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OPINION REVIEW

Non-alcoholic fatty liver disease in irritable bowel syndrome: More than a coincidence?

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Abstract

Irritable bowel syndrome (IBS) and non-alcoholic fatty liver disease (NAFLD) are amongst the most common gastrointestinal and liver conditions encountered in primary and secondary care. Recently, there has been interest in the apparent co-incidence of NAFLD in patients with IBS mainly driven by improved understanding of their shared risk factors and pathophysiology. In this paper we summarize the shared risk factors which include; overlapping nutritional and dietary factors as well as shared putative mechanisms of pathophysiology. These include changes in the gut microbiome, gut permeability, immunity, small bowel bacterial overgrowth and bile acid metabolism. This paper describes how these shared risk factors and etiological factors may have practical clinical implications for these highly prevalent conditions. It also highlights some of the limitations of current epidemiological data relating to estimates of the overlapping prevalence of the two conditions which have resulted in inconsistent results and, therefore the need for further research. Early recognition and management of the overlap could potentially have impacts on treatment outcomes, compliance and morbidity of both conditions. Patients with known IBS who have abnormal liver function tests or significant risk factors for NAFLD should be investigated appropriately for this possibility. Similarly, IBS should be considered in patients with NAFLD

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and symptoms of abdominal pain associated with defecation, an altered bowel habit and bloating.

Key Words: Irritable bowel syndrome; Non-alcoholic fatty liver disease; Metabolic syndrome; Obesity; Prevalence; Pathophysiology

Core Tip: Irritable bowel syndrome (IBS) and non-alcoholic fatty liver disease (NAFLD) are amongst the most common gastrointestinal and liver conditions encountered in primary and secondary care. There has been interest in the apparent co-incidence of NAFLD in patients with IBS mainly driven by improved understanding of their shared risk factors and pathophysiology. In this paper we summarize the shared risk factors which include; overlapping nutritional and dietary factors as well as shared putative mechanisms of pathophysiology. Physicians should be aware of the possibility of co-existence of IBS and NAFLD and consider investigating patients with IBS or NAFLD with clinical features of the other condition.

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INTRODUCTION

Irritable Bowel syndrome (IBS) is a disorder of gut-brain interaction (DGBI) resulting in recurrent abdominal pain associated with defecation and an altered bowel habit. Patients are considered to have IBS when they fulfill the Rome IV diagnostic criteria which include an altered bowel habit (constipation, diarrhea or a mix of both), associated with frequent abdominal pain and abdominal bloating or distension for at least 6 mo prior to diagnosis[1]. A recent systematic review and meta-analysis has shown a worldwide prevalence of IBS of 9.2% with significant regional variability[2]. In the United Kingdom, DGBIs such as IBS are very common, and account for around a third of gastroenterology outpatient referrals[3]. IBS can be debilitating often resulting in an increasing risk of anxiety or depression[4] with symptoms such as fecal incontinence that can be difficult to manage leading to poor quality of life and distress [5]. There is often significant clinician prejudice and frustration towards patients with IBS[6] resulting in unfair public perceptions and significant stigmatization[7].

Non-alcoholic fatty liver disease (NAFLD) is characterized by the accumulation of more than 5% of fat in the liver in the absence of a secondary cause. It is one of the major causes of liver disease worldwide and its pathogenesis is linked to metabolic syndrome, obesity and Type 2 diabetes. The population based prevalence of NAFLD is between 25%-44% but rises to 70% in patients with Type 2 diabetes[8,9]. NAFLD is recognized as a heterogeneous condition with variable rates of progression. In certain patients isolated steatosis leads to steatohepatitis and fibrosis, progressing ultimately to cirrhosis, decompensated liver disease and sometimes hepatocellular carcinoma. Population based screening studies have shown a prevalence of advanced fibrosis in 8% of patients rising to 27% in those with risk factors[10,11]. Unfortunately, the majority of patients are only diagnosed with liver disease when they present with advanced disease and many are of working age. Consequently, liver disease is responsible for the loss of 38000 and 22000 working life years, in men and women, respectively. NAFLD has been increasing in incidence in the western world with a predictable commensurate increase in liver transplant in both the United States and Europe[12-14].

There is increasing recognition that both IBS and NAFLD share a number of over-lapping risk and aetiological factors leading to growing interest in the possibility of an association between the two conditions. However, there is limited high quality data on the concomitance of IBS and NAFLD. As a result, IBS symptoms may not be routinely screened for in hepatology clinics and vice versa. Therefore, the aims of this article are

to summarize the current understanding of relevant overlapping patho-physiological and aetiological factors, and to highlight areas for future research and their clinical implications.

THE PREVALENCE OF CO-EXISTING IBS AND NAFLD

Table 1 summarizes the literature on the co-existing prevalence of IBS and NAFLD to date. Most studies have examined the incidence of NAFLD in previously diagnosed IBS. Unfortunately, a review of the literature of concomitant IBS and NAFLD revealed a very high variability in estimates of the prevalence from 12.9% to 74%, with significant differences in methodology in the diagnostic approaches for both conditions and the populations studied[15-17]. Amongst the reasons for this heterogeneity and variability include the change in the Rome criteria for IBS from Rome III, to the current Rome IV iteration, which is known to be more restrictive[18]. From a hepatology perspective, it is notable that all the studies to date have used raised liver transaminases, with a negative viral hepatitis screen, in the absence of excessive alcohol consumption, and abdominal ultrasound to diagnose NAFLD, which in the absence of objective liver fibrosis assessment could be considered sub-optimal.

Shin *et al*[16] found that the prevalence of presumed NAFLD was 12.9% in patients with diarrhoea predominant IBS (IBS-D) compared to 9.0% in patients with constipation predominant IBS (IBS-C), although the reasons for this apparent difference are unclear and merit further investigation. In an interesting study by Lee *et al*[19], rather than evaluating patients with a formal diagnosis of NAFLD, the authors assessed the incidence of elevated liver transaminases and the metabolic syndrome in patients with IBS, compared to an age and sex matched control group. Those with IBS were found to have a significantly higher alanine aminotransferase (ALT) (16.9% *vs* 7.7%; $P = 0.015$) and Gamma-glutamyl transferase (GGT) (24.1% *vs* 11.5%; $P = 0.037$) compared to the control group, and there was a significantly higher prevalence of metabolic syndrome in the IBS group (32.5% *vs* 12.7%; $P < 0.001$).

To our knowledge, there have only been three previous reports on the incidence of functional bowel symptoms in patients with NAFLD. Appleby *et al*[20] found that in 127 patients with NAFLD, 25% had chronic diarrhea, and 12% had features of bile acid diarrhoea with both being associated with a raised NAFLD fibrosis score. Furthermore, Singh *et al*[21] studied 632 patients in India diagnosed with fatty liver disease and found that 29.4% had co-existing clinical features of IBS. Similar findings were reported by Jones-Pauley *et al*[22] in a cross sectional study looking at IBS diagnosed by Rome IV criteria in 130 NAFLD patients and as many as 38 (29.2%) patients had IBS based on Rome IV criteria. Interestingly, depression and anxiety were found to be more prevalent in the IBS cohort, compared to the non-IBS cohort, indicating the detrimental effect of co-existing bowel symptoms may have on quality of life, and the resulting need for a multi-systems approach in NAFLD patients with IBS symptoms.

In summary, regardless of the iteration of the Rome IBS diagnostic criteria used and the highlighted limitations of the previous studies, the data summarized in Table 1 on the co-existing prevalence of IBS in patients with NAFLD consistently report a much higher prevalence of IBS than that reported in global prevalence studies using either Rome III or Rome IV diagnostic criteria[2].

OVERLAPPING ETIOLOGICAL FACTORS IBS AND NAFLD

Multiple etiological factors overlap between IBS and NAFLD leading to interest in possible associations including obesity, gut microbiome, dietary factors and immune mediated causes as illustrated in Figure 1.

OBESITY

NAFLD is intrinsically linked with obesity, diabetes and the metabolic syndrome. In obese populations, NAFLD has a prevalence of up to 95%[23]. Excess adipose tissue exhausting peripheral storage capacity resulting in deposition in the liver and increased insulin resistance is thought to be the main culprit for NAFLD pathogenesis [24]. Weight loss through diet and exercise reduces hepatic steatosis and fibrosis, and in 109 obese patients[25]. Lassailly *et al*[26] showed that bariatric surgery resolved non-

Table 1 Summarizes the literature on the co-existing prevalence of irritable bowel syndrome and non-alcoholic fatty liver disease to date

Author	Population studied	Study design	No. patients	Criteria for IBS diagnosis	IBS subtypes	Criteria for NAFLD diagnosis	Prevalence of NAFLD in IBS/ IBS in NAFLD	Outcomes
Hasanain *et al*[15]	IBS	Cross sectional study	100 patients with IBS	Rome III	IBS-C: 45%; IBS-D: 23%; IBS-M: 32%,	Ultrasound; No history of alcohol exposure; No exposure to steatogenic medications; Negative viral screen	74% of those with IBS had co-existing NAFLD	Moderate/severe NAFLD significantly associated with moderate/severe IBS (OR: 2.4, 95%CI: 1.3-62.7, P = 0.026)
Shin *et al* [16]	Healthy individuals *via* NHANES	Cross sectional study	2345 patients with IBS	Rome IV	IBS-C: 1023; IBS-D: 1322	Raised ALT or AST; Absence of excessive alcohol; Negative viral hepatitis screen	Prevalence of NAFLD in IBS-D: 12.9% (95%CI: 9.8-15.9); IBS-C: 9.0% (95%CI: 7.0-11.0)	NAFLD associated with diarrhoea *vs* normal bowel pattern (OR: 1.340, 95%CI: 1.007-1.784) and constipation (OR: 1.445, 95%CI: 1.028-2.031)
Arasteh *et al*[17]	IBS	Cohort study	1067 patients with IBS	Rome IV	IBS-D: 57 (5.3%); IBS-C: 380 (35.6%); IBS-U: 630 (59%)	Not documented	3.7%	Liver disease not associated with IBS (Coefficient: 0.26, OR: 1.30, 95%CI: 0.92-1.82)
Lee *et al*[19]	IBS *vs* control	Retrospective, cross sectional, case control study	83 IBS patients; 260 age and sex matched control	Rome III	IBS-C: 14.8%; IBS-D: 49.4%; IBS-M: 31.3%; IBS-U: 4.5%	Investigated raised ALT, GGT, AST and features of metabolic syndrome	16.9% of IBS patients had raised ALT; 24.1% had raised GGT	Significantly higher ALT in patients with IBS (16.9% *vs* 7.7%; P = 0.015); Significantly higher GGT in patients with IBS (24.1% *vs* 11.5%; P = 0.037); Significantly higher prevalence of metabolic syndrome in patients with IBS (32.5% *vs* 12.7%; P < 0.001)
Sarmini *et al*[73]	IBS *vs* control	Observational study	637942	Clinical diagnosis	Not documented	Not documented	Not available	Patients with IBS significantly more likely to develop NAFLD compared to non-IBS group (OR: 3.204, 95%CI: 3.130-3.279, P < 0.001)
Singh *et al* [24]	NAFLD	Retrospective analysis	632	Clinical diagnosis	Not documented	Ultrasound; Alcohol consumption < 20 g/d; Normal aetiological liver screen	186 (29.4%) patients with NAFLD had clinical diagnosis of IBS	IBS symptoms are highly prevalent in those with NAFLD
Jones-Pauley *et al* [22]	NAFLD	Cross-sectional study	130	Rome IV	Not documented	Not documented	38 (29.2%) patients with NAFLD met Rome IV IBS criteria	High prevalence of IBS in patients with NAFLD; Significant increase in prevalence of depression (18.4% *vs* 5.4%, P = 0.01) and anxiety (31.6% *vs* 9.8%, P = 0.002) in those with co-existing IBS compared to those with NAFLD without IBS

IBS: Irritable bowel syndrome; NAFLD: Non-alcoholic fatty liver disease; IBS-C: Constipation predominant IBS; IBS-D: Diarrhoea predominant IBS; IBS-M: Mixed IBS; IBS-U: Unsubtyped IBS; OR: Odds ratio; CI: Cumulative incidence; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase.

alcoholic steatohepatitis (NASH) within a year.

