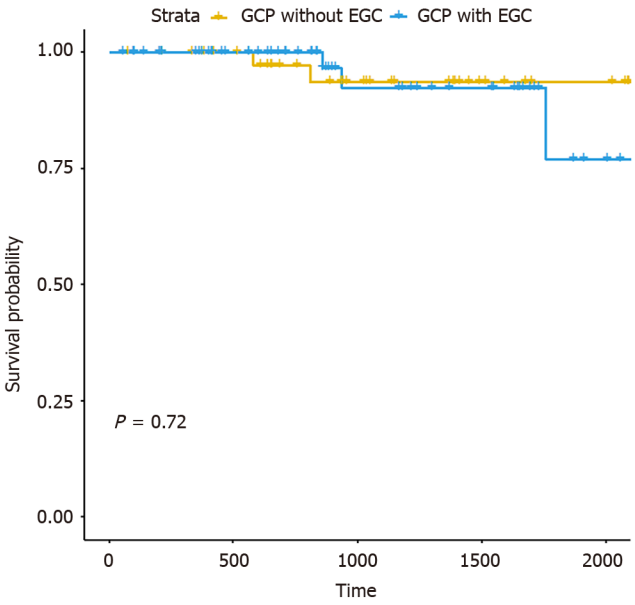

Figure 4 Survival analysis suggested that there was no statistical difference in recurrence between gastric cystica profunda groups with and without early gastric cancer (*P* = 0.72). GCP: Gastric cystica profunda; EGC: Early gastric cancer.

depression). Considering that EGC typically presents as mucosal lesions, it is evident that GCP featuring mucosal lesion types pose a heightened risk for EGC. An asymmetric expansion of glands in the mucosa and submucosa can lead to irregularities, resulting in the appearance of raised and depressed areas, often accompanied by erosion or ulcers. Consequently, the irregular morphology of GCP is deemed a high-risk factor for EGC. Whenever feasible, we recommend endoscopic resection for GCP, particularly when irregular morphology or mucosal lesion type is apparent, as this signifies a heightened risk of concurrent EGC.

The *en-bloc* resection and complete resection showed no difference between GCP with EGC and GCP without EGC groups. Additionally, there were no differences in complications, additional surgery, or recurrence between these two groups. These findings suggest that there is no disparity in the efficacy of endoscopic resection for GCP, regardless of the presence or absence of EGC. Therefore, similar to ESD for EGC with infiltration depth ≤ 500 μm, ESD emerges as a safe and effective minimally invasive treatment for GCP, irrespective of the presence of concurrent EGC.

To determine whether the irregular shape of GCP impacted *en-bloc* and complete resection rates, we compared the rates between groups with regular and irregular shapes. Our analysis revealed no statistically significant difference, suggesting that endoscopy can achieve *en-bloc* or complete resection even for GCPs with irregular shapes.

Despite the promising results, this study had certain limitations, including a small sample size and potential bias inherent in the retrospective design. Further research is imperative to gain a more comprehensive understanding of the natural progression of GCP and its malignant potential.

In summary, irregular shapes and mucosal lesion types observed during endoscopy might serve as high-risk factors for GCP with EGC. Future studies should aim to clarify the disease's natural progression and its malignant potential. Notably, ESD might be a secure and efficacious minimally invasive treatment, regardless of the presence of EGC.

CONCLUSION

The findings suggested that endoscopic resection might serve as an effective and minimally invasive treatment for GCP with or without EGC.

ARTICLE HIGHLIGHTS

Research background

Gastric cystica profunda (GCP) is an uncommon gastric lesion characterized by hyperplasia of connective tissues within the interstitium of the glands, involving the submucosal layer or even the muscularis propria of the stomach. Widespread chronic active or atrophic gastritis is considered a significant factor contributing to GCP. Patients with GCP may either be asymptomatic or present with non-specific digestive symptoms such as abdominal pain and belching. Due to the indistinct clinical characteristics and non-specific endoscopic manifestations, most clinicians have limited understanding of GCP. Additionally, GCP has been regarded as a potential premalignant lesion. Endoscopic identification of irregular shapes and mucosal lesion types may serve as high-risk factors for GCP associated with early gastric cancer (EGC). Irrespective of EGC presence, endoscopic submucosal dissection emerges as a secure and effective minimally invasive treatment.

Research motivation

Patients with GCP may either remain asymptomatic or present with non-specific digestive symptoms, including abdominal pain and belching. Owing to the unremarkable clinical characteristics and nonspecific endoscopic manifestations, most clinicians possess limited understanding of GCP. Furthermore, GCP has been regarded as a potential premalignant lesion; hence, the endoscopic diagnosis and early excision of GCP are deemed crucial. In this study, we conducted a retrospective analysis of 104 cases of GCP treated by endoscopic resection at our center from October 2011 to December 2022. Our analysis was based on their clinical manifestations, endoscopic findings, pathological results, and treatments. The primary objectives were to delineate the endoscopic features of GCP associated with EGC and to assess the impact of endoscopic resection on the diagnosis and treatment of GCP with EGC.

Research objectives

Given the limited literature and reports on GCP, our research might hold significance in raising awareness of GCP as a high-risk factor for EGC. Clinical differentiation from conditions such as hypertrophic gastritis, mesenchymal tumors, gastric cancer, and ectopic pancreas is crucial. Due to GCP's malignant potential, prompt removal through endoscopy or surgery is essential, coupled with regular postoperative follow-up. In this study, we delineated the endoscopic features of GCP and evaluated the impact of endoscopic resection on the diagnosis and treatment of GCP.

Research methods

This retrospective study involved 104 patients with GCP who underwent endoscopic resection. Alongside demographic and clinical data, regular patient follow-ups were conducted to assess local recurrence.

Research results

Among the 104 patients diagnosed with GCP who underwent endoscopic resection, 12.5% had a history of previous gastric procedures. The primary site predominantly affected was the cardia (38.5%, n = 40). GCP commonly exhibited intraluminal growth (99%), regular presentation (74.0%), and ulcerative mucosa (61.5%). The leading endoscopic feature was the mucosal lesion type (59.6%, n = 62). The average maximum diameter was 20.9 ± 15.3 mm, with mucosal involvement in 60.6% (n = 63). Procedures lasted 73.9 ± 57.5 min, achieving complete resection in 91.3% (n = 95). Recurrence (4.8%) was managed *via* either surgical intervention (n = 1) or through endoscopic resection (n = 4). Final pathology confirmed that 59.6% of GCP cases were associated with EGC. Univariate analysis indicated that elderly males were more susceptible to GCP associated with EGC. Conversely, multivariate analysis identified lesion morphology and endoscopic features as significant risk factors. Survival analysis demonstrated no statistically significant difference in recurrence between GCP with and without EGC (P = 0.72).

Research conclusions

The findings suggested that endoscopic resection might serve as an effective and minimally invasive treatment for GCP with or without EGC.

Research perspectives

Further research is imperative to gain a more comprehensive understanding of the natural progression of GCP and its malignant potential.

FOOTNOTES

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ORIGINAL ARTICLE

Observational Study

Red cell distribution width/platelet ratio estimates the 3-year risk of decompensation in Metabolic Dysfunction-Associated Steatotic Liver Disease-induced cirrhosis

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Abstract

BACKGROUND

For compensated advanced chronic liver disease (cACLD) patients, the first decompensation represents a dramatically worsening prognostic event. Based on the first decompensation event (DE), the transition to decompensated advanced chronic liver disease (dACLD) can occur through two modalities referred to as acute decompensation (AD) and non-AD (NAD), respectively. Clinically Significant Portal Hypertension (CSPH) is considered the strongest predictor of decompensation in these patients. However, due to its invasiveness and costs, CSPH is almost never evaluated in clinical practice. Therefore, recognizing non-invasively predicting tools still have more appeal across healthcare systems. The red cell distribution width to platelet ratio (RPR) has been reported to be an indicator of hepatic fibrosis in Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD). However, its predictive role for the decompensation has never been explored.

AIM

In this observational study, we investigated the clinical usage of RPR in predicting DEs in MASLD-related cACLD patients.

METHODS

Fourty controls and 150 MASLD-cACLD patients were consecutively enrolled and followed up (FUP) semiannually for 3 years. At baseline, biochemical, clinical, and

Liver Stiffness Measurement (LSM), Child-Pugh (CP), Model for End-Stage Liver Disease (MELD), aspartate aminotransferase/platelet count ratio index (APRI), Fibrosis-4 (FIB-4), Albumin-Bilirubin (ALBI), ALBI-FIB-4, and RPR were collected. During FUP, DEs (timing and modaities) were recorded. CSPH was assessed at the baseline and on DE occurrence according to the available Clinical Practice Guidelines.

RESULTS

Of 150 MASLD-related cACLD patients, 43 (28.6%) progressed to dACLD at a median time of 28.9 months (29 NAD and 14 AD). Baseline RPR values were significantly higher in cACLD in comparison to controls, as well as MELD, CP, APRI, FIB-4, ALBI, ALBI-FIB-4, and LSM in dACLD-progressing compared to cACLD individuals [all $P < 0.0001$, except for FIB-4 (P: 0.007) and ALBI (P: 0.011)]. Receiving operator curve analysis revealed RPR > 0.472 and > 0.894 as the best cut-offs in the prediction respectively of 3-year first DE, as well as its superiority compared to the other non-invasive tools examined. RPR (P: 0.02) and the presence of baseline-CSPH (P: 0.04) were significantly and independently associated with the DE. Patients presenting baseline-CSPH and RPR > 0.472 showed higher risk of decompensation (P: 0.0023).

CONCLUSION

Altogether these findings suggest the RPR as a valid and potentially applicable non-invasive tool in the prediction of timing and modalities of decompensation in MASLD-related cACLD patients.

Key Words: Liver cirrhosis; Red blood cell distribution width; Red blood cell distribution width to platelet ratio; Translational Medicine; Prognostic biomarker

Core Tip: The availability of non-invasive tools predicting the first decompensation event (DE) in Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD)-related compensated advanced chronic liver disease (cACLD) context is still demanded. Red cell distribution width to platelet ratio (RPR) has been shown to predict fibrosis in MASLD. Herein, we demonstrate that: (1) RPR predicts the first DE in MASLD-cACLD; (2) RPR predicts acute decompensation as the first DE in these patients; and (3) Patients presenting baseline Clinically Significant Portal Hypertension and RPR > 0.472 show higher risk of 3-year decompensation occurrence. Overall, RPR predicts time and modalities of DE in MASLD-related-ACLD patients, presenting the potential to be a valuable, easy-to perform, non-invasive clinical index.

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INTRODUCTION

In the last decade, the progressive development of tools non-invasively assessing the degree of hepatic fibrosis in patients with chronic liver diseases (CLDs) has allowed the identification of cirrhosis at the earlier and asymptomatic stage of compensated advanced CLD (cACLD), revolutionizing the clinical management and conditioning the therapeutic interven-tions potentially impacting on prognosis[1,2].

For cACLD patients, the transition to decompensated advanced CLD (dACLD), represents a dramatic prognosis-affecting event as the liver-related mortality occurring almost exclusively after this watershed episode[3]. Based on the first decompensation event (DE), the transition to dACLD can occur through two modalities with relatively different long-term consequences: The more prognostically burdensome acute decompensation (AD); the more progressive non-AD (NAD)[4].

Metabolic dysfunction-associated Steatotic Liver Disease (MASLD), encompassing a spectrum of disease manifestations ranging from simple steatosis to steatohepatitis (MASH) and advanced fibrosis (AF), represents the most common cause of liver cirrhosis worldwide with a severe healthy and socioeconomic burden[5,6]. To make matters worse, recent evidence indicates that MAFLD/MASH-related cACLD may progress more rapidly than other etiologies and a relatively earlier decompensation has been reported in these patients[7,8]. Therefore, determining the probability of decompensation, as well as identifying individuals requiring intensive monitoring and timely interventions, appears paramount research challenge.

Clinically significant portal hypertension (CSPH) defined by a Hepatic Venous Pressure Gradient (HVPG) value 0 mmHg has been revealed as the strongest predictor of decompensation in several CLDs etiologies, including MASH[9]. However, HVPG measurement is a nuanced, not-routinely performed procedure with a highly operator-dependent

accuracy. Transient Elastography (TE)-assessed Liver Stiffness Measurement (LSM), Fibrosis-4 (FIB-4), Albumin-Bilirubin (ALBI), ALBI-FIB-4, aspartate aminotransferase (AST)/platelet (PLT) count ratio Index (APRI), Child-Pugh (CP) score, and Model for End-Stage Liver Disease (MELD), have been investigated as models non-invasively predicting decompensation[10-15]. Despite the encouraging results suggested by these findings, the development of prognostic tools including not-exclusively specialist parameters would have more appeal across healthcare systems.

Red cell distribution width (RDW) is a routinely assessed haematochemical parameter providing an analytical measure of the variability [Standard Deviation (RDW-SD) and Coefficient Variation (RDW-CV)] in the size of circulating erythrocytes whose applicability as an independent prognosis marker in cardiovascular, renal, and infectious conditions has been largely demonstrated[16]. In hepatic chronic disorders, regardless of the etiology, the perpetuation of liver injury promotes reactive oxygen species release and decreased antioxidant compounds production, determining a systemic oxidative stress imbalance and low-grade inflammation status leading to bone-marrow suppression, reduced erythropoietin functioning, and thus irregular/immature erythrocytes output[17]. In line with this, elevated RDW values have been evidenced in patients affected by viral-related and non-viral-related CLDs[17], and several findings have highlighted its usefulness as a prognostic index in CLDs of different etiologies[18,19]. However, the potential link with decompensation occurrence in cACLD individuals has never been investigated. In long-lasting CLDs, the portal hypertension-related pancytopenia determining, among the other consequences, chronic anemia, and low platelet count, has constituted the pathophysiological rationale to reveal the role of RDW-to-PLT ratio (RPR) as an RDW-derivative non-invasively predicting hepatic AF[20]. In MASLD patients, RPR has been recently shown to reflect the severity of fibrosis, correlate with main non-invasive liver-fibrosis scoring systems, and accurately predict AF[21,22]. However, the role of RPR in the prediction of decompensation in terms of timing and relative modalities (AD or NAD) in MASLD-related cACLD patients has never been explored and, the availability of tools that accurately non-invasively predict and stratify the risk of decompensation still represents an unmet need.

In this study, by focusing on MASLD-related etiology, we aimed to evaluate the accuracy of the RPR in the prediction of 3-year first DE occurrence and relative modalities (NAD or AD) in cACLD patients.

MATERIALS AND METHODS

Experimental design

In this observational study, we consecutively enrolled patients affected by MASLD-related cACLD and a group of healthy controls. TE was adopted to non-invasively assess LSM and analytically define cACLD. The Alcohol Use Disorders Identification Test questionnaire was used to assess alcohol consumption, to exclude from the enrollment patients potentially affected by alcoholic liver disease.

As detailed below, at the enrollment, anthropometrical and clinical data were collected. Further, a 10 mL venous blood sample was collected to assess the biochemical parameters. Finally, at the baseline, MASLD-related cACLD individuals received a non-invasive evaluation of the hepatic disease severity and liver function status by computing RPR, APRI, FIB-4, ALBI, ALBI-FIB-4, MELD, and CP scores. Patients were semiannually followed up (FUP) over 3 years to record the occurrence of the first DE and the relative modalities by recognizing, according to D'Amico *et al*[4], two distinct modalities of decompensation: NAD and AD[4]. Liver-related events (LREs) defining decompensation, as well as NAD- and AD-specific features are detailed below.

CSPH and RPR were assessed at baseline and when the first DE occurred by using evaluation methods reported in detail in the dedicated subparagraph.

The experimental design is reported in Figure 1.

The estimation of the accuracy of the RPR in the prediction of 3-year first DE occurrence in comparison to the currently available non-invasive composite tools (APRI, FIB-4, ALBI, ALBI-FIB-4, MELD, and LSM) represented the primary study outcome.

The estimation of the accuracy of the RPR in the prediction of AD (3-year first DE) occurrence in comparison to the currently available non-invasive composite tools (APRI, FIB-4, ALBI, ALBI-FIB-4, MELD, and LSM), as well as the investigation of the relationship between RPR and baseline-CSPH with a consensual risk-stratification on DE occurrence, were the secondary study outcomes.

Patients

This study is in compliance with the Declaration of Helsinki (1975) and has been approved by the ethical committee of the University of Campania Luigi Vanvitelli in Naples (prot. n. 417/2018).

In the present study (Figure 1), after signing the informed consent, we consecutively enrolled healthy subjects as the control group and patients affected by MASLD-related cACLD. Liver Transient Elastography criteria were adopted to determine cACLD according to the Baveno VI consensus: LSM values 15 kPa defined cACLD[23]. MASLD diagnostic criteria were: (1) Overweight or obesity, defined as body mass index (BMI) > 25 kg/m^2; (2) presence of type 2 diabetes mellitus (T2DM) and/or (3) presence of ≥ one metabolic risk abnormalities identified by waist circumference ≥ 102 cm in men (and ≥ 88 cm in women); blood pressure ≥ 130/85 mmHg (or specific drug treatment); plasma triglycerides (TG) ≥150 mg/dL (or specific drug treatment); plasma high-density lipoprotein (HDL) cholesterol < 40 mg/dL for men (and < 50 mg/dL for women) (or specific drug treatment); prediabetes [fasting plasma glucose (FPG) levels 100-125 mg/Dl] or 2-h post-load glucose levels 140-199 mg/dL or glycated hemoglobin 5.7%-6.4%; homeostasis model assessment for insulin resistance (HOMA-IR) score ≥ 2.5[6]. The enrollment was carried out at the Hepato-Gastroenterology Division of the University of Campania Luigi Vanvitelli between January and November 2019. Inclusion criteria were age between 18

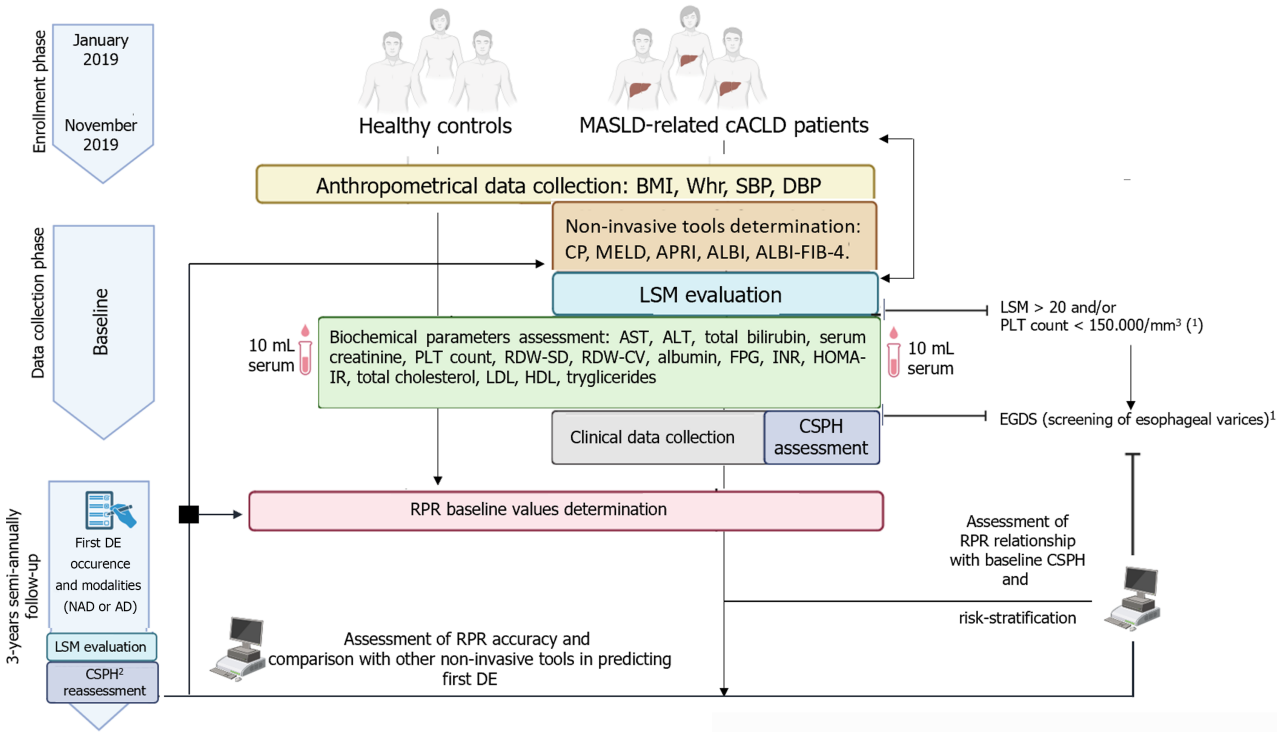

Figure 1 Experimental design. Clinically Significant Portal Hypertension Rule-in Liver Stiffness Measurement > 25 kPa. [1]Baveno VI criteria; [2]Baveno VII criteria. AD: Acute decompensation; NAD: Non-acute decompensation; LSM: Liver Stiffness Measurement; CSPH: Clinically Significant Portal Hypertension; MASLD: Metabolic Dysfunction-Associated Steatotic Liver Disease; cACLD: Compensated advanced chronic liver disease; BMI: Body mass index; Whr: Waist-to-hip ratio; SBP: Systolic; DBP: Diastolic blood pressure; CP: Child-Pugh; MELD: Model for End-Stage Liver Disease; APRI: Aspartate aminotransferase/platelet count ratio index; ALBI: Albumin-Bilirubin; FIB-4: Fibrosis-4; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International Normalized Ratio; PLT: Platelet; RDW: Red cell distribution width; FPG: Fasting plasma glucose; HOMA-IR: Homeostasis model assessment for insulin resistance; LDL: Low-density lipoprotein; HDL: High-density lipoprotein.

and 80 years and MASLD-related cACLD diagnosis. Exclusion criteria were the presence of hematological disorders (particularly, autoimmune hemolytic anemia, myelodysplastic syndrome, b-thalassemia, sickle cell anemia); chronic inflammatory diseases, acute or chronic kidney diseases, rheumatoid arthritis, systemic lupus erythematosus, autoimmune gastritis or other major systemic inflammatory diseases or tumors; ongoing infections; alcohol or drug abuse history; other etiologies of chronic liver damage; previous hepatocellular carcinoma diagnosis; ongoing chemotherapy, use of hepatoprotective drugs; decompensated liver cirrhosis (CP C) at the moment of the enrollment or in the previous 12 months, and psychological/psychiatric problems that could have invalidated the informed consent. At baseline, anthropometrical parameters collection included the determination of BMI by dividing the weight by the square of height (kg/m^2), and directly measured waist-to-hip ratio, systolic (mmHg), and diastolic blood pressure (mmHg). Clinical evaluation included the complete medical history collection and the assessment of alcohol consumption, smoking, drug abuse, comorbidities, and the concomitant therapies record [including Non-Selective Beta Blockers (*i.e.*, propranolol and carvedilol), whose administration was assessed also semiannually, during the follow-up medical examinations; Supplementary Table 1]. All the enrolled patients have undergone a 10 mL venous blood sample collection for the lab assessments. MASLD-cACLD-related patients were FUP every six months for 3 years and the occurrence of the first DE [time and modalities (NAD/AD)] was recorded. On the first DE, for each patient, RPR and CSPH were also reassessed.

Biochemical assessment

The evaluated biochemical data were AST, alanine aminotransferase (ALT), total bilirubin (TB), PLT, plasma albumin, International Normalized Ratio (INR), total cholesterol, HDL cholesterol, Low-density lipoprotein cholesterol, TG, insulin, and FPG. Insulin levels were measured enzymatically using commercially available kits (R&D Systems, Minneapolis, MN), AST, ALT, and glucose using a colorimetric assay kit (Amplite 13801/13803 and Thermo Fisher Scientific EIAGLUC). The HOMA-IR was calculated by using the formula: fasting insulin (μU/mL) × FPG (mmol/L)/22.5 [24].

RDW assessment

RDW was determined by using a suspension of blood cells passed through a small orifice along with an electric current of the Beckman Coulter analyzer (C11137 - DxI 9000 Analyzer, Beckman Coulter, Inc©). The individual blood element generates an impedance change in the orifice, which is directly proportional to the cell size. The system counts the individual cells and provides a size distribution. The RDW is then calculated at the 20% height level above the baseline of the Red Blood Cells histogram. In particular, the RDW-CV evaluates the volumetric distribution of red blood cells considering the coefficient of variation, while the RDW-SD defines the volumetric distribution concerning the standard

deviation.

Non-invasive validated tools assessing hepatic fibrosis and liver function

MELD score, which determines prognosis and prioritizes receipt of liver transplantation, incorporates 3 widely available laboratory variables including the INR, serum creatinine, and serum bilirubin. MELD was given by the formula: [9.57 × $\log_{10}$ (creatinine) + 3.78 × $\log_{10}$ (TB) + 11.2 × $\log_{10}$ (INR) + 6.43][14].

CP was evaluated using five clinical and laboratory criteria: Serum bilirubin (< 2 mg/dL: 1 point; 2-3 mg/dL: 2 points; > 3 mg/dL: 3 points), serum albumin (> 3.5 mg/dL: 1 point; 2.8-3.5 mg/dL: 2 points; < 2.8 mg/dL: 3 points), ascites (none: 1 point; grade 1-2: 2 points; grade 3: 3 points), and HE (none: 1 point; grade 1-2: 2 points; grade 3-4: 3 points)[25]. CP scoring system, broke down patients into three classes: CPA - good hepatic function (CP total range: 5-6), CPB - moderately impaired hepatic function (CP total range: 8-9), and CPC- advanced hepatic dysfunction (CP total range: 10-15)[25].

APRI was calculated by using the following validated formula: [(AST/upper limit of the normal AST range) + 100]/PLT count (10^3/mL)[26].

The ALBI score was calculated as [-0.085 × (albumin g/L) + 0.66 × $\log_{10}$ (TB mmol/L)][27]. FIB-4 score, a non-invasive estimation of liver scarring, was calculated by using the originally described formula[28]: Age × AST/PLT count (10^3/mL) × $ALT^{½}$. FIB-4 categories were: (1) Low risk for AF (< 1.45); (2) high risk for AF (> 3.25); or (3) indeterminate (1.45-3.25)[28].

The combined score ALBI-FIB-4 stratified patients as follows: I group of risk (ALBI ≤ -2.60 and FIB-4 ≤ 3.25); II group of risk (ALBI ≥ -2.60 and FIB-4 ≤ 3.25); III group of risk (ALBI ≤ -2.60 and FIB-4 ≥ 3.25); IV group of risk (ALBI ≥ -2.60 and FIB-4 ≥ 3.25)[29].

RPR was determined by using the formula: RDW-SD/PLT count (10^3/mL) 1000.

LSM

LSM was performed by using FibroScan® [version 502 (Echosens, Paris, France)] with M and XL probes[30]. We decided to use the XL probe when the ultrasound measured distance between the skin and the liver capsule resulted in greater than 2.5 cm and/or when the patient's BMI was > 30. FibroScan® was performed by an expert physician obtaining 10 acceptable measurements (defined as successful LSM), with the maximum number of attempts set at 20.

The criteria proposed by Boursier *et al*[30] were used to consider the measurement "very reliable" (IQR/M ≤ 0:1), "reliable" (0:1 < IQR/M ≤ 0:3 or IQR/M > 0:3 with LS median < 7:1 kilopascal), or "poorly reliable" (IQR/M > 0:3 with LS median ≥ 7:1 kPa[30,31].

LREs defining AD and NAD

LREs were ascites formation, hepatic encephalopathy (HE), jaundice, acute bacterial infections, and acute gastrointestinal bleeding. The onset of one (or more) LREs in cACLD patients defined the decompensation and thus the transition to dACLD. According to D'Amico *et al*[4], two distinct modalities of transition to decompensation were considered: (1) NAD was defined by slow/ grade 1 ascites formation, mild (grade 1 or 2) HE, or progressive jaundice in non-cholestatic cirrhosis; (2) AD was defined by grade 2/3 ascites within less than 2 wk, severe acute (*i.e.*, in patients with previous normal consciousness) HE, acute gastrointestinal bleeding, and any type of acute bacterial infection.

Evaluation and definition of CSPH

According to Baveno VI Criteria, for Esophagogastroduodenoscopy-(EGDS)-naïve patients, presenting baseline LSM values ≤ 20 kPa and/or a PLT count ≤ 150.000/mm^3 a screening EGDS was performed, while EGDS-not naive patients continued their regular surveillance endoscopy programs, according to the Clinical Practice Guidelines[23]. In all the cases, at the baseline, an EGDS proving esophageal varices defined CSPH. Baveno VII Criteria (CSPH-rule out if LSM ≤ 15 kPa and PLT count ≥ 150.000/mm^3, CSPH-rule in if LSM values ≥ 25 kPa)[32] were not available at the time of the enrollment and were exclusively used to reassess/confirm CSPH on the occasion of first DE occurrence, independently from the endoscopic surveillance programs for each patient (Figure 1).

Finally, the Japanese Research Society for Portal Hypertension Classification estimated the entity (F1; F2; F3) of varices [33].

Statistical analysis

Continuous data were described as mean and standard deviations, while categorical variables as *n* (%). The Kolmogorov-Smirnov test for normality was performed to evaluate if the parametric or non-parametric analysis should be applied. Mann-Whitney and t-test for independent groups, the Kruskal-Wallis test, or ANOVA test with posthoc Tukey analysis, in the case of non-normal or normal distribution respectively, were performed to compare the continuous variables. D% RPR [(RPR on the first DE - baseline RPR)/baseline RPR 100] and D% LSM [(LSM on the first DE - baseline LSM)/baseline LSM 100]} indicated RPR and LSM% variations during the study. Linear regression analysis was adopted to evaluate the relationship (R) between continuous variables. The area under the curve (AUC), estimated by receiving operator curve (ROC) analysis with the Youden index calculation for the identification of best cut-off values, integrally with the Chi-Square test for the sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) evaluation, was performed to evaluate the accuracy of RPR in the prediction of 3-year first DE and in the prediction of AD occurrence, as well as to estimate the accuracy of the RPR in comparison to other non-invasive composite tools (APRI, FIB-4, ALBI, ALBI-FIB-4, MELD, CP, and LSM) in the prediction of both these outcomes. The adjusted odds ratio (OR) of the study variables on the just mentioned events was calculated considering the confounding variables (sex, age,

BMI, diabetes, alcohol intake, the baseline/along the study administration of Non-Selective Beta Blockers) by using multinomial logistic regression models. Time-to-event analyses on DEs occurrence upper and under the RPR value ROC-analysis identified best cut-off was performed using the Kaplan-Meier method and the Log-rank test for the curve comparison considering a *P* value < 0.05 as statistically significant. Statistical significance was defined as $P < 0.05$ in a two-tailed test with a 95%CI. SPSS® *vs* 18.0 was used to perform the analysis. The sample size was estimated by Logistic Regression analysis (p0: 0.15; p1: 0.23; alfa: 0.05; power: 0.8) testing whether the variable (RPR) is a significant predictor of the binary (0/1) outcome (y = decompensation) performed by using wp. logistic function of STATA18 for macOS software.

RESULTS

A total of 150 MASLD-related-cACLD patients and 40 healthy controls were consecutively enrolled in this study. The baseline demographic data, anthropometric indexes, biochemical parameters, and non-invasive tools for liver-functional status and hepatic fibrosis assessment (CP, MELD, LSM, FIB-4, APRI, RPR, ALBI, and ALBI-FIB-4) are reported in Tables 1-4. The baseline prevalence of T2DM, primary hypertension, and dyslipidemia in the MASLD patients was respectively 54.6% ($n = 82$), 50.6% ($n = 76$), and 32 % ($n = 48$).

Prediction of decompensation

During a median follow-up of 36 (IQR: 35-36) months, 43 (28.6%) of 150 cACLD patients progressed to dACLD at a median time of 28.9 (95%CI: 27.20-32.80) months.

In 3 (21.4%) dACLD patients, community-acquired acute bacterial infections (2 Urinary Tract Infections and 1 Pneumonia) were recognized as the precipitants of decompensation configuring AD events. However, in 40 (93%) of the decompensating patients, no specific triggers could be identified. Overall survival following the first decompensation was 79.8% at 3 years. Detailed data about the first DE and relative modalities of decompensation (NAD *vs* AD) are described in the next subparagraph.

Tables 5-7 report the baseline demographic data, anthropometric indexes, and biochemical parameters, for remaining-cACLD and progressing-dACLD patients.

Patients transiting to dACLD presented significantly higher baseline RPR values in comparison to controls and not-decompensating individuals (all $P < 0.0001$; Figure 2A), as well as MELD ($P < 0.0001$), CP ($P < 0.0001$), LSM ($P < 0.0001$), APRI ($P < 0.0001$), FIB-4 (P: 0.007), and ALBI (P: 0.011) baseline values were significantly increased in dACLD individuals compared to patients remaining compensated (Figure 2B).

Linear regression analysis revealed the positive correlation between baseline RPR values and the others tools (CP: $r = 0.74$, 95%CI: 0.661- 0.807; MELD: $r = 0.75$, 95%CI: 0.679- 0.817; FIB-4: $r = 0.66$, 95%CI: 0.643-0.714; APRI: $r = 0.88$, 95%CI: 0.843-0.914; LSM: $r = 0.94$, 95%CI: 0.927-0.961; ALBI: $r = 0.51$, 95%CI: 0.491-0.564 ALBI-FIB-4: $r = 0.74$, 95%CI: 0.668-0.811; all $P < 0.0001$, except ALBI, P: 0.017; Figure 3).

ROC analysis with the Youden index calculation for the identification of best cut-off values revealed 0.472 as the RPR threshold (AUC: 0.95; sensitivity: 86.9%; specificity: 90.7%; NPV: 73.5%; PPV: 95.8%; $P < 0.0001$) in the prediction of 3-year first DE, as well as a superior RPR predictive accuracy compared to APRI (AUC: 0.88), FIB-4 (AUC: 0.72), MELD (AUC: 0.81), CP (AUC: 0.79), LSM (AUC: 0.88), ALBI (AUC: 0.90), and ALBI-FIB-4 (AUC: 0.93; all $P < 0.0001$; Figure 4; Table 8). The RPR predictive accuracy was not statistically significantly different between male and female patients (AUC male: 0.93 *vs* AUC female: 0.91; P: 0.071). For patients presenting baseline RPR values 0.472, the Kaplan-Meier Log-Rank Test analysis on the first DE occurrence revealed a significantly elevated risk of this event [hazard ratio (HR): 13.62, 95%CI: 7.11-15.8; $P < 0.0001$], as well as a different median time of decompensation and a higher incidence ratio rate (IRR) in comparison to individuals presenting a baseline RPR < 0.472 [RPR < 0.472 *vs* RPR ≥0.472; Median time of decompensation: 28.6 months *vs* 26.4 months ($P < 0.0001$); IRR: 8.24% *vs* 24.5% ($P < 0.0001$) (Figure 5)]. In patients progressing to the decompensation, the following variables were significantly associated with the first DE occurrence: Bilirubin (OR: 1.32; 95%CI: 1.09-1.47; P: 0.03), albumin (OR: 0.71; 95%CI: 0.45-0.80; $P < 0.0001$), RDW-SD (OR: 1.32; 95%CI: 0.98-1.41; P: 0.02), PLT (OR: 0.88; 95%CI: 0.78-0.93; P: 0.03), CP (OR: 1.88; 95%CI: 1.53-1.97; P: 0.03), MELD (OR: 1.51; 95%CI: 1.12-1.70; P: 0.02), LSM (OR: 1.87; 95%CI: 1.58-2.02; P: 0.04), ALBI (OR: 3.45; 95%CI: 3.02-3.67; $P < 0.0001$), ALBI-FIB-4 (OR: 2.90; 95%CI: 2.74-3.09; $P < 0.0001$), RPR (OR: 5.14; 95%CI: 4.98-5.3; $P < 0.0001$), and the presence of CSPH (defined by the evidence of esophageal varices) (OR: 4.31; 95%CI: 3.98-34.76; $P < 0.0001$; Supplementary Table 2).

The multinomial logistic regression analysis, performed by considering the confounding variables (sex, age, BMI, diabetes, alcohol intake, the baseline/along the study administration of Non-Selective Beta Blockers), revealed the RPR (adjusted OR: 1.91; 95%CI: 1.72-1.98; P: 0.002) and the presence of baseline-assessed CSPH (adjusted OR: 1.84; 95%CI: 1.72-1.91; P: 0.04) significantly and independently associated with the outcome (Supplementary Table 2 and Figure 6).

Prediction of AD

Of 43 cACLD patients progressing to dACLD, a first DE defining NAD and AD was respectively observed for 29 (NAD: 67.4 %) and 14 (AD: 32.5%) individuals. Tables 9-13 reports in detail the first DEs with the relative modalities for AD-decompensating and NAD-decompensating patients, as well as the relative baseline anthropometric indexes, biochemical parameters, and non-invasive tools for liver-functional status and hepatic fibrosis assessment (CP, MELD, LSM, FIB-4, APRI, ALBI, and RPR; Table 13). Consistently, AD-decompensating patients presented significantly higher baseline CP, MELD, APRI, LSM, ALBI, and RPR values in comparison to NAD-decompensating individuals (Table 13).

Table 1 Demographic data of the study population (baseline)

	Healthy subjects (*n* = 40)	cACLD patients (*n* = 150)	*P* value
Male [*n* (%)]	23 (57.5)	88 (58.7)	NS[1]
Female [*n* (%)]	17 (42.5)	62 (41.3)	NS[1]
Age (mean ± SD)	57.10 ± 17.03	63.15 ± 11.45	NS[2]
Child-Pugh Grade A [*n* (%)]	NA	107 (71.3%)	/
Child-Pugh Grade B [*n* (%)]	NA	43 (28.7%)	/

[1]Chi-square test.
[2]Mann-Whitney *U* test.
Statistically significant differences ($P < 0.05$) are reported in bold. NA: Not-assessed (or not-appliable); NS: Not statistically significant; cACLD: Compensated advanced chronic liver disease.

Table 2 Anthropometric indexes of the study population (baseline)

Variables (mean ± SD)	Healthy subjects (*n* = 40)	cACLD patients (*n* = 150)	*P* value[1]
BMI (kg/m^2)	24.97 ± 2.17	32.61 ± 23.94	< 0.0001
WhR	0.81 ± 0.05	1.56 ± 3.02	< 0.0001
Systolic blood pressure (mm/Hg)	115.3 ± 9.73	130.7 ± 12.57	< 0.0001
Diastolic blood pressure (mm/Hg)	74.67 ± 10.42	87.33 ± 8.58	0.003

[1]Mann-Whitney *U* test.
Statistically significant differences ($P < 0.05$) are reported in bold. BMI: Body mass index; WhR: Waist to hip ratio; cACLD: Compensated advanced chronic liver disease.

ROC analysis with the Youden index calculation for the identification of best cut-off values revealed ≥ 0.894 as the RPR threshold (AUC: 0.94; sensitivity: 93.1%; specificity: 85.7%; NPV: 85.71%; PPV: 93.1%; $P < 0.0001$) in the prediction of AD as first DE, as well as superior RPR accuracy compared to APRI (AUC: 0.88), FIB-4 (AUC: 0.75), MELD (AUC: 0.73), CP (AUC: 0.82), LSM (AUC: 0.85), ALBI (AUC: 0.77), and ALBI-FIB-4 (AUC: 0.79; all P <0.0001; Figure 7 and Table 14).

The multinomial logistic regression analysis, performed by considering the confounding variables (sex, age, BMI, diabetes, alcohol intake, the baseline/along the study administration of Non-Selective Beta Blockers), revealed the RPR baseline values (adjusted OR: 2.11; 95%CI: 1.72-2.22; *P*: 0.03), the presence of baseline-assessed CSPH (adjusted OR: 2.04; 95%CI: 1.92-2.11; *P*: 0.003), and the entity of varices (adjusted OR: 1.98; 95%CI: 1.79-2.06; *P*: 0.073) as the variables significantly and independently associated with the outcome (Supplementary Table 3).

Therefore, considering these relevant findings, individuals presenting baseline CSPH were considered as "high-risk of decompensation" patients and included in a further sub-analysis investigating the relationship between RPR, liver disease progression, CSPH, and decompensation.

RDW/PLT ratio, liver disease progression, portal hypertension, and risk of decompensation

Regarding RPR modifications and liver disease progression, a statistically significant positive correlation between D% RPR and D% LSM was highlighted (R: 0.84; 95%CI: 0.732-0.91; $P < 0.000$; Supplementary Figure 1). Concerning CSPH evaluation, of 150 patients, 71 (47.3%) underwent a screening EGDS [41 (57.7%) because of PLT-count-established Baveno VI criteria, 10 (14.1%) because of LSM-established Baveno VI Criteria, and 20 (28.2%) because of both criteria][23]. According to Baveno VII Criteria (32), CSPH was non-invasively assumable in 7 of 10 patients presenting LSM values 25 kPa; however, given the non-availability of these criteria at the time of the enrollment, an EGDS was performed. EGDS revealed the presence of varices in 52 (73%) of individuals (38: F1 varices; 14: F2 varices). Twenty of 150 cACLD individuals showed esophageal varices in anamnesis (14: F1 varices; 6: F2 varices). Hence, a total of 78 individuals (52%) were baseline-CSPH free and, of these, 12 (15.3%) progressed to decompensation; a total of 72 (48%) presented baseline-CSPH, and, of these, 21 (29.1%) progressed to decompensation [with 11 (37.9%) presenting AD as the first DE]. In a mirror way, the prevalence of baseline CSPH in decompensating patients was significantly higher in patients progressing to dACLD in comparison to individuals remaining compensated (*P*: 0.0001), and in AD-decompensating subjects in comparison to NAD-decompensating patients (*P*: 0.0035; Supplementary Figure 2). On the first DE, independently from the endoscopic surveillance programs, the Baveno VII CSPH-rule in criteria[32] were adopted and CSPH was assumable in 41 (95.3 %) of decompensating patients.