The association between IBS and obesity is more unclear[27]. Aro *et al*[28] found a significant association between the obesity and IBS symptoms such as abdominal pain and diarrhoea using the Abdominal Symptom Questionnaire as well as a positive association between obesity and a formal diagnosis of IBS. However, these have not been confirmed in several other studies[29-31]. Interestingly, Lee *et al*[30] found visceral abdominal adiposity was associated with increased risk of IBS-D. There is evidence that IBS is more prevalent in patients who are obese[32]. Schneck *et al*[33]

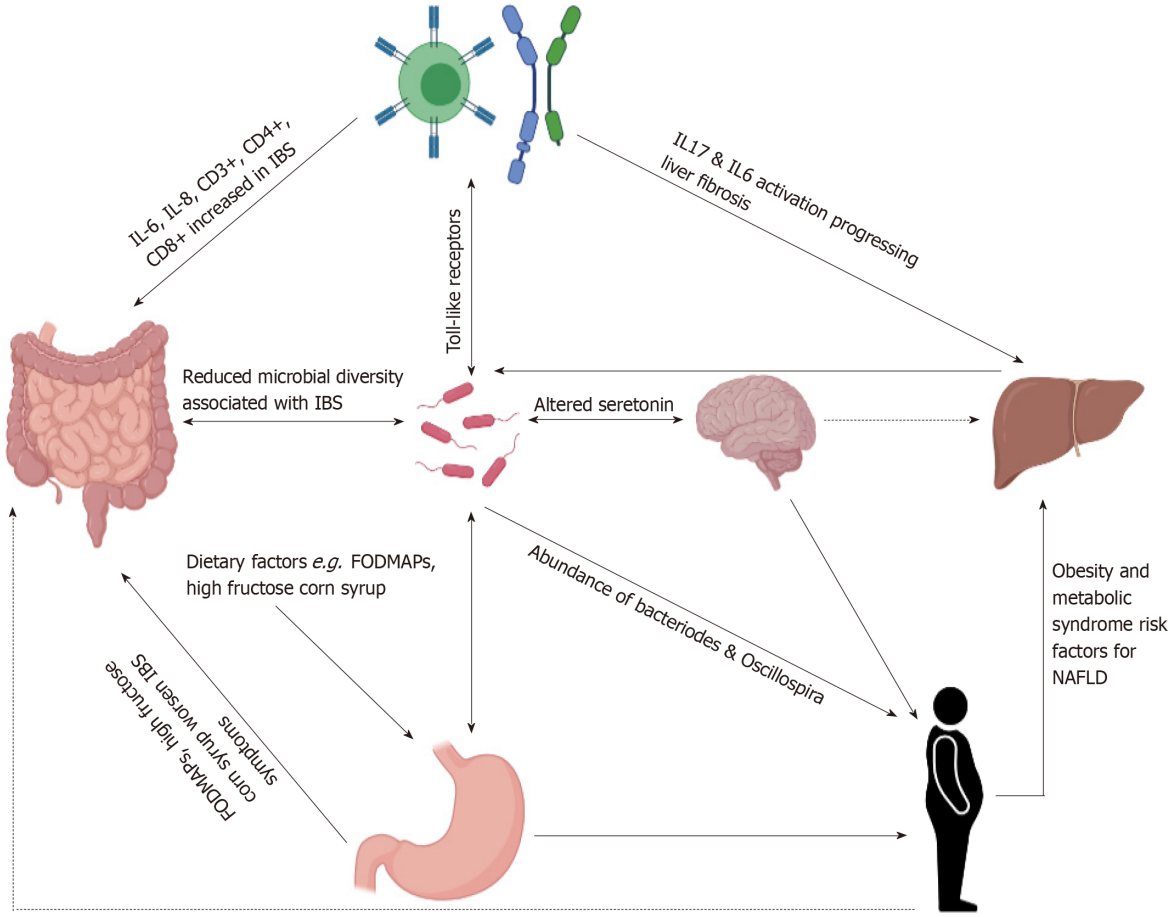

Figure 1 Schematic illustration summarizing associations and co-existing etiologies of irritable bowel syndrome and non-alcoholic fatty liver disease. IBS: Irritable bowel syndrome; NAFLD: Non-alcoholic fatty liver disease; IL: Interleukin.

described a cohort of patients with obesity undergoing bariatric surgery of which 30% fulfilled Rome III criteria for IBS. Further evidence for role of obesity in IBS is supported by the observation that increased visceral adiposity enhances perception of luminal stimuli, dysmotility and abdominal pain[34]. Higher body mass indexes have been associated with accelerated colonic and rectosigmoid transit and increased stool frequency[35]. Furthermore, weight loss through diet or bariatric surgery has been shown to improve symptoms[32,36]. Aasbrenn *et al*[37] prospectively analyzed the effect of a weight loss program on bowel symptoms using the IBS severity scoring system (IBS-SSS) and Gastrointestinal Symptom Rating Scale and found that there were significant improvements in the IBS-SSS in patients with IBS compared to those without.

MICROBIOME

The gut microbiota plays a vital role in the intestinal barrier function, metabolism of nutrients and development of immune tolerance and response. Dysregulation of the microbiome has been shown to be a component for the development of both NAFLD and IBS[38].

Long-term perturbation of the gut microbiota has been shown to contribute to metabolic syndrome and fatty liver disease[39]. Several mechanisms have been proposed on how the gut microbiota results in NAFLD development. This includes increased intestinal permeability leading greater lipopolysaccharide exposure to the host. This, in turn, results in toll like receptor (predominantly TLR4) activation of the innate immune system, causing liver inflammation as they are transported from the gut to the liver. Additionally, microbially produced metabolites, such as lactate and ethanol, can directly activate inflammatory cascades within the liver. Enterohepatic bile acid homeostasis is important for multiple processes, including fat absorption,

inflammation, immunity and microbial diversity. Significant differences have been noted in bile acid composition in metabolic diseases associating with progression of NAFLD[38,40].

Patients with hepatic steatosis and NASH have been shown to have increased *Proteobacteria, Enterobacteriaceae, Escherichia* and *Citrobacter* with reductions in abundance of *Rikenellaceae, Ruminococcaceae, Anaerosporobacter* and *Coprococcus*[39,40] Reductions in *Bifidobacteria* have also been observed and *Bifidobacteria* possibly reduce gut wall permeability to lipopolysaccherides, suggesting a relationship with the development of disease[39]. Interestingly, Frost *et al*[39] followed up patients who had incidental findings of fatty liver or diabetes and found changes in *Clostridium XIVa* as a result of dysbiosis with a strong association for increasing fatty acid biosynthesis. Type 2 diabetes is also noted to result in increased gut permeability. Aron-Wisnewsky *et al* [40] found significant overlap in microbial signatures between patients with NAFLD and NASH with obesity and diabetes, finding changes in abundance of *Oscillospira* and *Bacteriodes.* Further evidence on the importance of the gut microbiome in metabolic syndrome, is shown by fecal microbiota transplant being associated with a temporary improvement in peripheral insulin resistance[41].

Changes in intestinal microbial diversity is also thought to contribute to the development of IBS as the microbiota impacts on intestinal motility and sensitivity. Some patients with IBS have been shown to have changes in the *Fermicutes*-to-*Bacteriodes* ratio, reduced *lactobacilli* and *bifidobacterial* as well as reduced microbial diversity[38,42].

The gut-brain-microbiome axis is known to have an important role in glucose regulation. Gut microbiota modulation produces changes in the immune, neurotransmitter and monoaminergic activity of this axis. Serotonin secretion affects motility, pain perception but also plays a role in mood control[43]. NAFLD and the gut-brain axis may also be inter-related. There is evidence that depression is associated with NAFLD. However, disentangling the multiple contributors to depression in multifactorial disease states (as often seen in patients with metabolic syndrome) can be exceptionally difficult[44,45].

Dysregulation of the microbiome itself can lead to poor glycaemic control, acting through nitric oxide formation which affects the neuronal response to gut hormone Glucagon-like peptide-1 (GLP-1)[46]. The GLP-1 receptor antagonist, Semaglutide, has been shown to reduce liver fat and NASH resolution in patients with NAFLD[47]. It has also been used to treat weight loss and type-2 diabetes mellitus[48]. Given the known functions of GLP-1 on the gut microbiota, the effect seen in these studies may well be related to beneficial alterations in microbiome composition[49].

DIETARY FACTORS

Dietary factors have been shown to be integral to the man-agement of both IBS and NAFLD. Weight loss through diet and exercise is the mainstay of NAFLD management. Adherence to a Mediterranean diet reduces hepatic steatosis and achieves a greater weight loss in patients with NAFLD[50]. By contrast, patients with IBS have been shown to have a poorer adherence to a Mediterranean diet than healthy controls[50], a dietary factor which may therefore be relevant in the development of NAFLD in those with IBS. There is also some evidence that conservative weight loss can help IBS symptoms. Aasbrenn *et al*[37] found that a weight loss program resulted in a significant improvement in IBS symptoms as assessed by IBS-SSS questionnaires and Gastrointestinal Symptom Rating Scale[37].

Certain food groups appear to worsen IBS symptoms and contribute to NAFLD development. High fructose corn syrup (HFCS) is a disaccharide which is frequently used in artificial sweeteners, processed, canned and baked goods worldwide. HFCS has been shown to induce IBS symptoms through increased osmotic pressure and bacterial fermentation resulting in gas production, abdominal bloating and pain[51]. HFCS has also been shown to downregulate the insulin signaling pathway which would contribute to the pathogenesis of NAFLD[52]. Fructose consumption has also been shown to increase intestinal permeability potentially leading to the development of both NAFLD and IBS through the processes already outlined[53].

Certainly more research into the dietary implications on NAFLD and IBS is needed. Many patients with IBS notice that 'healthy' foods such as fruit and vegetables can make their symptoms worse and this results in some of them adopting a more 'unhealthy' diet which may lead to weight gain. There is evidence that a low FODMAP diet with excludes some fruits and vegeatables improves IBS symptoms

however to the authors' knowledge, there is a paucity of data on the effects of a low FODMAP diet on the progression of NAFLD.

IMMUNE MEDIATED FACTORS

Chronic inflammation is a critical driver of progressive disease in NAFLD and significant advances have been made to understand the role of inflammation[54,55]. The role of toll-like receptors (TLRs) and macrophage activation has already been discussed. Additionally, Natural killer cells and natural killer T cells contribute to inflammation by releasing cytokines and reactive oxygen species[56]. Tumor necrosis factor (TNF)-α, alongside other cytokines and growth factors, have also been shown to possible have a role in the development of NAFLD and NASH, in both animals and humans[38]. TNF-α in combination with interleukin (IL)-6 stimulates the production of leptin activating neutrophils and the innate immune system[38]. In addition, adaptive immune responses drive NASH as hepatic infiltration of B cells and CD4 and CD8 T cells exacerbate parenchymal injury and inflammation[56]. B cells play a profibrogenic role involving the stimulation of hepatic stellate cells and liver macrophages[57]. CD4+ T cells differentiate to type-17 T helper cells, producing IL-17 which has been implicated in the progression of NAFLD[58]. The balance of the adaptive immune cellular compartment within the liver can transition from a pro-resolution composition to pro-inflammatory subset, driving disease and fibrosis.

In IBS, a similar chronic low-grade inflammatory picture has also been described. The innate immune system is implicated with an increased number of mast cells throughout the intestines in some patients[59]. The adaptive immune response is also important with CD3+, CD4+ and CD8+ T cells increased in intestines and blood of patients with IBS[38]. Interestingly, an increase in IL-6 and IL-8 with reduced anti-inflammatory cytokines has been seen in serum of IBS patients[59]. The role of TLRs is also felt to be important with IL-6 and other cytokines acting through this mechanism [38]. TNF-α can act on the nervous system to cause hypersensitivity, gastric hypomotility and nausea[59].

SMALL INTESTINAL BACTERIAL OVERGROWTH

Small intestinal bacterial overgrowth (SIBO) can cause abdominal pain, bloating and chronic diarrhea. Although an area of controversy due to conflicting evidence, a number of previous studies have suggested that some patients with IBS have a relatively high prevalence of SIBO[60,61]. A recent metanalysis has shown that patients with IBS were more likely to test positive for SIBO than healthy controls[61]. Further circumstantial evidence for the gut-brain-microbiome-liver axis can be drawn from the effects of the non-absorbable antibiotic Rifaximin in both IBS and in liver disease. Whilst the mechanism is unclear, improvement in IBS symptoms have been demonstrated in patients in randomized controlled trials of Rifaximin[62,63]. Rifaximin is also often used to treat SIBO[64], a condition which has been shown to affect cognitive function in a subset of patients who present with brain fog[65]. Interestingly, treatment with Rifaximin has recently been shown in brain imaging studies to alter neuronal connectivity and increase cognitive flexibility through its effect on the gut microbiome particularly in beta and theta frequencies with a particular focus on the insular cortex, a region known to be affected in patients with IBS [66]. Furthermore, Rifaximin has an immunomodulatory action counteracting the pro-inflammatory response seen in gut microbiota dysbiosis[67]. In liver disease, Rifaximin is an established treatment for hepatic encephalopathy, with its effects attributed to alterations in the gut microbiome and resultant positive effects on cognitive function. Specifically in patients with biopsy proven NASH, Rifaximin has also been shown to reduce insulin resistance, inflammation and NAFLD fat scores[68]. Therefore, the effects of Rifaximin are multifactorial including reduced endotoxemia, modulation of inflammatory cytokines, and intestinal permeability as well as changing functional brain connectivity[62,66].

Further overlapping evidence for SIBO in this context comes from the obesity literature. There is evidence that obesity reduces gut motility, which may predispose to SIBO due to stasis, and plausibly this is thought to damage barrier function, which can result in bacterial translocation and altered gut-liver axis[53]. Furthermore, changes in the gut-liver axis may well a result of increased intestinal permeability. A high prevalence of SIBO has been observed in obese subjects however the association

between NAFLD and SIBO is less clear[53]. Studies have found the prevalence of SIBO in NAFLD to range from 39%-60% albeit in small numbers of patients. However, more recently, some research found 8% of NAFLD patients in their cohort had SIBO and there was no evidence that SIBO was associated with a higher risk of fibrosis[69-71].

BILE ACID DIARRHOEA

Bile acid malabsorption is a cause of chronic diarrhea and has been shown to be associated with an increased NAFLD fibrosis score. Hepatic bile acid production is regulated by Fibroblast growth factor 19 (FGF19) and Farnesoid-X-receptor (FXR) and obeticholic acid (a FXR agonist) has shown therapeutic potential in both bile acid related diarrhea and NAFLD[20]. Appleby *et al*[20] found that increased hepatic bile acid production and diarrhea were associated with an increased NAFLD score. Of further relevance to the link with NAFLD, bile acid diarrhoea has also been shown to be associated with raised body mass index[72]. This is therefore an important point to be considered in clinical practice when evaluating patients with suspected overlapping IBS and NAFLD, as up to a third of patients meeting the criteria for IBS-D have been shown to have bile salt malabsorption when investigated[72], and this condition should therefore be excluded in the context of watery diarrhea.

APPLICABILITY TO CLINICAL PRACTICE

Pulling this together, there is consistent evidence to show that IBS and NAFLD have a similar pathogenesis and therefore applying this to clinical practice, physicians should be aware that NAFLD may co-exist silently in patients with IBS and vice versa. Patients with IBS and incidental findings of elevated liver enzymes or with risk factors for NAFLD should be considered for non-invasive liver screening through ultrasound and appropriately available non-invasive fibrosis assessment using FIB-4 scoring, enhanced liver fibrosis testing or mechanical liver stiffness measurement.

Conversely, patients with NAFLD may not admit to the debilitating symptoms of IBS due to stigma or feeling that their symptoms are not relevant to their liver consultation. Screening for positive clinical features of IBS and targeted treatment for both conditions in unison may aid compliance with treatment, improve quality of life and ultimately improve morbidity.

However, as highlighted in this review, there is a lack of large, high quality cross-sectional data on the incidence of IBS in NAFLD patients and vice versa. To date, studies have been limited to the use of ultrasound and blood tests to diagnose NAFLD, however there is a lack of data that quantifies a fibrosis score which may be useful to correlate with IBS severity. From the currently available data (summarized in Table 1), whilst there is a suggestion that the IBS-D sub-type may be more common than IBS-C in patients with NAFLD, whether this is a genuine finding merits further evaluation in studies which have excluded bile salt malabsorption with appropriate investigations given its apparent independent association with NAFLD.

CONCLUSION

IBS and NAFLD are common conditions that can have significant effects on both physical and mental health[73], as well as significant healthcare and socioeconomic implications. There is some evidence that patients with IBS are more likely to develop NAFLD, and there are multiple different pathophysiological mechanisms that could contribute to both conditions, however more data is needed. Until such data clarifies this picture, the possibility of these conditions existing concomitantly should be considered proactively and investigated appropriately.

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REVIEW

Liver-side of inflammatory bowel diseases: Hepatobiliary and drug-induced disorders

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Abstract

Hepatobiliary disorders are among the most common extraintestinal manifestations in inflammatory bowel diseases (IBD), both in Crohn's disease and ulcerative colitis (UC), and therefore represent a diagnostic challenge. Immune-mediated conditions include primary sclerosing cholangitis (PSC) as the main form, variant forms of PSC (namely small-duct PSC, PSC-autoimmune hepatitis overlap syndrome and IgG4-related sclerosing cholangitis) and granulomatous hepatitis. PSC is by far the most common, presenting in up to 8% of IBD patients, more frequently in UC. Several genetic foci have been identified, but environmental factors are preponderant on disease pathogenesis. The course of the two diseases is typically independent. PSC diagnosis is based mostly on typical radiological findings and exclusion of secondary cholangiopathies. Risk of cholangiocarcinoma is significantly increased in PSC, as well as the risk of colorectal cancer in patients with PSC and IBD-related colitis. No disease-modifying drugs are approved to date. Thus, PSC management is directed against symptoms and complications and includes medical therapies for pruritus, endoscopic treatment of biliary stenosis and liver transplant for end-stage liver disease. Other non-immune-mediated hepatobiliary disorders are gallstone disease, whose incidence is higher in IBD and reported in up to one third of IBD patients, non-alcoholic fatty liver disease, pyogenic liver abscess and portal vein thrombosis. Drug-induced liver injury (DILI) is an important issue in IBD, since most IBD therapies may cause liver toxicity; however, the incidence of serious adverse events is low. Thiopurines and methotrexate are the most associated with DILI, while the risk related to anti-tumor necrosis factor-α and anti-integrins is low. Data on hepatotoxicity of newer drugs approved for IBD, like anti-interleukin 12/23 and tofacitinib, are still scarce, but the evidence from other rheumatic diseases is

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reassuring. Hepatitis B reactivation during immunosuppressive therapy is a major concern in IBD, and adequate screening and vaccination is warranted. On the other hand, hepatitis C reactivation does not seem to be a real risk, and hepatitis C antiviral treatment does not influence IBD natural history. The approach to an IBD patient with abnormal liver function tests is complex due to the wide range of differential diagnosis, but it is of paramount importance to make a quick and accurate diagnosis, as it may influence the therapeutic management.

Key Words: Inflammatory bowel diseases; Hepatobiliary disorders; Primary sclerosing cholangitis; Drug-induced liver injury; Biological drugs; Viral hepatitis

Core Tip: Hepatobiliary disorders are commonly associated with inflammatory bowel diseases (IBD) and represent a management challenge. They include (1) Immune-mediated diseases that can coexist with IBD, mainly primary sclerosing cholangitis; (2) Other non-immune-mediated disorders like gallstone disease; (3) Liver injury induced by drugs used in IBD; and (4) Risks related to concomitant viral hepatitis B and C. All these conditions are summarized in this review, according to the latest literature evidence and the current clinical practice guidelines.

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INTRODUCTION

Hepatobiliary disorders are common extraintestinal manifestations of inflammatory bowel diseases (IBD) and may occur in both Crohn's disease (CD) and ulcerative colitis (UC). The range of IBD-associated hepatobiliary disorders is wide and can underlie different pathogenetic mechanisms. They include diseases with immune-mediated pathogenesis, which typically have a course independent of intestinal activity, the most common being primary sclerosing cholangitis (PSC); variant form of PSC, like small-duct PSC, must also be considered. Other non-immune-mediated conditions include gallstone disease, whose incidence is increased in IBD patients, non-alcoholic fatty liver disease (NAFLD), pyogenic liver abscess and portal vein thrombosis. Drug-induced liver diseases is another important chapter, since several drugs used in IBD, mainly thiopurines, methotrexate and anti-tumor necrosis factor-α (anti-TNF) may induce liver toxicity. Concomitant viral hepatitis B and C in IBD is also a relevant issue, particularly hepatitis B reactivation under immunosuppressive therapy; however, the recent introduction of potent antiviral drugs for both the infections and the spread of the anti-hepatitis B virus vaccine (HBV) contributed to significantly lower the risk. The diagnosis of such hepatobiliary conditions is of great importance, since they may influence the management and therapeutic approach to IBD, contraindicate the use of some therapies, or prevent the evolution towards the end stage of liver disease. The main hepatobiliary disorders, which are discussed in this review, are summarized in Table 1. A proposed practical approach to abnormal liver function tests (LFT) in a patient with IBD is presented in Figure 1.

IMMUNE-MEDIATED CONDITIONS

PSC

PSC is the most common hepatobiliary manifestation associated with IBD. It is a rare, idiopathic, chronic cholestatic syndrome characterized by chronic inflammation, fibrosis and finally destruction of intra- and/or extra-hepatic bile ducts. PSC is a

Table 1 Main features of hepatobiliary manifestations associated with inflammatory bowel diseases

Hepatobiliary manifestation	Main features
Immune-mediated	
PSC	The most frequent (50%-80% of PSC patients have IBD, and 2%-8% of IBD patients have PSC)
	No medical treatment approved. Therapies directed towards PSC complications
	Increased risk of cholangiocarcinoma and colorectal cancer (surveillance needed)
Small duct PSC	Histological evidence of PSC, but normal cholangiogram
	More benign disease course than classic PSC (cholangiocarcinoma risk not increased)
PSC-AIH overlap syndrome	Coexistence of biochemical and histological features of AIH and PSC-associated biliary tract alterations
	Better response to steroids and immunosuppressants than PSC
IgG4-related sclerosing cholangitis	Part of the IgG4-related systemic disease
	Characterized by histological evidence of IgG4+ plasma cells infiltrate
	Good response to steroids
Granulomatous hepatitis	Rare, generally in Crohn's disease
	Autoimmune or drug-induced pathogenesis
	Good response to steroids
Non-immune-mediated	
Gallstone disease	Incidence increased in IBD, more in Crohn's disease
	Bile salts malabsorption underlying the pathogenesis
NAFLD	Not strictly associated with IBD; similar risk factors in the general population
	Higher NAFLD prevalence in patients with severe IBD activity
Pyogenic liver abscess	Rare, mainly in Crohn's disease
	Penetrating disease, steroid treatment and malnutrition are risk factors
Portal vein thrombosis	Increased risk in IBD, especially during severe disease flare and after surgery. Prophylactic treatment indicated in these settings
DILI	
Aminosalicylates	Low risk of DILI
	LFT monitoring not necessary
Thiopurines	DILI quite frequent (prevalence of about 3%); both dose-independent and dose-dependent toxicities are possible
	Regular LFT monitoring indicated
Methotrexate	DILI quite frequent, with a prevalent dose-dependent mechanism
	Regular LFT monitoring indicated
	Folic acid supplementation indicated during treatment
Anti-tumour necrosis factor-α	Low risk of DILI, mainly with infliximab
	LFT monitoring not necessary
Anti-integrins	Low risk of DILI
	LFT monitoring not necessary
Anti-interleukin 12/23	Low risk of DILI
	LFT monitoring not necessary
Tofacitinib	Data in IBD still scarce
	Alanine aminotransferase elevation quite frequent in rheumatoid arthritis, but generally mild
Hepatitis B reactivation	A relevant concern
	Antiviral therapy indicated in HBsAg positive patients

	LFT monitoring indicated in HBsAg negative/anti-HBc positive patients
	Vaccination indicated in naïve patients
Hepatitis C reactivation	Not a relevant concern

IBD: Inflammatory bowel diseases; PSC: Primary sclerosing cholangitis; LFT: Liver function tests; HBsAg: Hepatitis B surface antigen; DILI: Drug-induced liver injury; NAFLD: Non-alcoholic fatty liver disease; AIH: Autoimmune hepatitis.