Regarding RPR and CSPH, baseline RPR values were significantly higher in patients presenting baseline CSPH compared to individuals without esophageal varices ($P < 0.04$), and the prevalence of baseline CSPH in decompensating patients was significantly higher in patients presenting RPR baseline values > 0.472 (the best cut-off; Supplement-ary

Table 3 Biochemical parameters of the study population (baseline)

Variables (mean ± SD)	Healthy subjects (*n* = 40)	cACLD patients (*n* = 150)	*P* value[1]
AST (IU/L)	31.30 ± 10.14	48.74 ± 54.09	**< 0.0001**
ALT (IU/L)	39.37 ± 17.57	70.02 ± 15.05	**< 0.0001**
Bilirubin (μmol/L)	15.98 ± 1.74	25.86 ± 7.53	**< 0.0001**
PLT count (mm^3)	242.6 ± 42.76	155.7 ± 61.56	**< 0.0001**
RDW-CV (%)	14.40 ± 2.28	21.04 ± 15.20	**< 0.0001**
RDW-SD (fL)	40.19 ± 4.48	56.27 ± 10.54	**< 0.0001**
Albumin (g/L)	44.2 ± 0.29	26.35 ± 8.48	**< 0.0001**
INR	1.02 ± 0.38	1.78 ± 1.11	NS
HOMA-IR	1.77 ± 0.54	3.15 ± 1.52	**< 0.0001**
Insulin (μu/mL)	7.03 ± 1.52	11.67 ± 3.259	**< 0.0001**
FPG (mg/dL)	100.7 ± 9.35	120.9 ± 17.48	**< 0.0001**
Total cholesterol (mg/dL)	135.2 ± 42.07	185.2 ± 44.12	**< 0.0001**
HDL (mg/dL)	95.93 ± 27.29	42.37 ± 9.92	**< 0.0001**
LDL (mg/dL)	44.73 ± 9.67	126.1 ± 39.78	**< 0.0001**
Tryglicerides (mg/dL)	109.5 ± 32.14	150.6 ± 63.39	**0.002**
Creatinine (mg/dL)	0.97 ± 0.23	1.48 ± 3.88	**0.03**

[1]Mann-Whitney *U* test.

Statistically significant differences ($P < 0.05$) are reported in bold. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PLT: Platelets count; CV: Coefficient Variation; RDW: Red-cell distribution width; INR: International normalized ratio; HOMA-IR: homeostasis model assessment for insulin resistance; NS: Not statistically significant; cACLD: Compensated advanced chronic liver disease.

Table 4 Non-invasive tools for liver disease severity assessment of the study population (baseline)

Variables (mean ± SD)	Healthy subjects (*n* = 40)	cACLD patients (*n* = 150)	*P* value
LSM (kPa)	NA	19.67 ± 3.39	/
APRI	NA	1.75 ± 0.28	/
FIB-4	NA	3.11 ± 1.78	/
ALBI	NA	-2.378 ± 0.63	/
ALBI-FIB-4	NA	1.44 ± 0.99	/
Child-Pugh	NA	6.24 ± 1.23	/
MELD	NA	7.74 ± 2.69	/
RDW (fL)/PLT ratio	0.17 ± 0.03	0.458 ± 0.27	/

LSM: Liver stiffness measurement; APRI: Aspartate aminotransferase/platelet count ratio index; MELD: Model for End-stage Liver Disease; FIB-4: Fibrosis-4; NA: Not-assessed (or not-appliable); cACLD: Compensated advanced chronic liver disease.

Figure 3). Consistently, RPR baseline values progressively increased with the severity of esophageal varices ($P < 0.0001$), and a direct positive correlation between RPR and esophageal varices severity (no varices = 0; 1: F1; 2: F2) was also highlighted ($P < 0.0001$; R: 0.80; Supplementary Figure 4). Relevantly, individuals presenting baseline CSPH and RPR values > 0.472 showed a significantly elevated risk (HR: 3.10, 95%CI: 1.481-6.125; *P*: 0.0023) and IRR (57.5% *vs* 25%) of decompensation in comparison to baseline-CSPH individuals presenting lower RPR values supporting the following risk-stratification: (1) "High risk of decompensation" (baseline CSPH and RPR < 0.472); and (2) "very high-risk of decompensation" (baseline CSPH and RPR 0.472; Figure 8).

Table 5 Comparison of demographic data between patients remaining compensated and individuals progressing to decompensation during the follow-up period

	Patients remaining compensated (*n* = 107)	Patients progressing to decompensation (*n* = 43)	*P* value
Male [*n* (%)]	66 (61.7)	22 (51.2)	NS[1]
Female [*n* (%)]	41 (38.3)	21 (48.8)	NS[1]
Age (mean ± SD)	61.81 ± 10.99	66.47 ± 12.01	NS[2]
Child-Pugh Grade A [*n* (%)]	78 (72.9)	29 (67.5)	NS[1]
Child-Pugh Grade B [*n* (%)]	29 (27.1)	14 (32.5)	NS[1]

[1]Chi-square test.
[2]Mann-Whitney *U* test.
Statistically significant differences ($P < 0.05$) are reported in bold. NS: Not statistically significant.

Table 6 Comparison of anthropometric indexes between patients remaining compensated and individuals progressing to decompensation during the follow-up period

Variables (mean ± SD)	Patients remaining compensated (*n* = 107)	Patients progressing to decompensation (*n* = 43)	*P* value[1]
BMI (kg/m^2)	33.58 ± 2.28	30.18 ± 3.13	NS
WhR	1.79 ± 0.83	1.01 ± 0.13	NS
Systolic blood pressure (mm/Hg)	130.5 ± 13.49	131.2 ± 10.05	NS
Diastolic blood pressure (mm/Hg)	87.85 ± 8.85	86.05 ± 7.83	NS

[1]Mann-Whitney *U* test.
Statistically significant differences ($P < 0.05$) are reported in bold. BMI: Body mass index; WhR: Waist to hip ratio. NS: Not statistically significant.

DISCUSSION

The irrepressible spreading of MASLD worldwide[5], in synergy with the evidence that MASLD/MASH-related cirrhosis may more rapidly progress to dACLD[7,8], remark the identification of tools predicting the decompensation in these patients as an absolute global priority. Up to now, in scientific literature, various emerging findings suggested the RPR as a predictor of severe fibrosis and cirrhosis in MASLD[21,22]. However, the link between RPR and liver decompensation in MASLD patients has never been investigated.

In the present observational study, we investigated the accuracy of RPR in the prediction of 3-year first DE occurrence in MASLD-related cACLD patients as a non-invasive tool stratifying the risk of decompensation in this setting. For this purpose, 40 controls and 150 MAFLD-cACLD patients were enrolled and followed semi-annually for 3 years. At baseline, MAFLD-cACLD individuals received a complete liver-disease status assessment including the determination of MELD, CP, APRI, ALBI, FIB-4, ALBI-FIB-4, LSM, and RPR; DE were subsequently recorded along the entire follow-up.

As expected, RPR values were shown significantly higher ($P < 0.0001$) in ACLD patients in comparison to healthy controls. Moreover, RPR and the baseline values of all the other non-invasive tools appeared significantly (all $P < 0.0001$, except for FIB-4, P: 0.007 and ALBI, P: 0.011) increased in patients progressing to decompensation in comparison to subjects who completed the follow-up remaining compensated. In line with these findings, a direct positive linear relationship between baseline RPR values and the other non-invasive tools was also highlighted and, consistently with the pre-existing evidence exploring predominantly the RPR role in the prediction of hepatic fibrosis[21], the correlation between RPR and LSM emerged as the most strict (R: 0.94). However, in comparison to all the other non-invasive tools (MELD, CP, APRI, ALBI, FIB-4, ALBI-FIB-4, and LSM), ROC analysis with the Youden index calculation evidenced a significantly higher accuracy [AUC: 0.95; $P < 0.0001$] of RPR in the prediction of 3-year first DE occurrence, without statistically significant differences between male and female MASLD individuals. RPR optimal cut-off (≥ 0.472) was also highlighted, as well as the relatively excellent prognostic performance suggested by very high levels of sensitivity (86.9%), specificity (90.7%), and an elevated (95.8%) PPV of decompensation.

Relevantly, patients presenting baseline RPR values ≥ 0.472 showed an elevated risk (HR: 13.62) of decompensation at 3 years (median time of decompensation of 26.4 months), with an IRR for first DE occurrence significantly higher in comparison to individuals presenting baseline RPR values under the 0.472 threshold.

Emerging evidence has revealed that, according to the pattern of the first DE, the transition to dACLD can occur through two modalities with relatively different long-term repercussions: The prognostically burdensome AD; the progressive NAD[4]. Although AD has been reported as an event occurring more frequently in already decompensated

Table 7 Comparison of biochemical parameters between patients remaining compensated and individuals progressing to decompensation during the follow-up period

Variables (mean ± SD)	Patients remaining compensated (*n* = 107)	Patients progressing to decompensation (*n* = 43)	*P* value[1]
AST (IU/L)	29.28 ± 8.55	32.50 ± 27.15	NS
ALT (IU/L)	50.13 ± 17.5	54.86 ± 31.4	NS
Bilirubin (μmol/L)	23.64 ± 4.74	32.97 ± 7.10	NS
PLT count (mm^3)	183 ± 48.77	87.60 ± 28.15	**< 0.0001**
RDW-CV (%)	15.66 ± 3.53	34.41 ± 23.05	**< 0.0001**
RDW-SD (fL)	53.05 ± 8.91	64.30 ± 10.07	**< 0.0001**
Albumin (g/L)	35.02 ± 7.41	32.48 ± 1.54	NS
INR	1.26 ± 0.36	1.89 ± 0.27	NS
HOMA-IR	2.94 ± 1.57	3.66 ± 1.26	NS
Insulin (μu/mL)	11.50 ± 3.36	12.09 ± 2.98	NS
FPG (mg/dL)	121 ± 18.09	120.9 ± 16.07	NS
Total cholesterol (mg/dL)	185.2 ± 39.71	175.3 ± 54.09	NS
HDL (mg/dL)	43.23 ± 9.82	40.13 ± 9.97	NS
LDL (mg/dL)	125.9 ± 37.9	126.7 ± 44.51	NS
Tryglicerides (mg/dL)	145.8 ± 53.29	162.4 ± 82.99	NS
Creatinine (mg/dL)	1.13 ± 0.91	2.13 ± 1.07	**0.02**

[1]Mann-Whitney *U* test.

Statistically significant differences ($P < 0.05$) are reported in bold. AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, PLT: Platelets count; CV: Coefficient variation; RDW: Red-cell distribution width; INR: International normalized ratio; HOMA-IR: homeostasis model assessment for insulin resistance; n.s.: Not statistically significant.

Table 8 Receiving operator curve features using cut-off values > 0.472 as the red-cell distribution width to platelet ratio threshold in the prediction of 3-year first decompensation event

	Value	95%CI
Relative risk[1]	3.630	2.416 to 5.842
Reciprocal of relative risk	0.2755	0.1712 to 0.4140
Sensitivity	0.8692	0.7923 to 0.9204
Specificity	0.9070	0.7840 to 0.9632
Positive predictive value	0.9588	0.8987 to 0.9838
Negative predictive value	0.7358	0.6042 to 0.8356

[1]Koopman asymptotic score.

patients, when representing the first DE, it may severely impact the prognosis[4]. Therefore, the prediction of AD was based on a solid rationale and not fueled by *horror vacui*, representing a concrete aim of our research. To the best of our knowledge, in fact, our study is the first to assess the accuracy of a tool in the prediction of AD in cACLD patients. Concerning this, we demonstrated that modalities (AD *vs* NAD) of the first decompensation can be predicted by using RPR: An RPR ≥ 0.894 was shown as the threshold more accurately predicting AD (PPV: 93.1%). Moreover, ROC analysis also revealed the superiority of RPR in comparison to the other non-invasive tools (MELD, CP, APRI, ALBI, FIB-4, ALBI-FIB-4, and LSM) in the prediction of this outcome.

Altogether these findings suggest the RPR is a valid and potentially applicable non-invasive tool in the prediction of timing and modalities of decompensation in MASLD-related cACLD patients.

Table 9 Type of first decompensation event of non-acute and acute decompensating patients

Type of first decompensation event	NAD-decompensating patients (*n* = 29)	AD-decompensating patients (*n* = 14)
(A) Slow/ grade 1 ascites formation [*n* (%)]	14 (48.3)	/
(B) Mild (grade 1/2) hepatic encephalopathy [*n* (%)]	6 (20.7)	/
(C) Jaundice in non-cholestatic cirrhosis [*n* (%)]	9 (31)	/
A + B/A + C	2/5	
(D) Grade 2/3 ascites within less than 2 wk [*n* (%)]	/	5 (35.7)
(E) Severe acute[a] hepatic encephalopathy [*n* (%)]	/	4 (28.5)
(F) Acute gastrointestinal bleeding [*n* (%)]	/	2 (14.3)
(G) Acute bacterial infection	/	3 (21.5)

[a]In patients with previous normal consciousness.
AD: Acute decompensation; NAD: Non-acute decompensation.

Table 10 Demographic baseline data of non-acute and acute decompensating patients

	NAD-decompensating patients (*n* = 29)	AD-decompensating patients (*n* = 14)	*P* value
Male [*n* (%)]	16 (55.2)	6 (42.9)	NS[1]
Female [*n* (%)]	13 (44.8)	8 (57.1)	NS[1]
Age (mean ± SD)	64.79 ± 12.34	69.93 ± 10.91	NS[2]
Child-Pugh Grade A [*n* (%)]	9 (31.1)	3 (21.4)	NS[1]
Child-Pugh Grade B [*n* (%)]	20 (68.9)	11 (78.6)	NS[1]

[1]Chi-square test.
[2]Mann-Whitney *U* test.
Statistically significant differences ($P < 0.05$) are reported in bold; NS: Not statistically significant; AD: Acute decompensation; NAD: Non-acute decompensation.

Table 11 Anthropometric indexes demographic baseline data of non-acute and acute decompensating patients

Variables (mean ± SD)	NAD-decompensating patients (*n* = 29)	AD-decompensating patients (*n* = 14)	*P* value[1]
BMI (kg/m^2)	29.57 ± 3.17	31.43 ± 2.72	NS
WhR	0.99 ± 0.11	1.05 ± 0.17	NS
Systolic blood pressure (mm/Hg)	130.3 ± 9.81	132.9 ± 10.59	NS
Diastolic blood pressure (mm/Hg)	86.03 ± 8.59	86.07 ± 6.25	NS

[1]Mann-Whitney *U* test.
BMI: Body mass index; WhR: Waist to hip ratio. NS: Not statistically significant; AD: Acute decompensation; NAD: Non-acute decompensation.

The importance of predicting whether and how the patient affected by MASLD-related-cACLD will move to dACLD is related to various management aspects. First, decompensation constitutes a turning point in the natural history of ACLD, and an extremely relevant feature during the clinical course of cirrhosis, which should be managed as quickly and appropriately as possible, to improve the possibility of care; early detection of this transition phase would enable targeted therapeutic interventions, potential improving life expectancy, and improving their prognosis[34]. Secondly, it's also essential to highlight that risk of death strongly increases when a patient shifts to dACLD: 9.7 times as high as the risk in the general population, and it's double compared to cACLD subjects[35]. In these terms, the decompensation marks a significant worsening of patient prognosis from a median survival exceeding 12 years and a preserved quality of life in compensated patients to a median survival of 2-4 years in the decompensated stage with several socioeconomic and healthy repercussions: admission rate, hospital stay, and costs considerably increased in a stepwise manner after the first episode of AD[36]; hospitalizations for the dACLD increase by a third in the total healthcare costs compared to cACLD individuals[37].

Table 12 Biochemical parameters data of non-acute and acute decompensating patients

Variables (mean ± SD)	NAD-decompensating patients (*n* = 29)	AD-decompensating patients (*n* =14)	*P* value[1]
AST (IU/L)	27.93 ± 26.99	44.11 ± 11.26	**0.004**
ALT (IU/L)	40.10 ± 35.52	54.71 ± 51.23	NS
Bilirubin (μmol/L)	21.75 ± 5.32	26.94 ± 1.32	NS
PLT count (mm^3)	100.2 ± 24.37	61.57 ± 14.12	**< 0.0001**
RDW-CV (%)	26.16 ± 19.26	51.51 ± 21.23	**< 0.0001**
RDW-SD (fL)	54.39 ± 10.21	64.10 ± 10.14	**< 0.0001**
Albumin (g/L)	28.6 ± 2.21	21.8 ± 2.59	NS
INR	1.76 ± 0.66	1.96 ± 0.62	NS
HOMA-IR	3.44 ± 1.29	4.12 ± 1.09	NS
Total cholesterol (mg/dL)	195.4 ± 56.20	164.4 ± 44.17	NS
Tryglicerides (mg/dL)	167.4 ± 92.42	152.1 ± 60.71	NS
Creatinine (mg/dL)	1.27 ± 1.51	2.61 ± 1.22	**0.03**

[1]Mann-Whitney *U* test.
Statistically significant differences ($P < 0.05$) are reported in bold. ALT: Alanine aminotransferase; PLT: Platelets count; CV: Coefficient variation; RDW: Red-cell distribution width; INR: International normalized ratio; HOMA-IR: homeostasis model assessment for insulin resistance; NS: Not statistically significant; AD: Acute decompensation; NAD: Non-acute decompensation.

Table 13 Non-invasive tools for liver disease severity assessment of non-acute and acute decompensating patients

Variables (mean ± SD)	NAD-decompensating patients (*n* = 29)	AD-decompensating patients (*n* = 14)	*P* value[1]
LSM (kPa)	20.90 ± 2.09	28.04 ± 3.44	**< 0.0001**
APRI	1.60 ± 0.30	1.90 ± 0.38	**< 0.0001**
FIB-4	3.53 ± 1.75	3.86 ± 2.64	NS
ALBI	-1.98 ± 0.62	-1.66 ± 0.35	**0.03**
Child-Pugh	6.89 ± 0.97	7.42 ± 0.75	**0.046**
MELD	11.07 ± 3.35	13.79 ± 2.07	**0.011**
RDW (fL)/PLT ratio	0.668 ± 0.152	1.077 ± 0.253	**< 0.0001**

[1]Mann-Whitney *U* test.
Statistically significant differences ($P < 0.05$) are reported in bold. LSM: Liver stiffness measurement; APRI: Aspartate aminotransferase/platelet count ratio index; MELD: Model for End-stage Liver Disease FIB-4: Fibrosis-4; NS: Not statistically significant; AD: Acute decompensation; NAD: Non-acute decompensation.

Table 14 Receiving operator curve features using cut-off values > 0.894 as the red-cell distribution width to platelet ratio threshold in the prediction of acute decompensation as first decompensation event

	Value	95%CI
Sensitivity	0.9310	0.7804-0.9877
Specificity	0.8571	0.6006-0.9746
Positive predictive value	0.9310	0.7804-0.9877
Negative predictive value	0.8571	0.6006-0.9746

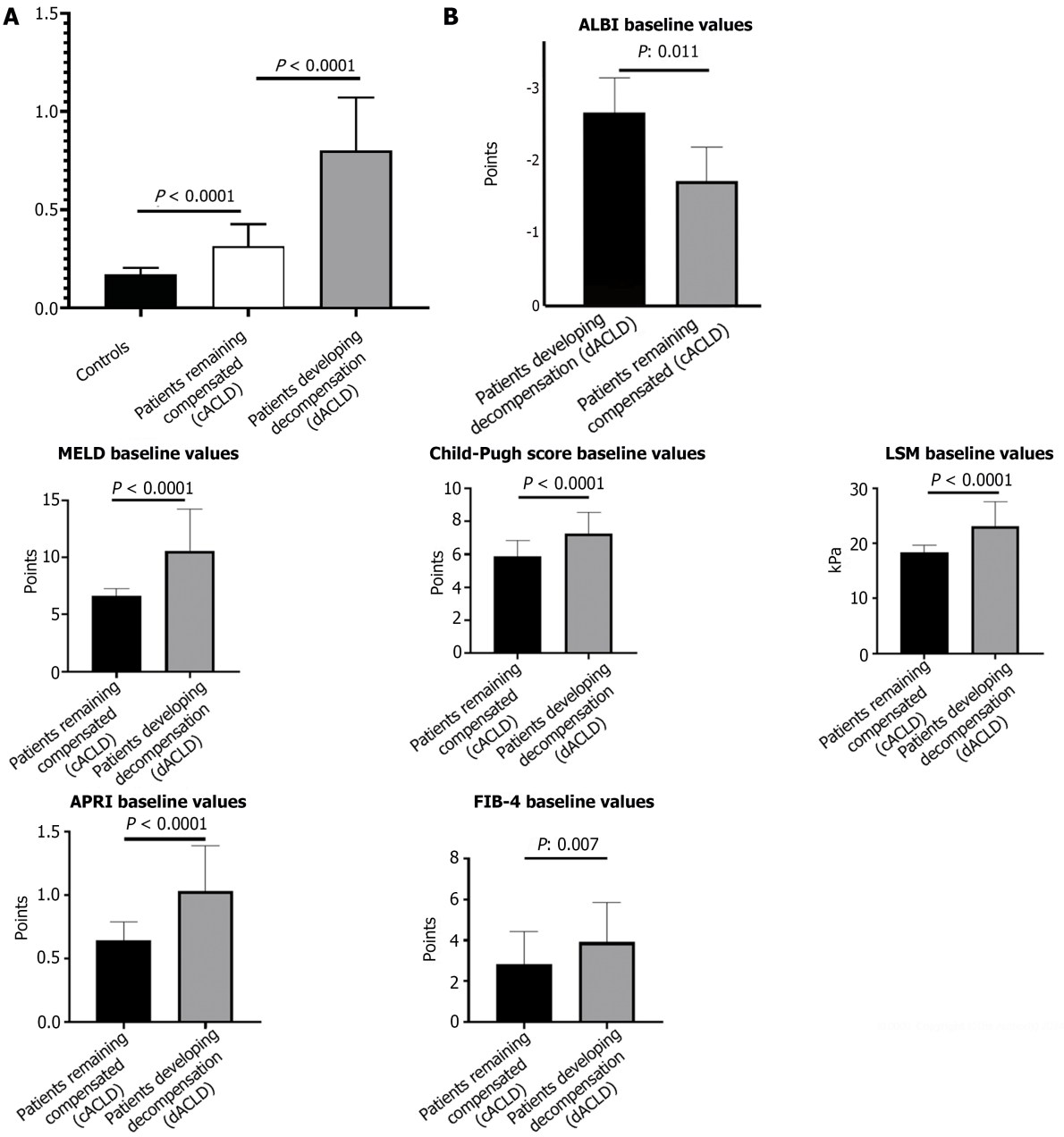

Figure 2 Comparison of red cell distribution width/platelet ratio and other non-invasive tools baseline values between compensated individuals and patients progressing to decompensation during the follow-up period. A and B: Cell distribution width/platelet ratio (A), other non-invasive tools (B). LSM: Liver Stiffness Measurement; CSPH: Clinically Significant Portal Hypertension; cACLD: Compensated advanced chronic liver disease; dACLD: Decompensated advanced chronic liver disease; MELD: Model for End-Stage Liver Disease; APRI: Aspartate aminotransferase/platelet count ratio index; ALBI: Albumin-Bilirubin; FIB-4: Fibrosis-4.

A plethora of studies have tried to explain which could be the most accurate predictor of decompensation in these patients[9,38]. The strongest predictor of transition to dACLD is, for values of ≥ 10 mmHg, the HVPG, well-studied as a marker of CSPH. However, due to the limitations related to justifying invasive HVPG measurement and its expensive costs, it is almost never evaluated in daily clinical practice in most centers[9,39]. However, while if for patients with viral- and alcohol-related cirrhosis, HVPG measurement is the gold-standard method to determine the presence of CSPH, in MASLD/MASH individuals the question is still widely debated[32,38]. Moreover, in patients with MASH- related cirrhosis, although an HVPG 10 mmHg remains strongly associated with the presence of clinical signs of portal hypertension, these signs can also be present in a small proportion of patients with HVPG values < 10 mmHg[32,38]. For all these reasons, the identification of other tools in this setting of patients is an unmet need and the availability of a non-invasive, easy to use, and not expensive index able to accurately predict the risk of decompensation could represent a revolutionary MASLD-management clinical weapon. In this sense, RPR appears an extremely useful and easy-to-adopt solution, both for its low invasiveness and costs, as it can be calculated by routine values available in daily clinical practice.

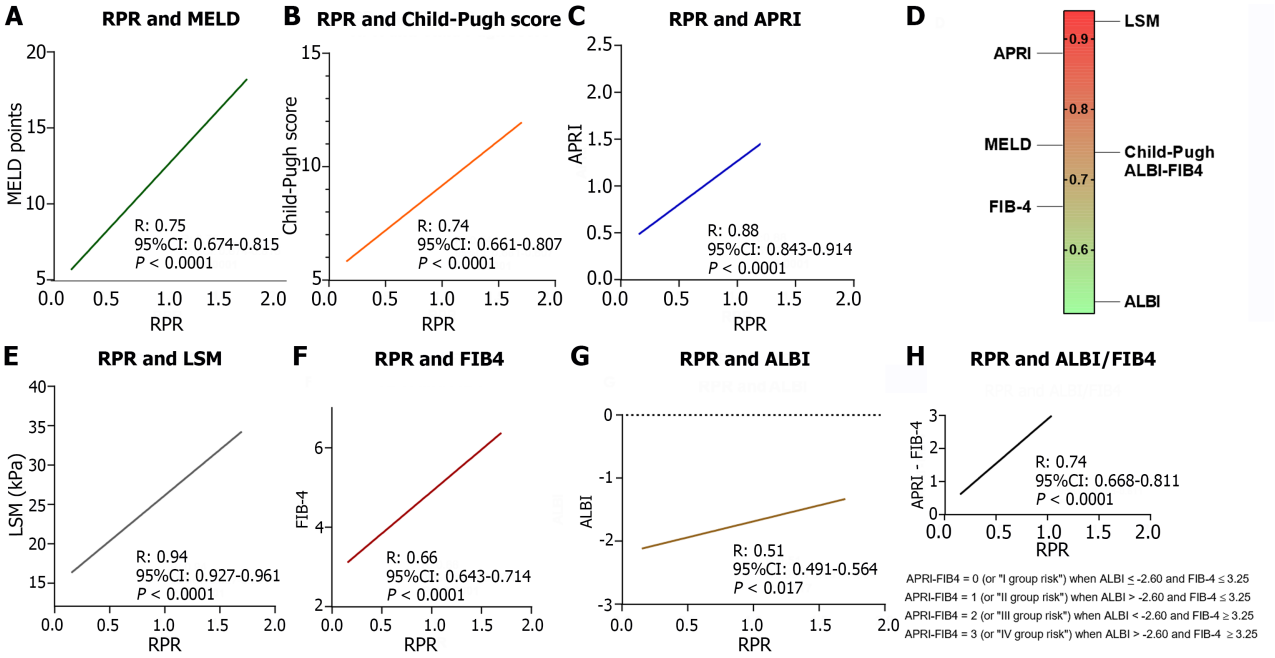

Figure 3 Relationship between baseline red cell distribution width/platelet ratio values and validated tools non-invasively assessing liver-function status and hepatic fibrosis. A, B, C, E, F, G and H: Linear regression of red cell distribution width to platelet ratio (RPR) and Model for End-Stage Liver Disease (A); RPR and Child-Push score (B); RPR and aspartate aminotransferase/platelet count ratio index (C); RPR and Liver Stiffness Measurement (E); RPR and Fibrosis-4 (FIB-4; F); RPR and Albumin-Bilirubin (ALBI; G); RPR and ALBI/FIB-4 (H). D: Heat map of R values revealed of to the linear regression analysis between baseline RPR others tools reported in panel A, B, C, E, F, G, H. LSM: Liver Stiffness Measurement; ALBI: Albumin-Bilirubin; FIB-4: Fibrosis-4; APRI: Aspartate aminotransferase/platelet count ratio index; MELD: Model for End-Stage Liver Disease; RPR: Red cell distribution width to platelet ratio.

Different research investigated the role of other non-invasive and routinely tools in the prediction of decompensation. Guha *et al*[12] in a recent study, also including patients with aetiologies other than MASLD, introduced a new model to predict the risk of decompensation in patients with compensated cirrhosis based on the combination of two (ALBI + FIB-4) previously identified scores: ALBI-FIB-4.

In our study, following the original ALBI-FIB-4 proposed group stratification, we compared the accuracy of RPR with ALBI-FIB-4 in the prediction of decompensation revealing a higher RPR performance in the prediction of this outcome (AUC: 0.95 *vs* AUC: 0.93). The NAFLD decompensation risk score (the Iowa Model) was recently developed to identify patients with MASLD at the highest risk of developing hepatic events using three variables-age, PLT count, and diabetes [15]. In a recent study including 249 MASLD patients, the AUC of the Iowa Model (0.88) was comparable to the FIB-4 (0.87) and higher than APRI (0.76)[15]. We herein decided to not perform a comparison RPR *vs* Iowa model, considering the new proposed MASLD diagnostic criteria[6] supporting the non-essential presence of diabetes to perform diagnosis, as many MASLD patients may present without this comorbidity. Rather, in our study, diabetes was included as a confounding variable in the multinomial logistic regression analysis.

The multinomial logistic regression analysis, performed by considering the confounding variables (sex, age, BMI, diabetes, alcohol intake, the administration of Non-Selective Beta Blockers), revealed besides the RPR, the baseline CSPH as a variable significantly associated with the outcomes (DE and AD). These findings constituted the *primum movens* to perform a sub-analysis investigating the relationship between RPR, liver disease progression, CSPH, and decompensation in our study. Consistently with the chronic nature of MASLD disorder, a significant positive correlation between RPR (DRPR) and LSM (DLSM) modifications was highlighted, suggesting RPR is dynamically influenced by the course of the hepatic disease.

The inclusion of CSPH assessment represented a crucial strength of our research: In fact, none of the other previously mentioned evidence reported the proportion of patients with varices, making uncertain whether patients were comparable regarding their likelihood of having CSPH and, therefore, of decompensating. In our study, baseline RPR values were significantly higher in patients with baseline CSPH ($P < 0.04$) and positively correlated with esophageal varices severity ($P < 0.0001$). The prevalence of baseline CSPH in decompensating patients was significantly higher in patients presenting RPR baseline values 0.472. Relevantly, individuals presenting baseline CSPH and RPR values 0.472 showed a significantly elevated risk (HR: 3.10, *P*: 0.0023) of decompensation in comparison to baseline-CSPH individuals presenting lower RPR values supporting the following risk-stratification: (1) "High risk of decompensation" (baseline CSPH and RPR < 0.472); and (2) "very high-risk of decompensation" (baseline CSPH and RPR 0.472). Considering the discrepant modalities of CSPH definition between baseline (EGDS-evidence of esophageal varices) and on first DE occurrence (CSPH assumption according to Baveno VII criteria) with a not-negligible number (61%) of patients avoiding/not undergoing surveillance endoscopy (*i.e.*, repetition, during the 3-years follow-up, of a new EGDS for patients presenting baseline CSPH) also due to SARS CoV2 pandemic-related logistic difficulties, the not-availability of HVPG data, and, even more relevant, the limited sample size of the sub-analysis, the RPR baseline accuracy in the prediction of

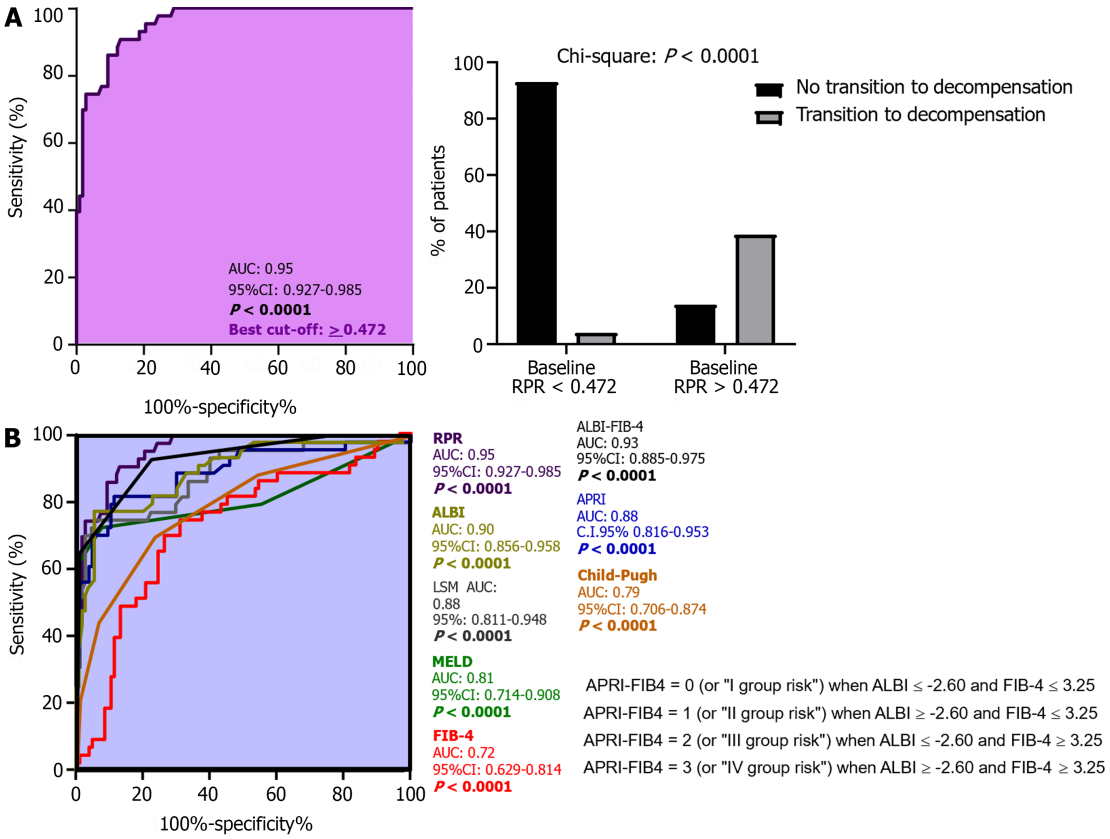

Figure 4 3-year decompensation predictive accuracy of red cell distribution width/platelet ratio and comparison with other non-invasive tools. A: Accuracy of baseline red cell distribution width to platelet ratio in predicting 3-years decompensation; B: Comparison with other non-invasive tools. ALBI: Albumin-Bilirubin; FIB-4: Fibrosis-4; APRI: Aspartate aminotransferase/platelet count ratio index; MELD: Model for End-Stage Liver Disease; RPR: Red cell distribution width to platelet ratio; AUC: Area under the curve.

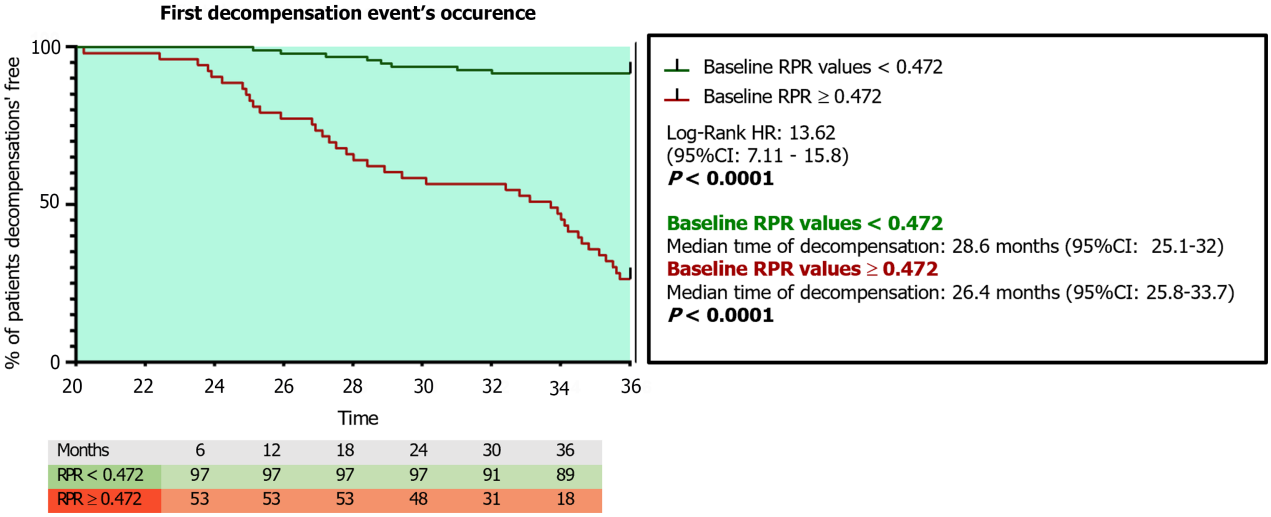

Figure 5 The first decompensation event's occurrence risk according to the baseline red cell distribution width/platelet ratio values. RPR: Red cell distribution width to platelet ratio.

baseline CSPH and CSPH development along the observational period did not represent an aim of our study and was herein not investigated. The PREDESCI trial evidenced the role of non-selective beta-blockers in the prevention of decompensation in patients with CSPH[40]. Considering this, after the inclusion of the administration of propranolol and carvedilol (recorded at the baseline and on every semiannual follow-up visit) in the logistic regression model, no influence on our predictive results was highlighted.

Our study presents some limitations. First, it is based on a single-center cohort of patients, so further prospective studies at multiple centers are required to validate the clinical use of RPR in validation cohorts. Second, our population, even if a representative MASLD cohort, could represent a relatively small sample size. Finally, the accuracy of RPR was

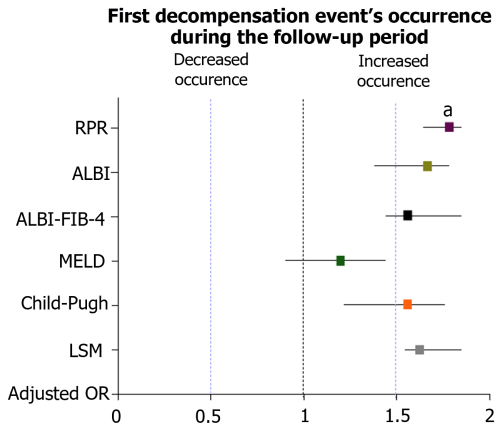

Figure 6 Adjusted odds ratios for non-invasive tools on the first decompensation event's occurrence. [a]*P* = 0.02. RPR: Red cell distribution width to platelet ratio; ALBI: Albumin-Bilirubin; FIB-4: Fibrosis-4; MELD: Model for End-Stage Liver Disease; LSM: Liver Stiffness Measurement; OR: Odds ratio.

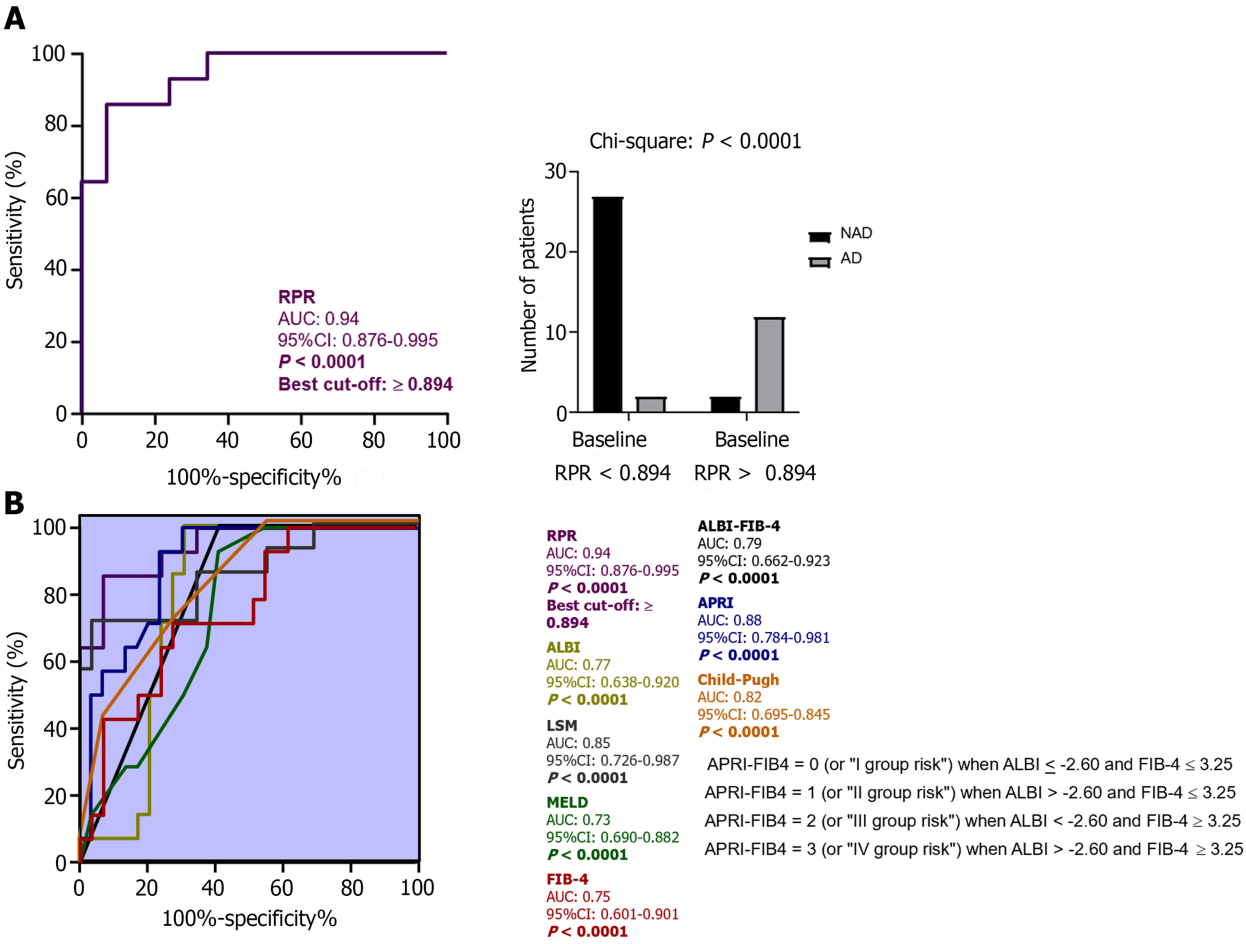

Figure 7 3-year acute decompensation predictive accuracy of red cell distribution width/platelet ratio and comparison with other non-invasive tools. A: Red cell distribution width/platelet ratio; B: Comparison with other non-invasive tools. AD: Acute decompensation; NAD: Non-acute decompensation; ALBI: Albumin-Bilirubin; FIB-4: Fibrosis-4; APRI: Aspartate aminotransferase/platelet count ratio index; MELD: Model for End-Stage Liver Disease; RPR: Red cell distribution width to platelet ratio; AUC: Area under the curve.

not compared with HVPG; unfortunately, in fact, the SARS CoV2 Lock-down negatively limited the availability of this tool in our center during the pandemic and we were able to collect HVPG data for a very restricted number of the enrolled patients.

As a final consideration, in the wake of our results and looking ahead to future scenarios, considering the elevated high risk of major cardiovascular events occurrence in MASLD patients[41], and the RDW well-consolidated association with cardiovascular diseases-related complications[42], it appears also reasonable to hypothesize the designation of studies investigating the potential relationship between the RPR and risk of cardiovascular acute events in MASLD individuals.