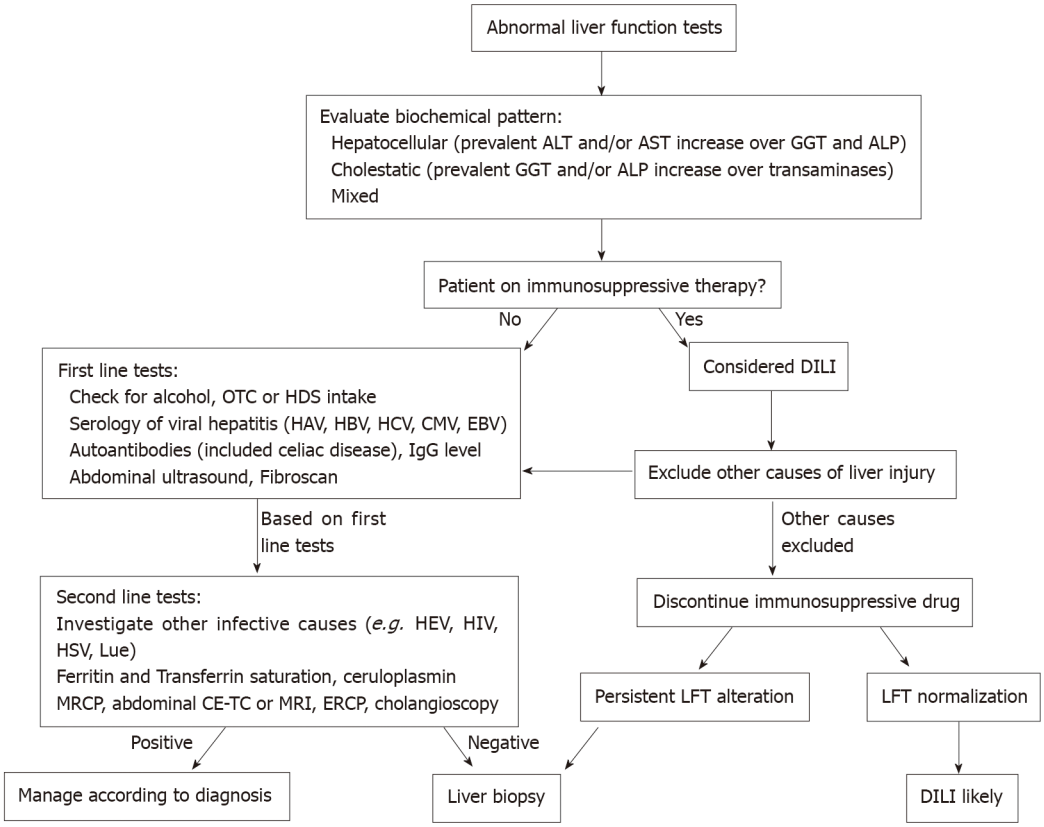

Figure 1 Mind map describing a practical approach to the inflammatory bowel disease patient with abnormal liver function tests. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; CE-CT: Contrast-enhancement computed tomography; CMV: Cytomegalovirus; DILI: Drug-induced liver injury; EBV: Epstein-Barr virus; GGT: Gamma-glutamyl transpeptidase; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDS: Herbal and dietary supplements; HEV: Hepatitis E virus; HIV: Human immunodeficiency virus; HSV: Herpes simplex virus; MRCP: Magnetic resonance cholangiopancreatography; MRI: Magnetic resonance imaging; OTC: Over-the-counter drugs.

progressive disease, leading to liver biliary cirrhosis and portal hypertension.

Epidemiology: According to a recent systematic review, the incidence and prevalence rates of PSC range from 0 to 1.3 *per* 100.000 inhabitants/year and from 0 to 16.2 *per* 100.000 inhabitants, respectively. There is a 2:1 male predominance and a peak of incidence between 30 to 40 years old[1]. PSC is commonly associated with IBD, with about 50%-80% of patients with PSC having concomitant IBD, more frequently UC[2], and about 2%-8% of patients with IBD having PSC[3]. PSC diagnosis usually precedes that of IBD, although PSC may be diagnosed many years after proctocolectomy for colitis[4].

Etiology: The exact etiology of PSC is unknown. A multifactorial pathogenesis has been proposed, in which genetic, immunological, and environmental factors contribute to the development of the disease. The increased risk of PSC in first-degree relatives suggests a genetic predisposition. Multiple human leukocyte antigen (HLA) haplotypes related to PSC susceptibility have been reported: HLA-B8, HLA-DRB1*0301 (DR3), HLA-DRB3*0101 (DRw52a) and HLA-DRB1*0401 (DR4)[5]. Interestingly, three UC susceptibility loci, harboring the genes *REL*, *IL2*, and *CARD9*, have been linked to PSC, supporting the association UC-PSC as a separate disease entity. However, genetic factors are implicated in a minority of PSC cases, clearly

emphasizing the predominant role of environmental risk factors in the overall disease liability[6,7]; colonic toxins, gut microbiota, portal bacteria and viral infections[6], are some of the main environmental determinants, which are discussed below. Based on the association between certain HLA haplotypes, the acute and chronic inflammatory infiltrate at histology, and given the association with several other autoimmune conditions, PSC has been classically considered an autoimmune disease[8]. Several autoantibodies may be present, including antinuclear antibodies in 24%-53%, smooth muscle antibodies in 13%-20%, and anti-perinuclear cytoplasmic antibodies (pANCA) in 65%-88% of patients[9]. However, none of these autoantibodies are reliable for diagnosis and there is no significant response of the disease to immunosuppressants. Chronic portal bacteremia is another important mechanism postulated: the bacterial translocation from the gut into the portal system can lead to biliary inflammation and recurrent cholangitis, probably through activation of the innate immune response in susceptible individuals[10]. Growing evidence suggests a relevant role of the gut microbiome in the pathogenesis of PSC, independently of IBD. Patients with PSC are characterized by a fecal overrepresentation of *Escherichia*, *Lactobacillus*, *Fusobacterium*, *Enterococcus* and *Ruminococcus*, and decreased populations of *Clostridium* cluster II, *Prevotella* and Bacteroides, compared to healthy individuals and patients with IBD alone[11-13]. Gut dysbiosis has been linked to an increase Gut dysbiosis has been linked to an increase in gut permeability and bacterial translocation that enter the enterohepatic circulation[14]. Other etiologic mechanisms such as ischemia and chronic viral infections have been postulated, but more evidence is needed.

Clinical presentation and diagnosis: Since most patients with PSC are asymptomatic at diagnosis, the disease is frequently suspected after routine liver biochemical tests. When the disease is symptomatic, the most common symptoms are pruritus, fatigue, right upper abdominal pain, and weight loss. Acute cholangitis is the first clinical manifestation of PSC in about 15% of cases[15]. Biochemical tests typically show a cholestatic pattern: An increased alkaline phosphatase (ALP) is the most frequent alteration, usually together with a raise of gamma-glutamyl transpeptidase. Notably, although an elevated ALP is a sensitive diagnostic marker, a normal level does not exclude PSC[6]. A high level of serum bilirubin is observed in an advanced stage of disease and is a marker of poor prognosis. Aminotransferases are often normal or mildly raised. As mentioned above, multiple autoantibodies, most frequently pANCA, have been associated with PSC, but they are not specific nor related to disease activity and prognosis[16]. Diagnosis is confirmed if the typical morphological alterations of biliary ducts are identified and causes of secondary sclerosing cholangitis are excluded. Magnetic resonance cholangiopancreatography (MRCP) should be the technique of choice for the investigation of suspected PSC, with a sensitivity and specificity for diagnosis of 0.86 and 0.94, respectively[17]. MRCP demonstrates diffuse, multifocal strictures and dilations of the intra- and extra-hepatic bile ducts. In about 40% of cases, the gallbladder and cystic duct are also involved[18]. Endoscopic retrograde cholangiopancreatography (ERCP) should be reserved for patients with biliary strictures requiring tissue acquisition (*e.g.* cytological brushing) or when therapeutic intervention is indicated (*e.g.* jaundice or acute cholangitis)[6]. In recent years, peroral cholangioscopy has emerged as a useful endoscopic tool in PSC management. It can provide a direct intraductal visualization, which allows guided biliary biopsies and can be helpful in distinguishing between benign and malignant strictures. A recent meta-analysis found a sensitivity and specificity of cholangioscopy-directed biopsies for all indications (*i.e.*, not limited to PSC) of 71.9% and 99.1%, respectively[19,20]; however, data on patients with PSC are still limited. Moreover, cholangioscopy has been recently used in the treatment of biliary stones in patients with PSC, with promising results[19]. Liver biopsy is not required to establish a diagnosis of a "classic" form of PSC. However, it is essential in presence of abnormal liver tests and normal cholangiogram to investigate small duct PSC, or in PSC patients with disproportionately elevated serum aminotransferase values to exclude PSC–autoimmune hepatitis (AIH) overlap syndrome. The most specific histological finding of PSC is periductal fibrosis with an "onion skin" pattern. In clinical practice, however, histological assessment is often non-specific, demonstrating general features of cholestasis that are similar to those find in primary biliary cirrhosis. Liver biopsy can also play a role in staging the disease and in defining the prognosis[6].

Complications and prognosis: PSC is a progressive disease that leads to severe complications involving liver, biliary tree and intestine. Fibrotic obliteration of intra-hepatic bile ducts finally evolves into liver cirrhosis, hepatic failure and portal hypertension. Disease progression towards end-stage liver disease is unavoidable in

most patients, and liver transplantation (LT) is considered the only curative treatment option[21]. In the literature, the median time from diagnosis to death or LT range from 7 to 22 years, with higher survival rates observed in overall PSC populations respected to cohorts of patients from liver transplant centers, which suffer from referral bias[22, 23]. In IBD patients, performing colectomy before PSC diagnosis was associated with lower risk of LT and death in a large cohort study in Sweden[14]. Portal hypertension is a frequent complication of PSC, and the presence of esophageal varices at diagnosis or history of variceal hemorrhage are considered predictors of worse prognosis[24]. PSC patients are at increased risk of cholangiocarcinoma (CCA), gallbladder carcinoma, hepatocellular carcinoma (HCC), and colorectal carcinoma (CCR). The estimated annual incidence of CCA in patients with PSC range from 0.5% to 1.5%[25, 26], with 20%-30% of CCA found synchronously at PSC diagnosis, and 50% of CCA occurring within 1 year[25]. According to a large international, multicentre, PSC cohort study (7121 patients from 37 countries), 10.9% of PSC patients developed a hepatopancreatobiliary malignancy, which was CCA in about 80% of cases[27]. Importantly, concomitant UC was a risk factor for future development of hepatopancreatobiliary malignancies[27]. Gallbladder cancer and HCC are less frequent complication of PSC, with a lifetime incidence of 3%-14% and 0.3%-2.8%, respectively[28]. An increased risk of CCR has been clearly demonstrated in patients with PSC-IBD, compared to patients with IBD or PSC alone. According to a recent meta-analysis of observational studies, patients with IBD and PSC were at increased risk of colorectal cancer compared with patients with IBD alone, with an odds ratio of 3.41 (95%CI: 2.13-5.48). Interestingly, stratification by IBD type revealed that PSC was a risk factor for colorectal cancer in patients with UC, but not in CD patients[29]. In addition, unlike in patients with UC alone, CCR risk in PSC-UC seems to manifest soon after the combined diagnosis, with a peak of incidence within the first 2 years of diagnosis[30]; thus, cancer surveillance is strongly recommended in PSC-UC, even in patients with ileal pouch-anal anastomosis (IPAA) after colectomy[31]. Finally, IBD patients with IPAA and concomitant PSC are at increased risk of pouchitis, with an almost double incidence at 10 years as compared to patients without PSC[32].