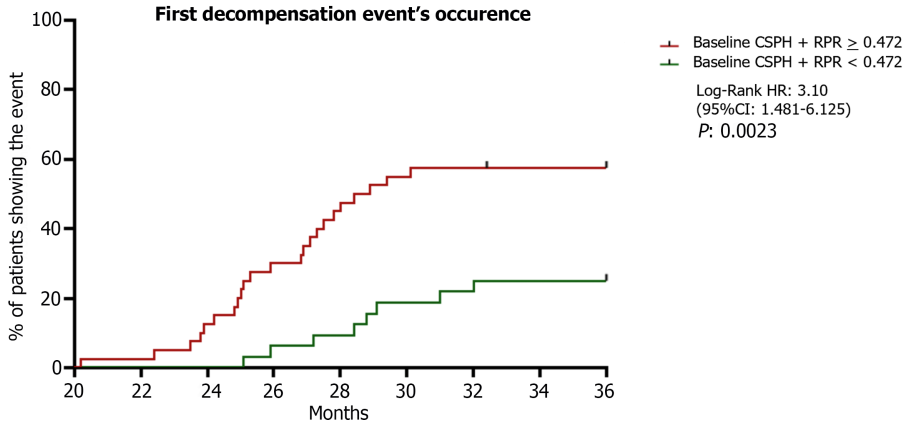

Figure 8 The first decompensation event's occurrence risk according to the baseline red cell distribution width/platelet ratio values and the presence of Clinically Significant Portal Hypertension. RPR: Red cell distribution width to platelet ratio; CSPH: Clinically Significant Portal Hypertension; HR: Hazard ratio.

The developing of tools simultaneously identifying MASLD subjects at higher risk of hepatic decompensation and acute cardiovascular events occurrence would represent a cornerstone element in the prognostic tailored management of these patients.

CONCLUSION

In the era of Precision Medicine, the development of tools non-invasively predicting decompensation in cACLD patients represents an unmet need and appears a paramount challenge for the hepatological research. Our study suggests RPR accurately predicts the time and modalities of decompensation in MASLD-related-ACLD patients, presenting the potential to be a valuable, easy-to perform, non-invasive clinical index.

ARTICLE HIGHLIGHTS

Research background

In clinical practice, the availability of non-invasive tools predicting the first decompensation event (DE) in Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD)-related compensated advanced chronic liver disease (cACLD) context is still an unmet need.

Research motivation

Red cell distribution width to platelet ratio (RPR) has been recently shown to predict fibrosis in MASLD patients; however, its role in predicting DE has never been explored.

Research objectives

Herein, we investigated the clinical usage of RPR in predicting DEs in MASLD-related cACLD patients.

Research methods

MASLD-cACLD patients were consecutively enrolled and followed up for 3 years. Biochemical, clinical, and Liver Stiffness Measurement were collected.

Research results

RPR accurately predicts [area under the curve (AUC): 0.94; best cut-off 0.472) the first DE in MASLD-cACLD. RPR accurately predicts acute decompensation (AD; AUC: 0.94; best cut-off 0.894) as the first DE in these patients. Patients presenting baseline clinically significant portal hypertension and RPR 0.472 show higher risk (hazard ratio: 3.10) of 3-year decompensation occurrence.

Research conclusions

Altogether these findings suggest RPR as a valid and potentially applicable non-invasive tool in the prediction of decompensation in MASLD-related cACLD patients.

Research perspectives

The potential availability of RPR as non-invasive, not expensive, and routinely assessable tool in the prediction of timing and modalities of decompensation in MASLD-cACLD patients could remodel the management of these patients.

FOOTNOTES

Co-first authors: Marcello Dallio and Mario Romeo.

Author contributions: Romeo M is responsible for guarantor of the article, conceptualization, methodology, investigation, and writing the original draft; Vaia P, Auletta S, Mammone S, Dallio M are responsible for conceptualization, methodology, formal analysis, investigation, and writing the original draft; Cipullo M, Niosi M are responsible for investigation, resources, data curation, and visualization; Naviglio S, Sapio L are responsible for reviewing the original draft; Ragone A is responsible for visualization; Federico A is responsible for conceptualization, data curation, supervision; all authors approved the final version of the manuscript. In light of the shared collaborative effort, as well as the distribution of responsibilities and burdens to complete the study, Dallio M and Romeo M were designated as co-first authors.

Institutional review board statement: The study was reviewed and approved by the Comitato Etico Università degli Studi della Campania "Luigi Vanvitelli" -Azienda Ospedaliera Universitaria "Luigi Vanvitelli"- AORN "Ospedali dei Colli" Institutional Review Board (Approval No. 417).

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: The authors declare no competing interests.

Data sharing statement: The data presented in this study are available on request from the corresponding author.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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ORIGINAL ARTICLE

Prospective Study

Gastrointestinal contrast-enhanced ultrasonography for diagnosis and treatment of peptic ulcer in children

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Abstract

BACKGROUND

The detection rate of peptic ulcer in children is improving, with development of diagnostic procedures. Gastroscopy is the gold standard for the diagnosis of peptic ulcer, but it is an invasive procedure. Gastrointestinal contrast-enhanced ultrasonography (CEUS) has the advantages of being painless, noninvasive, nonradioactive, easy to use, and safe.

AIM

To investigate the clinical value of CEUS for diagnosis and treatment of peptic ulcer in children.

METHODS

We investigated 43 children with digestive tract symptoms in our hospital from January 2021 to June 2022. All children were examined by routine ultrasound, gastrointestinal CEUS, and gastroscopy. The pathological results of gastroscopy were taken as the gold standard. Routine ultrasonography was performed before gastrointestinal CEUS. Conventional ultrasound showed the thickness of the gastroduodenal wall, gastric peristalsis, and the adjacent organs and tissues around the abdominal cavity. Gastrointestinal CEUS recorded the thickness of the gastroduodenal wall; the size, location and shape of the ulcer; gastric peristalsis; and adjacent organs and tissues around the abdominal cavity. The results of routine ultrasound and gastrointestinal ultrasound were compared with those of gastroscopy to evaluate the diagnostic results and coincidence rate of routine ultrasound and gastrointestinal CEUS. All children received informed consent from their guardians for CEUS. This study was reviewed and approved by the hospital medical ethics committee.

RESULTS

Among the 43 children, 17 (15 male, 2 female) were diagnosed with peptic ulcer

by gastroscopy. There were 26 children with nonpeptic ulcer. There were eight cases of peptic ulcer and 35 of nonpeptic ulcer diagnosed by conventional ultrasound. The diagnostic coincidence rate of peptic ulcer in children diagnosed by conventional ultrasound was 79.1% (34/43), which was significantly different from that of gastroscopy (P = 0.033). It indicates that the coincidence rate of gastrointestinal contrast-enhanced ultrasound and gastroscope is low. Fifteen cases of peptic ulcer and 28 of nonpeptic ulcer were diagnosed by CEUS. The diagnostic coincidence rate of peptic ulcer in children was 95.3% (41/43). There was no significant difference between CEUS and gastroscopy (P = 0.655). It indicates that the coincidence rate of gastrointestinal contrast-enhanced ultrasound and gastroscope is high.

CONCLUSION

Gastrointestinal CEUS has a high coincidence rate in the diagnosis of peptic ulcer in children, and can be used as a preliminary examination method.

Key Words: Contrast-enhanced ultrasound; Peptic ulcer; Children; Gastrointestinal tract; Abdominal pain; Acoustic contrast agent

Core Tip: In this study, routine gastrointestinal ultrasound and contrast-enhanced ultrasonography (CEUS) in children were compared with gastroscopy. The clinical coincidence rate between gastrointestinal CEUS and gastroscopy was higher, which provided a new examination method for pediatricians to screen upper gastrointestinal diseases. This method is painless, noninvasive, nonradioactive, simple to operate, accepted by children and parents, and can be used as a preliminary screening method for children with epigastric pain. It is expected to be an effective supplement to gastroscopy and provide a reference for clinical selection of appropriate treatment.

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INTRODUCTION

Diseases of the digestive system are common in childhood, and peptic ulcer is also common in clinical practice. However, the clinical symptoms of peptic ulcer in children are not typical and there is a lack of specific symptoms and signs in the early stage. Children cannot accurately express conscious symptoms or it is difficult to accurately describe the location and nature of the symptoms, resulting in missed diagnosis or misdiagnosis of peptic ulcer. Gastroscopy can directly observe the gastric and duodenal mucosa and the degree of pathological changes, which is the gold standard for diagnosis of gastrointestinal diseases[1]. However, as gastroscopy is an invasive method, clinicians and parents have some concerns about whether children can tolerate the examination process, and its safety[2]. At present, with the improvement of ultrasonic image resolution and the continuous improvement and development of ultrasonic diagnostic technology, contrast-enhanced ultrasonography (CEUS) is safe, simple and noninvasive, which makes the acceptance of patients higher[3].

This was a analysis of 43 children with digestive tract symptoms treated in our hospital from January 2021 to June 2022, to explore the clinical diagnostic value of routine ultrasound and gastrointestinal CEUS in children with peptic ulcer.

MATERIALS AND METHODS

General information

We investigated 43 children with gastrointestinal symptoms in our hospital from January 2021 to June 2022. All patients were examined by routine ultrasound, gastrointestinal CEUS, and gastroscopy. Eight patients (all male) with peptic ulcer were diagnosed by routine ultrasound, The age was 8-15 years, with an average of 10.8 ± 2.5 years. Fifteen patients (13 male, 2 female) with peptic ulcer were diagnosed by gastrointestinal CEUS. The age was 8-15 years, with an average of 11.4 ± 2.3 years. The above cases all had different degrees of upper gastrointestinal symptoms, such as epigastric fullness, nausea, vomiting, and epigastric pain. Some children showed periodic epigastric pain, empty abdominal pain or nocturnal pain, intermittent black stools, and anemia. The above children were compared with those who were examined by gastroscopy.

Gastroscopy

The gastroscopy method was based on the consensus of experts on gastroscopy and colonoscopy for children in Europe [4].

Routine ultrasound

Color Doppler ultrasound was performed using a Philips EPIQ7 color Doppler ultrasound diagnostic instrument (Philips, Netherlands), L12-5 Linear array probe, at a frequency of 5-12 MHz. The patients ate a light diet the day before the inspection, and avoided food that can produce gas and is not easy to digest. Patients fasted for 8 h and refrained from drinking for > 4 h before examination. Contrast agent produced by Huqingtang (Hangzhou, China) was chosen. Before taking the contrast agent, the contraindications such as gastrointestinal perforation, acute gastric dilatation and intestinal obstruction were eliminated by whole abdominal scan. The contrast agent was added to 150-200 mL water at 35-45 °C, stirred well, and hot water at > 90 °C was added to make 400-500 mL. This was stirred again and set aside. After cooling to a suitable temperature, the patient took the fluid orally. The dose depended on the age of the child: 3-10 years, 200-400 mL; 10-15 years, 400-500 mL.

CEUS

CEUS was performed by the same ultrasound physician on the same machine, and they did not know the pathological results of the child before the examination. Mainly in sitting, supine and right supine positions, a series of vertical and transverse and oblique scans were performed in the left middle and upper abdomen. There were the following scanning sections: (1) Cardia and lower esophagus (Figure 1A). The probe was placed obliquely under the left costal region near the xiphoid process and rotated to the left and rear to obtain the long-axis sonogram of the lower esophagus and cardia, and then the cross-exchange scan was performed to obtain the short-axis section and sonogram of the cardia and lower esophagus; (2) gastric fundus (Figure 1B). The probe was tilted to the left quarter rib and rotated to the left, posterior and upper, with an angle range of 0–80°. This section showed the fundus sonogram more completely; (3) gastric body (Figure 1C). The long axis of the gastric body can be displayed when the probe is positioned longitudinally on the left upper abdomen, and the short axis of the gastric body can be displayed when the probe is moved horizontally on the left upper abdomen; (4) gastric angle (Figure 1D). The probe was placed horizontally on the abdomen and scanned continuously around the umbilical cord to obtain a sonogram similar to the "double ring sign". The double-ring junction was the cross section of the gastric angle, the left ring was the gastric body, and the right ring was the gastric antrum; (5) gastric antrum (Figure 1E). The long axis of the probe was placed obliquely between the navel and the right upper abdomen, and the longest acoustic image of the gastric cavity was obtained by scanning at different angles. Moving left and right or up and down in this direction, yielded a complete image of the long axis of the gastric antrum. By positioning the probe in the long axis section of the gastric antrum, the complete short axis image of the gastric antrum was obtained by continuous cross scanning; (6) gastric coronal oblique section (Figure 1F). The probe was placed obliquely between the periumbilical region and the left upper abdomen, and a continuous lateral scan to the right front showed a clear gastric coronal oblique section; and (7) duodenum (Figure 1G). The probe was longitudinally placed in the right upper abdomen, its upper end rotated 60° to the right and 30° to the left, and the lower end of the probe was relatively fixed. A more complete duodenal sonogram was obtained in this range. We observed the duodenal bulb, and the descending, horizontal and ascending duodenum.

Typical CEUS findings of peptic ulcer

We observed local thickening of the gastric wall, and interruption and depression of the gastric mucosa at the bottom of the ulcer. The diameter of the ulcer was 5-10 mm, the shape was fairly regular, the edge was symmetrical and slightly raised, and it had a crater-like appearance. The thickened gastric wall at the base of the depression and around it showed low echo. The concave surface of the mucous membrane was flat. Peptic ulcer showed speckled hyperecho under CEUS. The local peristalsis of the gastric wall was weakened or disappeared. Duodenal bulbar ulcer showed localized thickening of the intestinal wall, deformed bulb, poor fluid filling, localized depression on the surface of duodenal bulbar ulcer with a diameter of 10 mm, and strong echo spots on the surface. These are typical CEUS and gastroscopy findings of peptic ulcer (Figures 2 and 3).

Statistical analysis

SPSS version 16.0 was used for statistical analysis. The numerical data (diagnostic results, coincidence rate, and diagnostic accuracy) were tested by χ^2 test and expressed as percentages. $P < 0.05$ indicated a significant difference.

RESULTS

Seventeen cases (15 male, 2 female) of peptic ulcer were diagnosed by gastroscopy. Fifteen children were positive for *Helicobacter pylori (H. pylori)* antibody and had different degrees of abdominal pain. Hemoglobin decreased by varying degrees in laboratory examination. Hemoglobin level was 51-105 g/L, with an average of 82.1 ± 13.8 g/L. There were also 26 cases of nonpeptic ulcer.

Routine ultrasound diagnosed eight cases of peptic ulcer, 35 cases of nonpeptic ulcer and there was nine missed diagnoses. The results are compared with gastroscopy in Table 1.

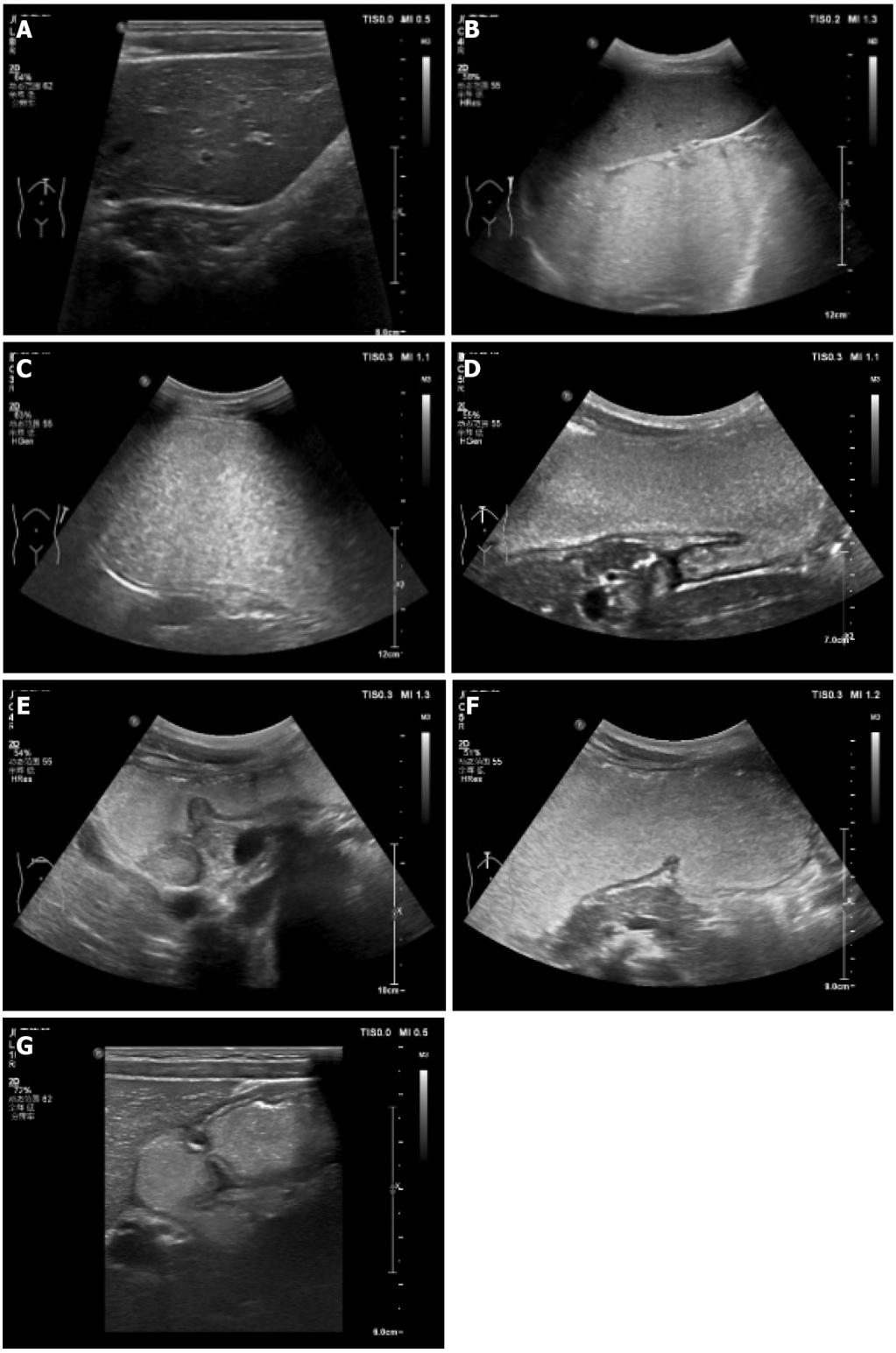

Figure 1 Contrast-enhanced ultrasound scanning. A: Section of cardia and lower esophagus; B: Section of gastric fundus; C: Section of the gastric body; D: Gastric angle section; E: Antrum section; F: Gastric coronal oblique section; G: Duodenal section.

Gastrointestinal CEUS diagnosed 15 cases of peptic ulcer, 28 cases of nonpeptic ulcer, and there were two missed diagnoses. The results are compared with gastroscopy in Table 2.

DISCUSSION

Peptic ulcer refers to chronic ulcer in the stomach and duodenum. It is a common disease in adults that is mainly caused by the erosion of gastrointestinal mucosa by gastric acid and pepsin. In the past, it was considered that peptic ulcer was

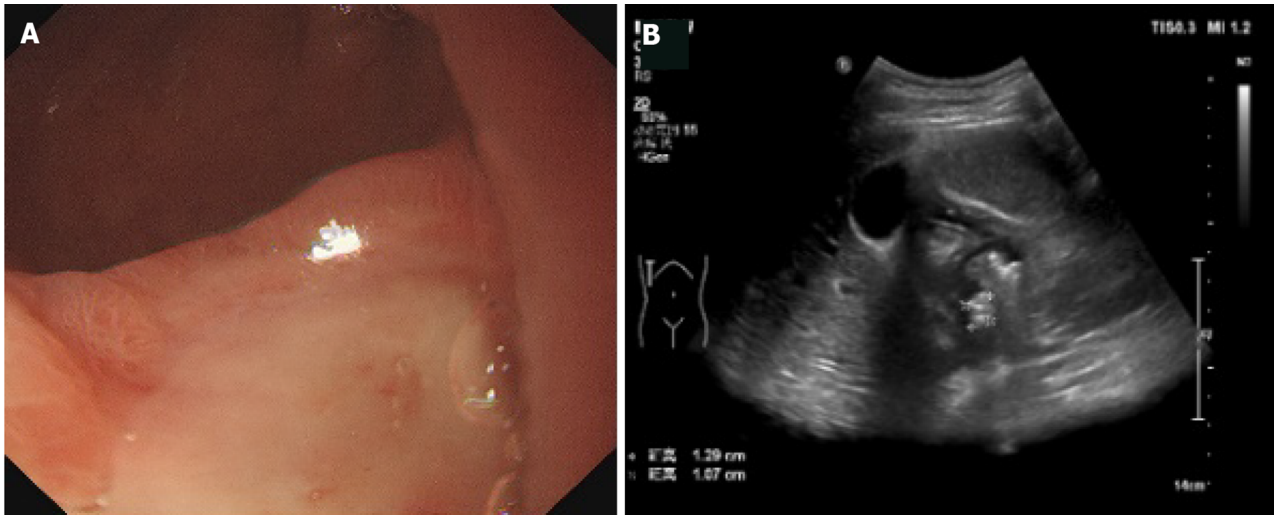

Figure 2 A 14-year-old male with duodenal ulcer diagnosed by gastroscopy. A: Gastroscope indicated a large ulcer on the anterior wall of the bulb, covered with thick white moss, congestion and edema of the surrounding mucosa; B: Contrast-enhanced ultrasonography showed that the shape of duodenal bulb was irregular, the area was small, and there were hyperechoic plaques, 13 mm × 11 mm × 13 mm in size, on the anterior wall of the duodenum.

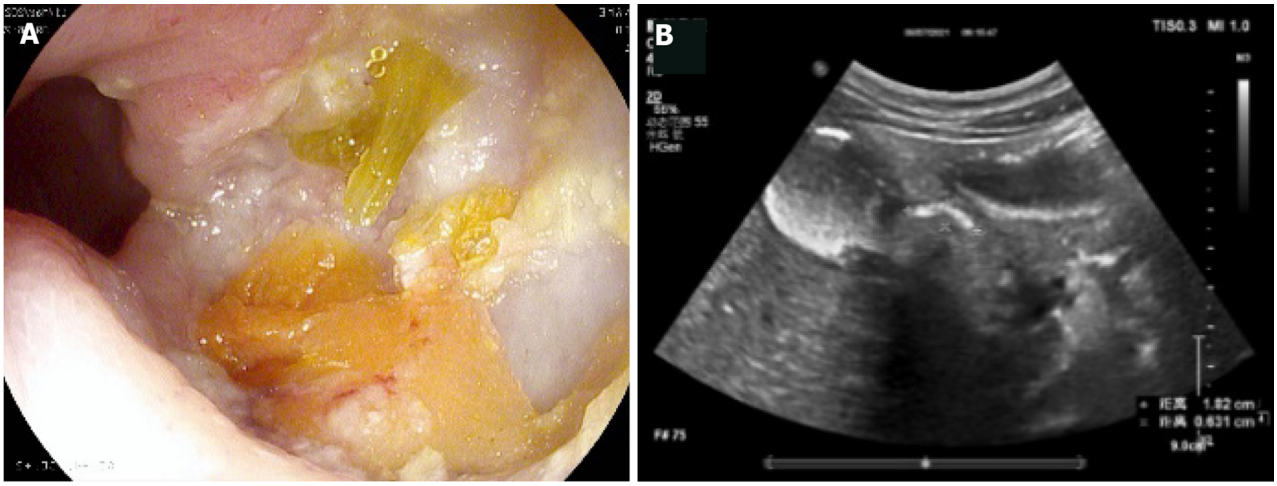

Figure 3 A 14-year-old female with duodenal ulcer diagnosed by gastroscopy. A: Gastroscopy indicated that there was a large ulcer in the duodenal bulb, covered with thick yellow and white moss, congestion and edema of the surrounding mucosa; B: Gastrointestinal contrast-enhanced ultrasonography showed that the shape of the duodenal bulb was irregular and the area was small; the anterior wall of the duodenum showed a hyperechoic plaque of 18 mm 6 mm; the local wall showed hypoechoic thickening, and the mucosal fold of the bulbar wall slightly thickened.

rare in children, but the development of diagnostic procedures has increased detection rates continuously. The lifetime prevalence rate of peptic ulcer in the general population is 5%-10%, with an annual incidence rate of 0.1%-0.3%[5]. Peptic ulcer in children is caused by many factors. The common cause is *H. pylori* infection. Oral and fecal transmission is the main route. *H. pylori* infection is mainly acquired in childhood, and adults can transmit *H. pylori* to children. *H. pylori* infection is still highly prevalent in children and adolescents globally[6]. In this study, the number of children with *H. pylori* infection was 88%. Overseas studies have also shown that *H. pylori* is the main cause of peptic ulcer, and > 75% of duodenal ulcers and > 17% of gastric ulcers are associated with this infection[7]. In children with *H. pylori*-infected duodenal ulcers, the mucosal microbiota of the duodenal bulb is altered, characterized by an increased abundance of *H. pylori* and decreased abundance of *Clostridium* and *Streptococcus*, which possibly alters the biological function of the commensal microbiota through specific metabolic pathways[8]. A systematic review and meta-analysis that assessed the global prevalence of *H. pylori* infection found that more than half the population is infected[9]. Therefore, it is essential to develop a plan for early detection of *H. pylori* to reduce the risk of peptic ulcer[10].

In our study, there were more male than female children with peptic ulcer, which is consistent with a previous study [11]. This may be because boys exercise more, eat too much and eat fast, which leads to excessive and rapid gastric acid secretion, which in turn destroys the duodenal mucosal barrier. Girls have higher estrogen levels, which can stimulate the duodenal mucosa to secrete bicarbonate, enhance mucosal barrier function, inhibit gastric and duodenal juice secretion, and reduce pepsin activity, thus protecting gastrointestinal mucosa to reduce the occurrence of ulcers[12,13].

Table 1 Comparison of the results of routine ultrasound and gastroscopy

		Method (%)		Total	χ^2	*P* value
		Routine ultrasound	Gastroscopy			
Group	Positive	8 (18.60)	17 (39.53)	25 (29.07)	4.568	0.033[a]
	Negative	35 (81.40)	26 (60.47)	61 (70.93)		
Total		43	43	86		

[a]$P < 0.05$.

Table 2 Comparison of the results of gastrointestinal contrast-enhanced ultrasound and gastroscopy

		Method (%)		Total	χ^2	*P* value
		Gastrointestinal contrast-enhanced ultrasound	Gastroscopy			
Group	Positive	15 (34.88)	17 (39.53)	32 (37.21)	0.199	0.655
	Negative	28 (65.12)	26 (60.47)	54 (62.79)		
Total		43	43	86		

The clinical symptoms of peptic ulcer in children vary with age. Studies have shown that younger children may be irritable, eat a poor diet, and have gastroesophageal reflux but no significant weight gain, and older children may be characterized by abdominal pain, flatulence, hematemesis and black stools[14]. In this study, all children had varying degrees of abdominal pain, because most children cannot accurately describe the degree, location and duration of abdominal pain, it is easy to miss diagnosis or misdiagnose, resulting in delayed treatment, seriously affecting the health of children. Moreover, the proportion of perforation, massive hemorrhage, severe anemia and other serious complications of peptic ulcer in children is higher than in adults[15,16]. It has been reported that most cases of duodenal ulcer perforation are in teenagers; less than half the cases have a history of abdominal pain, and most of them have ulcer perforation at the beginning of acute disease, which may be related to the inability of children to accurately describe abdominal discomfort[17].

The results of gastroscopy are used as the gold standard for the diagnosis of peptic ulcer in children. However, gastroscopy is an invasive examination method. Because the anatomical structure of the upper digestive tract in children is different from that in adults, it is difficult to operate with the narrow gastrointestinal tract and thin gastrointestinal wall, and cooperation from children is difficult. It is reported that only 55% of children with gastrointestinal symptoms have abnormal gastroscopy results[18]. Age is also a risk factor for children in capsule endoscopy[19]. Children do not cooperate well with ordinary gastroscopy, and even painless electronic gastroscopy may have adverse reactions such as hypotension, myocardial ischemia, drug allergy, and arrhythmia. Therefore, ultrasound as a safe, simple, noninvasive and rapid examination method for screening peptic ulcer in children is particularly important. Due to the presence of intestinal gases and feces, the diagnostic quality of conventional gastrointestinal ultrasound may be affected, and the diagnostic sensitivity is lower in older or obese children. Gastrointestinal CEUS can eliminate the interference of gas and contents in the gastrointestinal cavity and improve the diagnostic value of gastroduodenal diseases by filling the gastrointestinal cavity with oral contrast agent.

A total of 43 children were included in this study; 17 cases of duodenal ulcer were diagnosed by gastroscopy, and eight cases were diagnosed by routine ultrasound. The diagnostic accuracy was 47.1% (8/17), and the coincidence rate was 79.1% (34/43). Ultrasonography showed thickening of the local intestinal wall of the duodenum, decreased echo, stiffness and decreased peristalsis. Routine gastrointestinal ultrasound has some limitations, and the coincidence rate of diagnosis is low. Transabdominal ultrasonography is an effective method for detecting peptic ulcer in low-weight children[20]. Therefore, sonographers should carefully evaluate indirect findings around the stomach or duodenum[21]. Fifteen cases were diagnosed by gastrointestinal CEUS, the diagnostic accuracy was 88.2% (15/17), and the coincidence rate was 95.3% (41/43). Ultrasound showed that the shape of the duodenal bulb was irregular, the area was small, the local intestinal wall showed hypoechoic thickening, the mucosal folds of the duodenal wall were thickened, the ulcer surface had local depression, and strong echo spots could be seen on the surface. Gastrointestinal CEUS missed diagnosis in two cases, which may be because the ulcer area was smaller, and the gastric and duodenal ulcer in children is more difficult to see than in adults[22]. The lesion is small, with a diameter of 3-4 mm, and the ulcer is superficial, the bottom is smooth, and the thickening of the gastric wall around the ulcer is not obvious, so it is easy to miss diagnosis. The occurrence of these missed cases shows the limitations of CEUS. It is less sensitive to small and superficial upper digestive tract ulcers and requires a high level of operator skill. However, compared with gastroscopy, CEUS has advantages in compliance, repeatability, incidence of complications and tolerance in children.

Our study had some limitations. Only a few cases were selected. Only the examination results and coincidence rate were analyzed, and the correlation between the size, location, age, weight and diagnostic accuracy of ulcer was not studied in depth. The children with peptic ulcer were not re-examined to evaluate the clinical treatment effect and

supervise the recurrence.

CONCLUSION

Gastrointestinal CEUS in children has high accuracy, which provides pediatricians with a new and simple method for screening upper gastrointestinal diseases in children, which is easily accepted by children and parents, and is an effective supplement to gastroscopy. For children with recurrent abdominal pain and other upper gastrointestinal symptoms with unknown etiology, gastrointestinal CEUS should be performed to provide a reference for clinical selection of appropriate treatment.

ARTICLE HIGHLIGHTS

Research background

In a larger study, we will investigate the correlation between the size, location, age, weight and diagnostic accuracy of ulcers, and re-examine the children with peptic ulcer after regular treatment by gastrointestinal contrast-enhanced ultrasonography (CEUS).

Research motivation

CEUS has advantages in compliance, repeatability, incidence of complications, and tolerance of children, and has a high coincidence rate for clinical diagnosis. it can be used as a preliminary screening method for children with epigastric pain and an effective supplement to gastroscopy.

Research objectives

This study found that the diagnostic coincidence rate of conventional ultrasound was lower than that of gastrointestinal CEUS, and the results of gastrointestinal CEUS and gastroscopy were highly consistent, which confirmed that gastrointestinal CEUS had some advantages. The research on gastrointestinal CEUS in children was supplemented and improved. At present, gastrointestinal CEUS is not widely used in children, and it is necessary to establish the norms and standards of CEUS examination in children, which is helpful to better guide ultrasound physicians to carry out examination and improve the accuracy of examination.

Research methods

The contrast agents used in CEUS examination are food-grade contrast agents, which are safe, with no adverse effects or smell, and easy to drink. The contrast medium is a little sweet, easy for children to accept, and there is no need for intravenous sedative or general anesthesia. The examination process is painless, greatly reducing the anxiety of children and their families. The sound velocity and impedance of the contrast medium are similar to those of the liver. After oral administration of the contrast medium, the stomach and duodenum show uniform, medium and high dotted echoes, and at the same time, the gastric emptying time is prolonged. Under the gastrointestinal transmission window, the gastrointestinal wall structure and its pathological changes can be displayed more clearly. CEUS can also observe gastric peristalsis and extragastric tissue, and improve the diagnosis of gastroduodenal diseases.

Research results

The main goal of this study was to find the most suitable preliminary screening method for the diagnosis of peptic ulcer in children. For children who have contraindications for gastroscopy, CEUS can be a new option. For children with recurrent abdominal pain and other upper gastrointestinal symptoms with unknown etiology, gastrointestinal CEUS can provide a reference for clinical selection of appropriate treatment. For children with peptic ulcer who have been diagnosed and received regular drug treatment, the curative effect can be observed and evaluated repeatedly.

Research conclusions

The common examination methods for upper gastrointestinal ulcer in children include upper gastrointestinal X-ray barium meal examination, gastroscopy, gastric computed tomography, and gastric CEUS. In children, it is particularly important to find a simple, noninvasive examination method. CEUS is simple and noninvasive, the examination process is not painful, and there is no need for sedation or anesthesia, especially for children. It is expected to become a routine examination method for the diagnosis of digestive diseases in children.

Research perspectives

The detection rate of peptic ulcer in children is increasing, with developments in diagnostic procedures. Most children show abdominal pain, but cannot accurately describe it, so it is easy to miss diagnosis, misdiagnose, and delay treatment. Gastroscopy is the gold standard for the diagnosis of peptic ulcer, but it is an invasive examination. To maximize the diagnostic efficiency and reduce the risk, gastrointestinal CEUS screening is feasible before gastroscopy as an effective supplement to gastroscopy.

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FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Erlotinib combination with a mitochondria-targeted ubiquinone effectively suppresses pancreatic cancer cell survival

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Abstract

BACKGROUND

Pancreatic cancer is a leading cause of cancer-related deaths. Increased activity of the epidermal growth factor receptor (EGFR) is often observed in pancreatic cancer, and the small molecule EGFR inhibitor erlotinib has been approved for pancreatic cancer therapy by the food and drug administration. Nevertheless, erlotinib alone is ineffective and should be combined with other drugs to improve therapeutic outcomes. We previously showed that certain receptor tyrosine kinase inhibitors can increase mitochondrial membrane potential ($\Delta\psi_m$), facilitate tumor cell uptake of $\Delta\psi_m$-sensitive agents, disrupt mitochondrial homeostasis, and subsequently trigger tumor cell death. Erlotinib has not been tested for this effect.

AIM

To determine whether erlotinib can elevate $\Delta\psi_m$ and increase tumor cell uptake of $\Delta\psi_m$-sensitive agents, subsequently triggering tumor cell death.

METHODS

$\Delta\psi_m$-sensitive fluorescent dye was used to determine how erlotinib affects $\Delta\psi_m$ in pancreatic adenocarcinoma (PDAC) cell lines. The viability of conventional and patient-derived primary PDAC cell lines in 2D- and 3D cultures was measured after treating cells sequentially with erlotinib and mitochondria-targeted ubiquinone (MitoQ), a $\Delta\psi_m$-sensitive MitoQ. The synergy between erlotinib and MitoQ was then analyzed using SynergyFinder 2.0. The preclinical efficacy of the two-drug combination was determined using immune-compromised nude mice bearing PDAC cell line xenografts.

RESULTS

Erlotinib elevated $\Delta\psi_m$ in PDAC cells, facilitating tumor cell uptake and mitoch-

ondrial enrichment of $\Delta\psi_m$-sensitive agents. MitoQ triggered caspase-dependent apoptosis in PDAC cells in culture if used at high doses, while erlotinib pretreatment potentiated low doses of MitoQ. SynergyFinder suggested that these drugs synergistically induced tumor cell lethality. Consistent with *in vitro* data, erlotinib and MitoQ combination suppressed human PDAC cell line xenografts in mice more effectively than single treatments of each agent.

CONCLUSION

Our findings suggest that a combination of erlotinib and MitoQ has the potential to suppress pancreatic tumor cell viability effectively.

Key Words: Pancreatic cancer; Erlotinib; Mitochondria-targeted ubiquinone; Mitochondria; Combination therapy

Core Tip: In this study, we demonstrated that epidermal growth factor receptor inhibitor erlotinib increases mitochondrial membrane potential in pancreatic cancer cells, priming the tumor cells to mitochondria-targeted ubiquinone (MitoQ) sensitivity. Our data show that the combination of erlotinib and MitoQ can effectively and synergistically suppress pancreatic cancer cells *in vitro* and *in vivo*.

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INTRODUCTION

Pancreatic cancer is a highly aggressive and poorly prognostic disease with an overall five-year survival rate of 12.5%[1,2]. Many molecular alternations have been identified and evaluated for their potential as a therapeutic target in pancreatic cancer[3]. Nevertheless, there is still an urgent need to develop an effective treatment for pancreatic cancer.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) abnormally activated in many epithelial tumors, including non-small cell lung cancer (NSCLC)[4], glioblastoma[5], colon cancer[6], breast cancer[7], and pancreatic cancer[8]. Aberrantly activated EGFR promotes tumor cell proliferation and survival by activating critical signaling pathways such as the Ras/extracellular signal-regulated kinase, phosphatidylinositol-3 kinase/protein kinase B, and mammalian target of rapamycin pathways[9]. EGFR overexpression is a critical process for facilitating pancreatic tumorigenesis[10]. As such, the EGFR inhibitor erlotinib (Tarceva®) has been approved for treating pancreatic cancer. However, unlike its successful use for NSCLC as monotherapy[11], erlotinib is not as effective as monotherapy but is combined with gemcitabine to treat advanced pancreatic cancers[12-14]. Clinical trials have shown that erlotinib, in combination with capecitabine and chemoradiation, can also improve the survival of patients with resected pancreatic cancer[15,16]. Therefore, it is important to identify a combination therapy strategy to use erlotinib for effective pancreatic cancer therapy.

Certain RTK inhibitors (TKIs) affect mitochondrial activity, and this characteristic can be exploited to design a novel therapeutic strategy. For example, we previously showed that vandetanib and cabozantinib increase mitochondrial membrane potential ($\Delta\psi_m$) in the RTK REarrangement during Transfection-mutated thyroid tumor cells and subsequently facilitate tumor cell uptake and retention of $\Delta\psi_m$-sensitive mitochondria-targeted agents, including triphenylphosphonium (TPP)-mitochondria-targeted carboxy-proxyl (MitoCP) and mitochondria-targeted ubiquinone (MitoQ)[17]. This resulted in the accumulation of MitoCP and MitoQ in the tumor cell mitochondria, disrupting mitochondrial homeostasis and ultimately causing tumor cell death[17,18]. Of note, MitoQ is currently used as a dietary supplement due to its beneficial effects on mitochondrial bioenergetics[19,20] and vascular function[21-23], although this compound has been found to suppress tumor cells derived from the thyroid[17], skin[24], breast[25-27], and pancreas[28]. Therefore, selectively facilitating tumor cell enrichment of MitoQ using a TKI could be an effective strategy for tumor suppression. This strategy has not been tested for erlotinib.

Erlotinib is a TKI that targets multiple RTKs, including EGFR[29]. In this study, we tested a hypothesis that erlotinib increases $\Delta\psi_m$ in pancreatic tumor cells, hence priming the tumor cells to MitoQ sensitivity. Our data show that the combination of erlotinib and MitoQ can effectively and synergistically suppress pancreatic cancer cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell culture and reagents

The human pancreatic cancer cell line MiaPaCa-2 (ATCC) and pancreatic cancer cells-1 (PANC-1, ATCC) were maintained in Dulbecco's minimal essential medium (DMEM, Gibco, Thermo Fisher Scientific,No. 11965) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, No. 16000-044) and 100 U/mL of penicillin-streptomycin (Gibco, Thermo Fisher Scientific, No. 15140) per mL. Patient-derived pancreatic cell lines MCW462 and MCW670 were previously described[30,31]. They were maintained in DMEM (Invitrogen, Thermo Fisher Scientific, No. 11330032) supplemented with 6% FBS, 100 U of penicillin-streptomycin, 50 µL of 100 µg/mL of epidermal growth factor (Thermo Fisher Scientific, No. PHG0311), 2 mL of bovine pituitary extract (Gibco, No. 13028014), 2 µg/mL of hydrocortisone (MilliporeSigma, St. Louis, MO, No. H0888) and 70 µL of insulin (Gibco, No. 12585014). For organoid culture, 10,000 cells were plated onto 24 well plates precoated with 200 µL Matrigel (Corning, Tewksbury, MA, No. 356231) and maintained with the DMEM medium mixed with Matrigel at 10% of final volume (0.8-1.1 mg/mL), as instructed by the manufacturer. Hypoxic cell culture was carried out in a humidified incubator with 1% O_2, 5% CO_2 and 94% N_2. All experiments were performed using cells within ten passages from the acquisition point. Erlotinib was purchased from LC Laboratories (Woburn, MA, No. E-4997). Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (ZVAD) and gemcitabine were purchased from Selleckchem (Houston, TX, No. S7023 and No. S1714). MitoCP was obtained from Dr. Balaraman Kalyanaraman (Biophysics, Medical College of Wisconsin). MitoQ [(10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl) decyl) triphenyl phosphonium]was obtained from MitoQ Ltd. (Auckland, New Zealand). Tetramethyl-rhodamine methyl ester (TMRM) was purchased from Invitrogen (No. T668). 4%-20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels, 10 well, 30 µL were purchased from Bio-Rad (Hercules, CA, No. 4568093).

Cell viability and cell cycle analyses

The half maximal inhibitory concentration (IC_{50}) values and the combination drug effects were determined by crystal violet staining (Fisher Chemical, Thermo Fisher Scientific, No. C58125). Briefly, cells in 96 well-plates were fixed in formaldehyde (Fisher Chemical, No. BP531), stained with 0.05% crystal violet for 30 min, washed with water three times, air-dried, and incubated in 200 µL methanol (VWR Chemicals BDH®, Radnor, PA, No. BDH1135) for 10 min at room temperature before measuring absorbance at 540 nm. The IC_{50} values in organoid cultures were determined using SYTOX™ Green (Invitrogen, No. S7020) following the manufacturer's instruction. To determine apoptosis, cells were co-stained with annexin V (Invitrogen, No. A35122) and propidium iodide (Invitrogen, No. P1304MP) according to the manufacturer's instruction. Flow cytometry was performed using BD® LSR II Flow Cytometer (Franklin Lakes, NJ). FCS data were analyzed by FCS EXPRESS software (De Novo Software, Los Angeles, California).

Detection of mitochondrial membrane potential using TMRM

Cells were incubated with culture medium with 2 nM TMRM (Invitrogen, No. T668) in 24-well plates for 30 min in a humidified incubator at 37 °C, collected by trypsinization, resuspended in phosphate-buffered saline containing 0.5% bovine serum albumin, and analyzed using Guava EasyCyte flow cytometry system. Data were analyzed by FCS EXPRESS software as described previously[27].

Immunoblotting

Mitochondrial fractions were extracted using the Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific, No. 89874). Total cell lysates were prepared by harvesting cells in a lysis buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), and the protease and phosphatase inhibitor cocktails 2 and 3 (MilliporeSigma, No. P8340, P5726, and P0044, respectively). Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, No. 23227). Protein samples were resolved on the SDS-polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride membrane filter (Bio-Rad, No. 1620177). After transfer, membranes were blocked in a buffer containing 0.1 M Tris (pH 7.4), 0.9% NaCl, 0.1% Tween 20, and 5% nonfat dry milk for 1 h at 25 °C. Membranes were then incubated with an appropriate antibody overnight at 4 °C with agitation at the following dilutions: Poly (ADP-ribose) polymerase (PARP) (cell signaling, No. 9542) 1: 2000; cleaved lamin A (cell signaling, No. 2035) 1: 1000; Caspase 9 (cell signaling, No. 9502T) 1: 2000; Caspase 3 (cell signaling, No. 14220); E2F1 (Thermo Fisher Scientific, No. MS-879) 1: 2000; pRb (cell signaling, No. 9307); cytochrome c oxidase subunit IV (COX IV, cell signaling, No. 4850) 1: 2000; $p21^{CIP1}$ (Santa Cruz Biotechnology, No. sc-56335) 1:1000, $p27^{KIP1}$ (Santa Cruz Biotechnology, No. sc-1641) 1:1000; hypoxia-inducible factor 1 subunit alpha (HIF1α, Cell Signaling, No. 14179) 1:1000 and β-actin (MilliporeSigma, No. A2228) 1:5000. Chemiluminescence signals of immunoblots were visualized by SuperSignal West Pico (No. 34079) and Femto (No. 23227) chemiluminescence kits (Pierce), captured by ChemiDoc XRS+ (Bio-Rad), and analyzed by Image Lab software (Bio-Rad) for densitometry.

Tumor xenografts

A total of 5 × 10^6 PANC-1 cells suspended in 100 µL of Hank's balanced salt solution (Gibco, No. 14025) mixed with Extracellular Matrix Gel (MilliporeSigma, No. E6909) at 1:1 ratio was inoculated subcutaneously into the rear flanks of 6-week-old female athymic nude (*nu*/*nu*) mice (The Jackson Laboratory, Bar Harbour, ME, No. 007850). Once palpable, tumors were measured using Vernier calipers twice a week. Tumor volumes were calculated using the formula: Length × width × height × 0.5236. When tumor volumes reached 50 mm^3, mice were sorted into 4 groups of 7 animals to achieve equal tumor size distribution in all treatment groups. Mice were treated with vehicle, MitoQ, erlotinib, and the combination of two compounds (combo), respectively. Drugs dissolved in 100 µL vehicle (1:12 mixture of Dimethyl

sulfoxide /15% β-cyclodextrin) were orally administered by gavage daily for 4 d, followed by one day break. Four cycles of this treatment were conducted. The control group received only the vehicle, the MitoQ group received 20 mg drug/kg body weight/dose, the erlotinib group received 25 mg drug/kg body weight/dose, and the combination group received two doses of erlotinib followed by two doses of MitoQ in a cycle. Ethical endpoints were when tumor size reached 2000 mm^3. At the end of the experiments, animals were euthanized by CO_2 asphyxiation, and tumor tissues were harvested. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin, No. AUA00001327.

Statistical analysis

Unless otherwise specified, all graphs represent the mean ± the standard error of mean from biological replicates ($N \geq 3$). Statistical significance was determined by one-way or two-way analysis of variance with Bonferroni post-tests and two-tailed unpaired Student's *t*-test using PRISM (Graph-Pad Software, La Jolla, CA). IC_{50} was determined by PRISM. *P* values of < 0.05 were considered statistically significant.

RESULTS

MitoQ can suppress the viability of pancreatic ductal adenocarcinoma cells

We determined how MitoQ affects pancreatic adenocarcinoma (PDAC) cell viability in a cell line panel that included the conventional PDAC cell lines PANC-1 and MiaPaCa-2 and the patient-derived PDAC cell lines MCW462 and MCW670. As determined by the crystal violet viability assay, 48 h MitoQ treatment significantly decreased cell viability in the two-dimensional cell culture with the IC_{50} values determined below 1 μM in all cell lines (Figure 1A and B). In contrast, the functional moiety of MitoQ CoQ10 (ubiquinone) did not suppress cell viability, while the vehicle moiety TPP decreased cell viability mildly only at higher doses (Figure 1A). The organoid culture model is more physiologically relevant[32], and we also examined MitoQ responsiveness of MCW462 cells in organoid cultures. As determined by Sytox Green viability assay, MitoQ, but not CoQ10 and TPP, consistently decreased the viability of MCW462 cells in the organoid culture (Figure 1C). However, the IC_{50} value was higher in the three-dimensional culture. These data demonstrate that MitoQ can suppress PDAC cell viability, for which the mitochondrial targeting of its functional moiety might be critical.

MitoQ can induce caspase-dependent apoptosis in pancreatic ductal adenocarcinoma cells

To determine the molecular mechanism by which MitoQ suppresses PDAC cell viability, we performed the annexin V/propidium Iodide co-staining assay. We found that, if used at a high dose, MitoQ increased annexin V positive population in MiaPaCa-2 and MCW670 cultures (Figure 2A). However, the control CoQ10 and TPP did not show similar effects. This effect of MitoQ was significantly blocked by the pan-caspase inhibitor ZVAD (Figure 2A). Moreover, Western blot analysis of total lysates of these cells revealed that MitoQ, but not TPP or CoQ10, notably increased the cleavages of PARP and lamin A in a dose-dependent manner (Figure 2B). The cleavage of these proteins is a bonafide marker of caspase-dependent apoptosis[33]. Indeed, 5 μM MitoQ increased the cleavage of caspases 9 and 3 in MiaPaCa-2 cells while depleting these enzymes in MCW670 cells (Figure 2B). We also found that MitoQ decreased E2F1, the S-phase transcription factor[34], and the phosphorylation of its regulator Rb while increasing the cyclin-dependent kinase inhibitor $p27^{KIP1}$ without notably affecting $p21^{CIP1}$ levels in these cells (Figure 2B). These data suggest that MitoQ can suppress PDAC cell viability by inducing caspase-dependent apoptosis and partly by suppressing a few critical regulators for cell cycle progression.

Erlotinib can increase mitochondrial membrane potential and facilitate mitochondrial enrichment of mitochondrial membrane potential-sensitive agents in pancreatic ductal adenocarcinoma cells

We determined whether erlotinib can increase $\Delta\psi_m$ in PDAC cells. In the cultures of multiple PDAC cell lines, erlotinib treatment significantly increased cells stained with TMRM, a $\Delta\psi_m$-sensitive fluorescent dye, in a dose-dependent manner within 48 h (Figure 3A and B). Given this data, we determined whether erlotinib can increase the mitochondrial enrichment of a $\Delta\psi_m$-sensitive agent using the TPP-conjugated superoxide dismutase mimetic MitoCP as a tool compound. The CP moiety of MitoCP is a 5-membered nitroxide free radical that can form a covalent conjugate with thiol proteins, which can be detected by a TPP-specific antibody[17,35]. When the mitochondrial extracts from MitoCP-treated PANC-1 and MCW462 cells were analyzed for the effects of 24 h erlotinib pretreatment, western blotting revealed much higher levels of TPP-protein adducts in erlotinib-pretreated cells (Figure 3C). These data suggest that erlotinib can increase $\Delta\psi_m$ and, subsequently, mitochondrial enrichment of a $\Delta\psi_m$-sensitive agent in PDAC cells.

Erlotinib and MitoQ can synergistically suppress the viability of pancreatic ductal adenocarcinoma cells in culture

Given the data above, we asked whether erlotinib can synergize with MitoQ to suppress PDAC cell viability. As determined in PANC-1, MCW462, and MCW670 cells, a robust viability loss was induced when these cells were sequentially treated with erlotinib for the first 24 h and MitoQ for the subsequent 48 h (Figure 4A). Of note, our analysis using SynergyFinder 2.0[36] suggested that these two agents synergistically induced the viability loss (Figure 4B). Importantly, tumor cells in a physiological microenvironment are generally under a hypoxic condition[37]. We, therefore, determined whether the erlotinib-MitoQ combination can produce a similar synergistic effect under a hypoxic culture condition. To this end, we used PANC-1 cells maintained in the human plasma-like medium with 1% O_2, under which condition HIF1α expression substantially increased (Figure 4C). Under this hypoxic culture condition, the combination of erlotinib and

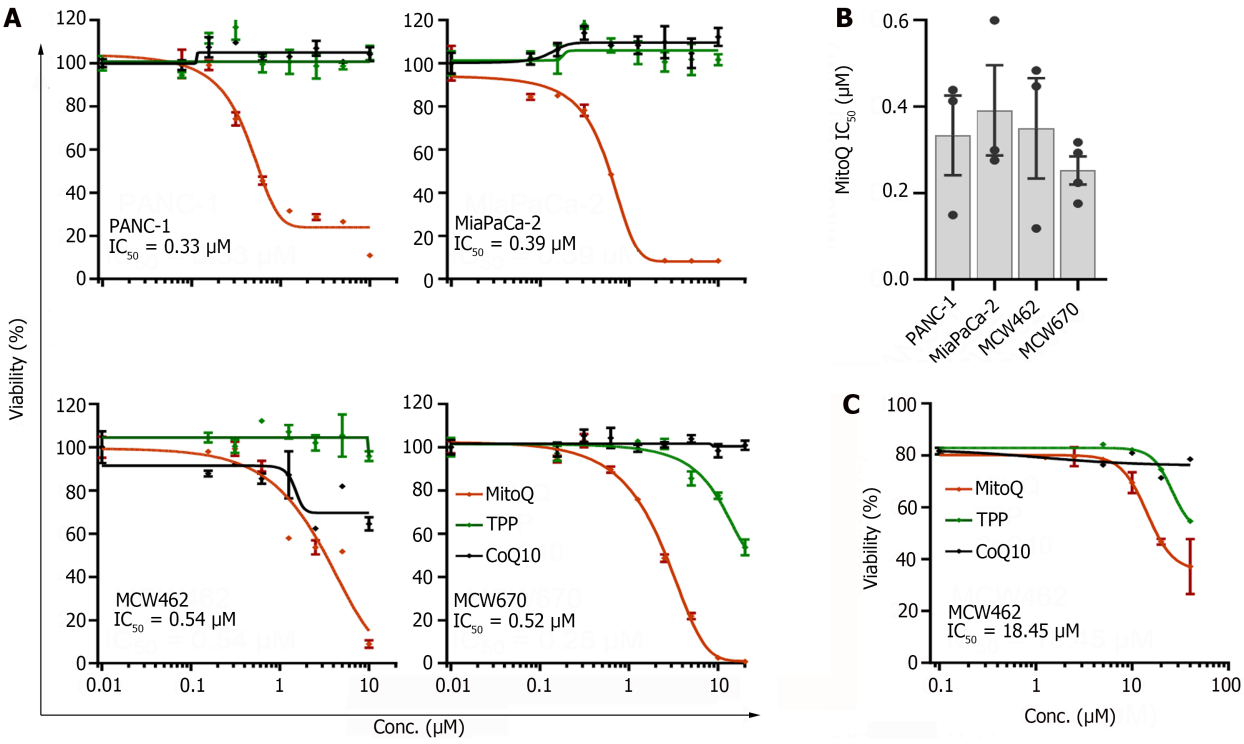

Figure 1 Mitochondria-targeted ubiquinone can suppress the viability of pancreatic adenocarcinoma cells. A: pancreatic adenocarcinoma (PDAC) cells in 12 well plates were treated with increasing doses of mitochondria-targeted ubiquinone (MitoQ), CoQ10, and triphenyl-phosphonium (TPP) for 48 h prior to determining cell viability by crystal violet staining. CoQ10 is the functional moiety of MitoQ and TPP is the vehicle moiety. 462 and 670 denote MCW462 and MCW670 cell lines, respectively; B Summary of the half maximal inhibitory concentration values determined for different PDAC cells treated and analyzed as described; C: MCW462 cells in organoid cultures were treated with increasing doses of MitoQ, CoQ10, and TPP for 48 h prior to determining cell viability by Sytox Green assays. Data (mean ± SEM, $n \geq 3$) are expressed as the percentage of untreated controls. TPP: Triphenyl-phosphonium; MitoQ: Mitochondria-targeted ubiquinone; IC_{50}: Half maximal inhibitory concentration.

MitoQ consistently suppressed the viability of PANC-1 cells (Figure 4D), through a synergistic effect as suggested by SynergyFinder 2.0 (Figure 4E). These data strongly suggest that erlotinib can synergize with MitoQ to suppress PDAC cells.

A combination of erlotinib and MitoQ effectively suppresses the growth of pancreatic cancer cells xenografts in mice

PANC-1 cells express relatively higher EGFR[38]. Given this information and the relatively high synergy score in this cell line (Figure 4), we used immune-compromised nude mice bearing PANC-1 xenografts to determine the preclinical efficacy of the erlotinib and MitoQ combination. These mice were subjected to 4 cycles of treatments in which erlotinib and MitoQ were orally administered singly or in combination (depicted in Figure 5A). For the drug combination, a cycle consisted of 2 d erlotinib treatment followed by 2 d MitoQ treatment and one drug holiday (Figure 5A), which is similar as the schedule that we previously used for preclinical evaluation of the vandetanib and MitoCP combination for thyroid cancer[17]. To compare mono- and combination therapies, we used erlotinib at 25 mg/kg/dose, a lower dose than the highly potent doses (50 mg/kg once daily) in the other preclinical models[39,40]. Likewise, we used MitoQ at 20 mg/kg/dose, which did not significantly suppress tumor growth in a preclinical breast cancer model, albeit decreasing tumor metastasis[26]. Consistent with the *in vitro* data above, the sequential administration of erlotinib and MitoQ suppressed the growth of PANC-1 xenografts more effectively than the monotherapy using each agent, as determined by monitoring tumor volume changes (Figure 5B) and by measuring the sizes and weight of tumors harvested at the end of the treatment (Figure 5C and D). In contrast, these treatments did not cause a significant difference in animal body weights (Figure 5E). These data suggest that erlotinib can synergize with MitoQ *in vivo* to suppress the growth of PDAC cells.

DISCUSSION

Our data show that MitoQ can suppress PDAC cell survival if used at an effective dose. Similarly, tumor cells derived from thyroid, skin, and breast cancers have shown sensitivity to MitoQ[24,25,27]. Many tumor cells exhibit elevated steady-state $\Delta\psi_m$, which is required for adaptation to hypoxia, escape from anoikis, and enhanced invasiveness[41,42]. For this reason, MitoQ would accumulate higher in the mitochondria of tumor cells than those of most normal cell types[23], which might increase the chance for MitoQ to exert any adverse effect on tumor cells. Our data suggest that the property of erlotinib to increase $\Delta\psi_m$ in PDAC cells can be exploited to drive the mitochondrial enrichment of MitoQ in PDAC cells.

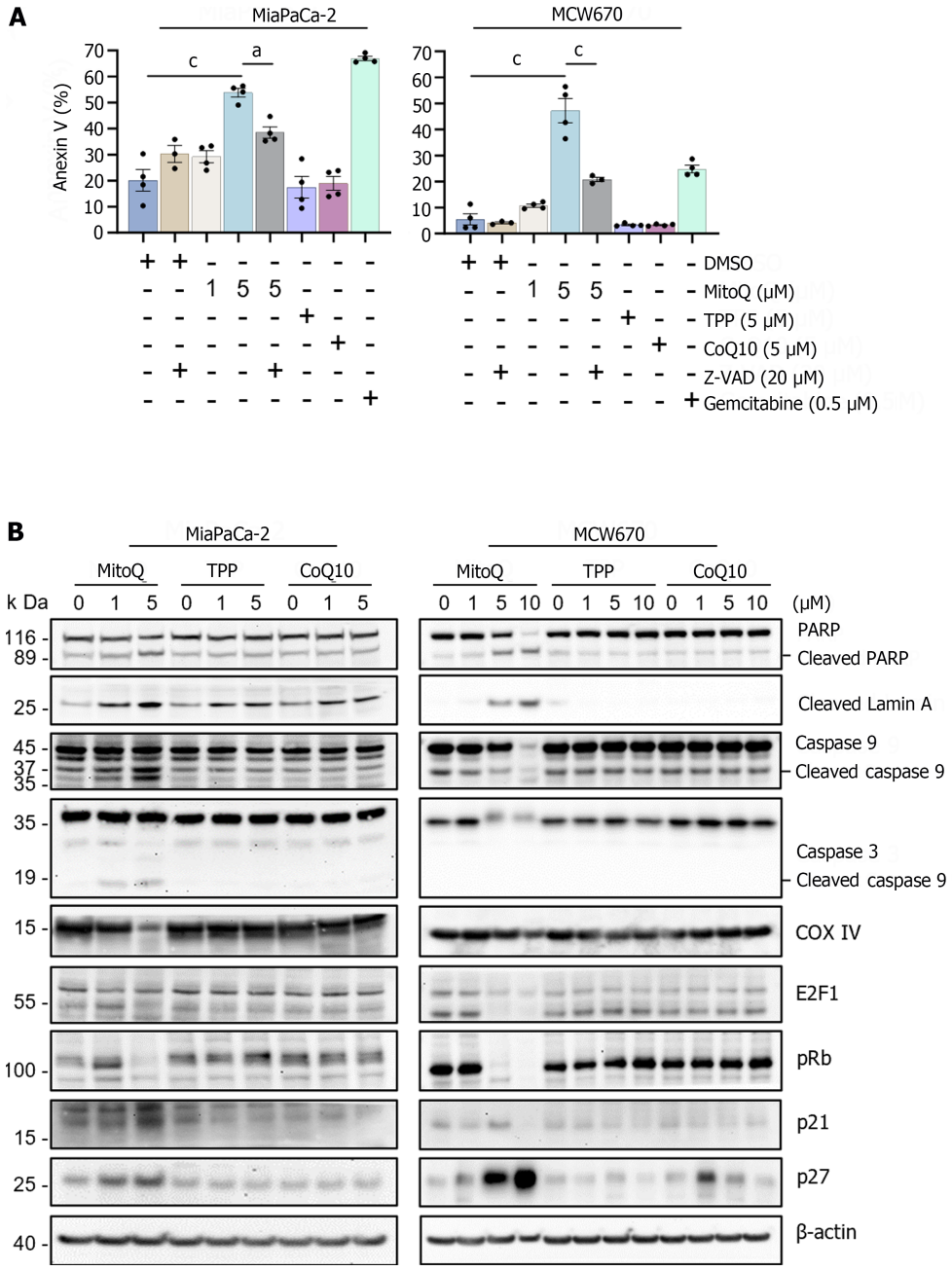

Figure 2 Mitochondria-targeted ubiquinone can induce caspase-dependent apoptosis in pancreatic adenocarcinoma cells. A: Apoptosis analysis of cells treated with mitochondria-targeted ubiquinone (MitoQ), with or without Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone, for 24 h. CoQ10 is the functional moiety of MitoQ and triphenyl-phosphonium is the vehicle moiety. Gemcitabine is the positive control for apoptosis induction. Data (mean ± SEM, $N \geq 3$) are expressed as the percentage of untreated controls. One-way ANOVA with Bonferroni post-tests; B: Western blot analysis of total lysates of cells treated as described. β-actin is the control for equal amounts of protein loading. $^{a}P < 0.05$. $^{c}P < 0.001$. MitoQ: Mitochondria-targeted ubiquinone; TPP: Triphenyl-phosphonium.

How does MitoQ kill tumor cells? Reactive oxygen species (ROS) produced in tumor cells are supposed to promote tumorigenesis, angiogenesis, metastasis, and chemoresistance[43,44]. Given this, tumor cells may be more susceptible to ROS scavenging. Indeed, an oxidized form of ubiquinone (BPM31510) is currently in clinical trial to treat solid tumors with a high ROS burden[45], and MitoQ may act in a similar fashion. In support of this notion, MitoQ can decrease ROS generation through the NRF 2/NAD(P)H quinone oxidoreductase 1 pathway, a key endogenous antioxidant defense mechanism[27,46,47]. Moreover, MitoQ was shown to inhibit redox signaling to prevent PDAC metastasis in mice[28]. However, in contrast, studies using bovine aortic endothelial cells demonstrated that MitoQ can also increase ROS generation in mitochondria[48,49]. Mechanistically, MitoQ can be reduced by complex II, but not by complexes I and III due to the bulkiness and positive charge of its TPP moiety that sterically hindered access to the proteins[50,51]. Therefore, MitoQ is anti- as well as pro-oxidant, depending on cell types. In either scenario, MitoQ over-enrichment would harm cells.

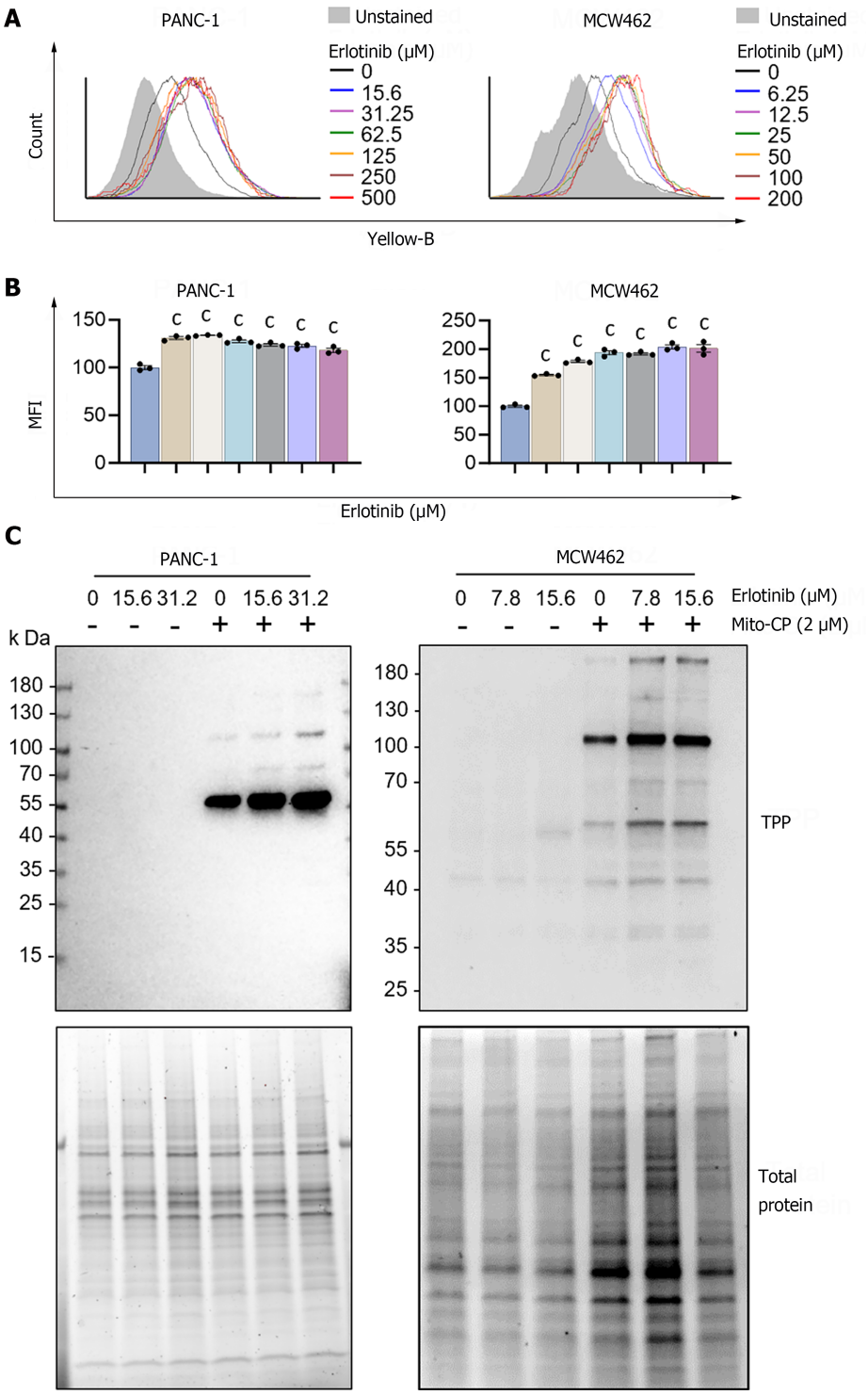

Figure 3 Erlotinib can increase mitochondrial membrane potential in pancreatic adenocarcinoma cells. A: Cells were treated with increasing concentrations of erlotinib for 2 d prior to staining with tetramethyl-rhodamine methyl ester (TMRM). Cellular TMRM retention was analyzed by flow cytometry measuring yellow fluorescence; B: Mean fluorescence intensities of TMRM-stained cells quantified by FCS Express software. Data are mean ± SEM ($n \geq 3$). One-way ANOVA with Bonferroni post-tests; C: Cells pretreated with erlotinib for 48 h were treated with 2 μM mitochondria-targeted carboxy-proxyl (MitoCP) for 1 h. Mitochondrial extracts of these cells were analyzed by Western blotting to detect the formation of MitoCP adducts using the antibody specific to the triphenyl-phosphonium moiety of MitoCP. Total proteins in the extracts were visualized in the stain-free gel as the control for equal protein loading. $^{c}P < 0.001$. MitoCP: Mitochondria-targeted carboxy-proxyl; TPP: Triphenyl-phosphonium.

How does erlotinib increase $\Delta\Psi_m$? Although not widely known, EGFR can translocate to mitochondria and regulate different mitochondria processes[52-55]. For example, EGFR translocation to the outer membrane of mitochondria promotes mitochondrial fission by disturbing the polymerization of the mitochondria fusion protein mitofusin 1 through direct interaction, which promotes the metastatic potential of NSCLC cells[55]. EGFR can directly bind to cytochrome c oxidase subunit II (COXII) in mitochondria[54], which leads to COXII phosphorylation and activity loss[56,57]. Increased COXII activity is implicated in hyperpolarization of mitochondria under conditions such as ischemia-reperfusion[58].

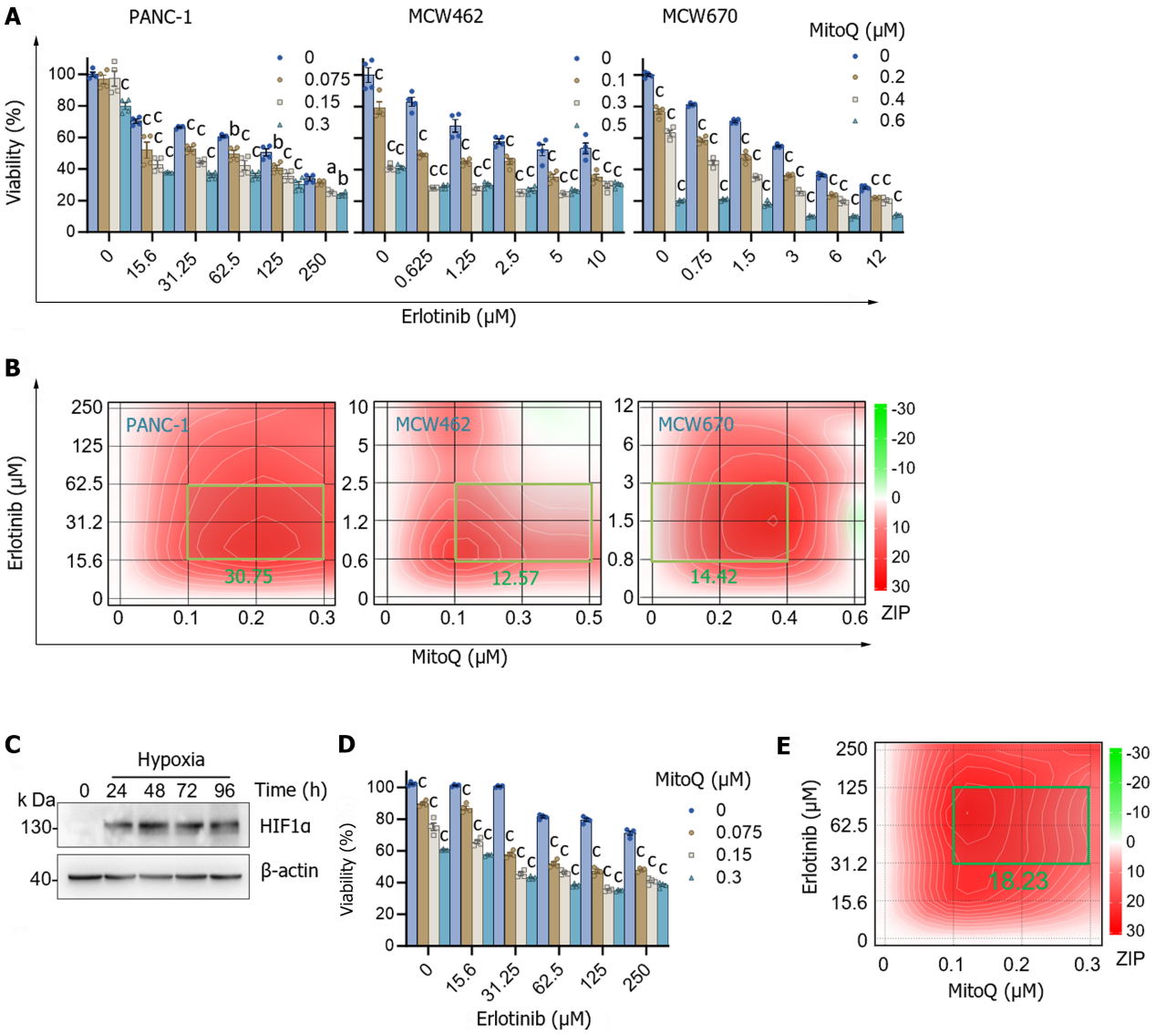

Figure 4 Erlotinib can synergize with mitochondria-targeted ubiquinone to suppress the viability of pancreatic adenocarcinoma cells. A: Cells, pretreated with different concentrations of erlotinib, were treated with different doses of mitochondria-targeted ubiquinone (MitoQ) for 48 h prior to crystal violet viability assay. Data (mean ± SEM, $n \geq 3$) are expressed as the percentage of untreated controls; B: SynergyFinder plotting of the viability data. The ZIP (zero interaction potency) scores are indicated for the most synergistic areas; C: Western blot analysis of PANC-1 cells maintained in the human plasma-like medium with 1% O_2. β-actin is the control for equal protein loading; D: PANC-1 cells pretreated with erlotinib for 24 h were treated with MitoQ for 48 h in the human plasma-like medium with 1% O_2 prior to crystal violet viability assay. Data (mean ± SEM, $n \geq 3$) are expressed as the percentage of untreated controls; E: SynergyFinder plotting of the viability data. The ZIP score is indicated for the most synergistic area. $^{a}P < 0.05$. $^{b}P < 0.005$. $^{c}P < 0.001$, Two-way ANOVA with Bonferroni post-tests. MitoQ: Mitochondria-targeted ubiquinone.

Because erlotinib abolished COXII inhibition by EGFR[59], a COXII regulation may underlie erlotinib-induced elevation of $\Delta\Psi_m$ in PDAC cells. Alternatively, EGFR may use a signaling pathway to regulate $\Delta\Psi_m$. For example, a deficiency of mechanistic target of rapamycin (mTOR) complex 2 and mTOR inhibition can cause $\Delta\Psi_m$ elevation in different cell types, including lung and breast tumor cells[60-62]. Because EGFR regulates mTOR[63,64], it may be possible that erlotinib affects $\Delta\Psi_m$ *via* the mTOR pathway. Although we hypothesize that $\Delta\Psi_m$-dependent mitochondrial over-enrichment of MitoQ is the primary mechanism underlying the synergy between MitoQ and erlotinib, we also appreciate additional possibilities. For example, MitoQ can induce mitophagy *via* the PTEN-induced kinase 1/Parkin RBR E3 ubiquitin-protein ligase pathway[65,66], and erlotinib can induce autophagy through p53 nuclear translocation, AMP-activated protein kinase activation, and mTOR suppression[67,68]. Therefore, it is also possible that MitoQ and erlotinib synergize in the context of autophagic cell death. Interrogation of these mechanisms remains as future studies.

CONCLUSION

Although MitoQ has been tested for its clinical potential for different diseases[21-23], it has not been tested for cancer therapy in a clinical trial setting. Based on the data in this study, we suggest that MitoQ can potentially suppress PDAC

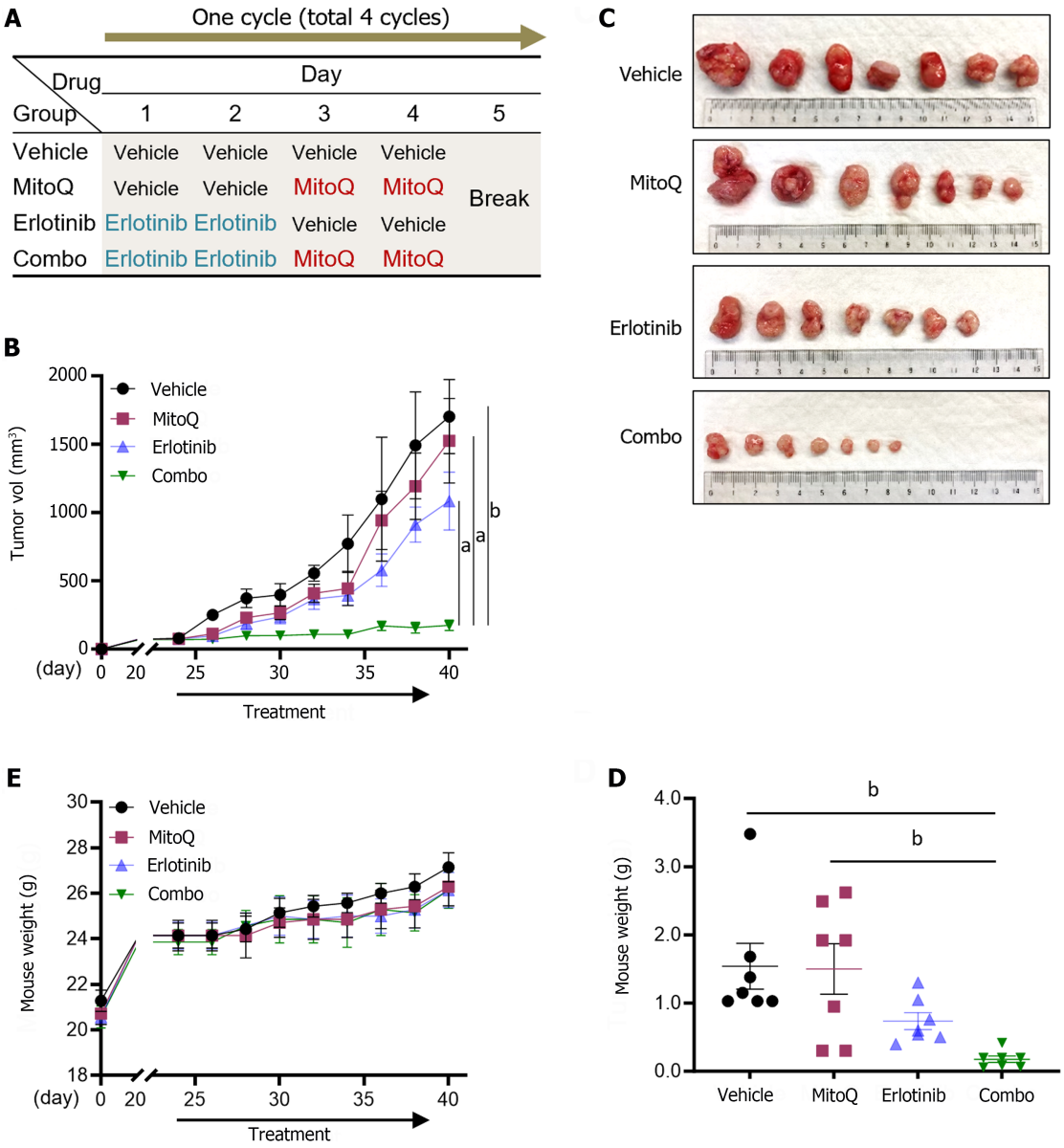

Figure 5 An erlotinib and mitochondria-targeted ubiquinone combination effectively suppresses PANC-1 xenografts in mice. A: Treatment schedule. Detailed information is provided in Materials and Method; B: Changes in tumor size (mean ± SEM, $N = 7$). Two-way ANOVA with Bonferroni post-tests; C: Images of tumors collected at the end of treatment; D: Weights of tumors; E: Body weight changes (mean ± SEM, $N = 7$) monitored during the treatment. [a]$P < 0.05$. [b]$P < 0.005$. MitoQ: Mitochondria-targeted ubiquinone.

cell survival and that its combination with erlotinib is a candidate strategy to target PDAC cells selectively. MitoQ has additional potential benefits because antioxidants can support the physical fitness of patients undergoing chemotherapy [69,70]. In support of this notion, MitoQ improved muscle atrophy and weakness in colon cancer cell line tumor-bearing mice[70]. Studies have shown that TKIs, including ponatinib, regorafenib, sunitinib, imatinib, and sorafenib, can also affect $\Delta\Psi_m$, although their effects vary depending upon cell types[61,71-73]. Our findings lay a foundation to evaluate these TKIs in a similar context for the combination therapy concept.

ARTICLE HIGHLIGHTS

Research background

We previously showed that receptor tyrosine kinase inhibitors such as cabozantinib and vandetanib can increase mitochondrial membrane potential ($\Delta\psi_m$) in tumor cells and, thus, facilitate mitochondrial enrichment of $\Delta\psi_m$-sensitive agents in tumor cells. This effect can disrupt mitochondrial homeostasis and trigger tumor cell death.

Research motivation

Pancreatic cancer is one of the most lethal tumors, demanding highly effective molecular therapies. Although the

epidermal growth factor receptor inhibitor erlotinib has been approved for pancreatic adenocarcinoma (PDAC), its efficacy is limited as monotherapy, and it is often used in combination with other drugs. We sought to determine whether erlotinib can be combined with a mitochondria-targeted agent for PDAC suppression.

Research methods

We measured cell viability by performing cell death assays in 2D and 3D cultures of conventional and patient-derived primary PDAC cell lines. We determined how erlotinib affects $\Delta\psi_m$ in PDAC cells using $\Delta\psi_m$-sensitive fluorescent dyes and by measuring protein adduct formation with mitochondria-targeted carboxy-proxyl (MitoCP) in mitochondria. We examined the effect of erlotinib and mitochondria-targeted ubiquinone (MitoQ) combination by measuring cell viability and analyzing synergy. We determined the preclinical efficacy and physiological relevance of the drug combination using immune-compromised nude mice bearing PDAC cell line xenografts.

Research results

Erlotinib elevated $\Delta\psi_m$ in PDAC cells and facilitated mitochondrial enrichment of the triphenyl-phosphonium (TPP)-conjugated agents. While MitoQ single treatment triggered caspase-dependent apoptosis in PDAC cells, its combination with erlotinib synergistically induced PDAC cell death. Consistent with these data, the drug combination suppressed human PDAC cell line xenografts in mice more effectively than a single treatment of each agent.

Research conclusions

These data suggest that erlotinib elevated $\Delta\psi_m$ in PDAC cells and facilitated mitochondrial enrichment of the TPP-conjugated agents. The drug combination synergistically suppressed PDAC cells.

Research objectives

We aimed to determine whether erlotinib elevates $\Delta\psi_m$ in PDAC cells, increases tumor cell uptake of the TPP cation-conjugated ubiquinone MitoQ, and subsequently causes tumor cell death.

Research perspectives

These data suggest that the erlotinib and MitoQ combination may have therapeutic potential for pancreatic cancer.

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FOOTNOTES

Co-first authors: Pui-Yin Leung and Wenjing Chen.

Author contributions: Leung PY designed and performed research, and analyzed data; Chen W designed and performed research, analyzed data, and wrote the paper; Sari AN performed research and analyzed data; Wu PK developed methodology; Sitaram P performed research and analyzed data; Tsai S secured funding for this study; Park JI conceived and designed research, wrote the paper, secured funding, and supervised the project. All authors were involved in the critical review of the results and have contributed to, read, and approved the final manuscript. Leung PY and Chen W contributed equally to this work as co-first authors. Designating these two authors as co-first authors accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper.

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Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin (AUA00001327).

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Data sharing statement: All data needed to evaluate the conclusions in the paper are present in the paper. Any additional information required to reanalyze the data reported in this paper is available from the corresponding authors upon request. Reagents and materials produced in this study are available pending a completed Materials Transfer Agreement.

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ORIGINAL ARTICLE

Basic Study

Milk fat globule epidermal growth factor 8 alleviates liver injury in severe acute pancreatitis by restoring autophagy flux and inhibiting ferroptosis in hepatocytes

Qing Cui, Hang-Cheng Liu, Wu-Ming Liu, Feng Ma, Yi Lv, Jian-Cang Ma, Rong-Qian Wu, Yi-Fan Ren

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Abstract

BACKGROUND

Liver injury is common in severe acute pancreatitis (SAP). Excessive autophagy often leads to an imbalance of homeostasis in hepatocytes, which induces lipid peroxidation and mitochondrial iron deposition and ultimately leads to ferroptosis. Our previous study found that milk fat globule epidermal growth factor 8 (MFG-E8) alleviates acinar cell damage during SAP *via* binding to αvβ3/5 integrins. MFG-E8 also seems to mitigate pancreatic fibrosis *via* inhibiting chaperone-mediated autophagy.

AIM

To speculate whether MFG-E8 could also alleviate SAP induced liver injury by restoring the abnormal autophagy flux.