Treatment: Treatment of PSC associated with IBD does not differ from PSC without IBD. To date, no medical treatments have been demonstrated to modify the course of "classic" PSC. In particular, ursodeoxycholic acid (UDCA) has shown to improve LFT in several studies, but two meta-analyses and a large multicentre study failed to show benefit from UDCA towards important clinical outcomes (*e.g.* complications and death) in patients with PSC[33,34]. Despite previous studies suggested a role of UDCA in prevention of cancer (CCR or CCA) in PSC, more recent meta-analyses and a randomized control trial did not confirm this effect[35,36]. UDCA is not currently recommended by PSC guidelines for either the treatment or cancer prevention[6,37]. Despite the presumed immune-mediated pathogenesis of the disease, corticosteroids and immunosuppressants are not recommended as well[6]. Thus, treatments goals in PSC are directed to the control of symptoms and management of complications, such as varices, liver decompensation, cholangitis, jaundice, pruritus, and malignancies. Endoscopic interventions, mainly ERCP, are a mainstay of PSC management, and specific guidelines have been published from collaboration of European Society of Gastrointestinal Endoscopy and European Association for the Study of the Liver (EASL)[38]. Main indications of ERCP in PSC are acute cholangitis, treatment of dominant strictures and suspicion of CCA. LT is a potential resolutive therapy in PSC patients with end-stage liver disease. Other disease-specific indications are intractable pruritus, recurrent cholangitis, and limited cases of very early stage of CCA[3]. A single-center experience from the Mayo Clinic reported survival rates after LT for PSC-related end-stage liver disease of 86% at 5 years and 70% at 10 years[39]. Recurrence of PSC after LT is a concern, occurring in 12%-37% of cases and causing significant impact on long term graft and recipient survival[40].

Variant forms of PSC

Small duct PSC: A minority of patients with cholestatic biochemistry and typical liver histology with concentric 'onion skin' fibrosis around the bile ducts, but with entirely normal cholangiogram, was first described by Wee and Ludwig[41] in 1985; they coined the term "small duct PSC". In a large multicentre study, 81% of patients with small-duct PSC had IBD, predominantly UC (78%) compared to CD (21%). In this study, none of the patients developed CCA or other intestinal malignancies during a median follow-up of 13 years, but 28% of them shown evidence of progression to large duct PSC at repeated cholangiography[42]. In a large bicentric study from United

Kingdom and Norway, only 12% of small duct PSC patients either required LT or died, compared to 47% of patients with "classic" PSC[43].

Overlap between PSC and AIH: PSC/AIH overlap syndrome is a rare disorder characterized by concomitant occurrence of the biochemical and histological features of AIH and the cholangiography abnormalities found in PSC. In a cohort of 211 PSC patients from United States, according to the International AIH group scoring system, AIH was diagnosed as "definite" in 1.4% and "probable" in 6% of patients[44]. An Italian cohort of PSC/AIH patients showed a lower mean age at presentation and higher alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values compared to "classic" PSC[45]. There is also a strong association between PSC/AIH and IBD; according to a recent systematic review, IBD was present in 44% of PSC/AIH patients, that was UC in 68% of cases[46]. Patients with an established diagnosis of AIH who also have IBD should be evaluated for concomitant PSC. Patients with PSC/AIH seem to benefit from treatment with immunosuppressive medications and have a better prognosis compared to patients with PSC alone[45].

IgG4-related sclerosing cholangitis: IgG4-related sclerosing cholangitis (IgG4-SC) is the biliary manifestation of the multi-organ inflammatory IgG4-related disease. Diagnosis requires histological evidence of IgG4+ plasma cells infiltrate (> 10 *per* high-power field), imaging of biliary tract involvement (which may be indistinguishable from the "classic" PSC), elevated serum IgG4 levels (> 135 mg/dL), evidence of other organ involvement and response to steroid treatment[47]. Autoimmune pancreatitis is the most frequent organ involvement associated with IgG4-SC, being present in > 90% of cases[48]. An increase in serum IgG4 is reported in 9%-22% of patients with PSC overall[48], making it difficult to distinguish a PSC with high serum IgG4 levels from a "true" IgG4-SC. EASL Cholestatic Liver Disease Guidelines recommends measurement of serum IgG4 in all patients with large-duct PSC at diagnosis[37]. While association with IBD is prevalent in PSC, this is rarely seen in IgG4-SC; high serum IgG4 levels have been observed in about 5% of IBD patients[49]. Unlike in PSC, response to steroid treatment in IgG4-SC is excellent. However, relapse after steroid withdrawal is common[50]; in these cases, second-line treatments include immunomodulators and rituximab[50,51].

AIH

AIH is an immune-mediated chronic liver disease characterized by hepatocellular inflammation, necrosis and progression to cirrhosis. The clinical presentation varies from persistent mild elevation of AST and ALT to fulminant forms of acute hepatitis. Mean age at presentation shows a bimodal pattern with one peak during childhood/teenage years and another between the 4th and 6th decade of life. The diagnosis of AIH must be suspected in presence of autoantibodies (mainly antinuclear, smooth muscle, soluble liver antigen/liver pancreas and liver/kidney microsomal type 1 antibodies), IgG elevation, consistent liver histology and exclusion of other forms of hepatitis[52]. Despite most of the data about AIH/IBD coexistence comes from studies focusing on PSC and AIH/PSC overlap syndrome, a higher prevalence of AIH has been found in patients with IBD, compared to subjects without IBD. In the cross-sectional study by Halling *et al*[53], AIH was more frequent in males and females with IBD compared with matched controls without IBD, with an odds ratio of 7.8 and 17.9, respectively[53]. Another study by Perdigoto *et al*[54] found a 16% prevalence of UC in patients with AIH, 42% of whom had also PSC features at cholangiography[54]. In this study, patients with colitis failed treatment for AIH more commonly and progressed to cirrhosis more frequently; similar results emerged from the study by Perdigoto *et al*[54].

Granulomatous hepatitis

Granulomatous hepatitis is a rare complication of IBD, with only a few cases of IBD-associated granulomatous hepatitis reported in literature[55-57]. It occurs more frequently in CD and can underlie an autoimmune pathogenesis or be induced by mesalamine or sulfasalazine therapy[58]. Clinical manifestations include fever, hepatomegaly and increase in cholestatic enzymes, although patients can be completely asymptomatic[59]. Response to corticosteroid therapy is generally good; methotrexate may be considered as second-line therapy in patients relapsing after steroids[60]. Prognosis is usually benign[61].

NON-IMMUNE-MEDIATED DISORDERS

Gallstone disease

Several studies and a meta-analysis showed a prevalence of cholelithiasis in CD ranging from 8% to 34%, with a 2- to 5-fold increased risk compared to the general population[62-68]. Three studies also evaluated UC patients, reporting a prevalence of gallstone disease of 4%-10%; only one of these found a significantly higher risk compared to a population without UC[62], while the other two studies, including the aforementioned meta-analysis, did not demonstrate this increased risk[64-66]. Most studies relied on abdominal ultrasound to diagnose the lithiasis. A recent case-cohort study on a large cohort of IBD patients reported an incidence of cholelithiasis of 5.21/1000 persons/year, compared to a 3.49/1000 persons/year incidence of a matched non-IBD cohort ($P < 0.001$); the significance was also maintained by differentiating CD and UC[69]. Another case-control study reported an incidence of gallstone disease in CD and UC of 14.35/1000 persons/year and 7.48/1000 persons/year, respectively, that were significantly higher than those of the matched control populations[70]. In all studies assessing both CD and UC, prevalence of gallstone disease was higher in CD compared to UC. Among the risk factors, ileal disease location, previous ileal resection and long-standing disease were the most frequently associated with gallstone disease in IBD[62-64,67,68,70]. The pathogenesis of cholelithiasis in IBD patients is usually attributed to bile salts malabsorption at the terminal ileum; this leads to a decrease in the total bile acid pool, leading to supersaturated bile in gallbladder, which predispose to stone formation[71,72]. Lapidus and Einarsson[71] reported that patients with ileal resection due to CD are characterized by lower cholesterol saturation, but increased bilirubin concentration in fasting duodenal bile, compared to healthy controls; therefore, these patients seem not predisposed to the formation of cholesterol stones, but rather at risk of developing pigment stones[71].

NAFLD

NAFLD refers to a clinical and pathological syndrome that includes a spectrum of histological findings ranging from benign steatosis to non-alcoholic steatohepatitis. Non-alcoholic steatohepatitis is defined by histological evidence of hepatic steatosis associated with inflammation, and can progress to hepatic fibrosis and cirrhosis. A recent meta-analysis reported a worldwide prevalence of NAFLD of 25% in the general population[73], a prevalence that seems to be worryingly increasing over time [74]. In the literature, the prevalence of NAFLD in patients with IBD is variable. Two recent meta-analyses reported a pooled prevalence of NAFLD in IBD of 27.5%[75] and 32%[76]; older age, obesity, type 2 diabetes, longer IBD duration and previous surgery were the main risk factors associated with the development of NAFLD[77]. A further meta-analysis specifically addressing the role of IBD treatment on the risk of NAFLD found no significant association between medications of all types (*i.e.*, steroids, biological agents, immunomodulators, methotrexate) and the risk of developing NAFLD[78]. Several studies also reported a higher prevalence of NAFLD among IBD patients with severe disease activity at the time of liver evaluation, compared to mild-moderate IBD cases[77,79,80].

Pyogenic liver abscess

Pyogenic liver abscesses are rarely seen in IBD, with only a few cases reported in literature, mainly in CD. A nationwide case-cohort study from Taiwan reported an incidence of pyogenic liver abscess in IBD patients of 6.7 cases/10000 persons/year, which was significantly higher compared to controls without IBD[81]. Clinical manifestations include fever, chills, anorexia, weight loss and abdominal pain with right upper quadrant tenderness, which can mimic an IBD flare and lead to a diagnostic delay. Moreover, hepatic abscesses have been reported as the initial presentation of CD in several cases[82,83]. Risk factors predisposing to liver abscesses in IBD include abdominal surgery, fistulizing disease, intra-abdominal abscess, malnutrition, and corticosteroid treatment[84]. Dissemination from intra-abdominal abscesses and portal bacteremia secondary to impaired intestinal permeability are the most involved pathogenic mechanism[84].

Portal vein thrombosis

IBD patients are at increased risk of venous thromboembolism (VTE)[85]. In two studies on large cohorts of IBD patients with a follow-up time over 10 years, thromboembolic complications were reported in about 1% of patients, with an incidence of VTE of 2.6/1000 persons/year[85,86]. Porto-mesenteric venous system is a

frequent site of thrombosis in IBD and is a potentially catastrophic complication, which may lead to bowel ischemia or infarction and to acute or chronic portal hypertension; the mortality rate range between 3%-25%[86,87]. Incidence is higher during disease flares and after surgical procedures[88-90], and prophylactic treatment with low-molecular-weight heparin in severely active disease is indicated by guidelines to reduce the risk of thromboembolism[91]. However, about 30%-50% of thrombosis occurs in remission phases of the disease[92-94], indicating that factors other than inflammatory status can be involved in the pathogenesis of the thrombotic event. Immobilization, extensive colonic disease, central catheters, corticosteroids, and smoking are other known prothrombotic risk factors[90,95]. A hematologic prothrombotic condition can be found in up to 40% of portal vein thrombotic events in IBD, hyperhomocysteinemia being the most frequently found[95]. Thrombocytosis is frequently seen during IBD flares and may result from systemic inflammatory activity and/or iron-deficiency anemia[96]; however, no data on a possible association between thrombocytosis and VTE in IBD is available to date, since large clinical studies addressing this association are still lacking[97]. Moreover, IBD are associated with significant changes in circulating levels of various coagulation factors, as result of an imbalance between procoagulant and anti-coagulant pathways. Specifically, higher levels of prothrombin fragment 1 and 2, fibrinogen, factors V and VIII, thrombin-antithrombin complex, plasmin-α2-antiplasmin complex, and an impairment of the protein C pathway have been described in IBD[97-99]. Specific mutations in clotting factors, *e.g.* Factor V Leiden, are rare, but important to be identified as they may indicate long-term anticoagulant treatment[100]. European Crohn's and Colitis Organization (ECCO) guidelines recommend appropriate screening for prothrombotic condition after IBD diagnosis and anticoagulant treatment in accordance with international guidelines[95].

DRUG-INDUCED LIVER DISEASE IN IBD

The therapeutic armamentarium for the treatment of IBD is gradually expanding. This certainly offers greater potential for therapeutic benefit, but the risk of hepatotoxicity is a concern. Although the overall risk of serious adverse events is low, cases of drug-induced liver injury (DILI) have been reported for most drugs used in IBD, and some therapies carry a significant risk of liver toxicity. DILI induced by IBD drugs can be allergic/idiosyncratic (dose-independent) or related to hepatotoxins (typically dose-dependent). In addition, some drugs can cause hepatotoxicity with more than one pathogenic mechanism. According to EASL guidelines, the exclusion of other causes of hepatotoxicity is necessary for the diagnosis of DILI, and recovery after drug discontinuation is an important criterion for the causality assessment[101] (Figure 1). The following paragraphs will describe the association between the main drugs used in IBD and the risk and type of DILI.

Aminosalicylates

Sulfasalazine was the first aminosalicylate approved for the induction and maintenance of remission in mild-to-moderate UC. Within the bowel, sulfasalazine is cleaved into sulfapyridine and 5-aminosalicylic acid, most called mesalamine. Sulfapyridine, a sulfa-containing antibacterial agent, is then absorbed from the colon into the bloodstream, transported to the liver, and acetylated; acetylation was reported to be genetically programmed, with slow acetylators having higher levels of free sulfasalazine and more drug-induced adverse events[102]. Mesalamine is minimally absorbed and largely excreted in the stools and is primarily responsible for the anti-inflammatory effect on the colon. The introduction of the various mesalamine formulations has almost completely supplanted the use of sulfasalazine in UC, while the utility of aminosalicylates in CD remains unclear[91,103]. Both sulfapyridine and mesalamine are rarely associated with liver injury. According to the United Kingdom's Committee on the Safety of Medicines, from 1991 to 1998 the incidence of hepatitis in patients treated with mesalamine was 3.1 cases *per* million, compared to 6 cases *per* million in patients treated with sulfasalazine[104]. A French pharmacovigilance study on mesalamine microgranules (Pentasa®) reported 0.79 cases of LFT elevations *per* million treatment days over a 2-year period[105]. The toxic effect almost always occurs within the first 2 mo of treatment, and LFT normalize in most cases after drug discontinuation[105]. For sulfasalazine, sporadic cases of granulomatous hepatitis or fulminant hepatitis have been reported[106-108]. Due to this low risk of hepatotoxicity, a close monitoring of liver chemistries is not necessary in patients treated with

aminosalicylates.

Thiopurines

Azathioprine (AZA) and its metabolite 6-mercaptopurine (6-MP) are two thiopurine analogues widely used for the treatment of IBD. Main indication of AZA and 6-MP is the maintenance of remission in steroid-dependent CD and UC[91,103]. Purine analogues act as DNA synthesis inhibitors by antagonizing endogenous purines, and lead to both cytotoxic and immunosuppressive effects[109]. Overall, adverse events due to thiopurines are frequent and occur in 15%-40% of patients, leading to dose reduction or drug withdrawal[110]. Thiopurine-related adverse events are classified into dose-independent (or allergic/idiosyncratic) and dose-dependent. The former are thought to be immune-mediated and include rash, fever, arthralgia, and pancreatitis; the latter include myelotoxicity as the main manifestation. Thiopurine-induced hepatotoxicity can be both dose-dependent and independent, based on the pathogenetic mechanism involved[111,112]. Dose-independent liver toxicity usually occurs within 3 mo of therapy and includes hypersensitivity and idiosyncratic reactions[111]; type of hepatotoxicity can be described as acute hepatocellular hepatitis, with prevalent increase of aminotransferase levels, acute cholestatic hepatitis, with prevalent increase of serum ALP, or mixed[113,114]. Other less frequent findings include peliosis hepatis, hepatic sinusoidal dilatation, veno-occlusive disease, perisinusoidal and portal fibrosis, and nodular regenerative hyperplasia[113]. Thiopurine-related DILI has been related to thiopurine metabolites. After absorption, AZA is metabolized in the liver to 6-MP, which undergo a complex metabolization by three enzymes; one of them is the thiopurine S-methyltransferase (TPMT), that lead to 6-methylmercaptopurine (6-MMP) formation. 6-MMP is a non-effective metabolite which is important in hepatotoxicity development[109]. Approximately 15%–20% of IBD patients treated with thiopurines demonstrate hypermethylation (or shunting), a phenomenon due to a high TPMT activity that leads to preferential methylation of 6-MP to 6-MMP over bioactivation to thioguanine nucleotides (TGNs); the usual definition of hypermethylation is a ratio of 6-MMP to TGNs of > 11. Subtherapeutic TGNs level results in a poor response to therapy, while a high 6-MMP level (> 5700 pmol/8 × 10^8 erythrocytes) has been correlated with a 3-fold increased risk of liver toxicity[115]. Allopurinol is a xanthine oxidase inhibitor that prevents the breakdown of thiopurines into thiouric acid (TUA), thus increasing the bioavailability of 6-MP. Several studies have demonstrated that the combination of low dose thiopurine, *i.e.* 25%–50% of the standard dose, with 100 mg of allopurinol corrects hypermethylation in patients who have experienced thiopurines-induced hepatotoxicity or who have had a poor response to thiopurines treatment[116,117]. However, Shaye *et al*[118] showed that about 90% of patients with 6-MMP > 5700 pmol/8 × 10^8 erythrocytes have no hepatotoxicity and almost 40% of subjects with hepatotoxicity had 6-MMP levels below this cut-off[118]. Moreover, a recent case-control study and a meta-analysis failed to demonstrate any correlations between *TPMT* gene polymorphisms and hepatic adverse events in IBD patients[119,120]. The reported frequency of thiopurine-related hepatotoxicity varies widely among studies, ranging from to 3% to 17%[108,115,121,122]; a systematic review by Gisbert *et al*[113] reported a mean prevalence of thiopurine-induced liver injury of 3%, with a mean annual rate of 1.4%[113]. In a prospective cohort study, abnormal liver function (defined by ALT or ALP levels > 50% the upper normal limit) occurred in 13% of patients, while hepatotoxicity (defined by ALT or ALP levels greater than twice the upper normal limit) developed in 10% [111]. CD, liver steatosis and concomitant steroid therapy are reported risk factors for liver injury during thiopurine therapy[108,111,123]. It has been shown that most cases of thiopurine-induced liver injury completely resolved after dose reduction, while the need to discontinue therapy only occurred in about 3%-4% of cases[111,118,124]. Switching from AZA to 6-MP in the case of AZA-induced DILI is a possible strategy, which is effective in resolving the liver toxicity in 71%-87% of cases[114,125]. Despite an optimal frequency has not yet been established, regular monitoring of blood tests should be performed for the entire duration of thiopurine treatment, more frequently in the first 3 mo of therapy[113,126,127]. British Society of Gastroenterology (BSG) guidelines on IBD recommend the monitoring of full blood count and LFT at 2, 4, 8 and 12 wk of thiopurine therapy, and every 12 wk thereafter[127].

Methotrexate

Methotrexate (MTX) is a folic acid analogue with inhibitory activity against many enzymes in the metabolic pathway of folic acid. MTX inhibits production of thymidylate, purines, and methionine and leads to accumulation of adenosine, which has a potent anti-inflammatory activity. These actions inhibit cellular proliferation and

tissue migration, and decrease production of inflammatory mediators[128]. MTX is currently indicated for the maintenance of remission in steroid-dependent CD[129], while its role in UC is still controversial[130]. The hepatotoxic potential of MTX is well known. A meta-analysis of clinical trials on IBD patients treated with MTX reported a pooled incidence rate of abnormal hepatic aminotransferase levels, which the author defined as up to a 2-fold increase over the upper limit of the normal, of 1.4 *per* 100 person-months. The rate of hepatotoxicity, defined as aminotransferase levels greater than a 2-fold over the upper normal limit, was 0.9 *per* 100 person-months. The rate of withdrawal of MTX due to these abnormalities was 0.8 *per* 100 person-months[112,131]. Alcohol intake is a main risk factor for MTX-induced hepatotoxicity and should be strictly avoided. Other potential risk factors are obesity, diabetes mellitus and chronic viral hepatitis[112,131,132]. Folic acid supplementation has been correlated with reduction of methotrexate-induced hepatic adverse events and is therefore recommended[133]. Regular liver chemistry tests are recommended for the monitoring of hepatotoxicity, every 2 wk for the first 2 mo and then every 2-3 mo[134]; the drug should be stopped if transaminases exceed twice the upper normal limit[127]. Although liver biopsy was previously indicated after an MTX-treatment cumulative dose ≥ 1.5 g, this practice is no longer recommended by current rheumatologic guidelines[134], this is based on recent evidence that show a low incidence of liver injury in patients receiving a chronic low dose of MTX[112]. In a retrospective study on 87 IBD patients with a mean MTX cumulative dose of 1813 mg, 76% of patients maintained normal liver chemistry tests throughout MTX therapy; a liver biopsy was performed in 11 patients after a cumulative dose ≥ 1.5 g and found no case of moderate or severe fibrosis[112]. Another study evaluating 20 liver biopsies after a cumulative methotrexate dose of ≥ 1.5 g (mean dose 2.6 g) found mild histological abnormalities in 95% of patients; abnormal liver chemistry tests were present in 30% of patients and did not correlate with histological toxicity[135]. However, liver biopsy should be performed in cases of persistent alteration of transaminases, especially in case of no reduction after lowering the drug dose. Transient elastography is a promising tool for the monitoring of liver fibrosis in MTX-treated patients and can be useful in selecting patients for liver biopsy[136].

Biological agents

Anti-TNF-α: Since its introduction in the 1990s, anti-TNF-α antibody therapy has revolutionized the treatment of IBD. Anti-TNFs, which include infliximab, adalimumab, golimumab and certolizumab pegol, are approved for the treatment of moderate-to-severe CD and UC and demonstrated high efficacy in the induction and maintenance of both clinical and endoscopic remission[91,103]. Several types of anti-TNF-related adverse events have been reported, mostly of infectious, auto-immune and tumoral types. DILI caused by anti-TNF is uncommon, mostly mild and related to infliximab. However, cases of liver failure requiring transplantation has rarely been reported[137-139]. Shelton *et al*[140] evaluated the incidence of liver enzyme elevation in a large cohort of IBD patients treated with anti-TNF: Only 102 out of 1753 patients (6%) developed ALT elevation, and in about half of cases this could clearly be linked to an alternative etiology. Infliximab was the involved anti-TNF in 96% cases. Compared to a control population of anti-TNF-treated patients without liver enzyme elevation, no difference in concomitant immunomodulator therapy, body max index, age and gender was found. The majority of patients with ALT elevation continued anti-TNF, most of them normalizing the liver enzyme during the follow-up. In 10 patients switching to a second anti-TNF was performed, without recurrence of liver injury [140]. Ghabril *et al*[141] identified 34 cases of DILI related to anti-TNF used for a variety of auto-immune conditions from a review of the United States DILI Network database and PubMed research. The drug presumed to have caused DILI was infliximab in 76% of cases. The liver injury was scored as mild-to-moderate in 93% of cases. Fifteen of the 17 patients undergoing liver biopsy showed clear features of autoimmunity. All patients improved after discontinuation of the anti-TNF[141]. The mechanism underlying liver toxicity remains to be elucidated. Infliximab-related hepatitis seems to be sustained by an immune-mediated mechanism, mimicking the characteristics of AIH type I, although a direct liver damage cannot be ruled out[112]. Currently, Food and Drug Administration classifies infliximab as a Most-DILI-concern drug, adalimumab as a Less-DILI-concern drug, and golimumab and certolizumab as Ambiguous-DILI-concern drug[142]. The current consensus recommends the use of infliximab in selected cases of patients with significant liver disease, and that treatment should be discontinued or avoided in patients with transaminases above three times the upper limit of normal[143].