METHODS

SAP was induced in mice by 2 hly intraperitoneal injections of 4.0 g/kg L-arginine or 7 hly injections of 50 μg/kg cerulein plus lipopolysaccharide. *mfge8*-knockout mice were used to study the effect of MFG-E8 deficiency on SAP-induced liver injury. Cilengitide, a specific αvβ3/5 integrin inhibitor, was used to investigate the possible mechanism of MFG-E8.

RESULTS

The results showed that MFG-E8 deficiency aggravated SAP-induced liver injury in mice, enhanced autophagy flux in hepatocyte, and worsened the degree of ferroptosis. Exogenous MFG-E8 reduced SAP-induced liver injury in a dose-dependent manner. Mechanistically, MFG-E8 mitigated excessive autophagy and inhibited ferroptosis in liver cells. Cilengitide abolished MFG-E8's beneficial effects in SAP-induced liver injury.

CONCLUSION

MFG-E8 acts as an endogenous protective mediator in SAP-induced liver injury. MFG-E8 alleviates the excessive autophagy and inhibits ferroptosis in hepatocytes by binding to integrin αVβ3/5.

Key Words: Autophagy flux; Ferroptosis; Liver injury; Milk fat globule epidermal growth factor 8; αvβ3/5 integrins; Acute pancreatitis

Core Tip: Serum milk fat globule epidermal growth factor 8 (MFG-E8) concentration is negatively correlated with inflammatory severity in acute pancreatitis (AP) patients. Deficiency of MFG-E8 would aggravate the hepatic inflammatory response, and exacerbates the imbalance of liver autophagy flux caused by AP. Eventually leading to ferroptosis. Recombinant MFG-E8 administration restored autophagy flux, reduced ferroptosis and alleviated liver injury in AP in a dose-dependent manner. MFG-E8 alleviates the excessive autophagy and inhibits ferroptosis in hepatocytes possibly by binding to integrin αVβ3/5.

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INTRODUCTION

Severe acute pancreatitis (SAP) is a systemic disease characterized by pancreatic self-digestion and a systemic inflammatory response[1,2]. Compared to the self-limiting nature of mild AP, multiple organ dysfunction syndrome (MODS) caused by SAP develops cascade exacerbations without intervention, and it is one of the leading causes of death in the emergency department of digestive diseases[3,4]. As an organ adjacent to the pancreas, the liver is often the first echelon of external pancreatic attack caused by SAP. The metabolic and exocrine dysfunction caused by liver injury further aggravates the failure of other vital organs, forming a second hit for the patient. The invasion of peripancreatic necrosis products combined with hematogenous spread of intestinal bacteria results in destruction of the liver parenchyma[5,6]. Disorders of liver cell metabolism and exocrine function further aggravate the failure of the lung, kidney and other important organs, forming the second hit after the onset of SAP[7,8].

Autophagy is a catabolic process in which autophagic lysosomes degrade most cytoplasmic contents[9]. Stable autophagic flux participates in maintaining the normal homeostasis of cells, such as hepatocytes. Autophagy involves proteins encoded by a group of autophagy-related genes (ATGs)[10]. Mammalian autophagy related protein 16 like protein 1 (ATG16L1) contains a coiled-coil domain with an amino terminal and multiple carboxy-terminal WD repeats. ATG16L1 has the ability to lipidate LC3-binding sites and maintain autophagy circulation[11,12]. Abnormal protein expression of ATGs combined with increased LC3I to LC3II transformation and depletion of autophagy substrate transporters (*e.g.*, P62) suggests hyperactivation of intracellular autophagy.

Excessive autophagy leads to ferroptosis, which is a new type of iron-dependent programmed cell death that is different from apoptosis and necrosis[13-15]. The main mechanism of ferroptosis is that under the action of divalent iron or ester oxygenase, unsaturated fatty acids with high expression on the cell membrane are catalyzed to produce lipid peroxidation, thus inducing cell death. In addition, a decrease in glutathione peroxidase 4 (GPX4), the core enzyme regulating the antioxidant system (glutathione system), is observed[16-18]. Disturbances in intracellular homeostasis, including hyperactivity of autophagy, thereby lead to ferroptosis. Active ferroptosis does not promote the recovery of cell function but aggravates the damage. Recent evidence suggests that ferroptosis contributes to acute and chronic liver injury and that it may be one of the major culprits of liver cell death[19-21]. Therefore, we focused on the changes in hepatocyte autophagy and ferroptosis in liver injury caused by SAP and explored possible therapeutic targets.

Milk fat globule epidermal growth factor 8 (MFG-E8) is a secreted lipophilic glycoprotein that contains an RGD motif and interacts with integrins[22,23]. MFG-E8 is involved in a variety of intercellular signaling pathways and enhances the phagocytosis of inflammatory cells and apoptotic cells by macrophages[24-26]. Our previous study confirmed that MFG-E8 maintains cellular homeostasis by suppressing endoplasmic reticulum stress (ERS) in pancreatic exocrine acinar cells and protects against SAP[27,28]. ERS has been reported to play an important role in promoting intracellular autophagy disorder and inducing ferroptosis[29]. MFG-E8 also plays a protective role in nonalcoholic fatty liver disease[30]. However, whether MFG-E8 also plays a role in liver injury caused by SAP remains unknown. Therefore, the present study aimed to clarify the specific role of MFG-E8 in SAP-induced liver injury and its regulation of homeostasis in damaged liver cells.

MATERIALS AND METHODS

Patients

In total, 134 AP patients (age ≥ 18 years) who were treated at the First Affiliated Hospital of Xi'an Jiaotong University from January 2018 to January 2019 were included in this study. AP was diagnosed according to the International Atlanta Symposium on Acute Pancreatitis[1]. The present study was approved by the Ethics Committee of First Affiliated Hospital of Xi'an Jiaotong University.

Experimental animals

MFG-E8 knockout mice purchased from Shanghai Model Organisms Center (Shanghai, China) were used in this study. The litter wild-type mice were used as the control group in this experiment. The male adult C57BL/6J mice used in the exogenous MFG-E8 experiment were purchased from the Animal Experimental Center of Xi'an Jiaotong University (Xi'an, China). All laboratory animals were housed in a standard animal laboratory facility that simulates day and night on 12-h cycles. The mice had free access to water and food, and each cage was equipped with its own ventilation system. Before experimental intervention, all mice were randomly divided into groups with six mice per group and fasted for one night. The study protocol was approved by the Institutional Animal Care and Use Committee of the Ethics Committee of Xi'an Jiaotong University Health Science Center.

Experimental SAP models

L-arginine-SAP was induced by 2 hly intraperitoneal injections of 4.0 g/kg L-arginine (Sigma Aldrich, Shanghai, China). At 2 h after the last injection of L-arginine, normal saline (vehicle) or 5, 10, or 20 μg/kg MFG-E8 (RD System, Inc. Minnesota, United States) was administered through intraperitoneal injection. To determine the role of the αvβ3/5 integrins in the effect of MFG-E8 on the liver, cilengitide (20 mg/kg, SELLECK, Texas, United States), a specific αvβ3/5 integrin inhibitor[31,32], was administered through intraperitoneal injection at 1 h after the last injection of L-arginine. The animals were anesthetized by inhalation of isoflurane and sacrificed at 69 h after MFG-E8 treatment (*i.e.*, 72 h after the first injection of L-arginine). Serum and hepatic tissue samples were collected.

Cerulein + lipopolysaccharide (LPS)-SAP was induced by 7 hly injections of cerulein (50 μg/kg). LPS (10 mg/kg, L8880, Solarbio, Beijing, China) was added to the last cerulein injection. At 30 min after the second injection of cerulein, 20 μg/kg MFG-E8 was administered intraperitoneally. Blood and tissue samples were harvested at 4 h after the last injection of cerulein (*i.e.*, 11 h after the first injection of cerulein).

Detailed methods for the following procedures and antibody information are provided in the Supplementary materials: Hematoxylin and eosin (H&E) staining; enzyme-linked immunosorbent assay; granulocyte myeloperoxidase (MPO) assessment; detection of glutathione (GSH) and malondialdehyde (MDA) levels; TdT-mediated dUTP Nick-End Labeling (TUNEL) staining; transmission electron microscopy; western blot analysis; antibodies and statistical analysis.

RESULTS

Serum MFG-E8 concentration is negatively correlated with inflammatory severity in AP patients

Systemic inflammation caused by AP often leads to MODS and is the culprit of liver damage. Our previous study found that the concentration of MFG-E8 in the serum of AP patients is inversely proportional to the severity of the disease[27]. In the present study, we further explored the potential correlation between serum MFG-E8 concentration and the severity of systemic inflammation. A total of 134 AP patients were included in this study. Of these patients, 57 patients (42.5%) had biliary disease, 5 patients (3.7%) had alcohol misuse, 37 patients (27.6%) had hyperlipidemia and 35 patients (26.1%) had other causes. Local complications, organ failure and in-hospital mortality occurred in 35 patients (26.1%), 19 patients (14.2%), and 1 patient (0.7%), respectively (Supplementary Table 1).

White blood cells (WBCs) and procalcitonin (PCT) are commonly used circulating markers of inflammation. As shown in Figure 1A and B, serum MFG-E8 concentrations were negatively correlated with serum WBC counts (r = -0.356, P < 0.01) and PCT concentrations (r = -0.404, P < 0.01), suggesting an inverse relationship between serum MFG-E8 levels and inflammatory severity. Furthermore, serum MFG-E8 concentrations were negatively correlated with MODS scores[33] (r = -0.338, P < 0.01) in AP patients (Figure 1C). These results suggested that AP patients with low concentrations of MFG-E8 are more prone to sepsis and MODS.

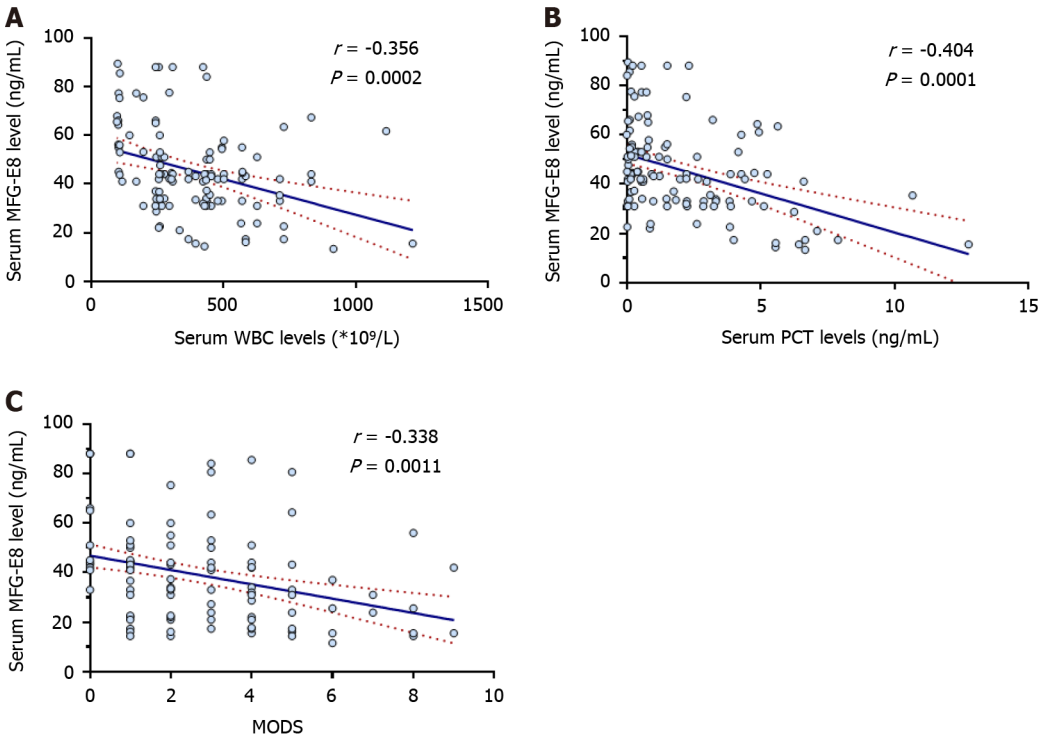

Figure 1 Serum milk fat globule epidermal growth factor 8 concentration was negatively correlated with the inflammatory severity in acute pancreatitis patients. Blood samples from 134 acute pancreatitis patients were collected, and serum milk fat globule epidermal growth factor 8 (MFG-E8) levels were measured. A: Correlation analysis of serum MFG-E8 levels and serum white blood cell levels; B: Correlation analysis of serum MFG-E8 levels and serum procalcitonin levels; C: Correlation analysis of serum MFG-E8 levels and multiple organ failure syndrome scores. WBC: White blood cells; PCT: Procalcitonin; MODS: Multiple organ failure syndrome; MFG-E8: Milk fat globule epidermal growth factor 8.

MFG-E8 deficiency aggravates hepatic injury in experimental SAP

SAP was induced in mice by intraperitoneal injection of L-arginine. Compared to control mice, the liver of SAP mice was significantly damaged with structural disorder of liver lobules, vacuoles in liver cells and accumulation of red blood cells in hepatic sinusoids (Figure 2A and B). However, knockout of *mfge8* gene resulted in more significant liver damage in SAP mice, and the degree of liver damage in MFG-E8-deficient mice was 0.3 times worse than that in wild-type mice after quantification of the pathological score ($P < 0.05$, Figure 2A and B). The area of liver necrosis in MFG-E8-deficient mice was twice as much as that in wild-type mice ($P < 0.05$, Figure 2C).

Our previous studies confirmed that SAP leads to impaired intestinal mucosal barrier function, resulting in intestinal bacteria transfer into the blood and inducing multiple organ infections[34]. To examine whether the liver is also secondary to inflammation in experimental SAP, we examined the expression levels of a range of cytokines in the liver. As shown in Figure 2D and E, the expression levels of tumor necrosis factor (TNF)-α and MPO were significantly higher than normal in the liver of SAP mice, which was exacerbated by the loss of MFG-E8 expression. These results suggested that MFG-E8 deficiency may reduce the anti-inflammatory ability of the liver in experimental SAP ($P < 0.05$). Consistent with the pathological scores, there was a greater proportion of apoptotic cells labeled with TUNEL fluorescence in MFG-E8-deficient SAP mice, which was more than 1.5 times that of wild-type SAP mice ($P < 0.05$, Figure 2F and G).

MFG-E8 deficiency aggravates hepatic autophagy and ferroptosis in experimental SAP

Excessive autophagy is secondary to the damage of hepatocytes caused by external physical and chemical factors, and the hyperactive autophagic flux further promotes the imbalance of cellular homeostasis[35,36]. Therefore, we detected the expression levels of the autophagy-related proteins, ATG16L1, P62 and LC3B in the liver. ATG16L1 played a crucial role in determining the lipidylation site of LC3 and promoting the maturation of autophagosomes[11] (Figure 3A), while the lack of MFG-E8 increased the level of ATG16L1 in the liver of SAP mice ($P < 0.05$, Figure 3B and C). P62 and LC3 cooperatively constitute the ability of autophagosomes to form, degrade and transport degradation substrates[37]. As shown in Figure 3B and C, SAP resulted in abnormal expression levels of P62 and LC3 in mice, and the deletion of MFG-E8 aggravated the disorder of autophagic flux ($P < 0.05$).

Ferroptosis is a type of cell death closely related to autophagy[38]. To investigate the potential role of ferroptosis in liver injury caused by SAP, we examined the expression levels of two biomarkers of ferroptosis, namely, prostaglandin-endoperoxide synthase 2 (PTGS2) and GPX4. The results showed that SAP resulted in a slight increase in PTGS2 and a significant decrease in GPX4 in the livers of wild-type mice. In MFG-E8-deficient mice, SAP resulted in a significant increase in PTGS2 and almost no GPX4 expression ($P < 0.05$, Figure 3D and E). Ferroptosis is characterized by iron accumulation, lipid peroxidation and antioxidant system damage[18,29,39], therefore, we detected the expression levels of MDA and GSH. As expected, MFG-E8 deficiency aggravated the elevated MDA levels in the liver and increased GSH

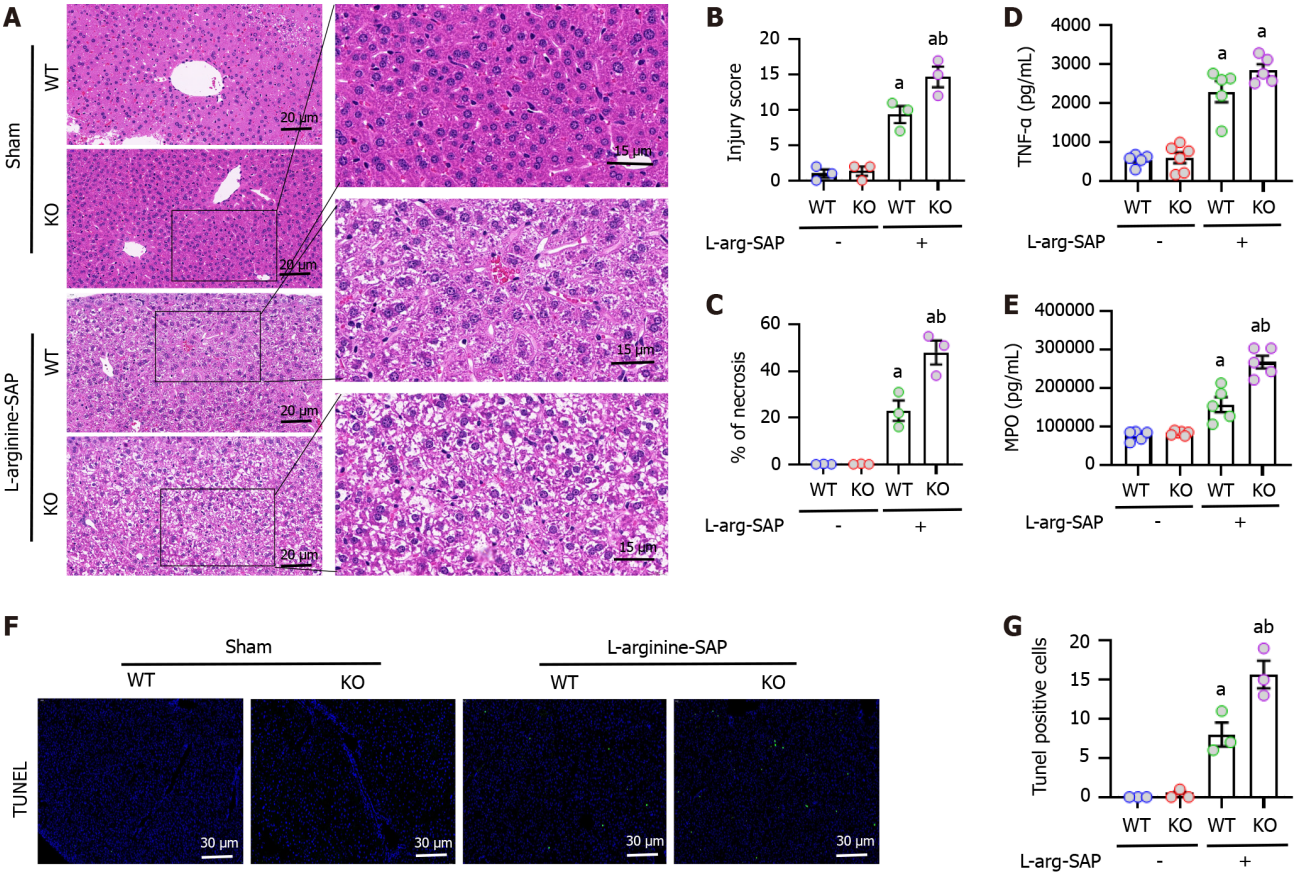

Figure 2 Milk fat globule epidermal growth factor 8 deficiency aggravated hepatic injury and inflammation in experimental severe acute pancreatitis. In mice, acute pancreatitis (AP) was induced by 2 hly intraperitoneal injections of 4.0 g/kg L-arginine. The animals were sacrificed at 72 h after the first injection of L-arginine. Blood and tissue samples were collected. A: Representative photos of hematoxylin and eosin staining in the liver (400 × or 1000 ×); B: Hepatic injury scores; C: Percentages of necrotic areas; D: Tumor necrosis factor-α levels in the liver; E: Myeloperoxidase levels in the liver; F and G: Representative photos of TdT-mediated dUTP Nick-End Labeling (TUNEL) staining (400 ×) and quantitative of TUNEL staining. *n* = 3-6/group, error bars indicate the SEM; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* vehicle group. SAP: Severe acute pancreatitis; KO: Knockout; WT: Wild type; TNF-α: Tumor necrosis factor-α; MPO: Myeloperoxidase; TUNEL: TdT-mediated dUTP Nick-End Labeling.

consumption (Figure 3F and G). Furthermore, SAP caused an increase in oxygen free radical products in mouse liver tissue, and *mfge8* knockdown also increased the accumulation of reactive oxygen species products (Figure 3H).

MFG-E8 administration mitigates liver injury and restores hepatocyte homeostasis in experimental SAP

To further investigate the potential protective effect of MFG-E8 on liver injury caused by SAP, recombinant MFG-E8 (5, 10, or 20 μg/kg) was administered 2 h after the second injection of L-arginine. Similar to the results shown in Figure 2A, L-SAP resulted in extensive liver damage in mice with a visual field of hepatic vacuolation and hepatic lobules barely discernible under light microscopy (Figure 4A). Intraperitoneal injection of recombinant MFG-E8 not only restored the changes in liver microstructure ($P < 0.05$, Figure 4A-C) but also reduced the levels of TNF-α and MPO in the liver parenchyma ($P < 0.05$, Figure 4D and E). The beneficial effects of MFG-E8 were dose dependent. Similarly, 10 μg/kg or 20 μg/kg MFG-E8 reduced the number of TUNEL-positive cells by 45% ($P > 0.05$) or 85% ($P < 0.05$), respectively (Figure 4F and G). Intraperitoneal injection of MFG-E8 also alleviated liver damage caused by cerulein + LPS-induced SAP in a dose-dependent manner ($P < 0.05$, Supplementary Figure 1A-C).

As expected, 10 μg/kg (low dose) exogenous MFG-E8 partially restored the expression levels of proteins associated with autophagic flux ($P > 0.05$, Figure 5A and B) and ferroptosis ($P > 0.05$, Figure 5C and D) in damaged hepatocytes, while 20 μg/kg MFG-E8 almost completely restored the expression levels of these proteins ($P < 0.05$, Figure 5A-D). The restoration of MDA and GSH production as well as the repair of reactive oxygen species level in hepatocytes also indicated that exogenous MFG-E8 reduced iron accumulation, lipid peroxidation and antioxidant system damage of liver cells in a dose-dependent manner ($P < 0.05$, Figure 5E-G). We then observed morphological changes in mitochondria under electron microscopy. SAP results in increased electron density of the mitochondrial matrix and destruction of the mitochondrial crest and mitochondrial membrane in hepatocytes, and 20 μg/kg MFG-E8 also relieved these mitochondrial morphological abnormalities (Figure 5H). This evidence suggested that exogenous MFG-E8 supplementation may alleviate liver injury and promote hepatocyte homeostasis in experimental SAP.

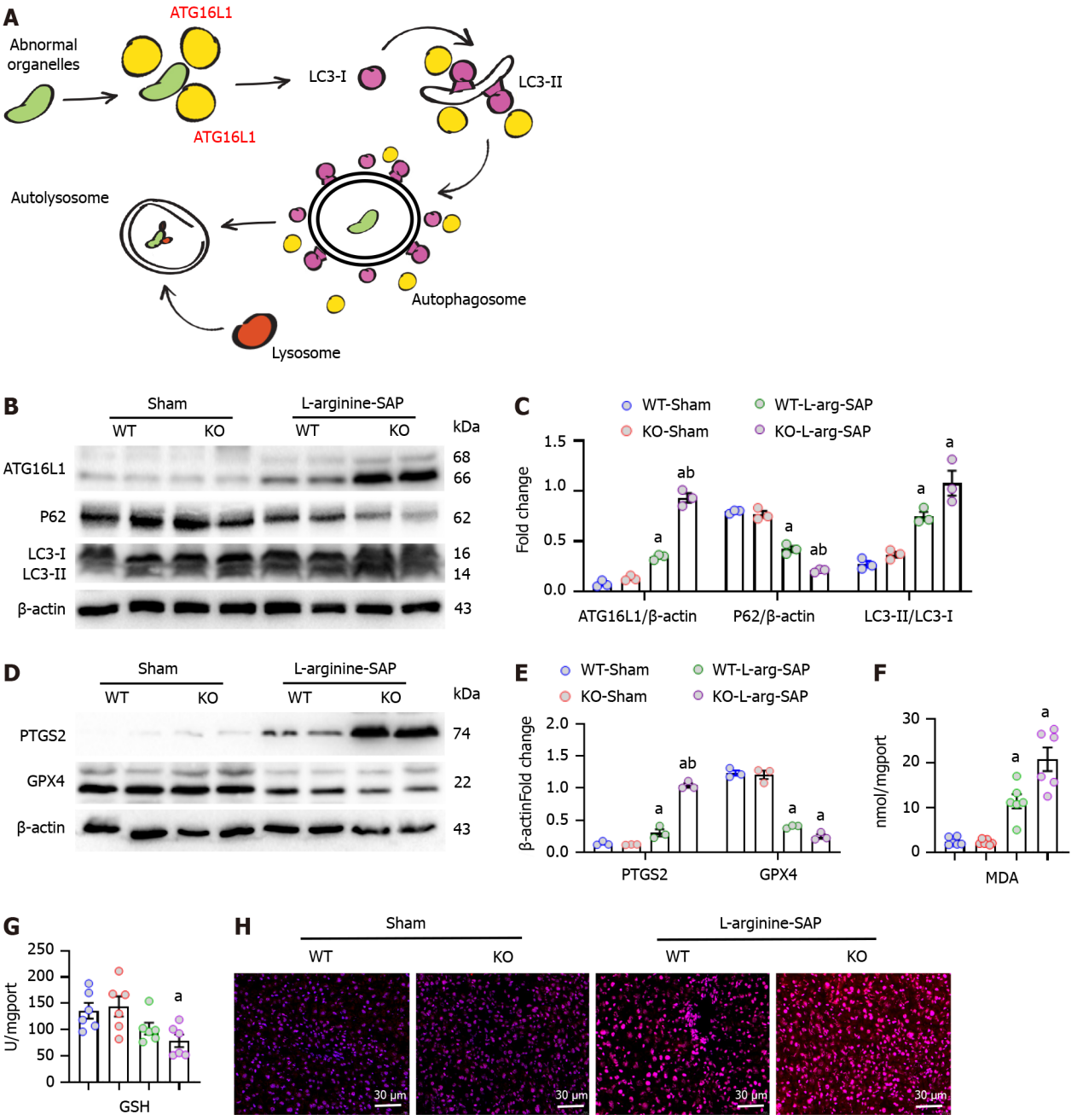

Figure 3 Milk fat globule epidermal growth factor 8 deficiency aggravated hepatic autophagy and ferroptosis in experimental severe acute pancreatitis. In mice, severe acute pancreatitis was induced by 2 hly intraperitoneal injections of 4.0 g/kg L-arginine. The animals were sacrificed at 72 h after the first injection of L-arginine. Blood and tissue samples were collected. A: Autophagy related protein 16 like protein 1 (ATG16L1) assists LC3 in the formation of autophagosomes; B-E: Western blot analysis and quantitative of the expression of ATG16L1, P62, LC3, prostaglandin-endoperoxide synthase 2 and glutathione peroxidase 4 in the liver; F: Malondialdehyde levels in the liver; G: Glutathione levels in the liver; H: Representative images of DHE staining in the pancreas. n = 3-6/group, error bars indicate the SEM; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* vehicle group. SAP: Severe acute pancreatitis; KO: Knockout; WT: Wild type; ATG16L1: Autophagy related protein 16 like protein 1; PTGS2: Prostaglandin-endoperoxide synthase 2; GPX4: Glutathione peroxidase 4; MDA: Malondialdehyde; GSH: Glutathione.

MFG-E8 protects the liver from damage through integrins αVβ3/5

Previous evidence suggests that MFG-E8 is involved in signal transduction mainly by binding the αVβ3 and αVβ5 integrins[23,26,40]. To investigate whether the protective effect of MFG-E8 on the liver is also αVβ3/5 integrin-mediated, we utilized cilengitide, a specific αVβ3/5 integrin inhibitor, to block the possible target of MFG-E8.

H&E staining and damage quantification showed that cilengitide counterbalanced the protective effect of MFG-E8 on the liver of SAP mice ($P < 0.05$, Figure 6A-C). Similarly, TNF-α ($P < 0.05$, Figure 6D) and MPO ($P < 0.05$, Figure 6E) in liver were also increased after cilengitide addition, reaching almost the same level as those in vehicle-treated mice ($P > 0.05$). The number of TUNEL-positive cells significantly increased after cilengitide was added and even reached 1.1 times that in vehicle-treated mice ($P > 0.05$, Figure 6F and G).

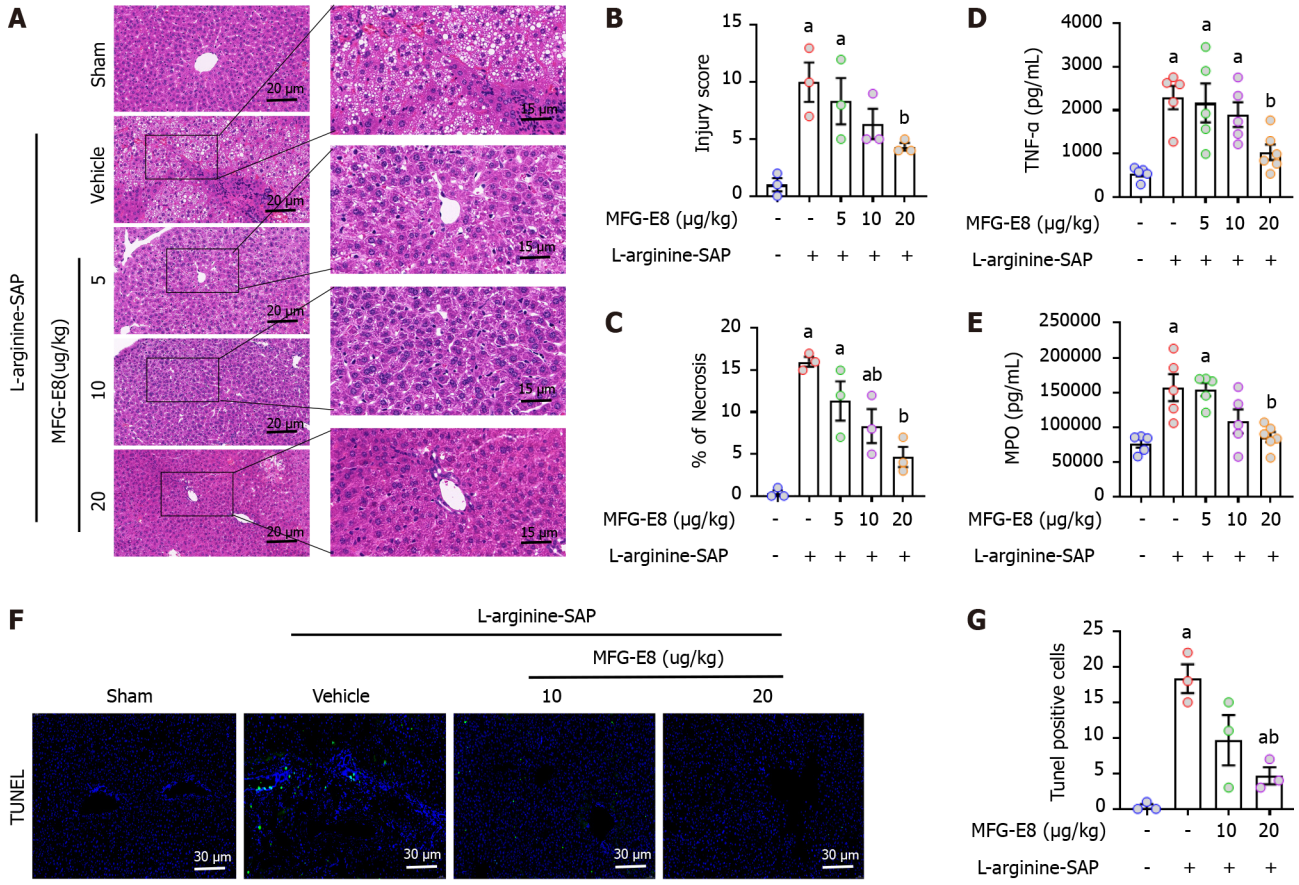

Figure 4 Milk fat globule epidermal growth factor 8 administration mitigates liver Injury in experimental severe acute pancreatitis. In mice, severe acute pancreatitis was induced by 2 hly intraperitoneal injections of 4.0 g/kg L-arginine. At 2 h after the last injection of L-arginine, normal saline (vehicle) or 5, 10, or 20 μg/kg milk fat globule epidermal growth factor 8 (MFG-E8) was administered through intraperitoneal injection. The animals were sacrificed at 69 h after MFG-E8 treatment (*i.e.*, 72 h after the first injection of L-arginine). Blood and tissue samples were collected. A: Representative photos of hematoxylin and eosin staining in the liver (400 × or 1000 ×); B: Hepatic injury scores; C: Percentages of necrotic areas; D: Tumor necrosis factor-α levels in the liver; E: Myeloperoxidase levels in the liver; F and G: Representative photos of TdT-mediated dUTP Nick-End Labeling (TUNEL) staining (400 ×) and quantitative of TUNEL staining. n = 3-6/group, error bars indicate the SEM; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* vehicle group. SAP: Severe acute pancreatitis; TNF-α: Tumor necrosis factor-α; MPO: Myeloperoxidase; TUNEL: TdT-mediated dUTP Nick-End Labeling; MFG-E8: Milk fat globule epidermal growth factor 8.

We next explored whether the restoration of autophagic flux and inhibition of ferroptosis by MFG-E8 were also mediated by integrins αVβ3/5. As shown in Figure 7A-D, after the addition of cilengitide, the effect of exogenous MFG-E8 on ATG16L1, P62, LC3 and GPX4 almost disappeared, and the expression level of PTGS2 was approximately 1.2 times that of vehicle-treated mice ($P < 0.05$). Moreover, iron accumulation, mitochondrial ultramorphological abnormalities, lipid peroxidation and damage to the antioxidant system, which are the signature events of ferroptosis, were also intensified by the addition of cilengitide ($P < 0.05$, Figure 7E-G). These results suggested that MFG-E8 alleviates liver damage caused by SAP while inhibiting autophagic flux disorder and the exacerbation of ferroptosis through integrins αVβ3/5 (Figure 8).

DISCUSSION

In the present study, we found that the lack of MFG-E8 aggravated liver damage and the imbalance of hepatocyte homeostasis caused by SAP. Intraperitoneal injection of exogenous MFG-E8 restored excessive autophagy, reduced ferroptosis and alleviated liver injury by acting on integrins αVβ3/5. Moreover, the protective effect of exogenous MFG-E8 on the liver was dose dependent.

Autophagy flux disorder mediates intracellular dysfunction in a variety of pathological conditions[41,42]. As the site of synthesis, metabolism and secretion of various substances necessary for life, the active intracellular environment of hepatocytes is easily affected by excessive autophagy, resulting in homeostasis disorder[43,44]. The formation of autophagic vesicles requires a necessary pair of ubiquitin-like coupling systems, namely, ATG12-ATG5 and ATG8 (LC3)-phosphatidylethanolamine (LC3-PE)[45]. ATG16L1 provides a functional link between these two key autophagy ubiquitin-like coupling systems. ATG16L1 binds ATG5 of the ATG12-ATG5 connector to form an 800 kDa polymeric complex[46]. The ATG12-ATG-5-ATG16L1 complex is located in the preautophagosome membrane where it identifies the site of LC3 lipidation and catalyzes the reaction required for mature autophagosome formation[47,48].

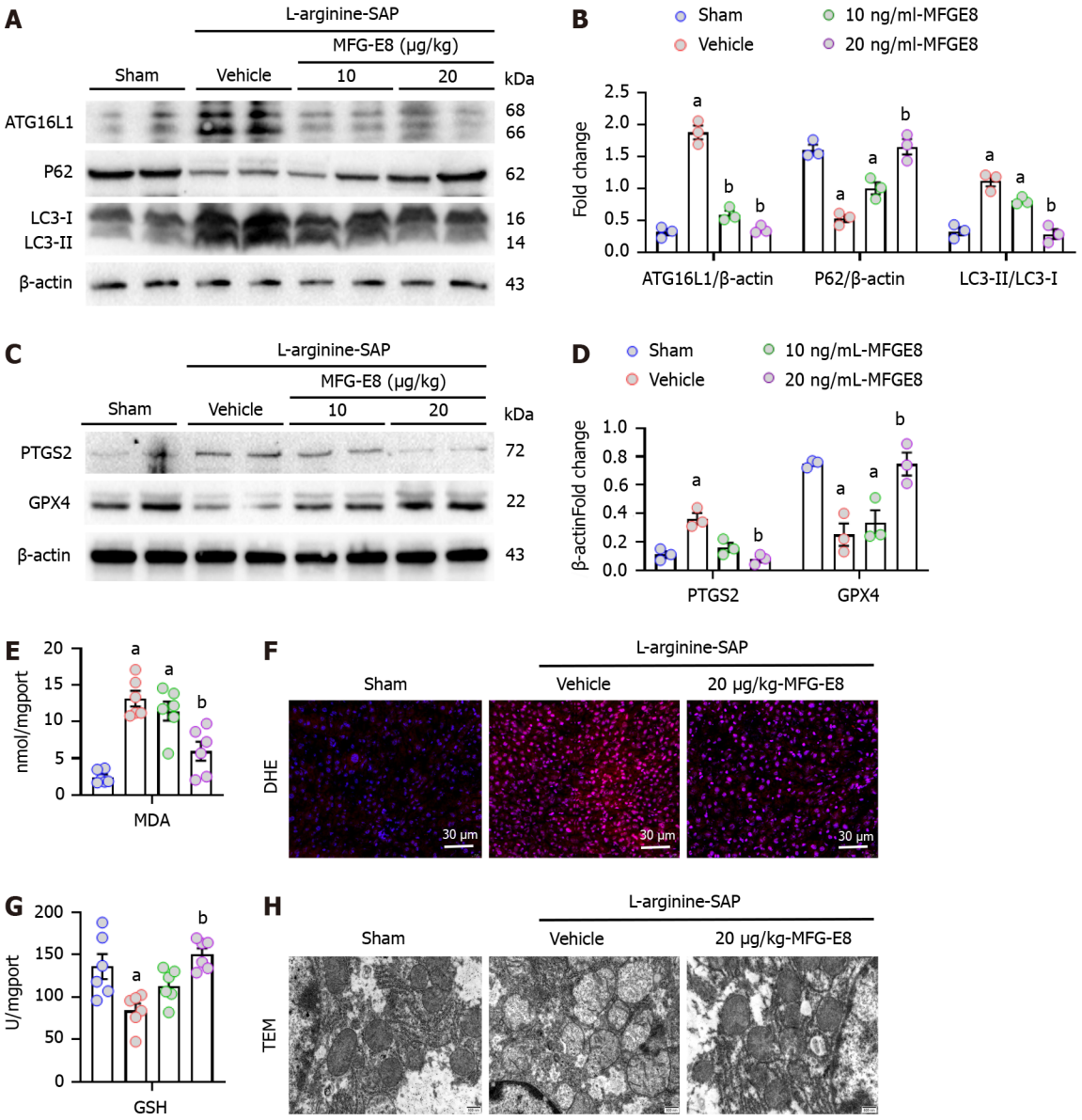

Figure 5 Milk fat globule epidermal growth factor 8 administration mitigates hepatic autophagy and ferroptosis in experimental severe acute pancreatitis. In mice, arginine-severe acute pancreatitis was induced by 2 hly intraperitoneal injections of 4.0 g/kg L-arginine. At 2 h after the last injection of L-arginine, normal saline (vehicle) or 10, or 20 μg/kg milk fat globule epidermal growth factor 8 (MFG-E8) was administered through intraperitoneal injection. The animals were sacrificed at 69 h after MFG-E8 treatment (*i.e.*, 72 h after the first injection of L-arginine). Blood and tissue samples were collected. A-D: Western blot analysis and quantitative of the expression of autophagy related protein 16 like protein 1, P62, LC3, prostaglandin-endoperoxide synthase 2 and glutathione peroxidase 4 in the liver; E: Malondialdehyde levels in the liver; F: Representative images of DHE staining in the pancreas; G: Glutathione levels in the liver; H: Ultrastructural changes of mitochondria in hepatocytes (electron microscopy). *n* = 3-6/group, error bars indicate the SEM; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* vehicle group. SAP: Severe acute pancreatitis; ATG16L1: Autophagy related protein 16 like protein 1; PTGS2: Prostaglandin-endoperoxide synthase 2; GPX4: Glutathione peroxidase 4; MDA: Malondialdehyde; GSH: Glutathione; MFG-E8: Milk fat globule epidermal growth factor 8.

Genome-wide association scans have revealed mutations in ATG16L1, a gene linked to Crohn's disease[49]. Mice abnormal expression of the ATG16L1 coiled-helix domain show impaired autophagosome formation and increased inflammatory cytokines, consistent with their role in inflammatory disease pathogenesis. Suballele ATG16L1 mice also show autophagy defects and intestinal Pan cell dysfunction similar to that found in Crohn's disease[50]. In the present study, we found that the lack of MFG-E8 aggravated the abnormal expression of ATG16L1 in hepatocytes caused by SAP and worsened the disorder of autophagic flux (abnormal expression of P62 and LC3). Exogenous MFG-E8 restored liver ATG16L1, P62 and LC3 levels in a dose-dependent manner. These results suggested that the moderating effect of MFG-E8 on the SAP-induced hepatic inflammatory response (increased TNF-α and MPO) may be achieved by restoring ATG16L1 and alleviating excessive autophagy.