Anti-integrins: Natalizumab is a monoclonal antibody that antagonizes both the integrins α4β1 and α4β7, which are necessary for the homing of lymphocytes to brain and gut, respectively. Natalizumab is therefore approved for the treatment of multiple sclerosis, and has been tested with good results in CD; however, the risk of JC virus-associated progressive multifocal leukoencephalopathy (PML) has limited its use in IBD[144-146]. Vedolizumab is a humanized monoclonal antibody which, unlike natalizumab, specifically inhibits α4β7 integrin, thus eliminating the risk of PML[147]. Vedolizumab is approved for the treatment of both moderate to severely active CD and UC since 2014[91,103]. To date, only sporadic cases of liver injury during vedolizumab therapy have been reported[148,149]. In the prelicensure trials, three patients developed hepatitis, although it is unclear whether the increase in transaminases indicated drug-induced or autoimmune etiology[150]. In the GEMINI-1 and GEMINI-2 phase III trials, no differences in LFT were found compared to placebo[151,152]. Therefore, vedolizumab is considered almost free from liver toxicity.

Anti-interleukin 12/23: Ustekinumab is a human monoclonal antibody directed against the p40 subunit, which is a component of both interleukin (IL)-12 and IL-23, allowing this drug to simultaneously inhibit both these cytokines. Ustekinumab has been recently approved as a second line therapy for moderate-to-severe CD and UC since 2016 and 2019, respectively[153,154]. Although current data are limited, liver injury related to ustekinumab seems to be very uncommon. In the phase III trial on CD, a similar rate of adverse events compared to placebo was reported, with no mention of hepatotoxicity[153]. According to the Clinical and Research Information on DILI database, mild-to-moderate serum aminotransferase elevation was reported in 0.5% to 1.4% of patients during ustekinumab therapy. However, this event was no more frequent than placebo and resolved without discontinuing the drug[155]. Risankizumab is a monoclonal antibody directed against p19 subunit of IL-23 and therefore selectively inhibit this cytokine. Phase II and III trials in IBD are ongoing and safety data are still limited[156].

Tofacitinib

Tofacitinib is an oral Janus kinase inhibitor and is the first drug of this class approved for the treatment of IBD, specifically UC since May 2018[157], while others are currently being tested in phase II and III trials[158]. Tofacitinib is indicated for the treatment of adult patients with moderately to severely active UC, who have had an inadequate response or who are intolerant to anti-TNF[127,158]. Data about hepatotoxic effects of tofacitinib mainly derive from rheumatoid arthritis, where a slight ALT elevation was reported in about 30% of patients, but elevation above 3 times the upper normal limit occurred in 1%-2% of patients[159-161]. Data regarding tofacitinib-induced liver toxicity in IBD are still limited. However, no increased incidence of liver injury has been reported either in the pivotal trial or in subsequent real-life studies[162-165].

IBD AND VIRAL HEPATITIS B AND C

In literature, the reported prevalence of hepatitis B surface antigen (HBsAg) and anti-HBc positivity in IBD patients ranges from 0.6%-5.7% and from 1.6%-41.6%, respectively, depending on the geographic area considered[166]. Despite previous studies reported a higher prevalence of HBV positivity in IBD patients compared to the general population, more recent studies indicated an equal or lower prevalence which tends to decrease over time, suggesting that preventive measures like vaccination, use of disposable materials and implementation of transfusion safety programs are effective[166,167]. The risk of viral reactivation is a major concern in HBV patients treated with immunosuppressants. This event is closely related both to the stage of the infection and the type of immunosuppressive drug used. HBV reactivation, defined as the increase in HBV viremia of more than 1 Log10 IU/mL, is characterized by a broad spectrum of clinical manifestations, that range from viremia without clinically relevant manifestations to fulminant life-threatening hepatitis[168]. For this reason, both ECCO and BSG guidelines recommend hepatitis B screening immediately after diagnosis of IBD, checking for HBsAg, anti-HBs, and anti-HBc[127,169]. If screening was not performed at the time of diagnosis, it should be performed before immunosuppressive therapy initiation[127,169]. HBsAg-positive/anti-HBc–positive patients carry the higher risk of reactivation, and should receive potent anti-viral agents (nucleoside/nucleotide analogues with high barrier to resistance)

Table 2 Management of patients with inflammatory bowel disease undergoing immunosuppressive therapy according to hepatitis B status

Hepatitis B status	Indications
HBsAg positive/anti-HBc positive (chronic hepatitis B)	Antiviral treatment (start 3-4 wk before and continue at least 12 mo after the immunosuppressive treatment)
HBsAg negative/anti-HBc positive (occult hepatitis B)	Liver function tests monitoring every 2-3 mo
HBsAg negative/anti-HBc negative/anti-HBs negative (naïve for hepatitis B)	Vaccination (indicated at diagnosis)
HBsAg negative/anti-HBc negative/anti-HBs positive	Check previous hepatitis B vaccination. Dose hepatitis B virus-DNA if uncertainty

HBsAg: Hepatitis B surface antigen.

such as tenofovir and entecavir. Prophylactic treatment should be started 3-4 wk before immunosuppressive therapy and continued until at least 12 mo after the end of treatment[169]. HBsAg-negative/anti-HBc–positive patients are considered to have occult infection and viral reactivation is rare in this group with types of immunosuppressants used in IBD; in this case, HBV viremia (HBV-DNA) should be checked every 2-3 mo during the treatment and antiviral treatment started when HBV-DNA is detected[169]. Hepatitis B vaccination in all seronegative patients at IBD diagnosis is recommended by ECCO guidelines[169], while BSG guidelines indicate vaccination in high-risk groups[127]. Anti-HBs level should be measured after vaccination to confirm response; however, a reduction in vaccination during immunosuppressive therapy (mainly immunomodulators and anti-TNF) has emerged from several studies[170] and a recent meta-analysis[171]. Indications for the management of the IBD patient undergoing immunosuppressive therapy according to HBV status are summarized in Table 2.

Hepatitis C prevalence in IBD is similar to the general population[168]. The risk of HCV reactivation under immunosuppressive therapies used in IBD is low[172,173]. Small case series reported successful treatment of hepatitis C with direct-acting antiviral (DAA) in patients on anti-TNF therapy[170] and no drug-drug interaction between DAA and anti-TNF has emerged[174]; thus, concomitant treatment with DAA and anti-TNF seems to be safe, although more studies specifically addressing this setting are needed.

CONCLUSION

Hepatobiliary disorders are frequently seen in IBD, and PSC represents the most common of them. A broad spectrum of pathogenic mechanisms may underlie the disorders, ranging from autoimmune conditions, metabolic diseases, infections up to drug toxicity, and two or more diseases can co-exist in the same patient. Moreover, liver disease severity can range from mild, which only requires monitoring over time, to liver failure, that may require LT. A step-by-step approach to the IBD patient with abnormal LFTs is extremely important to make the correct diagnosis, prevent complications, and identify those cases that warrant early and aggressive treatment. Finally, the diagnostic complexity often requires a multidisciplinary management involving gastroenterologist and hepatologist.

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REVIEW

Gastrointestinal and hepatic side effects of potential treatment for COVID-19 and vaccination in patients with chronic liver diseases

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Abstract

The outbreak of coronavirus disease 2019 (COVID-19) is a global pandemic. Many clinical trials have been performed to investigate potential treatments or vaccines for this disease to reduce the high morbidity and mortality. The drugs of higher interest include umifenovir, bromhexine, remdesivir, lopinavir/ritonavir, steroid, tocilizumab, interferon alpha or beta, ribavirin, fivapiravir, nitazoxanide, ivermectin, molnupiravir, hydroxychloroquine/chloroquine alone or in combination with azithromycin, and baricitinib. Gastrointestinal (GI) symptoms and liver dysfunction are frequently seen in patients with COVID-19, which can make it difficult to differentiate disease manifestations from treatment adverse effects. GI symptoms of COVID-19 include anorexia, dyspepsia, nausea, vomiting, diarrhea and abdominal pain. Liver injury can be a result of systemic inflammation or cytokine storm, or due to the adverse drug effects in patients who have been receiving different treatments. Regular monitoring of liver function should be performed. COVID-19 vaccines have been rapidly developed with different technologies including mRNA, viral vectors, inactivated viruses, recombinant DNA, protein subunits and live attenuated viruses. Patients with chronic liver disease or inflammatory bowel disease and liver transplant recipients are encouraged to receive vaccination as the benefits outweigh the risks. Vaccination against COVID-19 is also recommended to family members and healthcare professionals caring for these patients to reduce exposure to the severe acute respiratory syndrome coronavirus 2 virus.

Key Words: COVID-19 treatment; Gastrointestinal side effects; Hepatic side effects; COVID-19 vaccine; Chronic liver disease; Liver transplantation

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Core Tip: Gastrointestinal symptoms such as anorexia, dyspepsia, nausea, vomiting, diarrhea and abdominal pain are common among patients with coronavirus disease 2019 (COVID-19). Liver injury can be a result of systemic inflammation or cytokine storm, or due to the adverse drug reactions of different treatments. Regular monitoring of liver function is recommended. Patients with inflammatory bowel disease, chronic liver diseases or liver transplant recipients are encouraged to receive the COVID-19 vaccine, and the benefits will outweigh the risks in the vast majority of patients.

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INTRODUCTION

The outbreak of coronavirus disease 2019 (COVID-19) is a global pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It is a very contagious virus and has infected millions of people worldwide causing numerous deaths. There are many clinical trials investigating potential treatments or vaccines for this disease to reduce the high morbidity and mortality.

Drugs with potential utility include remdesivir, lopinavir/ritonavir (LPV/r), steroids, tocilizumab, interferon alpha or beta, ribavirin, hydroxychloroquine/chloroquine alone or in combination with azithromycin, and baricitinib. Gastrointestinal (GI) symptoms and liver dysfunction are frequently seen in COVID-19 which can make it difficult to differentiate disease manifestations from treatment side effects[1,2].

The common GI symptoms in patients with COVID-19 include anorexia, dyspepsia, nausea, vomiting, diarrhea and abdominal pain[3-11]. The pooled prevalence of GI symptoms is 17.6% according to a recent meta-analysis[12]. The hepatic manifestations of COVID-19 include elevated liver enzymes and less commonly elevated bilirubin levels. The incidence of liver injury ranges from 14.8% to 53% as indicated by abnormal alanine transaminase (ALT)/aspartate aminotransferase (AST) levels with slight elevation of bilirubin levels[2,7]. Patients with liver dysfunction also tend to have severe COVID-19, and the liver injury in these patients can be a result of systemic inflammation or cytokine storm, or due to the adverse drug reactions in severe COVID-19 patients who have been receiving different treatments. While cholangiocytes may contribute to hepatic regeneration and immune response, it has been suggested that bile duct epithelial cells play a greater role in hepatic injury due to SARS-CoV-2 infection than cholangiocytes do[13]. The aim of the current article is to review the GI and hepatic side effects associated with the potential agents for the treatment of COVID-19, focusing particularly on redemsivir, LPV/r and steroids which have shown beneficial effects in the treatment of COVID-19. COVID-19 vaccines are now available in many countries and an increasing number of people are getting vaccinated. We will discuss their side effects and the current views on whether patients with chronic liver diseases (CLD), liver transplantation or inflammatory bowel disease (IBD) should receive the vaccine.

COVID-19 TREATMENTS

The agents used for COVID-19 treatment can be classified according to the type of agents, such as antiviral, antiparasitic, antibacterial and immunomodulatory agents, or according to the site of action on the SARS-CoV-2 virus such as blocking the entry of virus, inhibition of viral replication and anti-inflammatory effect.

Viral entry can be blocked by proteins, peptides, or small molecule compounds that bind to the viral S protein, thereby preventing the virus from interacting with the host membrane. Examples are umifenovir and bromhexine[14].

Inhibitors of viral nucleic acid synthesis are the best represented class of antiviral drugs that suppress viral replication in host cells[15]. Examples include lopinavir-

ritonavir, remdesivir, ribavirin, chloroquine or hydroxychloroquine, favipiravir, nitazoxanide, ivermectin and molnupiravir.