Recent evidence suggests that ferroptosis plays a critical role in the development of nonneoplastic liver disease[51]. Ferroptosis is a new type of iron-dependent programmed cell death that is different from apoptosis, cell necrosis and autophagy. The main mechanism of ferroptosis is that under the action of divalent iron or ester oxygenase, unsaturated fatty acids with high expression on the cell membrane are catalyzed to produce lipid peroxidation, thus inducing cell death[18,52]. In addition, decreased expression of antioxidant systems (glutathione GSH and GPX4) has also been

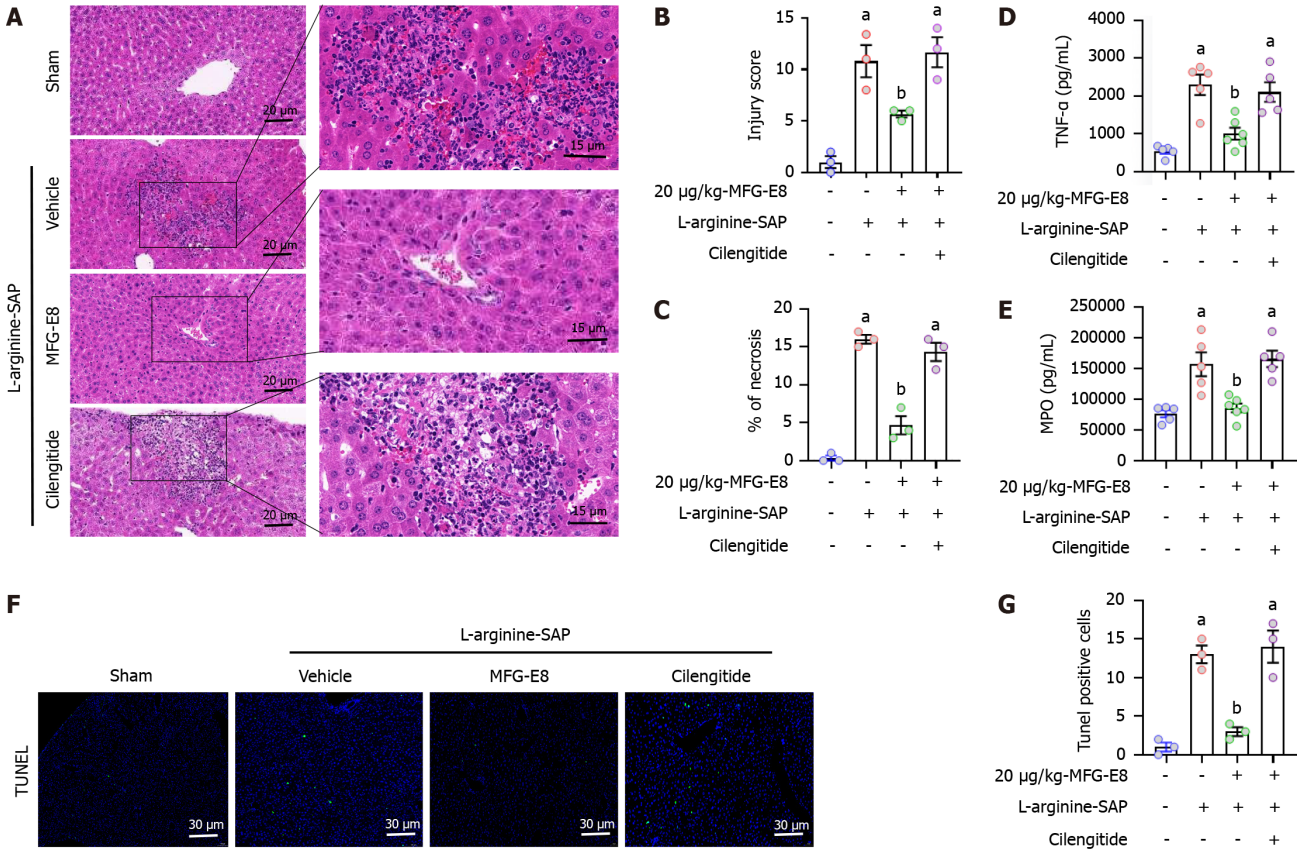

Figure 6 Milk fat globule epidermal growth factor 8 protects the liver from damage through the integrin αVβ3/5. In mice, severe acute pancreatitis was induced by 2 hly intraperitoneal injections of 4.0 g/kg L-arginine. At 2 h after the last injection of L-arginine, normal saline (vehicle) or 20 μg/kg milk fat globule epidermal growth factor 8 (MFG-E8) was administered through intraperitoneal injection. To determine whether the protective effect of MFG-E8 in liver is mediated through integrin αVβ3/5, 20 mg/kg cilengitide, a specific integrin αVβ3/5 antagonist, was administered intraperitoneally at 1 h before the injection of MFG-E8 (*i.e.*, 1 h after the last injection of L-arginine). The animals were sacrificed at 69 h after MFG-E8 treatment (*i.e.*, 72 h after the first injection of L-arginine). Blood and tissue samples were collected. A: Representative photos of hematoxylin and eosin staining in the liver (400 × or 1000 ×); B: Hepatic injury scores; C: Percentages of necrotic areas; D: Tumor necrosis factor-α levels in the liver; E: Myeloperoxidase levels in the liver; F and G: Representative photos of TdT-mediated dUTP Nick-End Labeling (TUNEL) staining (400 ×) and quantitative of TUNEL staining. n = 3-6/group, error bars indicate the SEM; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* vehicle group. SAP: Severe acute pancreatitis; TNF-α: Tumor necrosis factor-α; MPO: Myeloperoxidase; TUNEL: TdT-mediated dUTP Nick-End Labeling; MFG-E8: Milk fat globule epidermal growth factor 8.

observed with ferroptosis[53]. The effect of MFG-E8 on ferroptosis has not been reported in previous studies. In the present study, we found for the first time a possible link between MFG-E8 and ferroptosis in liver cells. It is possible that the protective effect of MFG-E8 on liver injury induced by SAP is also induced by alleviating ferroptosis, which is similar to the protective effect of (+)-clausenamide on the liver by alleviating ferroptosis as reported by Wang *et al*[54].

PTGS2, also known as cyclooxygenase-2, is a biomarker of ferroptosis similar to GPX4[29]. Ferroptosis is a form of programmed cell death induced by iron-dependent lipid peroxidation. GPX4 prevents rust by converting lipid hydroperoxides into nontoxic lipid alcohols[55]. The present study showed that oxidative stress in hepatocytes caused by lipid peroxidation resulted in mitochondrial membrane structure destruction. Electron microscopy showed that the mitochondria of hepatocytes of SAP mice showed significant swelling or even rupture, and iron ion deposition appeared in mitochondria. The use of exogenous MFG-E8 alleviates oxidative stress and restores mitochondrial morphology in hepatocytes, which is consistent with our recent findings on SAP. Moreover, MFG-E8 activates the focal adhesion kinase (FAK) - signal transduction and transcriptional activation factor 3 (STAT3) signaling pathway and alleviates the extent of mitochondrial damage during SAP. Together, these results indicate that MFG-E8 plays a crucial role in maintaining cellular homeostasis, such as mitochondrial function, lipid oxidation reactions and iron ion metabolism. However, the association of these cellular homeostasis and specific signaling pathways involved in MFG-E8 need to be further explored.

Our previous studies have shown that MFG-E8 maintains cellular homeostasis by alleviating ER stress in pancreatic exocrine acinar cells. The beneficial effects of MFG-E8 are mediated through activating the αVβ3/5 integrin-FAK-STAT3 signaling pathway[28]. The present study also explored the potential role of the αVβ3/5 integrin-FAK-STAT3 signaling pathway in the liver. However, the results showed that inhibition of MFG-E8 binding to integrins αVβ3/5 effectively antagonized the protective effect of MFG-E8 on SAP-induced liver injury, while blocking the FAK-STAT3 signaling pathway had almost no effect on the effect of MFG-E8 in the liver. Elucidating the specific molecular mechanism of the protective effect of MFG-E8 on hepatocytes is our future research direction.

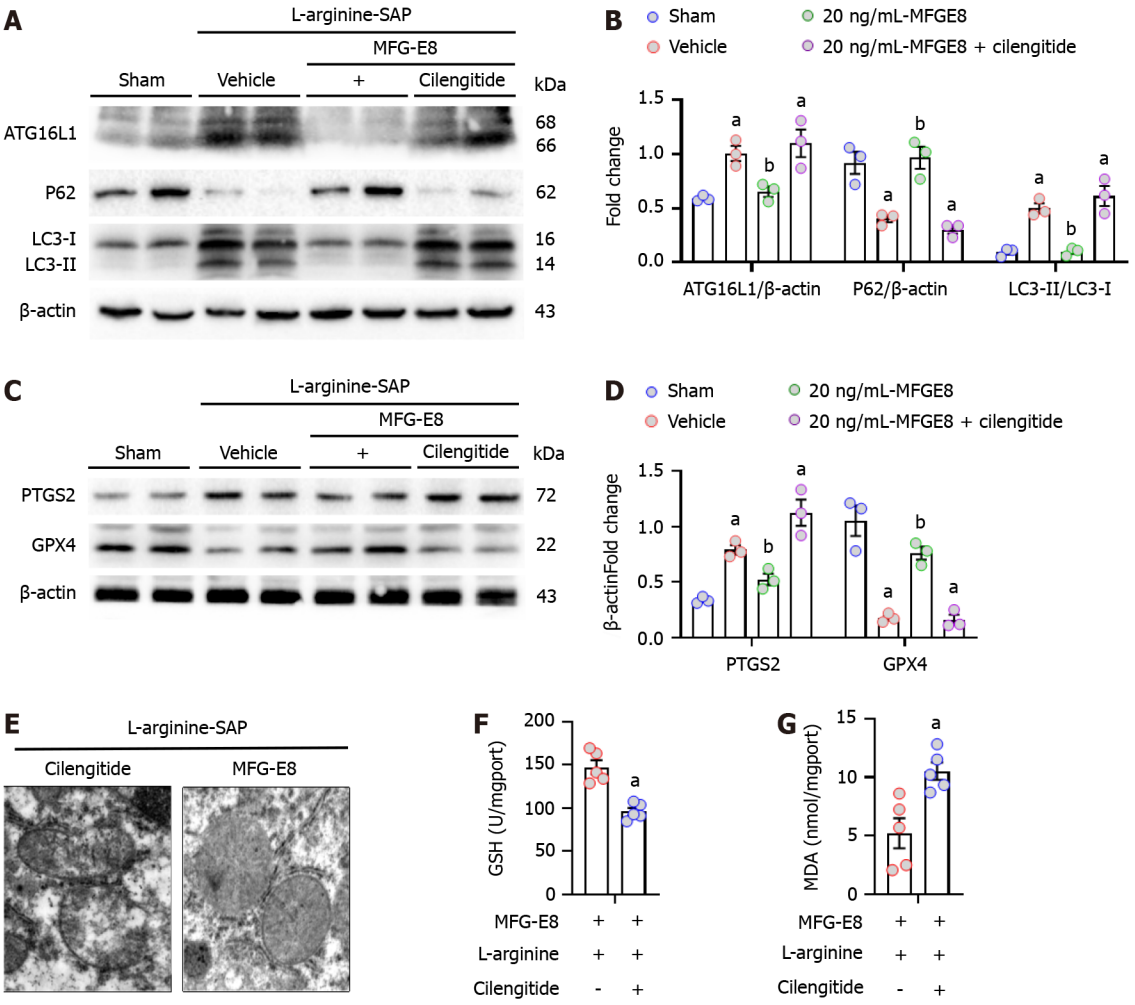

Figure 7 Milk fat globule epidermal growth factor 8 mitigates hepatic autophagy and ferroptosis through the integrin αVβ3/5. In mice, severe acute pancreatitis was induced by 2 hly intraperitoneal injections of 4.0 g/kg L-arginine. At 2 h after the last injection of L-arginine, normal saline (vehicle) or 20 μg/kg milk fat globule epidermal growth factor 8 (MFG-E8) was administered through intraperitoneal injection. To determine whether the protective effect of MFG-E8 in liver is mediated through integrin αVβ3/5, 20 mg/kg cilengitide, a specific integrin αVβ3/5 antagonist, was administered intraperitoneally at 1 h before the injection of MFG-E8 (*i.e.*, 1 h after the last injection of L-arginine). The animals were sacrificed at 69 h after MFG-E8 treatment (*i.e.*, 72 h after the first injection of L-arginine). Blood and tissue samples were collected. A-D: Western blot analysis and quantitative of the expression of autophagy related protein 16 like protein 1, P62, LC3, prostaglandin-endoperoxide synthase 2 and glutathione peroxidase 4 in the liver; E: Ultrastructural changes of mitochondria in hepatocytes (electron microscopy); F: Glutathione levels in the liver; G: Malondialdehyde levels in the liver. n = 3-6/group, error bars indicate the SEM; $^{a}P < 0.05$ *vs* sham group; $^{b}P < 0.05$ *vs* vehicle group. SAP: Severe acute pancreatitis; ATG16L1: Autophagy related protein 16 like protein 1; PTGS2: Prostaglandin-endoperoxide synthase 2; GPX4: Glutathione peroxidase 4; MDA: Malondialdehyde; GSH: Glutathione; MFG-E8: Milk fat globule epidermal growth factor 8.

The present study had several deficiencies, and additional studies are required. Due to the lack of clinical samples, we were unable to validate these results in patients with AP. TUNEL fluorescence staining showed that MFG-E8 also reduced SAP-induced apoptosis of liver cells. It remains unknown whether ferroptosis or apoptosis plays a more important role in SAP-induced liver death. Moreover, the role of MFG-E8 in ferroptosis has not been previously reported, and excessive autophagy can lead to ferroptosis[13,56]. Therefore, whether MFG-E8 directly regulates the occurrence of ferroptosis or indirectly alleviates ferroptosis by improving the homeostasis of autophagy flux remains to be explored further. Furthermore, in this study, we discussed the possible role of exogenous MFG-E8 in improving impaired liver function by binding integrin αVβ3/5. However, whether the intracellular MFG-E8 affects the liver injury caused by SAP and the specific mechanism remain obscure. Next, we will construct hepatocyte-specific *mfge8* gene modified mice (knockout or over-expression), and combine with *in vitro* experiments to further clarify the possible role of intracellular MFG-E8 in SAP-liver injury.

CONCLUSION

MFG-E8 alleviates excessive autophagy, inhibits ferroptosis in hepatocytes, and protects the liver from damage in SAP. The beneficial effects of MFG-E8 appears to be mediated through activating the αVβ3/5 integrin. These findings may provide a new perspective to reveal the role of MFG-E8 in SAP.

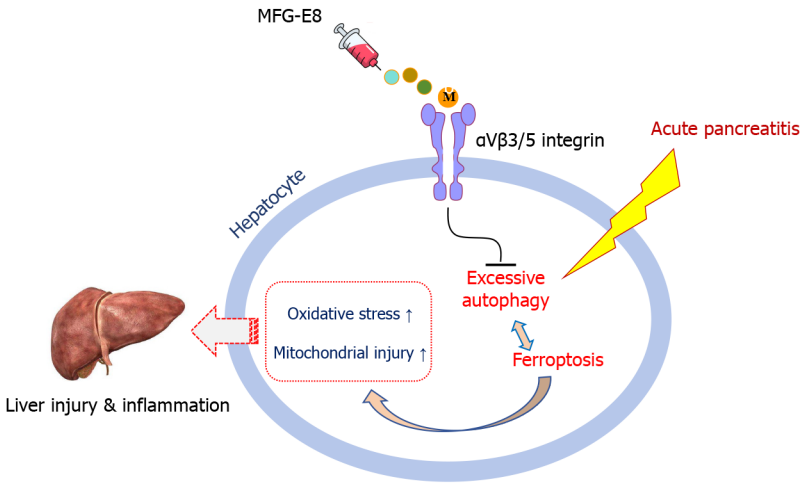

Figure 8 Graphical abstract. Exogenous milk fat globule epidermal growth factor 8 restored impaired autophagy, reduced ferroptosis and alleviated liver injury by acting on integrin αVβ3/5. MFG-E8: Milk fat globule epidermal growth factor 8.

ARTICLE HIGHLIGHTS

Research background

Liver injury is common in severe acute pancreatitis (SAP). Excessive autophagy often leads to an imbalance of homeostasis in hepatocytes, which induces lipid peroxidation and mitochondrial iron deposition and ultimately leads to ferroptosis. Our previous study found that milk fat globule epidermal growth factor 8 (MFG-E8) alleviates acinar cell damage during SAP *via* binding to αvβ3/5 integrins. MFG-E8 also seems to mitigate pancreatic autophagy in chronic pancreatitis. Whether MFG-E8 also alleviates damaged liver cells in SAP through a similar mechanism is unknown.

Research motivation

This study is to identify whether MFG-E8 could alleviate SAP induced liver injury by restoring the abnormal autophagy flux and alleviate mitochondrial damage like it in acute or chronic pancreatitis.

Research objectives

This study aims to investigate the role of MFG-E8 in SAP-related liver injury.

Research methods

Of the 134 AP patients (age ≥ 18 years) were included in this study. AP was diagnosed according to the International Atlanta Symposium on Acute Pancreatitis. SAP was induced in mice by 2 hly intraperitoneal injections of 4.0 g/kg L-arginine or 7 hly injections of 50 μg/kg cerulein plus lipopolysaccharide. *mfge8*-knockout mice were used to study the effect of MFG-E8 deficiency on SAP-induced liver injury. Cilengitide, a specific αvβ3/5 integrin inhibitor, was used to investigate the possible mechanism of MFG-E8.

Research results

Serum MFG-E8 concentration is negatively correlated with inflammatory severity in AP patients. MFG-E8 deficiency aggravated SAP-induced liver injury in mice, enhanced autophagy flux in hepatocyte, and worsened the degree of ferroptosis. Exogenous MFG-E8 reduced SAP-induced liver injury in a dose-dependent manner. Mechanistically, MFG-E8 mitigated excessive autophagy and inhibited ferroptosis in liver cells. Cilengitide abolished MFG-E8's beneficial effects in SAP-induced liver injury.

Research conclusions

Our findings suggested MFG-E8 acts as an endogenous protective mediator in SAP-induced liver injury. MFG-E8 alleviates the excessive autophagy and inhibits ferroptosis in hepatocytes by binding to integrin αVβ3/5.

Research perspectives

These findings may provide a new perspective to reveal the role of MFG-E8 in SAP and its regulation of homeostasis in damaged liver cells.

FOOTNOTES

Co-first authors: Qing Cui and Hang-Cheng Liu.

Author contributions: Cui Q, Liu HC, and Liu WM acquired and analyzed the data, wrote the paper; Ma F, Lv Y, Ma JC, and Wu RQ interpreted the data; Lv Y, Wu RQ, and Ren YF revised the paper; Ren YF designed and supervised the study; and all authors approved the final version of the article.

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SYSTEMATIC REVIEWS

Diagnostic and therapeutic role of endoscopic ultrasound in liver diseases: A systematic review and meta-analysis

Eyad Gadour, Abeer Awad, Zeinab Hassan, Khalid Jebril Shrwani, Bogdan Miutescu, Hussein Hassan Okasha

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Abstract

BACKGROUND

In hepatology, the clinical use of endoscopic ultrasound (EUS) has experienced a notable increase in recent times. These applications range from the diagnosis to the treatment of various liver diseases. Therefore, this systematic review summarizes the evidence for the diagnostic and therapeutic roles of EUS in liver diseases.

AIM
To examine and summarize the current available evidence of the possible roles of the EUS in making a suitable diagnosis in liver diseases as well as the therapeutic accuracy and efficacy.

METHODS
PubMed, Medline, Cochrane Library, Web of Science, and Google Scholar databases were extensively searched until October 2023. The methodological quality of the eligible articles was assessed using the Newcastle-Ottawa scale or Cochrane Risk of Bias tool. In addition, statistical analyses were performed using the Comprehensive Meta-Analysis software.

RESULTS
Overall, 45 articles on EUS were included (28 on diagnostic role and 17 on therapeutic role). Pooled analysis demonstrated that EUS diagnostic tests had an accuracy of 92.4% for focal liver lesions (FLL) and 96.6% for parenchymal liver diseases. EUS-guided liver biopsies with either fine needle aspiration or fine needle biopsy had low complication rates when sampling FLL and parenchymal liver diseases (3.1% and 8.7%, respectively). Analysis of data from four studies showed that EUS-guided liver abscess had high clinical (90.7%) and technical success (90.7%) without significant complications. Similarly, EUS-guided interventions for the treatment of gastric varices (GV) have high technical success (98%) and GV obliteration rate (84%) with few complications (15%) and rebleeding events (17%).

CONCLUSION
EUS in liver diseases is a promising technique with the potential to be considered a first-line therapeutic and diagnostic option in selected cases.

Key Words: Focal liver lesion; Liver abscess drainage; Fine needle aspiration; Gastric varices; Endoscopic ultrasound

Core Tip: This is an extensive systematic review to assess the efficacy and accuracy of the endoscopic ultrasound (EUS) in dealing with different liver pathologies. The EUS guided liver abscess drainage (EUS-AD) was highly accurate (90.7%) and very safe, with more than 90% of patients experienced no complications post EUS-AD. The safety profiles of the EUS guided aspiration and EUS guided biopsy was very promising with very low complication rate. EUS guided interventions is a safe and accurate procedure and this was demonstrated in different interventions such as EUS guided gastric varies obliteration which was successful in 84% with only 15% rebreeding risk.

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INTRODUCTION

Since its introduction in the 1980s, endoscopic ultrasonography (EUS) has emerged as a pivotal diagnostic and therapeutic tool, particularly for assessing a wide range of gastrointestinal (GI) and pancreatobiliary disorders[1,2]. Traditionally, EUS has not been commonly used to assess liver conditions. However, since its first publication in 1999 demonstrating the efficacy of EUS and fine-needle aspiration (EUS-FNA) in diagnosing focal liver lesions (FLL), the clinical utilization of EUS for evaluating the liver has gained interest[3]. Research has shown that owing to its ability to provide high-resolution images, EUS is valuable for detecting small liver lesions that often go unnoticed after transabdominal ultrasound (US) and computed tomography (CT)[4]. However, research on EUS for liver tumors often fails to provide details on the location of tumors within the liver segments. This may be because EUS anatomical segmentation of the liver is considered less significant.

EUS offers advantages that distinguish it from other diagnostic tools. EUS is performed by inserting the probe into the GI tract; therefore, it can provide close proximity to the target tissues[5]. This close proximity is particularly valuable for evaluating lesions within the GI wall, adjacent lymph nodes, and surrounding vasculature. It is also valuable in guiding FNA and fine-needle biopsy (FNB) for the collection of tissue samples from lesions and suspicious areas identified during the course of examination[6]. Furthermore, EUS can provide real-time imaging, which allows for dynamic assessment and precise localization of lesions[7].

Despite its advantages, evidence of the role of EUS in liver disease is limited. Therefore, this systematic review aimed to evaluate the diagnostic and therapeutic roles of EUS in liver disease.

MATERIALS AND METHODS

Information sources and searches

PubMed, Medline, Cochrane Library, Web of Science, and Google Scholar databases were comprehensively searched for all randomized and nonrandomized studies published from inception to October 2023. The bibliographies of potential articles were also scrutinized for additional studies. Studies with the following MeSH terms and keywords were retrieved from the electronic databases: (Endoscopic ultrasound OR endosonography OR EUS OR endoscopic ultrasound-guided fine needle aspiration OR EUS-FNA OR endoscopic ultrasound-guided fine needle biopsy OR EUS-FNB) AND (diagnosis OR diagnostic OR detection OR treatment OR interventional OR therapeutic) AND (hepatic OR liver). The gray literature and duplicates were not retrieved, as they would have interfered with the scientific purpose of the current study.

Eligibility criteria

Two independent reviewers scrutinized potential studies using predefined inclusion and exclusion criteria. Studies were eligible for review and analysis if they were full articles published in English, included human participants, or reported on the role of EUS in the diagnosis or treatment of liver diseases, including portal hypertension. On the other hand, articles that went against these criteria or were designed as case reports, systematic reviews, conference abstracts, and letters to the editors or reported the therapeutic and diagnostic role of EUS in extrahepatic structures such as bile duct and gall bladder were excluded. In the event of differences between the reviewers, a third reviewer was consulted to harmonize discrepancies.

Data extraction

Two impartial reviewers examined all included records and abstracted the data required for review and analysis into separate Excel files. Discrepancies in the extracted data were resolved through constructive discussions or by consulting a third reviewer. The extracted data included the Author ID (surname of the primary author and publication date), study design, study location (country), characteristics of the enrolled patients (sample size, sex distribution, mean/median age, and indication for conducting EUS/EUS-guided diagnostic tests), diagnostic tests used, intervention, treated liver disorder, and outcomes.

The outcomes of our study were divided into the therapeutic and diagnostic groups. The diagnostic endpoints included diagnostic accuracy and yield. Diagnostic accuracy was defined as the ratio of true positives to true negatives for an accurate cytological or histological diagnosis in the total number of patients. Therapeutic outcomes included procedure-related complications, technical and clinical success, gastric varices (GV) obliteration, and rebleeding.

Quality appraisal

Randomized and nonrandomized studies were included in the current review; therefore, quality assessment was performed using two different tools. The Newcastle-Ottawa scale was used to assess the methodological quality of non-randomized studies. This tool evaluates studies according to the selection, comparability, and outcome domains. For every domain, a maximum of one star was assigned for a fully answered criterion; otherwise, no stars were assigned. In the selection domain, a maximum of 4 stars could be attained, whereas a maximum of two and three stars could be achieved for the comparability and outcome domains, respectively.

On the other hand, bias assessment of randomized trials was performed using Cochrane's risk of bias (RoB) tool embedded within the Review Manager software. RoB was assessed based on selection, attrition, performance, reporting, and other biases. A low RoB was assigned to a domain that was sufficiently addressed within the study, whereas a high and unclear risk was assigned to domains that were not entirely addressed or had insufficient information to make a judgment.

Data synthesis

The comprehensive meta-analysis software (CMA V3) was used to conduct all statistical analyses in the present study. The random-effects model was used to pool the estimated weighted effect size and counter-anticipated heterogeneity. The inter-study heterogeneity was calculated using the I^2 statistics, of which values > 50% were regarded as significant [8]. Moreover, the effect sizes were calculated together with their 95% confidence intervals, and when possible, subgroup analyses were performed according to diagnostic tests or EUS-guided interventions.

RESULTS

Study selection

An extensive database search identified 1347 potential articles. Duplicate screening resulted in the exclusion of 495 duplicate studies. Subsequently, 716 records were eliminated based on title, abstract, and title screening, and 49 were not retrieved as they were either case reports, reviews, conference abstracts, or letters to the editor. Finally, 45 records were included and the remaining 42 were excluded for the following reasons: nine were published in different languages and 33 evaluated the diagnostic or therapeutic role of EUS in extrahepatic structures and other parts of the body (Figure 1).

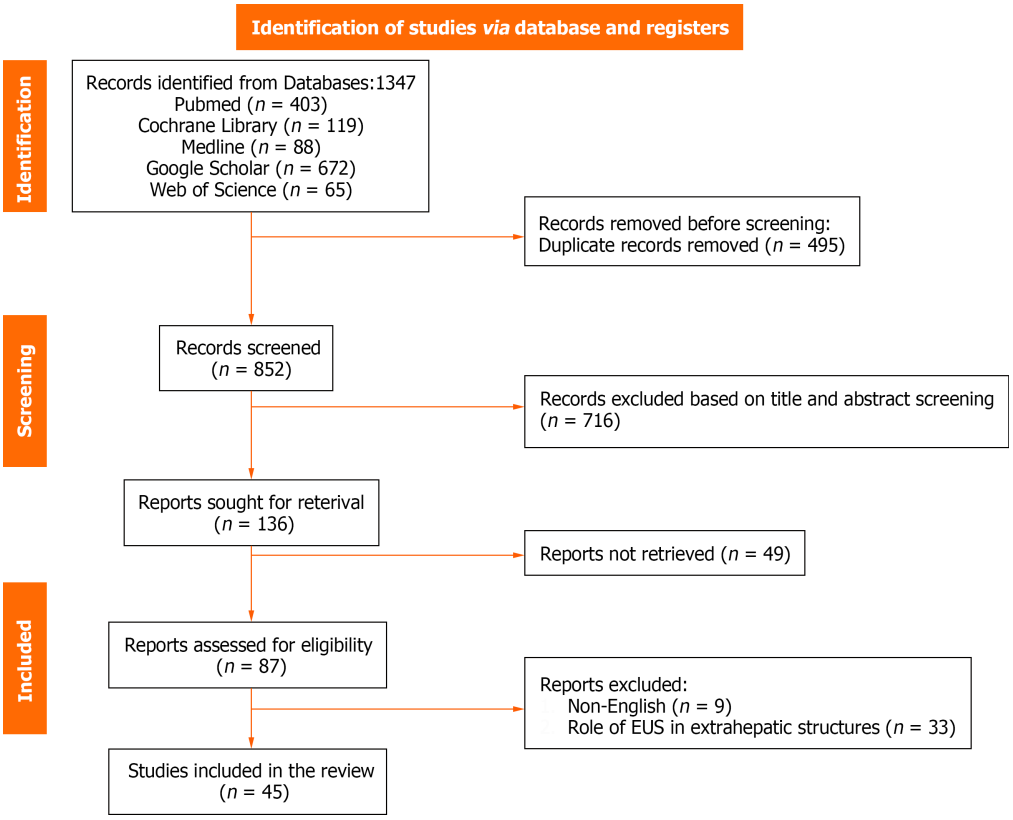

Figure 1 The preferred reporting items for systematic reviews and meta-analyses flow diagram for study selection. EUS: Endoscopic ultrasound.

Methodological quality and RoB assessment

Using the Newcastle–Ottawa scale and Cochrane RoB, we found that most studies were of good or fair quality. Table 1 presents the Newcastle-Ottawa scale results and Figure 2 summarizes the RoB.

Diagnostic role of EUS in the diagnosis of liver diseases

Twenty-eight studies reported the diagnostic role of EUS, of which 16 evaluated its value in detecting FLL, 10 in detecting parenchymal liver diseases (PLD), and two in detecting portal hypertension. Furthermore, all the studies were conducted in individual countries (11 in the United States, 2 in Japan, 3 in Romania, 2 in Turkey, 3 in Korea, 1 in Italy, 1 in Germany, 1 in India, 2 in China, and 1 in Egypt; Table 2).

Role of EUS in the detection of FLL

The cumulative analyses on the role of EUS in detecting FLL have shown an overall diagnostic accuracy rate of 92.4% (95%CI: 89.2 – 0.95). A subgroup analysis of the EUS diagnostic tests has shown that EUS alone had a diagnostic accuracy of 90.1%, whereas EUS-FNA and EUS-FNB had diagnostic accuracies of 93.4% and 98%, respectively. Furthermore, analysis of data from two studies has shown that Contrast-enhanced EUS (CEH-EUS) had a diagnostic accuracy of 94% for detecting FLL (Figure 3A).

Additionally, a safety analysis was performed to determine the safety of EUS-FNA and EUS-FNB in diagnosing FLL. Our subgroup analysis suggested that EUS-FNA had a complication rate of 2.9%, whereas the rate of complications when using EUS-FNA was 3.8% (Figure 3B).

Role of EUS in the detection of PLD

Seventeen studies assessing the value of EUS in detecting parenchymal liver disease reported an overall diagnostic accuracy of 96.6%. A subgroup analysis of data from these studies showed that EUS-FNA had a diagnostic accuracy of 96.6%, whereas EUS-FNB had a diagnostic accuracy of 97.6% for the detection of PLD (Figure 4A). Furthermore, a safety evaluation of these diagnostic tests has shown complication rates of 6.2% and 9.6% for EUS-FNA and EUS-FNB, respectively (Figure 4B).

Role of EUS in the detection of portal hypertension

Although studies on the role of EUS in portal hypertension are limited, we to identify two human studies evaluating the efficacy of EUS-guided portal pressure gradient (PPG) measurements. A meta-analysis of data from these studies revealed that 40 patients underwent EUS-PPG, with a technical success rate of 95.1% (Figure 5A). No complications related to this procedure have been previously reported.

Table 1 Methodological quality using the Newcastle-Ottawa scale

Ref.	Selection (/4)	Comparability (/2)	Outcome (/3)	Overall methodological quality
Ichim *et al*[9], 2022	3	1	2	Good
Minaga *et al*[10], 2021	2	1	1	Poor
Takano *et al*[11], 2021	3	1	2	Good
Ichim *et al*[12], 2020	3	1	2	Good
Facciorusso *et al*[13], 2021	3	1	3	Good
Chon *et al*[14], 2019	3	1	2	Good
Akay *et al*[15], 2021	3	1	3	Good
Chen *et al*[16], 2020	3	1	3	Good
Hollerbach *et al*[17], 2003	3	1	2	Good
Singh *et al*[18], 2007	2	1	2	Fair
tenBerge *et al*[19], 2002	2	1	2	Fair
Lee *et al*[20], 2015	3	2	1	Poor
Oh *et al*[21], 2018	3	2	2	Good
Singh *et al*[22], 2009	3	1	2	Good
Okasha *et al*[23], 2023	3	1	2	Good
Hasan *et al*[24], 2019	2	1	3	Good
Bhogal *et al*[25], 2020	3	1	3	Good
Diehl *et al*[26], 2015	2	1	2	Fair
Sundaram *et al*[27], 2023	4	1	2	Good
Saab *et al*[28], 2017	2	1	1	Poor
Sey *et al*[29], 2016	3	2	1	Poor
Shah *et al*[30], 2017	2	1	1	Poor
Sisman *et al*[31], 2020	2	1	2	Fair
Stavropoulos *et al*[32], 2012	3	2	1	Poor
Zhang *et al*[33], 2021	3	1	2	Good
Huang *et al*[34], 2017	3	1	2	Good
Ogura *et al*[35], 2016	3	1	2	Good
Tanikawa *et al*[36], 2023	3	1	2	Good
Tonozuka *et al*[37], 2015	2	1	2	Fair
Carbajo *et al*[38], 2019	3	1	2	Good
Nakaji *et al*[39], 2016	3	1	2	Good
Frost *et al*[40], 2018	2	1	2	Fair
Bhat *et al*[41], 2016	3	1	2	Good
Bick *et al*[42], 2019	3	1	2	Good
Binmoeller *et al*[43], 2011	3	1	2	Good
Bazarbashi *et al*[44], 2020	3	2	1	Poor
Mukkada *et al*[45], 2018	3	1	2	Good
Lee *et al*[46], 2000	2	2	2	Fair
Gubler *et al*[47], 2014	2	1	2	Fair
Kozieł *et al*[48], 2019	3	1	2	Good
Romero-Castro *et al*[49], 2013	4	2	2	Good

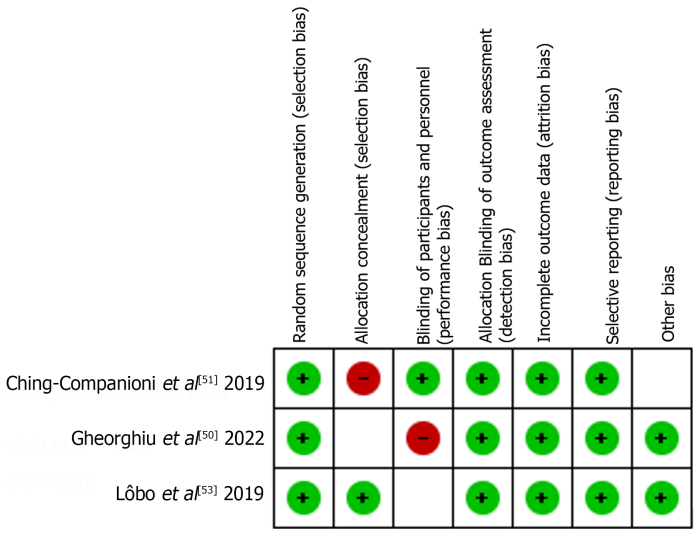

Figure 2 Risk of bias summary.

Therapeutic role of EUS in liver diseases

In the current review, the role of EUS in the treatment of liver diseases was reported in 17 studies. Four of these studies reported the efficacy of EUS-guided liver abscess drainage (EUS-AD), whereas two reported the value of EUS-guided interventions for the treatment of liver lesions. Additionally, 11 studies reported the therapeutic efficacy of various EUS-guided treatments of GV (Table 3).

Role of EUS in drainage of liver abscess

The efficacy of EUS-AD was reported in four studies[35-38]. A pooled analysis of data from these studies has shown that EUS-AD had a high technical (90.7%) and clinical success (90.7%; Figure 5B and C). Furthermore, two studies that included patients with hepatic abscesses reported that EUS-AD did not have any immediate or delayed complications.

Role of EUS in the treatment of solid liver lesions

The use of EUS to guide the treatment of FLL is a new and evolving field that has mostly been reported in case reports and animal studies. However, we identified two human studies[39,52] reporting the efficacy of EUS-guided interventions for solid liver lesions. Jiang *et al*[52] reported that EUS-guided therapy (ethanol injection, n = 10; iodine-125 seed brachytherapy, n = 13) was successful in most cases of left-sided liver tumors (23/25) without any procedure-related complications. Furthermore, complete tumor response was achieved in 65.2% of the patients, whereas partial response was achieved in 34.8%[52].

Nakaji *et al*[39] studied the efficacy of EUS-guided ethanol injections in the treatment of hepatocellular carcinoma (HCC). They found that the overall survival at 1, 2, and 3 years after the EUS-guided intervention was 91.7%, 75%, and 53.3%, respectively. Moreover, they reported two episodes of fever related to the procedure. However, no serious complications, such as intra-abdominal hemorrhage, abscesses, or bilomas were recorded[39].

Role of EUS in the management of GV

The role of EUS in GV treatment has not yet been fully established and remains an area of investigation. Therefore, we evaluated the efficacy and safety of EUS-guided interventions [cyanoacrylate (CYA), coil embolization, thrombin, and a combination of CYA and coil embolization] for GV. The pooled analyses revealed that EUS-guided interventions had a technical success rate of 98%. In addition, the rate of complication, GV obliteration, and rebleeding events were 15%, 84%, and 17%, respectively. Subgroup analyses of individual EUS-guided interventions are presented in Table 4.

DISCUSSION

This systematic review and meta-analysis summarizes the evidence for the therapeutic and diagnostic roles of EUS in hepatic diseases. The pooled analysis showed that EUS is an effective and safe tool for the diagnosis of FLL, PLD, and portal hypertension. We found that EUS-guided interventions were effective and safe for the treatment of liver diseases.

Diagnostic role of EUS

Despite the establishment of transabdominal US, CT, and magnetic resonance imaging as diagnostic tools for liver diseases, the use of EUS as a diagnostic and therapeutic modality has increased considerably in recent years. In our analysis, we found that EUS-guided liver biopsy (FNA and FNB) for parenchymal liver disease had high diagnostic accuracy (96.6%) and low complication rates (8.7%). This finding is consistent with that reported in the first meta-analysis

Table 2 Characteristics of studies on the role of endoscopic ultrasound in the diagnosis of liver diseases

Ref.	Study design	Study location	Participants characteristics				Diagnostic test	Outcomes
			Sample (*n*)	M/F	Age (yr)	Indication		
Ichim *et al*[9], 2022	Single-arm observational study	Romania	30	17/13	64.3	FLL	EUS-FNA	Diagnostic accuracy: 97% Complications: 1 patient
Minaga *et al*[10], 2021	Retrospective study	Japan	426	248/178	69 (63–75)	FLL	CEH-EUS	Diagnostic accuracy: 98.4%
Takano *et al*[11], 2021	Retrospective study	Japan	106	60/46	68 (32–87)	FLL	EUS-FNA	Diagnostic accuracy: 96% Complications: 1 patient
Ichim *et al*[12], 2020	Prospective study	Romania	48	27/21	66.3 (40–83)	FLL	EUS-FNA	Diagnostic accuracy: 98% Complications: None
Facciorusso *et al*[13], 2021	Retrospective study	Italy	116	70/46	NR	FLL	EUS-FNB	Diagnostic accuracy: 88.8% Complications: None
Chon *et al*[14], 2019	Retrospective study	Korea	58	35/23	68.1 (42–86)	FLL	EUS-FNB	Diagnostic accuracy: 89.7% Complications: 1 patient
Akay *et al*[15], 2021	Retrospective study	Turkey	25	15/10	62.73 ± 15.24	FLL	EUS-FNA	Diagnostic accuracy: 86.3% Complications: None
Gheorghiu *et al*[50], 2022	Prospective RCT	Romania	30	21/9	60 (37–84)	FLL	EUS-FNA and EUS-FNB	Diagnostic accuracy: 100% and 86.7% for EUS-FNB and EUS-FNA, respectively Complications: None
Chen *et al*[16], 2020	Retrospective study	China	38	35/3	55.7 ± 11.8	FLL	EUS-FNB	Diagnostic accuracy: 90% Complications: 3 patients
Hollerbach *et al*[17], 2003	Prospective study	Germany	41	NR	66 ± 7	FLL	EUS-FNA	Diagnostic accuracy: 94% Complications: 2 patients
Singh *et al*[18], 2007	Prospective study	United States	17	NR	56 (43–85)	FLL	EUS and EUS-FNA	Diagnostic accuracy: 65% and 94% for EUS and EUS-FNA, respectively Complications: None
tenBerge *et al*[19], 2002	Retrospective study		26	NR	NR	FLL	EUS-FNA	Diagnostic accuracy: 89% Complications: 6 patients
Lee *et al*[20], 2015	Retrospective study	Korea	21	9/12	63 (37–81)	FLL	EUS-FNB	Diagnostic accuracy: 90.5% Complications: None
Oh *et al*[21], 2018	Prospective study	Korea	30	19/11	66.5 (55.5–74)	FLL	CEH-EUS and CEH-EUS-FNA	Diagnostic accuracy: 80% and 86.7% for CEH-EUS and CEH-EUS-FNA, respectively Complications: None
Singh *et al*[22], 2009	Prospective study	United States	131	128/3	67 (45–86)	FLL	EUS and EUS-FNA	Diagnostic accuracy: 97% and 98% for EUS and EUS-FNA, respectively Complications: None
Okasha *et al*[23], 2023	Cross-sectional study	Egypt	43	32/11	56	FLL	EUS and EUS-FNA/FNB	Diagnostic accuracy: 94%, and 100% for EUS and EUS-FNA/FNB

								Complications: None
Ching-Companioni *et al* [51], 2019	Prospective RCT	United States	40	NR	NR	PLD	EUS-FNA and EUS-FNB	Diagnostic accuracy: 100% Complications: 13 patients
Hasan *et al*[24], 2019	Prospective study	United States	40	14/26	61 (46.7–68.2)	PLD	EUS-FNB	Diagnostic accuracy: 100% Complications: 9 patients
Bhogal *et al*[25], 2020	Retrospective study	United States	513	244/269	NR	PLD	EUS-FNA and EUS-FNB	Diagnostic accuracy: 99%
Diehl *et al*[26], 2015	Prospective study	United States	110	48/62	53 (9–87)	PLD	EUS-FNA	Diagnostic accuracy: 98% Complications: 1 patient
Sundaram *et al* [27], 2023	Retrospective study	India	74	37/37	44.5 (18–79)	PLD	EUS-FNA	Diagnostic accuracy: 97.3% Complications: 5 patients
Saab *et al*[28], 2017	Retrospective study	United States	47	16/31	54	PLD	EUS-FNB	Diagnostic accuracy: 100% Complications: 2 patients
Sey *et al*[29], 2016	Cross-sectional study	United States	75	24/51	51	PLD	EUS-FNB	Diagnostic accuracy: 82.7% Complications: 2 patients
Shah *et al*[30], 2017	Retrospective study	United States	24	NR	NR	PLD	EUS-FNB	Diagnostic accuracy: 96% Complications: 2 patients
Sisman *et al*[31], 2020	Retrospective study	Turkey	40	24/16	44 (22–72)	PLD	EUS-FNB	Diagnostic accuracy: 100% Complications: 2 patients
Stavropoulos *et al* [32], 2012	Prospective case series	United States	22	6/16	61 (32–79)	PLD	EUS-FNA	Diagnostic accuracy: 91% Complications: None
Zhang *et al*[33], 2021	Prospective study	China	12	9/3	NR	PH	EUS-PPG	Technical success rate: 91.7% EUS-PPG correlates well with HVPG (r = 0.923) Complications: None
Huang *et al*[34], 2017	Prospective study	United States	28	18/10	63 (30–80)	PH	EUS-PPG	Technical success rate: 100% EUS-PPG correlates well with clinical parameters of PH Complications: None

EUS: Endoscopic ultrasound; FLL: Focal liver lesions; CEH-EUS: Contrast-enhanced endoscopic ultrasound; FNA: Fine-needle aspiration; FNB: Fine-needle biopsy; NR: Not report; RCT: Randomized clinical trial; PLD: Parenchymal liver diseases; EUS-PPG: Endoscopic ultrasound-guided portal pressure gradient; PH: Portal hypertension; HVPG: Hepatic venous pressure gradient; M/F: Male/female.

of nine studies published between 2009 and 2016[54]. According to that meta-analysis, EUS-liver biopsy (EUS-LB) had an overall diagnostic yield of 93.9% and a complication rate of 2.3%. Similarly, a more recent meta-analysis evaluating the efficacy and safety of EUS-LB in patients with parenchymal liver disease and FLL revealed that EUS-LB had a high diagnostic yield (95%) and low adverse event rate (3%)[55]. The evidence from these studies and our analysis suggests that EUS-LB may be a safer diagnostic alternative for PLD. However, our subgroup analysis has shown that adverse events were more prevalent when using FNB needles than FNA needles (9.6% *vs* 6.2%). Therefore, high-quality randomized trials are needed to evaluate the safety of EUS-FNA compared with EUS-FNB in the diagnosis of PLD.

EUS is also a valuable diagnostic tool for FLL. EUS can provide high-resolution images of the liver anatomy, enabling the identification and characterization of focal lesions. In our analyses, we found that EUS-guided biopsy had an overall diagnostic accuracy of 92.4% and a low complication rate (3.1%). This finding is consistent with a previous review article reporting that the diagnostic yield of EUS-guided biopsy of FLL ranges from 89.7% to 100%[7]. Furthermore, our

Table 3 Characteristics of studies on the therapeutic role of endoscopic ultrasound

Ref.	Study design	Study location	Participant characteristics		Condition	Intervention	Outcomes
			Sample (*n*)	M/F			
Ogura *et al*[35], 2016	Retrospective study	Japan	27	20/7	Liver abscess	EUS-AD	Clinical success: 100% Technical success: 100% Complications: None
Tanikawa *et al* [36], 2023	Retrospective study	Japan	8	4/4	Liver abscess	EUS-AD	Clinical success: 87.5% Technical success: 87.5%
Tonozuka *et al* [37], 2015	Retrospective case series	Japan		NR	Liver abscess	EUS-AD	Clinical success: 100% Technical success: 100% Complications: None
Carbajo *et al*[38], 2019	Retrospective study	Spain	9	NR	Liver abscess	EUS-AD	Clinical success: 88.9% Technical success: 88.9%
Nakaji *et al*[39], 2016	Retrospective study	Japan	12	10/2	Solid liver lesions	EUS-guided ethanol injection	Complications: 2 Overall survival: 91.7%, 75%, and 53.3% at 1, 2, and 3 years
Jiang *et al*[52], 2016	Case series	China	26	17/9	Solid liver lesions	EUS-guided ethanol injection and iodine-125 brachytherapy	Complications: None
Frost *et al*[40], 2018	Case series	Ireland	8	7/1	GV	EUS-guided thrombin injection	Complications: None Obliteration: 75% Rebleeding: 1 patient
Bhat *et al*[41], 2016	Retrospective study	United States	152	97/55	GV	EUS-guided CYA and coil embolization	Technical success: 99% Obliteration: 93% Rebleeding: 20 patients Complications: 9 patients
Bick *et al*[42], 2019	Retrospective study	United States	104	62/42	GV	EUS-guided CYA	Obliteration: 79% Rebleeding: 12 patients Complications: 13 patients
Binmoeller *et al* [43], 2011	Retrospective study	United States	30	19/11	GV	EUS-guided CYA and coil embolization	Technical success: 100% Obliteration: 95.8% Rebleeding: 4 patients Complications: None
Bazarbashi *et al* [44], 2020	Prospective study	United States	40	27/13	GV	EUS-Guided coil embolization	Technical success: 100% Obliteration: 100% Complications: 1 patient
Lôbo *et al*[53], 2019	RCT	Brazil	32	13/19	GV	EUS-guided CYA and coil embolization	Complications: 13 patients Obliteration: 93.3%
Mukkada *et al* [45], 2018	Retrospective study	India	30	NR	GV	EUS-Guided coil embolization	Rebleeding: 6 patients
Lee *et al*[46], 2000	Prospective study	China	101	69/32	GV	EUS-guided CYA	Obliteration: 79.6% Complications: 22 patients Rebleeding: 19 patients

Gubler *et al*[47], 2014	Retrospective study	Switzerland	40	25/15	GV	EUS-guided CYA	Complications: 2 patients
Kozieł *et al*[48], 2019	Retrospective study	Poland	16	9/7	GV	EUS-guided CYA and coil embolization	Technical success: 94% Complications: 6 patients
Romero-Castro *et al*[49], 2013	Retrospective study	Germany	30	22/8	GV	EUS-guided coil embolization	Obliteration: 90.9% Complications: 1 patient Rebleeding: None

M/F: Male/female; NR: Not report; RCT: Randomized clinical trial; EUS-AD: Endoscopic ultrasound guided liver abscess drainage; GV: Gastric varices; CYA: Cyanoacrylate.

Table 4 Outcomes of endoscopic ultrasound-guided interventions in the management of gastric varices

Outcome	Cumulative analyses (95%CI)	Subgroup analyses (95%CI)			
		EUS-CYA	EUS-Coil	EUS-CYA + Coil	EUS-thrombin
Technical success	0.98 (0.92–0.99)	NR	0.96 (0.55–0.99)	0.98 (0.92–0.99)	NR
Obliteration	0.84 (0.79–0.88)	0.78 (0.70–0.85)	0.93 (0.71–0.99)	0.93 (0.88–0.97)	0.75 (0.38–0.94)
Complications	0.15 (0.07–0.28)	0.20 (0.07–0.44)	0.10 (0.02–0.31)	0.22 (0.04–0.69)	0.06 (0.003–0.51)
Rebleeding	0.17 (0.13–0.23)	0.26 (0.13–0.49)	0.08 (0.02–0.34)	0.16 (0.11–0.23)	0.13 (0.02–0.54)

EUS-CYA: Endoscopic ultrasound-cyanoacrylate; NR: Not report.

subgroup analysis has shown that both EUS-FNA and EUS-FNB used in sampling FLL had excellent diagnostic accuracy (93.4% and 98%, respectively). However, a recent prospective trial found that a 22G EUS-FNB had significantly better diagnostic accuracy than a 22G EUS-FNA for FLL (100% *vs* 83.3%)[50]. However, these findings cannot be used independently to guide the clinical diagnosis of FLL owing to various limitations. First, the trial was carried out in a single center and had a limited number of patients, indicating that it is not representative of all FLL cases worldwide. Second, cytology was not performed on the EUS-FNA samples; thus, the diagnostic accuracy of EUS-FNA may have decreased. Finally, rapid on-site or macroscopic on-site evaluation was not conducted; hence, it is possible that the diagnostic accuracy decreased.

In addition, the use of CEH-EUS for FLL examination has gained interest. Owing to the dual blood supply to the liver, US contrast agents help examine the FLL in the arterial, portal, and venous phases. A pooled analysis of data from two studies in our review has shown that CEH-EUS achieved a diagnostic accuracy of 94% without any reported complications. Therefore, CEH-EUS has the potential to be integrated into daily clinical practice for the detection of suspected FLLs and for maximizing the management of these patients. However, further studies are required to confirm these findings.

EUS has several clinical applications in portal hypertension, including assessment of GV, assessment of collateral veins, and measurement of hemodynamic changes. It is also valuable for direct measurement of the PPG, which reflects the severity of portal hypertension and is an excellent prognostic factor in hepatic disease[56]. The two human studies[33,34] in the current review have shown that EUS can be used to guide the measurement of PPG, with a technical success rate of 95.1% and minimal complications. Zhang *et al*[33] observed a strong correlation between EUS-PPG using a 22G FNA needle and the hepatic venous pressure gradient (Pearson correlation, $r = 0.93$). Therefore, EUS is safe and has a potential significance in the management and understanding of portal hypertension. However, larger clinical trials are needed to confirm these findings.

Therapeutic role of EUS

In addition to its use as a diagnostic tool, EUS plays an important role in the treatment of liver diseases. Percutaneous drainage (PCD) is considered the first-line therapy for liver abscess drainage because it is minimally invasive and has a considerably high technical success[57,58]. However, this is disadvantageous because external drainage and self-tube removal may cause patient discomfort. Therefore, EUS-AD was developed to address these challenges. Although the efficacy of EUS-AD has largely been examined in case reports[59-65], we identified four small case series. The pooled analysis of data from these studies has shown that it has a high clinical (90.7%) and technical success rate (90.7%), and no major complications. This finding has been supported by a previous review that found that EUS-AD has a technical success rate of 97.5% for draining liver abscesses that are difficult to access[64]. Therefore, EUS-AD is a safe and viable intervention, especially for abscesses inaccessible by PCD.

EUS has also been used to treat FLL using various techniques. However, this is a relatively new and expanding field, with the majority of information obtained from case reports and animal research. In the present study, only two studies

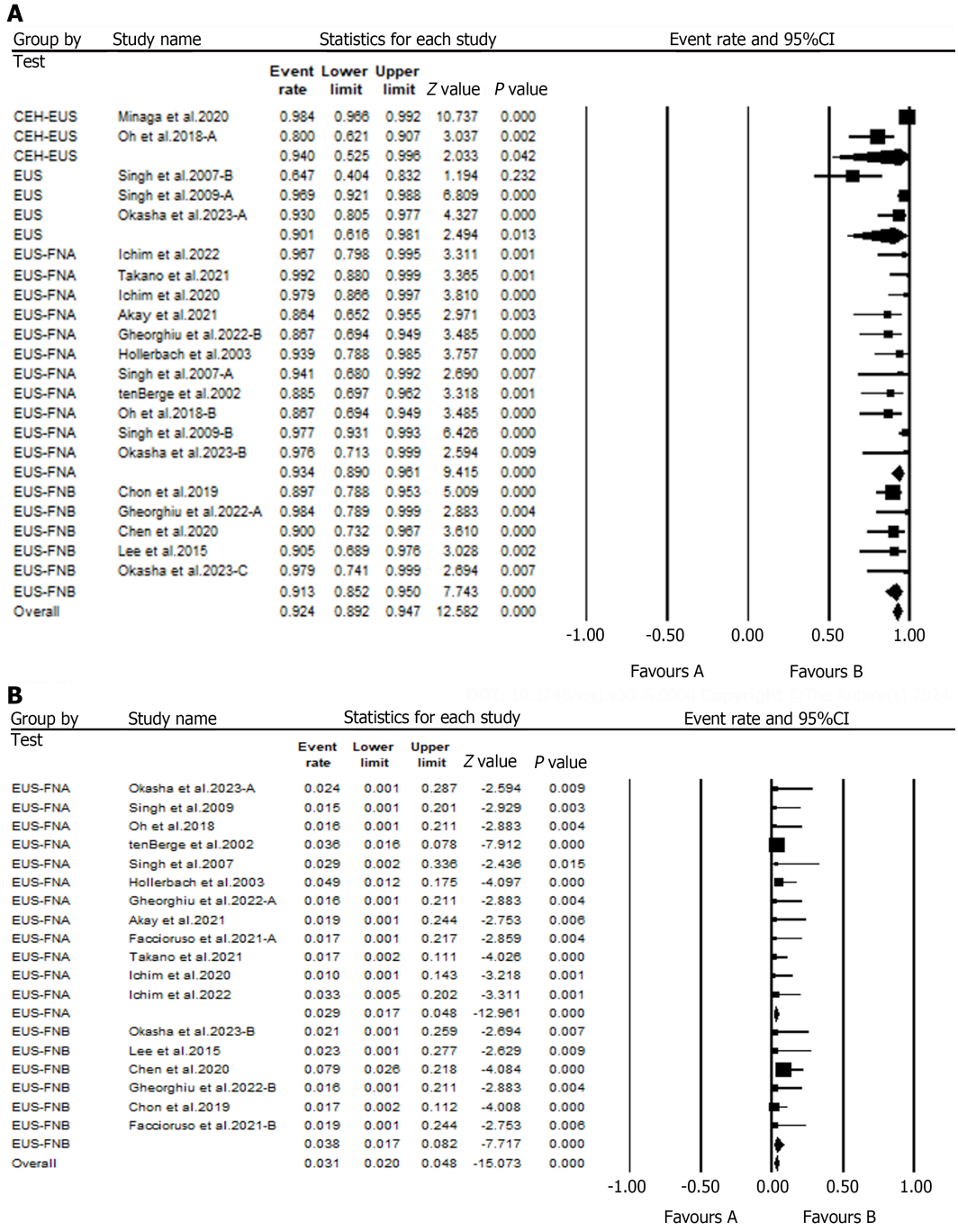

Figure 3 Forest plot of diagnostic and complications accuracy. A: Forest plot of diagnostic and accuracy in focal liver lesion detection (FLL); B: Forest plot of complications in FLL diagnosis. EUS: Endoscopic ultrasound; CEH-EUS: Contrast-enhanced endoscopic ultrasound; FNA: Fine-needle aspiration; FNB: Fine-needle biopsy.

reported EUS-guided interventions for solid liver lesions. A case series by Jiang *et al*[52] reported that EUS-guided iodine-125 brachytherapy was a safer and more effective treatment modality than EUS-guided ethanol injection for refractory left-sided liver lesions[52]. However, this finding warrants further large-scale clinical trials and comparative studies. In contrast, Nakaji *et al*[39] revealed that EUS-guided ethanol injection may be an effective and safe treatment option for early-stage HCC located in the caudate lobe[39].

GV in portal hypertension and cirrhosis can be catastrophic if not managed appropriately. Currently, therapeutic methods for managing GV include medical techniques, endoscopic interventions, and interventional radiology-guided procedures, such as transjugular intrahepatic portosystemic shunt and balloon retrograde transvenous obliteration. However, in recent years, EUS-guided interventions, such as EUS-guided coil embolization, thrombin, and CYA injections, have gained interest. Our pooled analysis has shown that EUS-guided interventions for GV had high technical success (98%), high obliteration rates (84%), low complications (15%), and low rebleeding events (17%). Furthermore, the subgroup analysis revealed that EUS-guided coil embolization alone was associated with fewer complications than EUS-guided CYA alone (10% *vs* 20%, respectively). Additionally, we noticed that combining CYA with coil embolization was associated with improved technical success, obliteration rates, and complication rates compared to EUS-guided CYA

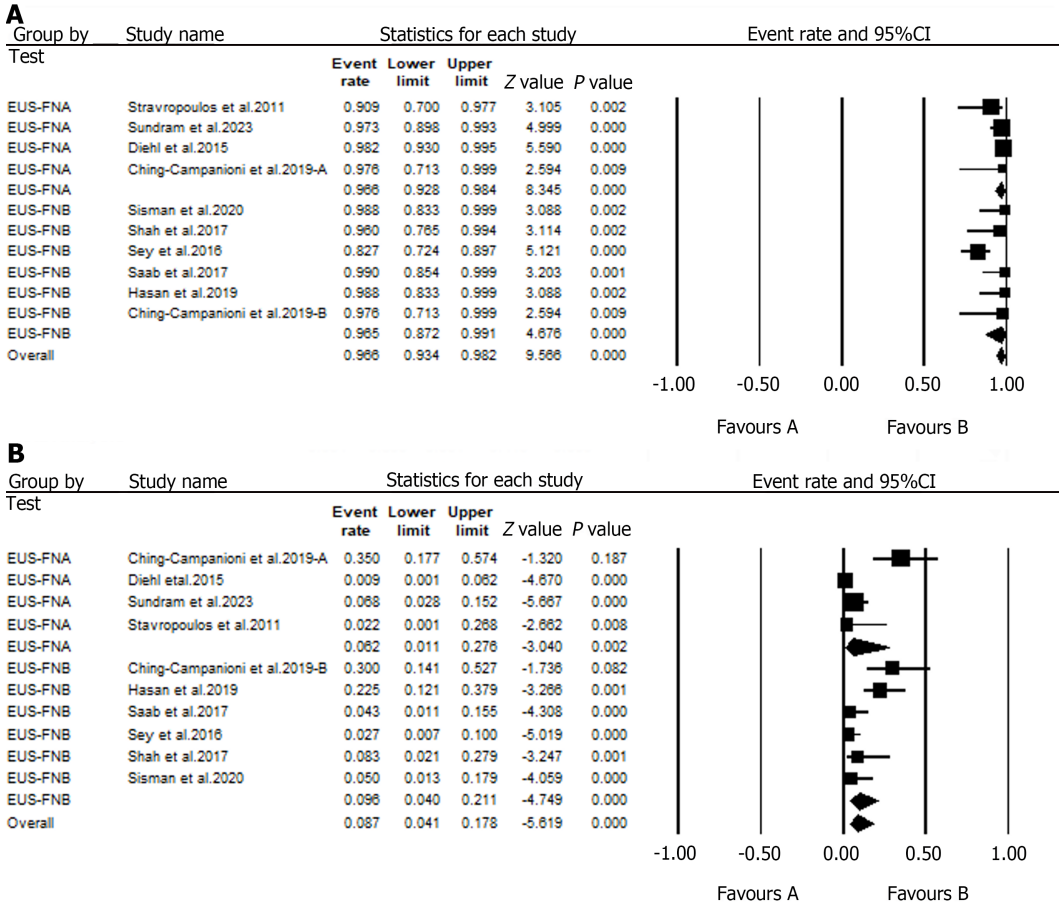

Figure 4 Forest plot of diagnostic accuracy and complications in parenchymal liver disease detection. A: Forest plot of diagnostic accuracy in parenchymal liver disease (PLD) detection; B: Forest plot of complications in PLD diagnosis. EUS: Endoscopic ultrasound; FNA: Fine-needle aspiration; FNB: Fine-needle biopsy.

alone.

Limitations

Similar to other scientific research articles, our review has several limitations that should be considered when interpreting our findings. First, we observed high inter-study heterogeneity in our statistical analysis, which may be due to the varied and limited sample sizes. However, we used a random-effects model to account for this heterogeneity and obtained conservative results. Second, most studies included in the present research were conducted in single centers; hence, they are not entirely representative of the general population and community. Third, most studies were retrospective or prospective in nature, indicating that they were subject to selection and confounding biases. Finally, conference abstracts and articles published in different languages were eliminated, indicating that the data from these studies improved the scientific and statistical power of the meta-analysis.

CONCLUSION

EUS plays a significant role in the diagnosis and treatment of hepatic disorders. Notably, EUS-LB with FNA or FNB provides excellent diagnostic precision for FLL and PLD. Accumulated evidence indicates that EUS-FNB may be more effective than EUS-FNA for FLL diagnosis, and the addition of contrast enhancement can improve the diagnostic accuracy of EUS. However, these findings need extensive validation through larger clinical trials and comparative studies. EUS-guided interventions tend to be effective in the treatment of liver abscesses, GV, and FLL, with reduced complication risks. Nevertheless, the potential efficacy of EUS-guided interventions requires further large-scale randomized trials.

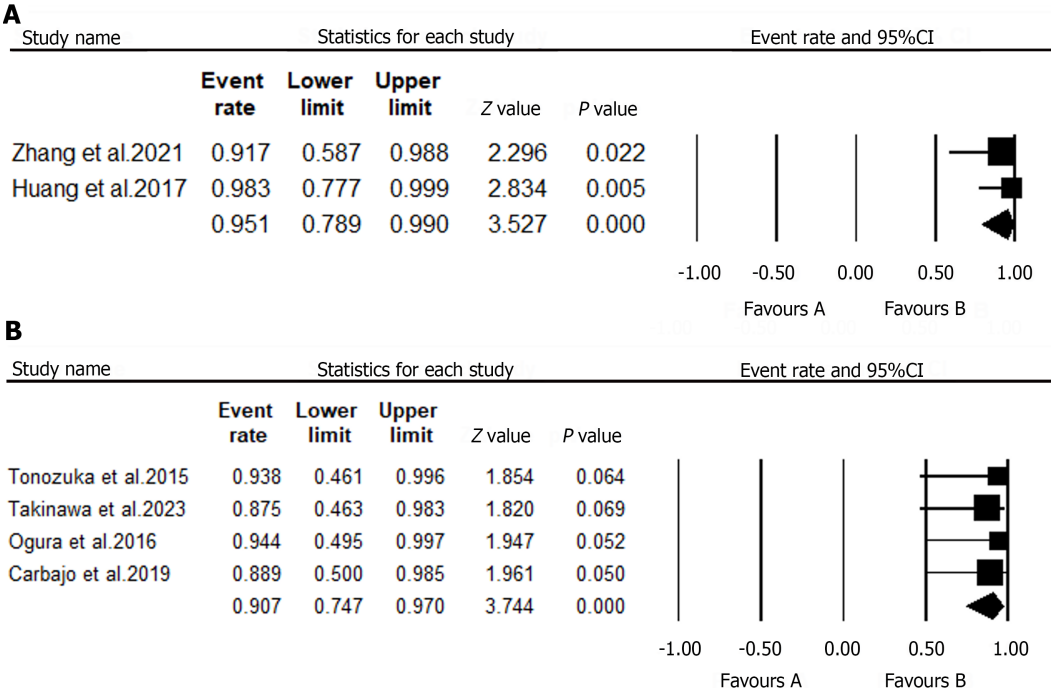

Figure 5 Forest plot of the technical success rate of endoscopic ultrasound. A: Forest plot of the technical success rate of endoscopic ultrasound (EUS) in detecting portal hypertension; B: Forest plot of the technical success rate of EUS-guided liver abscess drainage; C: Forest plot of the clinical success rate of EUS-guided liver abscess drainage.

ARTICLE HIGHLIGHTS

Research background

Endoscopic ultrasound (EUS) is a diagnostic and therapeutic procedure. The use of the EUS in the field of liver disease is recognizably increasing. However, the safety and efficacy are not well addressed.

Research motivation

We aimed to explore the safety and accuracy profile of the EUS in hepatology by comparing 28 articles evaluating the diagnostic role and 17 evaluating the therapeutic role of EUS.

Research objectives

To examine and explore the accuracy and efficacy of the role of the EUS in liver disease including the international aspects.

Research methods

We independently conducted an extensive systematic review using an electronic search on PubMed, Medline, Cochrane Library, Web of Science, and Google Scholar databases were extensively scoured for studies until October 2023. The methodological quality of the eligible articles was performed using the Newcastle-Ottawa scale or Cochrane's Risk of Bias tool. In addition, statistical analyses were performed with the comprehensive meta-analysis software.

Research results

The pooled analysis demonstrated that EUS diagnostic tests have an accuracy of 92.4% for focal liver lesions (FLL) and 96.6% for parenchymal liver diseases. In addition, the cumulative analyses showed that EUS-guided liver biopsies with either fine needle aspiration or fine needle biopsy have low complication rates when sampling FLL and parenchymal liver diseases (3.1% and 8.7%, respectively). Furthermore, analysis of data from four studies has shown that EUS-guided liver abscess has a high clinical (90.7%) and technical success (90.7%) without significant complications. Similarly, EUS-guided interventions for the treatment of gastric varices (GV) have a high technical success (98%) and GV obliteration rates (84%), with low complications (15%) and rebleeding events (17%).

Research conclusions

The role of EUS in the liver disease is well established with promising accuracy and efficacy profile. We found that EUS-guided interventions are effective and safe in treating liver diseases.

Research perspectives

EUS in liver diseases is a promising technique with the potential to be considered as a first-line therapeutic and diagnostic option in selected cases.

FOOTNOTES

Author contributions: Gadour E, and Okasha HH contributed to conceptualization; Gadour E, Awad A, Miutescu B and Okasha HH contributed to methodology; Hassan Z contributed to software; Okasha HH, Miutescu B and Gadour E contributed to validation; Gadour E contributed to formal analysis; Hassan Z, Shrwani KJ and Gadour E contributed to investigation; Awad A contributed to resources; Awad A and Okasha HH contributed to data curation; Awad A contributed to writing—original draft preparation; Gadour E, Okasha HH, Hassan Z, Awad A, Miutescu B and Shrwani KJ contributed to writing—review and editing; Hassan Z contributed to visualization; Okasha HH, and Gadour E contributed to supervision; Gadour E contributed to project administration; All authors have read and agreed to the published version of the manuscript.

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META-ANALYSIS

Metformin and pancreatic neuroendocrine tumors: A systematic review and meta-analysis

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Abstract

BACKGROUND

Most patients with advanced pancreatic neuroendocrine tumors (pNETs) die due to tumor progression. Therefore, identifying new therapies with low toxicity and good tolerability to use concomitantly with the established pNET treatment is relevant. In this perspective, metformin is emerging as a molecule of interest. Retrospective studies have described metformin, a widely used agent for the treatment of patients with type 2 diabetes mellitus (T2DM), to be effective in modulating different tumor-related events, including cancer incidence, recurrence and survival by inhibiting mTOR phosphorylation. This systematic review evaluates the role of T2DM and metformin in the insurgence and post-treatment outcomes in patients with pNET.

AIM

To systematically analyze and summarize evidence related to the diagnostic and prognostic value of T2DM and metformin for predicting the insurgence and post-treatment outcomes of pNET.

METHODS

A systematic review of the published literature was undertaken, focusing on the role of T2DM and metformin in insurgence and prognosis of pNET, measured through outcomes of tumor-free survival (TFS), overall survival and progression-

free survival.

RESULTS

A total of 13 studies (5674 patients) were included in this review. Analysis of 809 pNET cases from five retrospective studies (low study heterogeneity with I^2 = 0%) confirms the correlation between T2DM and insurgence of pNET (OR = 2.13, 95%CI = 1.56-4.55; P < 0.001). The pooled data from 1174 pNET patients showed the correlation between T2DM and post-treatment TFS in pNET patients (hazard ratio = 1.84, 95%CI = 0.78-2.90; P < 0.001). The study heterogeneity was intermediate, with I^2 = 51%. A few studies limited the possibility of performing pooled analysis in the setting of metformin; therefore, results were heterogeneous, with no statistical relevance to the use of this drug in the diagnosis and prognosis of pNET.

CONCLUSION

T2DM represents a risk factor for the insurgence of pNET and is a significant predictor of poor post-treatment TFS of pNET patients. Unfortunately, a few studies with heterogeneous results limited the possibility of exploring the effect of metformin in the diagnosis and prognosis of pNET.

Key Words: Pancreatic neuroendocrine tumors; Type 2 diabetes mellitus; Prognosis; Treatment; Metformin

Core Tip: Pancreatic neuroendocrine tumors (pNETs) are a challenge to diagnose and treat. Often curative treatments are not possible and additional therapy aimed at symptom relief and tumor cell growth inhibition is warranted. Unfortunately, a significant number of pNET patients do not respond to the above-mentioned medical treatments or show resistance. Therefore, exploring the risk factors and additional therapeutics is of importance. This systematic review and meta-analysis showed that in patients with type 2 diabetes mellitus (T2DM), the risk for pNET insurgence was significantly increased. In addition, T2DM was a significant predictor of poor tumor free survival. Results on the role of metformin in the setting of diagnosis and prognosis of pNET due to paucity of data and data heterogeneity failed to show statistical relevance of its use, although there are indices that it might positively impact the progression free survival.

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INTRODUCTION

The incidence of pancreatic neuroendocrine tumors (pNETs), especially non-functioning ones, increased more than 3-fold in the last few decades. Moreover, due to their relatively indolent course, the prevalence of pNETs is also rising, so they account for approximately 10% of all pancreatic neoplasms[1]. Unfortunately, due to an asymptomatic course, diagnosis is still challenging, and tumors are often diagnosed at the metastatic stage, with spread mainly to the liver[2,3], when curative treatments, such as surgical removal of primary tumors, are not feasible[4]. In the presence of metastases, surgical procedures are unfortunately more often palliative and additional therapy is warranted, including somatostatin analogs for symptom relief and antiproliferative effect[5,6], everolimus, mTOR inhibitor for inhibiting angiogenesis, proliferation, and potentiating tumor cell apoptosis[7], sunitinib, tyrosine kinase inhibitor, for angiogenesis inhibition[8] or where available nuclear treatment options[9]. Unfortunately, many pNET patients do not respond to or resist the above-mentioned medical treatments[10].

Most patients with advanced pNETs die due to tumor progression. Therefore, identifying new therapies with low toxicity and good tolerability to use concomitantly with the established pNET treatment is relevant. In this perspective, metformin is emerging as a molecule of interest[11-13].

Retrospective studies have described metformin, a widely used agent for the treatment of patients with type 2 diabetes mellitus (T2DM), to be effective in modulating different tumor-related events, including cancer incidence, recurrence and survival by inhibiting mTOR phosphorylation[14]. In pNET development, hyperactivation of PI3K/Akt signaling and activation of the mTOR pathway mediated through insulin-like growth factor-1 have been implicated to play a crucial role in carcinogenesis, thus providing the rationale for metformin use[15].

Moreover, although risk factors for pNET are still inconclusive, T2DM has been described as an essential contributor to tumor development, with a high incidence and prevalence of diabetes seen among pNET patients[16,17]. Indeed, the incidence of sporadic pNETs parallels that of T2DM, the highest in the fifth decade[18]. Moreover, T2DM, through chronic hyperglycemia, might accelerate tumor cell growth and spread, a mechanism seen in many cancer types[19], which might also negatively affect pNET prognosis[17], while metformin *in vitro* leads to inhibition of NET cell aggressiveness[20].

This systematic review evaluates the role of T2DM and metformin in the insurgence and post-treatment outcomes in patients with pNET. A pooled analysis was also performed according to the observed results.

MATERIALS AND METHODS

This systematic review was conducted and reported under the guidelines for systematic reviews and meta-analyses for prognostic factor studies and the PRISMA and AMSTAR-2 Guidelines[21,22].

Search strategy and eligibility criteria for studies

Objective: The main goal of this review was to systematically analyze and summarize evidence relating to the diagnostic and prognostic value of T2DM and metformin for predicting the insurgence and post-treatment outcomes of pNET.

Search strategy: Medline (PubMed) database was searched through June 2023 for relevant published original articles using the following keywords: (pancre AND neuroendocrine tumor) AND (diabetes OR T2DM OR mellit OR MODY OR DM2).

We also searched the reference lists of included studies. Two authors (Lai Q and Coppola A) independently reviewed the found records based on titles, abstracts, and the full text against the eligibility criteria (Figure 1). Consensus or a third reviewer (Cigrovski Berkovic M) resolved any conflict regarding study selection. Period of research: 01/01/2000-June 15, 2023.

Eligibility criteria: This review focused on retrospective and prospective observational studies that evaluated the diagnosis and the post-treatment outcomes in pNET adults over 18 years. Studies were included if they investigated the diagnostic or prognostic value of T2DM or metformin measured in pNET patients.

Case series, case reports, literature reviews or studies without adequate prognostic analyses were excluded. Studies were selected based on the PICOTS framework. No geographic or follow-up restrictions were applied. Only studies in the English language were considered. A limitation in the year of publication was applied, excluding all the studies before January 2000. If a study featured multiple eligible articles, we chose the most recent paper with the most significant number of participants and the most extended duration of follow-up.

Data extraction

Two independent reviewers (Lai Q and Coppola A) identified and collected data using the modified CHARMS-PF checklist[23]. Information extracted in each selected study included: First author (reference number), year of publication, country, period or study enrollment, design of the study, number of cases, number of controls, percentage of male sex, mean age, outcome measure, outcome value and 95%CI.

Risk of bias assessment

The Newcastle-Ottawa scale (NOS) was used to assess information on study quality; this scale varies from zero to a maximum possible score of nine and incorporates information on participant selection, outcome, exposure ascertainment, and the potential for confounding[24]. Two authors (Lai Q and Cigrovski Berkovic M) assessed the included studies. Any discrepancies were resolved by consensus or by a third reviewer (Sesa V).

Statistical analysis

Odds ratios (ORs) or hazard ratios (HRs) with the corresponding 95%CI were used for the outcomes. Only the data adjusted for potential confounders were used to realize the pooled analyses reported in the present study. A random effects model was used to account for heterogeneity among studies. Heterogeneity was assessed using the Higgins I^2 statistic[25]. An I^2 > 75% indicated high heterogeneity, 50%–75% moderate heterogeneity, and < 50% mild heterogeneity [26]. Forest plots were used to graphically display the effect size in each study and the pooled estimates. The heterogeneity of the different studies was graphically reported using the Galbraith plot and the Funnel plot. A P value < 0.05 was considered statistically significant. All analyses were conducted using STATA statistical package version 14.0 (StataCorp LLC, College Station, TX, United States).

RESULTS

Study selection process and study characteristics

The PRISMA flow diagram summarizes the study selection process (Figure 1). The search strategy identified 530 records and no records from reference lists. Records were screened based on the selection by title/abstract. Three hundred forty-seven records were excluded because they were irrelevant to the review question or did not adhere to the inclusion criteria. Of the remaining 183 eligible records, 170 full-text articles were discarded for several reasons (Figure 1). In detail, the reasons for discard were: Non-human study (n = 14), non-English (n = 24), editorial/Letter/case report/case series (n = 103), no text available (n = 2), review article (n = 15), study not relevant (n = 12).

Key characteristics of the included studies are illustrated in Tables 1 and 2.

Table 1 Characteristics of studies included in systematic review: Type 2 diabetes mellitus as risk factor

Ref.	Country	Period	Design	*n*	Controls	Male (%)	Mean age (yr)	Outcome measure	95%CI	NOS
Diagnosis										
Hassan *et al*[27], 2008	United States	2000-2006	Retro	160	924	55	54	OR	2.8 (1.5–5.2)	7
Halfdanarson *et al*[28], 2014	United States	2000-2011	Retro	273	602	54	59	OR	1.9 (1.3-2.9)	8
Valente *et al*[29], 2017	Europe	2013-2015	Retro	201	603	51	60	OR	2.1 (1.3-3.5)[1]	8
Giraldi *et al*[30], 2021	Italy	2014-2017	Retro	100	248	46	NA	OR	3.0 (1.2-7.9)	8
Feola *et al*[31], 2022	Italy	NA	Retro	75	210	NA	NA	OR	2.6 (1.3-5.2)	6
Treatment (PFS)										
Pusceddu *et al*[32], 2018	Italy	1999-2015	Retro	445	-	53	59	HR	1.0 (0.6-1.5)	8
Pusceddu *et al*[33], 2021	Italy	2006-2013	Pros	204	-	52	62	HR	1.6 (1.0- 2.8)	9
Treatment (TFS)										
de Mestier *et al*[34], 2020	France	2003-2018	Retro	268	-	40	55	HR	2.4 (1.2-4.5)	8
Sandini *et al*[35], 2020	Germany	2001-2017	Retro	417	-	56	58	HR	2.3 (1.3-4.2)	8
Fan *et al*[17], 2020	China	2006-2018	Retro	299	-	40	NA	HR	1.0 (0.6-1.8)	8
Tan *et al*[36], 2022	China	2009-2019	Retro	190	-	48	NA	HR	4.5 (1.2-10.3)	8
Treatment (OS)										
Fan *et al*[17], 2020	China	2006-2018	Retro	299	-	40	NA	HR	1.2 (0.5-2.7)	8
Awwad *et al*[37], 2022	Germany	1999-2009	Retro	120	-	NA	58	HR	3.2 (1.2-10.3)[2]	8
Zhang *et al*[38], 2022	China	2008-2020	Retro	335	-	43	NA	HR	2.7 (1.3-5.3)	8

[1]No recent onset.
[2]Only patients with metabolic syndrome (*n* = 32).
NOS: Newcastle–Ottawa Score; OR: Odds ratio; NA: Not available; HR: Hazard ratio; PFS: Progression-free survival; TFS: Tumor-free survival; OS: Overall survival.

None of the studies included was a randomized controlled trial (RCT); only one was prospective, and the remaining 12 were retrospective experiences. No study reported was balanced after propensity score analysis.

Studies were conducted between 2008 and 2022 in five countries: Italy (*n* = 4), China (*n* = 3), the United States (*n* = 2), Germany (*n* = 2), and France (*n* = 1). One study was a European multicentric study.

The study population ranged from 120 to 1084 participants. The total number of cases enrolled was 5674 cases. The mean patient age range was 54-62 years, and the percentage of males ranged from 40%-56%. Heterogeneous outcomes were reported in the different studies. Five studies explored the role of T2DM as a risk factor for the insurgence of pNET [27-31], while the remaining eight studies explored the role of T2DM in terms of post-treatment outcomes[32-38]. The post-treatment outcomes were also heterogeneous, including progression-free survival (PFS), tumor-free survival (TFS), and overall survival (OS).

As for the role of metformin, only five studies explored its role in the setting of pNET[17,27,29,32,37]. In detail, two studies reported the role of metformin in the insurgence of pNET[27,29], and the remaining three explored PFS, TFS, or OS[17,32,37].

Risk of bias assessment

As reported in Tables 1 and 2, studies selected for review showed a good NOS, ranging from 6-9.

T2DM results and pooled analyses

In all the studies exploring the role of T2DM as a risk factor for pNET insurgence, this disease always resulted as a risk factor[27-31]. A meta-analysis was performed to explore this aspect. In patients with T2DM, the risk for pNET insurgence was significantly increased (OR = 2.13, 95%CI = 1.56-4.55; $P < 0.001$). The heterogeneity of these studies was low, with an $I^2 = 0\%$ (Figure 2A). The low heterogeneity was graphically observable, also looking at the Galbraith and Funnel plots (Figure 3A and B).

Four studies explored the effect of T2DM in terms of post-treatment TFS[17,34-36]. The meta-analysis of HRs performed to explore this aspect showed that T2DM was a significant predictor of poor TFS (HR = 1.84, 95%CI = 0.78-

Table 2 Characteristics of studies included in systematic review: Metformin as risk factor

Ref.	Country	Period	Design	*n*	Controls	Male (%)	Mean age (yr)	Outcome measure	95%CI	NOS
Diagnosis										
Hassan *et al*[27], 2008	United States	2000-2006	Retro	160	924	55	54	OR	0.8 (0.3-2.5)	7
Valente *et al*[29], 2017	Europe	2013-2015	Retro	201	603	51	60	OR	1.4 (0.7-2.7)[1] 1.5 (0.7-3.4)[2]	8
Treatment (PFS)										
Pusceddu *et al*[32], 2018	Italy	1999-2015	Retro	445	-	53	59	HR	0.5 (0.3-0.8)	8
Treatment (TFS)										
Fan *et al*[17], 2020	China	2006-2018	Retro	299	-	40	NA	HR	0.8 (0.4-1.6)[3] 1.3 (0.6-3.0)[3]	8
Treatment (OS)										
Fan *et al*[17], 2020	China	2006-2018	Retro	299	-	40	NA	HR	0.7 (0.2-2.6)[4] 0.4 (0.1-2.8)[4]	8
Awwad *et al*[37], 2022	Germany	1999-2009	Retro	120	-	NA	58	HR	2.6 (0.7-7.0)[5]	8

[1]No insulin.
[2]Metformin and insulin.
[3]Only with metastases.
[4]No metastases
[5]Only patients with metabolic syndrome (n = 32).
NOS: Newcastle–Ottawa Score; OR: Odds ratio; NA: Not available; HR: Hazard ratio; PFS: Progression-free survival; TFS: Tumor-free survival; OS: Overall survival.

2.90; P < 0.001). The heterogeneity of these studies was intermediate, with an I^2 = 51% (Figure 2B). The intermediate heterogeneity was also graphically observable in the Galbraith and Funnel plots (Figure 3C and D).

Metformin results

The few studies exploring the different outcomes limited the possibility of performing pooled analyses in metformin. As reported in Table 2, heterogeneous results were reported, with no clear statistical relevance of the use of this drug in both the diagnosis and prognosis settings of pNET. Only one study showed a protective effect of metformin on the risk of PFS [32].

DISCUSSION

pNETs represent an increasingly diagnosed pancreatic pathology, with incidence rising with age[39].

According to population-based studies, 5-year survival of pNET patients, depending on patients' characteristics and the therapy used, ranges from 15% to 60%[40]. A minority of patients develop pNETs in association with inherited multiple endocrine neoplasia syndromes with known genetic mutations, while the majority of pNETs occur sporadically and knowledge of related risk factors is still insufficient. A study by Halfdanarson *et al*[28], investigating different risk factors among cases of low-grade pNETs, reported T2DM to be more common in pNET cases than matched controls (19% *vs* 11%, P < 0.001).

Our results performed on 3396 patients, including 809 pNET cases from five retrospective studies, confirm the correlation between T2DM and insurgence of pNET (OR = 2.13, 95%CI = 1.56-4.55; P < 0.001)[27-31]. Possible mechanisms are still speculative and involve both chronic hyperglycemia, which is a hallmark of T2DM and hyperinsulinemia. It seems that higher glucose availability to cancer cells, as present in T2DM, accelerates tumor growth, proliferation and metastatic spread, while hyperinsulinemia might further promote tumor growth through direct and indirect effects. As a direct effect, insulin stimulates glucose uptake and consumption by the pNET cells, stimulating their proliferation and indirectly, insulin displays mitogenic actions promoting cell division and spread and inhibiting apoptosis through the activation of the IR-IGF-1-receptor/PI3K/AKT/mTORC1 pathway. Moreover, hyperinsulinemia downregulates the expression of IGF-1-BPs, which, in turn, enhances the bioavailability of IGF-1 and promotes its binding to IGF1R, leading to tumor cell growth[41]. In addition, low-grade chronic inflammation accompanying T2DM can also create a beneficial tumor microenvironment, promoting pNET growth and spread[42,43]. A few studies (mainly retrospective) have also reported the correlation between T2DM and the prognosis of pNETs. According to a study by Fan and coworkers, in the case of concomitant T2DM and pNET, patients had a greater chance for metastatic disease and neural invasion[17], greater tumor size[44] and poor survival post-pancreatic surgery[35]. We analyzed the pooled data from 1174 pNET

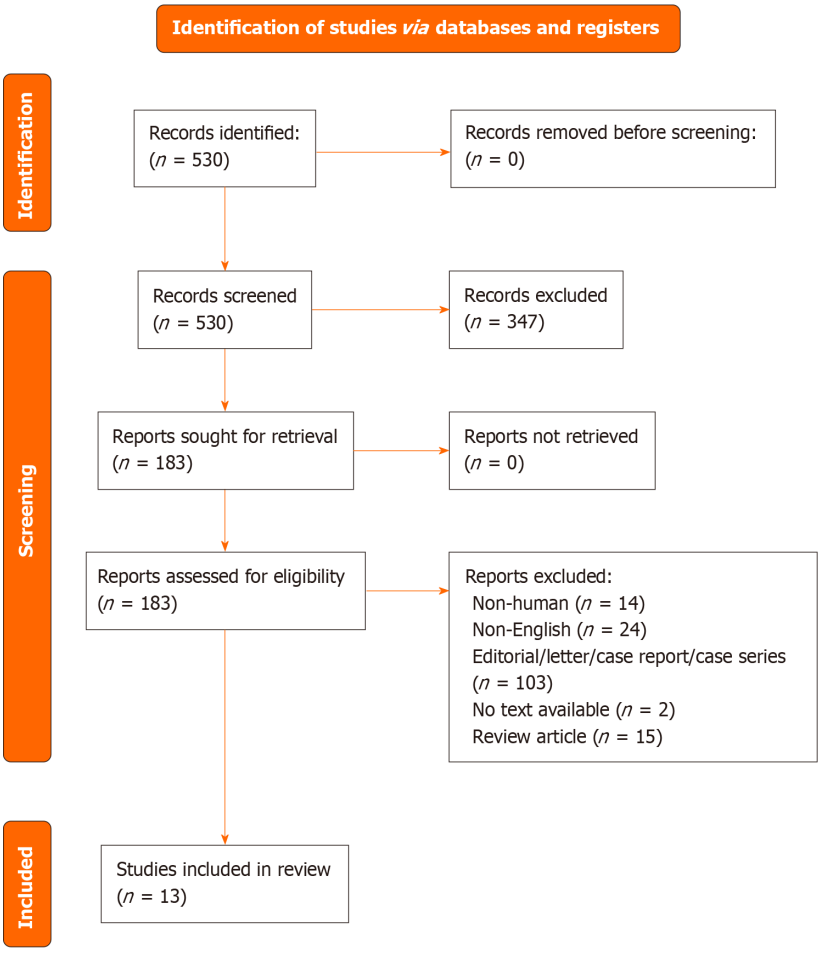


Figure 1 PRISMA 2020 flow diagram. Selection of studies to review the diagnostic and prognostic value of type 2 diabetes mellitus and metformin for predicting the insurgence and post-treatment outcome of pancreatic neuroendocrine tumor.

patients and found the correlation between T2DM and TFS in pNET patients (HR = 1.84, 95%CI = 0.78-2.90; $P < 0.001$), suggesting higher recurrence risk in case of concomitant T2DM[17,32-38].

As T2DM seems to be a risk factor for contracting pNET and potentially negatively impacts the patients' outcomes, studies exploring the role of anti-diabetic agents, specifically metformin, in similar settings are of importance. Metformin has been investigated as an anticancer agent in the setting of different cancer types. In the case of pancreatic adenocarcinoma, its use in diabetic patients was associated with reduced cancer risk, while data on patients' survival are still inconclusive but also suggestive of positive effects[45]. The possibility to repurpose metformin in case of pNET treatment is suggested by the results of a small study by Pusceddu *et al*[46] where 12 patients with advanced G 1–2 pNETs and concomitant T2DM (compared to 19 patients without T2DM) had a significantly longer PFS if treated with metformin on top of everolimus 10 mg daily in combination with octreotide LAR 30 mg i.m. every 28 d. Median PFS was 29 mo in patients with T2DM taking metformin compared with 11 mo in normoglycemic patients ($P = 0.018$)[46]. A more extensive multicentric Italian study involving 445 patients with advanced pNETs suggests metformin, probably irrespective of its dose, significantly prolongs PFS of patients with T2DM compared to other anti-diabetic drugs used on top of everolimus with or without somatostatin analogs (44.2 mo *vs* 20.8 mo), especially if introduced three months prior to standard anticancer treatment[32].

The *post hoc* analysis of the CLARINET study, including patients with advanced, non-functional entero-pancreatic NETs with an indolent course (both pNETs and intestinal NETS with a Ki67 ≤ 10%) treated with lanreotide or placebo also showed a favorable effect of metformin on the PFS of patients who had T2DM prior to study treatment and were randomized to the placebo arm. In this patient subgroup, PFS more than doubled compared to patients not receiving metformin. On the other hand, there was no additional benefit when metformin was added to patients treated with lanreotide[33].

Currently, evidence from prospective, randomized studies is still not available. Until the data from the ongoing clinical trial, MetNET1[47], a prospective, open-label, single-arm trial in which patients with advanced pNETs will receive metformin in combination with first-line somatostatin analogs and everolimus, also including patients without diabetes mellitus based on published preclinical data indicating that metformin also produces direct (cell-autonomous) antitumor effects, independent of glucose extracellular concentration becomes public there is no firm recommendation for its use in the setting of pNET[10,20].

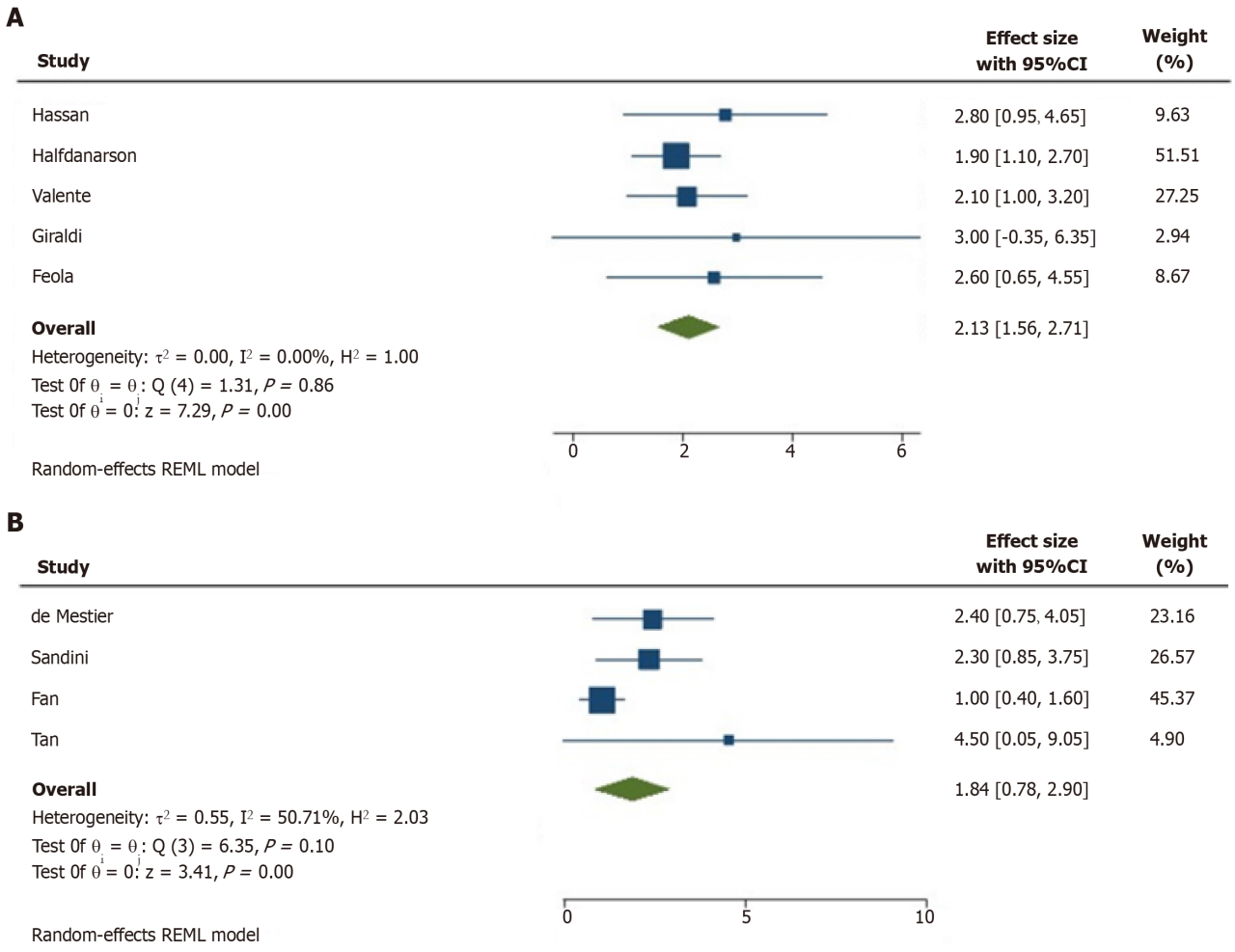


Figure 2 Association between type 2 diabetes mellitus and pancreatic neuroendocrine tumor. A: Forest plots of hazard ratios and 95%CIs for the association between type 2 diabetes mellitus (T2DM) and pancreatic neuroendocrine tumor (pNET) diagnosis; B: Forest plots of hazard ratios and 95%CIs for the association between T2DM and pNET tumor-free survival.

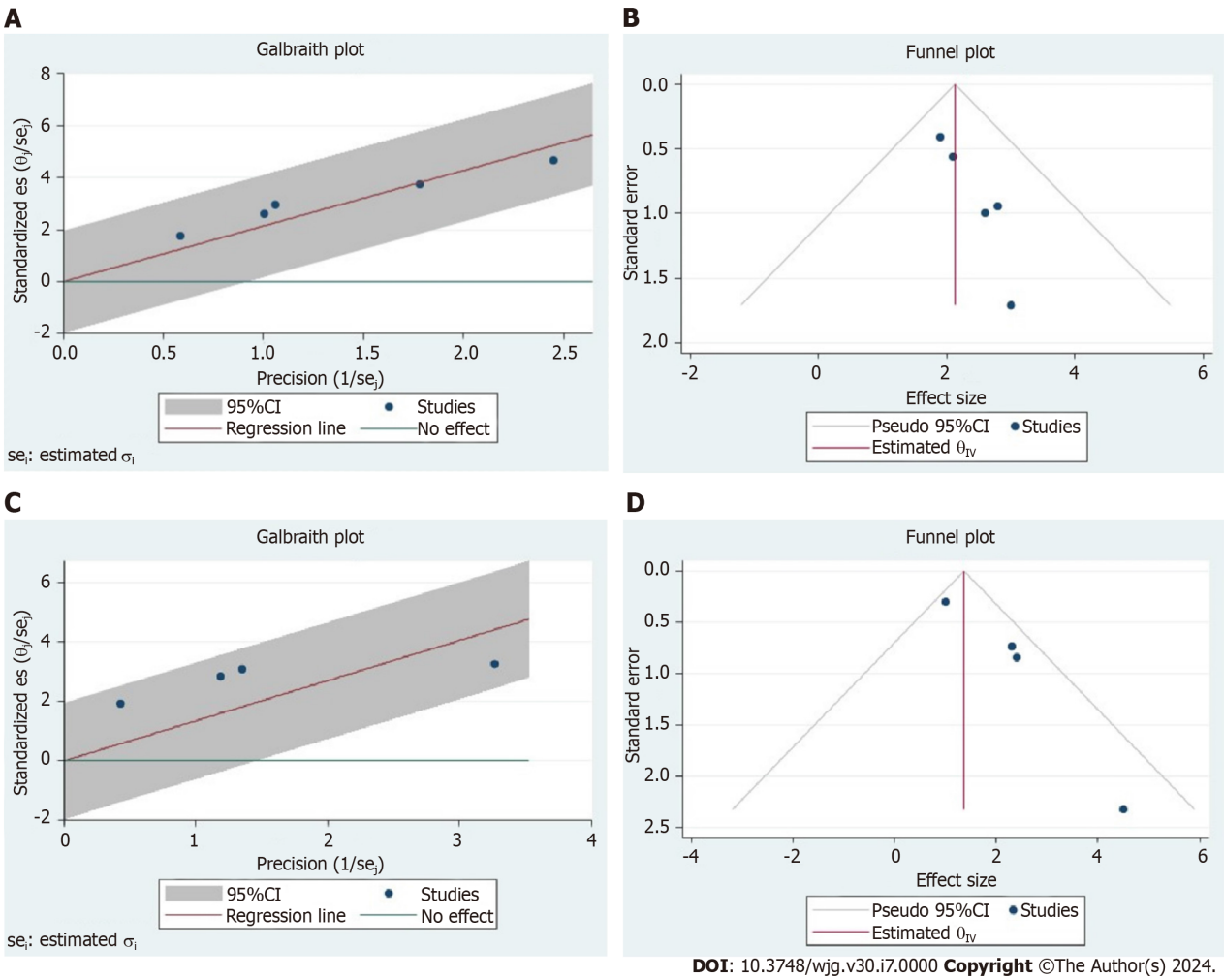
In this analysis, we could only include a small number of studies, limiting the possibility of performing pooled analysis. The heterogeneity of included studies enabled us to explore its relevance in the settings of diagnosis and prognosis of pNETs.

The present study presents some limitations. Only one prospective study was available, and no RCTs were present among the investigable studies. Therefore, heterogeneity across the studies and potential inclusion biases should be considered. Second, it was impossible to perform detailed pooled analyses concerning several outcomes due to the paucity of studies to consider. This limitation was particularly true in the case of metformin studies. Lastly, several potential confounders that are impossible to analyze should be considered, like the duration of T2DM, the concomitant use of insulin or the duration of anti-diabetic therapies. This type of data should be relevant in constructing meta-regressions, but unfortunately, these data were missing in several explored studies.

In conclusion, until results of RCTs, including patients with pNETs with or without concomitant T2DM receiving metformin in different proven anticancer treatments, become available, data on metformin effects in this setting is still inconclusive.

CONCLUSION

T2DM represents a risk factor for the insurgence of pNET and is a significant predictor of poor post-treatment TFS of pNET patients. Unfortunately, a few studies with heterogeneous results limited the possibility of exploring the effect of metformin in the diagnosis and prognosis of pNET.



DOI: 10.3748/wjg.v30.i7.0000

Figure 3 Heterogenity of the studies exploring the association between type 2 diabetes mellitus and pancreatic neuroendocrine tumor. A and B: Galbraith plot (A) and Funnel plot (B) reporting the heterogeneity of the studies exploring the association between type 2 diabetes mellitus (T2DM) and pancreatic neuroendocrine tumor (pNET) diagnosis; C and D: Galbraith plot (C) and Funnel plot (D) reporting the heterogeneity of the studies exploring the association between T2DM and pNET tumor-free survival.

ARTICLE HIGHLIGHTS

Research background

Advanced pancreatic neuroendocrine tumors (pNETs) are difficult to treat with low overall survival (OS). In pNET development, hyperactivation of PI3K/Akt signaling and activation of the mTOR pathway mediated through insulin-like growth factor-1 have been implicated to play a crucial role in carcinogenesis, thus providing the rationale for metformin use. Moreover, although risk factors for pNET are still inconclusive, type 2 Diabetes mellitus (T2DM) has been described as an essential contributor to tumor development, with a high incidence and prevalence of diabetes seen among pNET patients. This systematic review evaluates the role of T2DM and metformin in the insurgence and post-treatment outcomes in patients with pNET.

Research motivation

Regarding scarce data on pNET treatment and risk factors we wanted to investigate and analyze available data related to diagnostic and prognostic value of T2DM and metformin in association with pNET.

Research objectives

The main aim of this review was to systematically analyze and summarize evidence related to the diagnostic and prognostic value of T2DM and metformin for predicting the insurgence and post-treatment outcomes of pNETs.

Research methods

A systematic review of the published literature was undertaken, focusing on the role of T2DM and metformin in insurgence and prognosis of pNET, measured through outcomes of tumor-free survival (TFS), OS and progression-free survival.

Research results

A total of 13 studies (n = 5674 patients) were included in this review. Analysis of 809 pNET cases from five retrospective studies (low study heterogeneity with I^2 = 0%) confirms the correlation between T2DM and insurgence of pNET (odds ratios = 2.13, 95%CI = 1.56-4.55; P < 0.001). The pooled data from 1174 pNET patients showed the correlation between T2DM and post-treatment (TFS) in pNET patients (hazard ratio = 1.84, 95%CI = 0.78-2.90; P < 0.001). The study heterogeneity was intermediate, with I^2 = 51%. A few studies limited the possibility of performing pooled analysis in the setting of metformin; therefore, results were heterogeneous, with no statistical relevance to the use of this drug in the diagnosis and prognosis of pNET.

Research conclusions

T2DM represents a risk factor for the insurgence of pNET and is a significant predictor of poor post-treatment (TFS) of pNET patients. Unfortunately, a few studies with heterogeneous results limited the possibility of exploring the effect of metformin in the diagnosis and prognosis of pNET.

Research perspectives

Future research should further try to identify other risk factors and their influence on pNETs as well as the role of metformin in the diagnosis and prognosis of pNET.

FOOTNOTES

Author contributions: Lai Q and Cigrovski Berkovic M contributed to the conception and design of the study; Coppola A and Lai Q contributed to the acquisition of data; Lai Q, Coppola A, and Sesa V analyzed and interpreted the data; Mrzljak A and Cigrovski Berkovic M drafted the article; Lai Q critically revised the manuscript; all authors approved the final version.

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LETTER TO THE EDITOR

Complementary comments on metastatic liver lesions with exceptional and rare cases

Kemal Bugra Memis, Sonay Aydin

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Peer-review model: Single blind

Peer-review report's scientific quality classification

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Grade C (Good): C
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Abstract

Liver metastases can appear in different forms in magnetic resonance imaging. Contrary to popular belief, while radiologists report hypovascular or hypervascular metastatic lesions, exceptional examples may be detected in various tumors. The aim of this article is to improve this review by presenting rare and atypical examples of liver metastasis, as well as cases that might potentially be misdiagnosed as metastases during the process of differential diagnosis.

Key Words: Hepatic lesions; Magnetic resonance imaging; Liver metastases; Echinococcus alveolaris; Prostate adenocarcinoma; Appendix neuroendocrine tumor

Core Tip: Maino *et al* summarized the most frequent appearances of liver metastasis in detail. This letter adds to the mentioned literature with atypical examples and a potential misleading infectious cause, alveolar echinococcosis.

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TO THE EDITOR

Maino *et al*[1] recently published research suggesting the role of magnetic resonance imaging (MRI) in liver metastases. They reviewed the importance of MRI in the diagnosis and evaluation of liver metastases, as well as a description of their primary

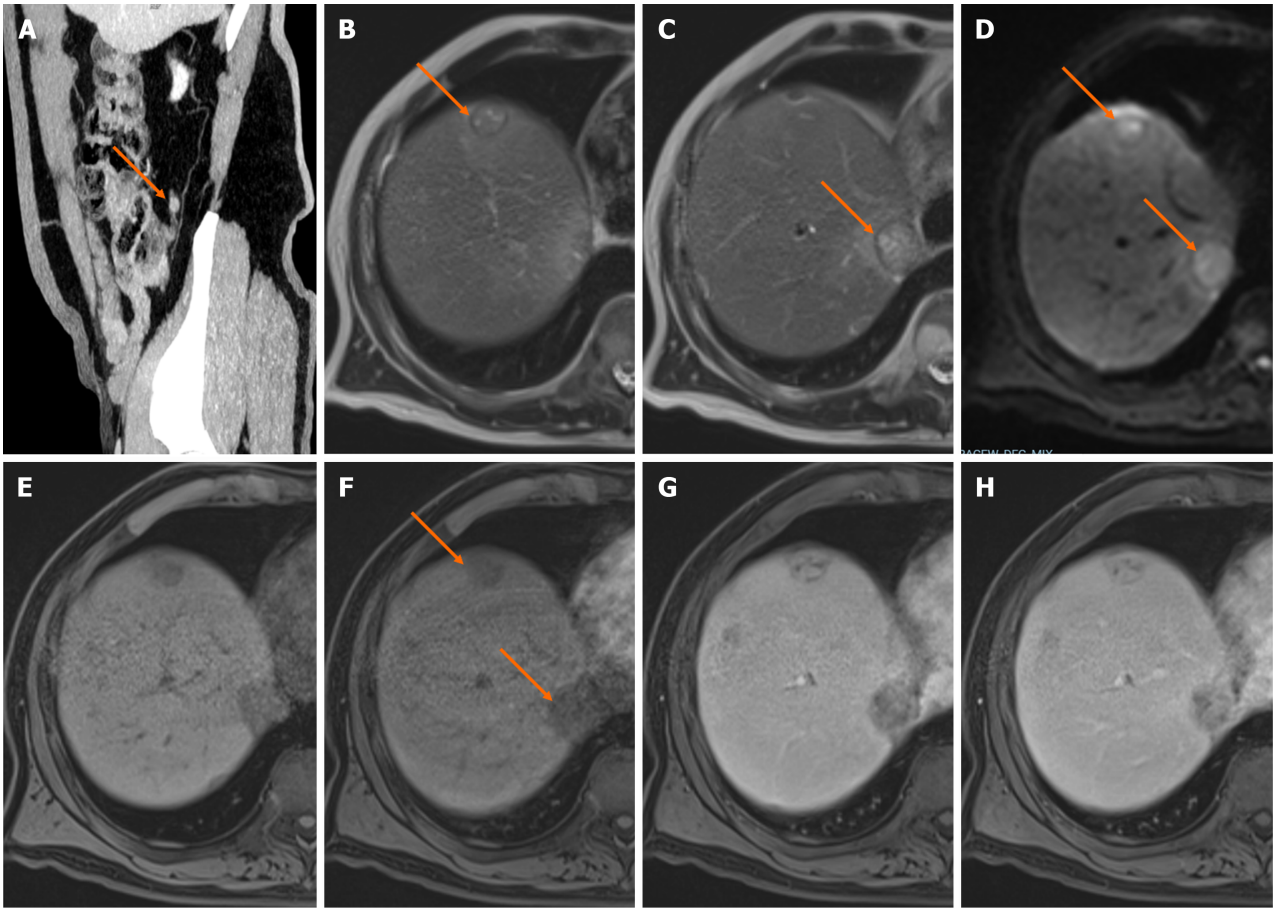

Figure 1 Liver metastases from an appendix neuroendocrine tumor. In Gd-DOTA-magnetic resonance imaging of a 60-year-old male patient. A: On sagittal MIP computed tomography images, there is an enhancing lesion located at the tip of the appendix (orange arrow); B and C: On consecutive T2-weighted images, lesions appear slightly hyperintense (orange arrows); D: Diffusion restriction in lesions on diffusion weighted imaging (orange arrows); E: Before contrast administration, lesions are hypointense; F: During the post-contrast late hepatic arterial phase, lesions appear hypovascular (orange arrows); G and H: Lesions are hyperintense on the portal-venous and delayed post-contrast phase.

imaging characteristics. Additionally, they described MRI protocols using contrast agents to better diagnose liver metastases. Furthermore, the study emphasises the added value of the most recent imaging tools as well as the usual and atypical appearance of liver metastases, which increases their effect[1]. This letter aims to contribute to this review by presenting rare and atypical examples of liver metastases.

According to the authors of this study, hypovascular lesions are the most common typical appearances of liver metastases, but some tumors will present with atypical appearances, such as hypervascular metastases. As mentioned in the literature, hypervascular liver metastases typically arise from hypervascular primary cancers such as neuroendocrine cancers, kidney cancer, melanoma, and thyroid cancer[1,2]. In 73% of patients, hepatic metastases of neuroendocrine tumors displayed a characteristic hypervascular appearance. However, it should be kept in mind that hypervascular neuroendocrine tumors may cause hypovascular liver metastasis[3]. As seen in Figure 1, neuroendocrine tumors should also be included in the differential diagnosis of hypovascular liver metastases.

The article by Ozaki *et al*[2] demonstrated the prevalence of synchronous liver metastasis in primary tumors originating from various organs. They determined that the pancreas had the highest prevalence (77.6%), whereas the prostate had the lowest prevalence (4.8%). We intended to make this unusual entity memorable with our case in Figure 2, which shows the metastasis of prostate adenocarcinoma to the liver.

When describing the visible liver lesions as metastases, a differential diagnosis between primary benign or malignant liver masses and infectious diseases should be made initially. While multiple liver abscesses are the most common among these infectious lesions, alveolar echinococcosis (AE), a rare parasitic disease that we present in Figure 3, can also be misdiagnosed as metastases. The WHO classification system for PNM, based on imaging findings, also emphasises AE as a potential alternative differential diagnosis for malignant liver masses. To distinguish AE from other tumors, serology findings and multiple imaging modalities (ultrasonography and computed tomography), and most importantly, keeping the diagnosis in mind, are required[4-6].

Our objective in this letter was to make a contribution to the literature with images of exceptional and rarely occurring cases in daily practice. All authors are in complete agreement with the information stated. The content of this manuscript is our original work and has not been published, in whole or in part, before or simultaneously with this submission.

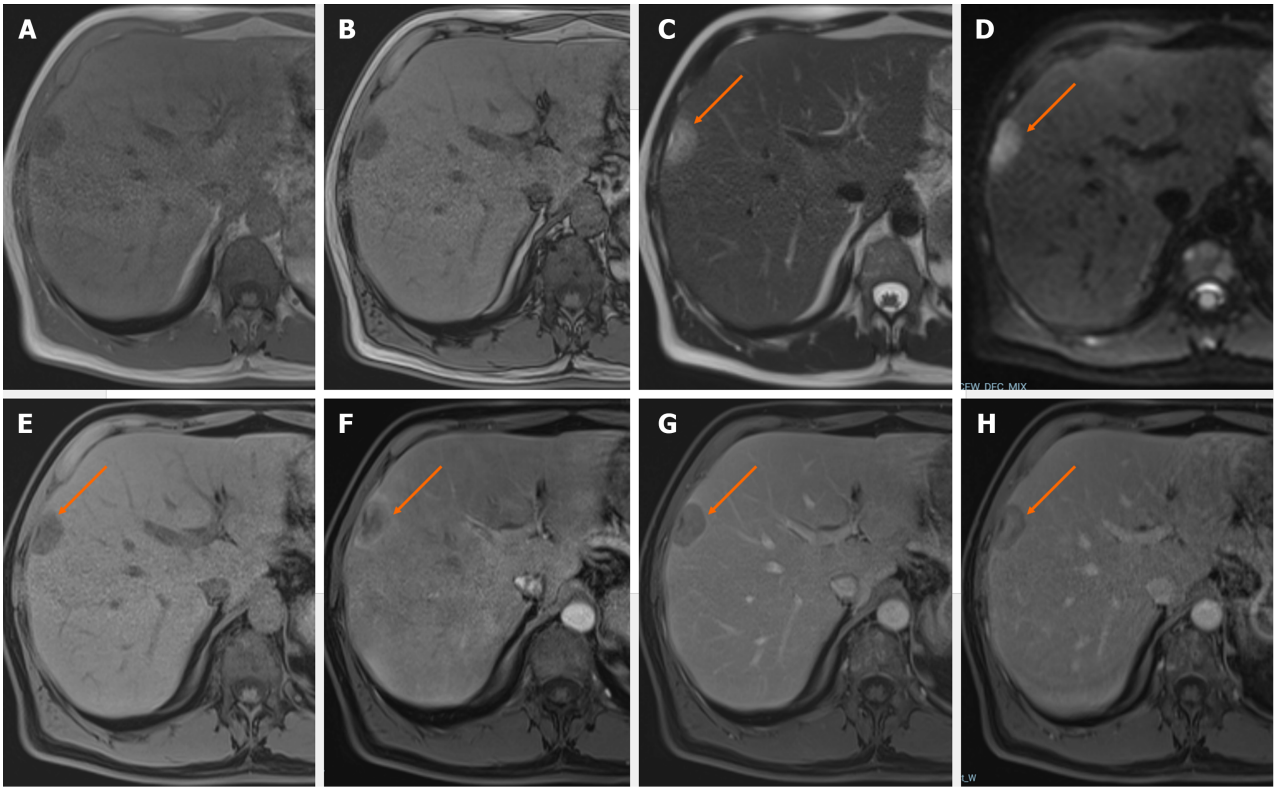

Figure 2 Liver metastases from prostate adenocarcinoma. In Gd-EOB-magnetic resonance imaging of a 52-year-old male patient. A: On in-phase images, there is a focal hypointense liver lesion; B: On out-of-phase images, the lesion persists hypointense compared to the healthy liver parenchyma; C: On T2-weighted images, the lesion appears slightly hyperintense (orange arrow); D: Diffusion restriction in the lesion on diffusion-weighted imaging (orange arrow); E: Before contrast administration, the lesion is hypointense (orange arrow); F: The lesion appears hypervascular due to peripheral rim-like hyperenhancement during the post-contrast late hepatic arterial phase (orange arrow); G: The lesion is hypointense on the portal-venous phase compared to the healthy liver parenchyma (orange arrow); H: On the hepatobiliary phase, low signal intensity of the lesion due to washout is observed, especially in the peripheral areas (orange arrow).

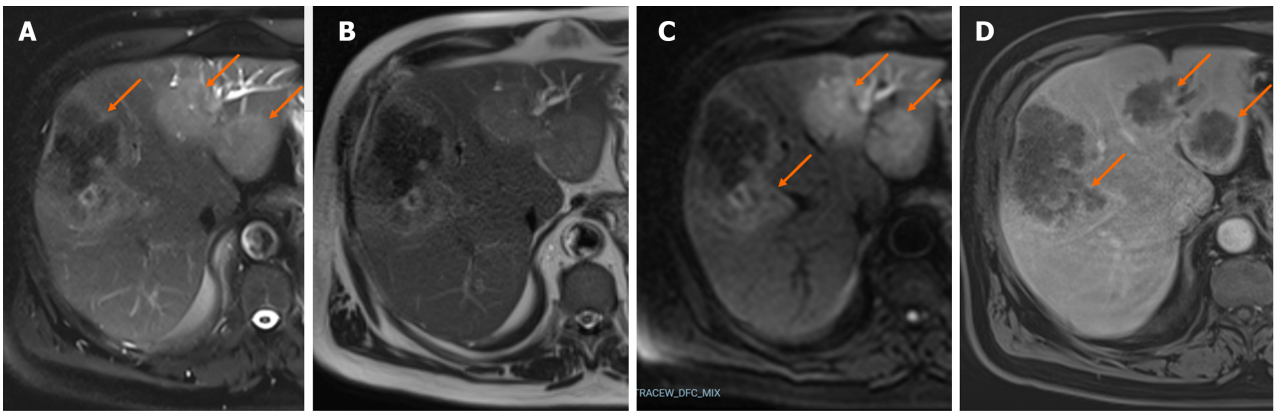

Figure 3 A case of Echinococcus alveolaris in the liver. In Gd-EOB-magnetic resonance imaging of a 69-year-old male patient. A and B: On with and without fat-suppressed T2-weighted images the lesions appear slightly hyperintense and hypointense areas in the central part of the largest lesion (orange arrows); C: Diffusion-weighted imaging reveals restriction of diffusion of the lesions (orange arrows); D: The lesions show peripheral rim-like enhancement on the hepatobiliary phase (orange arrows).

FOOTNOTES

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LETTER TO THE EDITOR

Endoscopic ultrasonography-related diagnostic accuracy and clinical significance on small rectal neuroendocrine neoplasms

Jun Weng, Yu-Fan Chen, Shu-Han Li, Yan-Hua Lv, Ruo-Bing Chen, Guo-Liang Xu, Shi-Yong Lin, Kun-Hao Bai

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Abstract

This research aimed to examine the diagnostic accuracy and clinical significance of endoscopic ultrasonography (EUS) in the context of small rectal neuroendocrine neoplasms (NENs). A total of 108 patients with rectal subepithelial lesions (SELs) with a diameter of < 20 mm were included in the analysis. The diagnosis and depth assessment of EUS was compared to the histology findings. The prevalence of NENs in rectal SELs was 78.7% (85/108). The sensitivity of EUS in detecting rectal NENs was 98.9% (84/85), while the specificity was 52.2% (12/23). Overall, the diagnostic accuracy of EUS in identifying rectal NENs was 88.9% (96/108). The overall accuracy rate for EUS in assessing the depth of invasion in rectal NENs was 92.9% (78/84). Therefore, EUS demonstrates reasonable diagnostic accuracy in detecting small rectal NENs, with good sensitivity but inferior specificity. EUS may also assist physicians in assessing the depth of invasion in small rectal NENs before endoscopic excision.

Key Words: Rectal neuroendocrine neoplasms; Endoscopic ultrasonography; Diagnosis; Depth of invasion

Core Tip: The diagnostic efficacy and clinical significance of endoscopic ultrasonography (EUS) on rectal neuroendocrine neoplasms (NENs) have not been well demonstrated owing to the infrequency of these particular disorders. In this manuscript, we compared the results of EUS with histology findings among a total of 108 patients with rectal subepithelial lesions with a diameter of < 20 mm. We found that EUS demonstrates reasonable diagnostic accuracy in detecting small rectal NENs, with good sensitivity but inferior specificity. EUS may also assist physicians in assessing the depth of invasion in small rectal NENs before endoscopic excision.

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TO THE EDITOR

Rectal neuroendocrine neoplasms (NENs) are diminutive but potentially malignant neoplasms. The management of NENs is contingent upon the dimensions of the tumor and the depth of its invasion. According to current consensus recommendations pertaining to the therapy of rectal NENs[1], it is recommended that endoscopic excision be considered as a viable approach for the treatment of small tumors (< 2 cm) that are limited to the mucosa or submucosa, since these tumors have a low propensity for metastatic dissemination. While the predominant kind of rectal subepithelial lesions (SELs) consists of NENs, there exists a diverse range of other types, including small gastrointestinal tumors (GISTs), lymphangiomas, and neurilemmomas. Additionally, non-tumor diseases such as endometriosis, duplication cysts, and inflammatory lesions may also be seen in this context. Differentiating NENs from SELs is of paramount significance for the proper implementation of treatment interventions. Preoperative endoscopic ultrasonography (EUS) has been demonstrated in identifying and assessing invasion depth in rectal NENs, a critical factor in choosing the most suitable treatment approach. Nevertheless, the diagnostic efficacy and clinical significance of EUS have not been well demonstrated owing to the infrequency of these particular disorders[2-8]. In this study, we investigated the diagnostic precision and clinical significance of EUS in relation to rectal NENs with a diameter of less than 20 mm.

A retrospective assessment was conducted on 108 cases of rectal SELs with a diameter of less than 20 mm, which were treated at Sun Yat-Sen University Cancer Center from January 2010 to June 2021, after getting approval from the Ethics Committee of the Institutional Review Board. All of the lesions were removed using an endoscopic procedure and then subjected to histological examination. The criteria for inclusion were: (1) The lesion was in the rectum, at a distance of less than 15 cm from the anus; (2) the lesion had a diameter of less than 20 mm; and (3) prior to the endoscopic excision, EUS and radiography did not reveal any evidence of local lymph node involvement or distant metastases. The exclusion criteria were: (1) Epithelial lesions, such as malignancy and adenoma, were eliminated from consideration in this study; and (2) no histological diagnosis. Prior to performing endoscopic excision, all patients underwent assessment *via* EUS to establish an EUS diagnosis and measure the depth of invasion. A blinded expert in EUS, who was blinded of the histology data, conducted a review of the ultrasonic pictures. The EUS expert then made a single diagnosis and evaluated the depth of invasion, which was then documented. The diagnosis and depth assessment of EUS was compared to the histology findings. Then diagnostic tests were used to determine the diagnostic accuracy of EUS. Prior to undergoing EUS and endoscopic excision procedures, all patients were duly informed about the possible risks and advantages associated with the interventions. Furthermore, they were required to submit written informed consent as a prerequisite for their participation. The techniques conducted in this study adhere to the guidelines outlined in the 1964 Helsinki Declaration and subsequent ethics that are pertinent to the research.

Table 1 presents a comprehensive overview of the clinical data analyses conducted on a cohort of 84 patients with rectal NENs. The mean ages of the participants in the study were 44.6 ± 13.4 years, while the average size of the lesions was 7.9 ± 3.2 mm (range, 3-20 mm). A total of 11 patients had positive vertical margins, leading to a R0 resection rate of 86.9%, with only one case in which total resection (R0) was achieved with vascular infiltration. Based on the mitoses and Ki-67 proliferation index categorization, it was determined that 89.3% (75/84) were categorized as G1, whereas 10.7% (9/84) were categorized as G2. In 7.1% (6/84) of cases, the lesions were limited to the mucosal layer, whereas in 92.9% (78/84), the lesions extended into the submucosal layer.

Table 2 displays the diagnostic outcomes of rectal SELs in 108 patients, as determined by EUS and histology. The prevalence of NENs in rectal SELs was 78.7% (85/108). The sensitivity of EUS in detecting rectal NENs was 98.9% (84/85), while the specificity was 52.2% (12/23). The positive predictive value was 88.4% (84/95), and the negative predictive value was 92.3% (12/13). The positive and negative likelihood ratios were 2.07 and 0.02, the overall diagnostic accuracy was 88.9%, and the Youden index was 0.51.

Table 3 presents a comparison of invasion depth for rectal NENs as evaluated by EUS and histology. Out of the total of 10 rectal NENs cases infiltrating the second layer (mucosa), as determined by EUS, five cases were limited to the mucosa, while the other 5 cases demonstrated invasion into the submucosa. Out of the total of 74 rectal NENs cases infiltrating the third layer (submucosa), as determined by EUS, only one case was limited to the mucosa, while the other 73 cases demonstrated invasion into the submucosa. The overall accuracy of EUS in assessing the invasion depth of rectal NENs was 92.9% (78/84).

Table 1 The characteristics of 84 lesions with rectal neuroendocrine neoplasms

	Total
Number	84
Age (yr)	44.6 ± 13.4
Gender (male, %)	60.0 (45/79)
Lesion size (mm)	7.9 ± 3.2
R0 resection (%)	86.9 (73/84)
Vascular invasion (%)	1.2 (1/84)
Histologic grade (%)	
G1	89.3 (75/84)
G2	10.7 (9/84)
Depth of invasion (%)	
Mucosa	7.1 (6/84)
Submucosa	92.9 (78/84)

Table 2 Findings of histology and endoscopic ultrasonography-based diagnosis of 108 rectal subepithelial lesions

EUS diagnosis	Histology diagnosis		Total
	NENs	Non-NENs	
NENs	84	11	95
Non-NENs	1	12	13
Total	85	23	108

EUS: Endoscopic ultrasonography; NENs: Neuroendocrine Neoplasms.

Table 3 Comparison of invasion depth for rectal neuroendocrine neoplasms *via* endoscopic ultrasonography and histology

EUS assessment	Histology diagnosis		Total
	Mucosa	Submucosa	
The 2th layer	5	5	10
The 3th layer	1	73	74
Total	6	78	84

EUS: Endoscopic ultrasonography; NENs: Neuroendocrine Neoplasms.

The current investigation revealed that EUS demonstrates a notable level of sensitivity, but accompanied by a comparatively lower level of specificity, when used for diagnosing rectal NENs. EUS accurately detected 98.9% (84/85) of rectal NENs, with just one case being misinterpreted as GIST. Out of the 23 additional rectal SELs, twelve cases (52.2%) were accurately identified and categorized as non-NENs. Moreover, eleven cases of non-NENs were inaccurately identified as NENs, including ten cases of inflammatory lesions and one case of neurilemmoma. Hence, the presence of inflammatory nodules localized into the second or third layer may potentially lead to misdiagnosis as NENs due to their comparable acoustic symptoms. Additionally, our study revealed that EUS has reasonable accuracy in assessing the depth of invasion in rectal NENs. Nevertheless, in cases where the rectal NENs were assessed by EUS and found to be limited to the mucosa, there was a significant likelihood of inaccurate determination of the depth of invasion, indicating a shallower depth. According to the findings of our research, a significant proportion (92.9%) of rectal NENs demonstrated invasion into the submucosal layer. Therefore, in cases where rectal NENs are determined to be limited to the mucosa using EUS assessment, it is still necessary to do submucosal dissection to obtain a complete resection.

This study has limitations that should be taken into consideration. Since magnetic resonance imaging (MRI) and EUS are widely used in the diagnosis and evaluation of rectal NENs, it is meaningful to compare the results between MRI and EUS. However, it is with regret that rectal NENs included in our study were all less than 2 cm and the vast majority of

them were not examined by MRI. Besides, artificial intelligence-assisted endoscopic diagnosis has been research hotspot. Therefore, it is suggested that future research can introduce artificial intelligence to further improve the diagnostic value of EUS on rectal NENs.

In summary, EUS demonstrates acceptable diagnostic precision in identifying rectal NENs, exhibiting a commendable level of sensitivity. However, it displays a less desirable level of specificity, which poses difficulties in distinguishing NENs from other SELs, particularly inflammatory nodules. EUS may provide valuable assistance in assessing the depth of invasion for rectal NETs prior to endoscopic excision.

FOOTNOTES

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Co-corresponding authors: Kun-Hao Bai and Shi-Yong Lin.

Author contributions: Bai KH, Lin SY, Xu GL designed the research; Li SH, Lv YH and Chen RB collected the data; Weng J, Chen YF, Li SH did the analysis; Weng J, Chen YF, Bai KH and Lin SY prepared the manuscript draft; Bai KH and Li SH provided research support and revised the manuscript. All authors read and approved the final manuscript. Weng J and Chen YF contributed equally to this work as co-first authors. Bai KH and Lin SY contributed equally to this work as co-corresponding authors. The reasons are as follows. First, the research was performed as a collaborative effort, and the designation of co-first authors and co-corresponding authors authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. Second, Weng J and Chen YF contributed efforts of equal substance for the data analysis and preparing manuscript draft. Bai KH and Lin SY contributed efforts of equal substance for designing the research, preparing manuscript draft, providing research support and revising the manuscript. In summary, we believe that both designating Weng J and Chen YF as co-first authors and designating Bai KH and Lin SY as co-corresponding authors are fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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