The RNA-dependent RNA polymerase (RdRp) is found in the core of the coronavirus replication machinery, nsp12 protein, and has an important role in the viral life cycle[16]. Inhibition of RdRp is a possible target for therapeutic interventions. Examples of RdRP inhibitors include favipiravir and ribavirin.

Excessive inflammatory responses and cytokine release are found in patients with severe cases of COVID-19. This mechanism contributes to the worsening of the disease and stimulates lung and other systemic injuries. The early modulation of these responses can reduce the risk of acute respiratory distress[17]. Examples of agents that target the inflammatory response include steroids, tocilizumab [an anti-interleukin (IL)-6 monoclonal antibody] and baricitinib. The mechanisms of agents used for the treatment of COVID-19 are shown in Figure 1.

AGENTS AGAINST THE ENTRY OF VIRUS

Umifenovir

Umifenovir is used for the treatment of some enveloped and non-enveloped viral infection. It can also effectively block SARS-CoV-2 entry into cells and inhibits post-entry stages of infection[18]. The efficacy of the drug was assessed in an open-label randomized controlled trial (RCT). One hundred patients were randomly assigned to two treatment groups receiving either hydroxychloroquine followed by LPV/r or hydroxychloroquine followed by umifenovir[19]. The primary outcome was hospitalization duration and clinical improvement 7 d after admission.

Umifenovir significantly improved clinical and laboratory parameters including peripheral oxygen saturation, intensive care unit (ICU) admission rate, duration of hospitalization, white blood cell (WBC), and erythrocyte sedimentation rate when compared with LPV/r. The duration of hospitalization in the umifenovir group was significantly shorter than in the LPV/r arm (7.2 d *vs* 9.6 d; $P = 0.02$)[19].

Nausea, vomiting and liver function test (LFT) derangements are the major GI and hepatic abnormalities that can occur in patients receiving umifenovir. Clinicians should use the drug with caution in those patients with hepatic impairment.

Bromhexine

SARS-CoV-2 invades the human body through the angiotensin-converting enzyme 2 (ACE-2)/transmembrane protease serine 2 (TMPRSS2). In addition to host cell entry, TMPRSS2 is involved in the maturation and release of the virus, which ultimately increase the viral infectivity[20]. Therefore, a possible useful therapeutic approach for COVID-19 is the inhibition of TMPRSS2[21].

Bromhexine has strong inhibitory effect on TMPRSS2 and can be used to block pulmonary virus infection[22]. Therefore, it may exert a protective effect against COVID-19-induced acute lung injury. The effect and safety of bromhexine was assessed in patients with mild or moderate COVID-19 who were randomly assigned to a bromhexine group or a control group at a 2:1 ratio[22]. The primary end points were the time to clinical recovery and the rate of deterioration after initiation of medications.

There were no significant differences in the outcomes between the two treatment groups. The side effects include LFT derangement (38.9%), gingivitis (11.1%), insomnia (11.1%), headache (5.6%), and elevated WBCs in urine (5.6%). However, all side effects were mild and no patient stopped the treatment because of the adverse effects[22].

Another randomized, open-label clinical trial study involving 78 patients was performed to assess the efficacy of bromhexine. Patients were randomized to the bromhexine group or the control group. The primary outcomes were the rate of ICU admissions, intubation and then mechanical ventilation, and 28-d mortality[23]. When compared with the standard treatment group, the bromhexine-treated group showed a significant reduction in ICU admissions (5.1% *vs* 28.2%, $P = 0.006$), intubation (2.6% *vs* 23.1%, $P = 0.007$) and death (0 *vs* 5, $P = 0.027$)[23].

INHIBITORS OF VIRAL REPLICATION

LPV/r

LPV/r is a co-formulation of two structurally related protease inhibitor (PI) antiret-

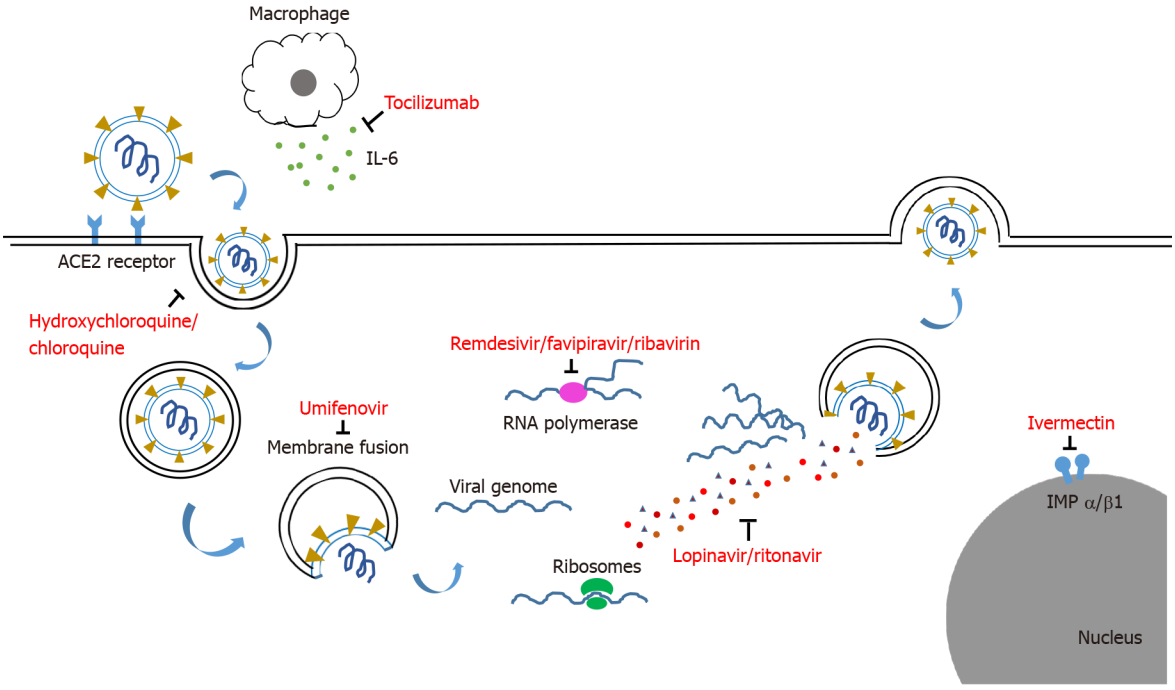

Figure 1 The mechanism of potential treatment of coronavirus disease 2019. ACE: Angiotensin-converting enzyme; IL-6: Interleukin-6.

roviral agents widely used to treat HIV infections[24]. Ritonavir substantially increases the half-life of lopinavir by inhibiting cytochrome P450 (CYP) isoenzyme 3A4[25]. PIs prevent cleavage of gag and gag-pol protein precursors in infected cells, arresting maturation and inhibiting the formation of infectious virions, thereby preventing subsequent waves of infection[26].

Lopinavir demonstrated *in vitro* inhibitory activity against SARS-CoV and Middle East respiratory syndrome coronavirus[27-29]. Addition of LPV/r to ribavirin in treating SARS patients showed a reduction of adverse outcomes [death or development of acute respiratory distress syndrome (ARDS) requiring intensive care] compared to ribavirin alone[30]. Conflicting results of published data have stirred controversy concerning the use of LPV/r in COVID-19 patients. Cao *et al*[31] conducted a RCT in Wuhan, China to assess the efficacy and safety of LPV/r in 199 severe COVID-19 patients. Patients were randomly assigned in a 1:1 ratio to receive either LPV/r (400/100 mg, orally) twice daily or supportive care alone. Treatment with LPV/r was not associated with a difference from standard care in the time to clinical improvement [hazard ratio (HR) for clinical improvement, 1.31; 95% confidence interval (CI): 0.95 to 1.80]. The 28-d mortality rate and the percentages of patients with detectable viral RNA at various time points were similar. In a modified intention-to-treat analysis, which excluded three patients with early death, antiviral treatment shortened the median time to clinical improvement by 1 day compared with standard care (15 d *vs* 16 d, HR, 1.39; 95%CI: 1.00 to 1.91)[31]. Another RCT included 86 patients with mild to moderate disease; the use of LPV/r did not shorten the time of positive-to-negative conversion of SARS-CoV-2 nucleic acid in respiratory specimen, nor symptoms or radiological improvement[32]. On the other hand, Yan *et al*[33] reported data from a retrospective study including 129 non-critically ill patients with COVID-19. They showed that the median duration of SARS-CoV-2 shedding in the LPV/r treatment group was 22 d [interquartile range (IQR) 18-29], which was significantly shorter than in group that did not receive LPV/r treatment (28.5 d, IQR 19.5-38) (log-rank P = 0.009). Subgroup analysis revealed that the administration of LPV/r treatment within 10 d of symptom onset, but not later administration, could shorten the duration of SARS-CoV-2 RNA shedding compared with no LPV/r treatment[33]. Ye *et al*[34] studied the clinical efficacy of LPV/r in 47 patients and showed that patients in the active treatment group returned to normal body temperature in a shorter time compared with the control group (4.8 ± 1.94 d *vs* 7.3 ± 1.53 d, P = 0.0364).

GI adverse events were common in patients receiving LPV/r. The most common GI adverse event in patients receiving LPV/r was diarrhea (occurring in 20% of patients); others included nausea, vomiting abdominal pain and gastroenteritis[35]. In the study by Cao *et al*[31], 14% of patients were unable to complete the full 14-d course of LPV/r

because of GI adverse events (Table 1). In the study by Li *et al*[32], one patient withdrew from the study due to severe diarrhea. Twice-daily dosing of LPV/r is associated with a reduced frequency of moderate to severe diarrhea compared with once daily [36]. The majority of patients who develop diarrhea can be managed conservatively and may not require antidiarrheal treatment[37]. Hypokalemia, secondary to diarrhea or emesis, should be treated according to standard local protocols[38]. If patients develop significant adverse effects, lower dosages of LPV/r (*e.g.*, 200/100 mg twice a day) can be considered, with the understanding that lower doses may not markedly alleviate toxicities[34].

Ritonavir use is associated with a 5-fold higher incidence of severe hepatotoxicity compared with other PIs[39]. Hepatitis including elevation of AST, ALT, and gamma-glutamyl transferase levels has been reported in 3.5% of patients taking LPV/r, according to the package insert[35]. This drug is principally metabolized by the hepatic CYP3A4 isoenzyme[40] and therefore, caution should be exercised when administering this drug to patients with hepatic impairment. Safety data on LPV/r use in patients with cirrhosis do exist[41]. Coinfection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) increases the risk hepatotoxicity and patients with such infections should be monitored closely[42]. Patients with severe liver disease such as cirrhosis or those with significant elevation of liver enzyme were excluded from RCTs [31,32]. Concomitant use of tenofovir with LPV/r is not recommended since this will lead to elevated levels of tenofovir. Physicians may consider switching from tenofovir to entecavir during treatment with LPV/r.

Remdesivir

Remdesivir was initially under clinical development for the treatment of Ebola virus disease[43]. It is a monophosphoramidate prodrug of an adenosine analog, which is then metabolized in cells to an active nucleoside triphosphate that inhibits viral RdRp early in the viral infectious cycle. It has demonstrated antiviral activity against coronavirus including SARS-CoV-2[44-47]. Other potential antiviral mechanisms involve lethal mutagenesis and chain termination[48,49].

Remdesivir was used to treat the first case of COVID-19 infection in the United States[3]. Thereafter, numerous clinical trials focusing on its efficacy and safety have been published. In a multicenter RCT led by Beigel *et al*[50] including 1059 hospitalized patients with evidence of lower respiratory tract involvement, remdesivir was administered intravenously as a 200-mg loading dose on day 1, followed by 100-mg daily on days 2 through 10 or until hospital discharge or death. Patients who received treatment had a shorter time to recovery than patients who received placebo (median 11 d *vs* 15 d; rate ratio for recovery, 1.32; 95%CI: 1.12 to 1.55; $P < 0.001$). Recovery was defined as patients not requiring supplemental oxygen or ongoing medical care except for infection-control reasons. Mortality was numerically lower in the treatment group than the placebo group, but the difference was not significant (HR for death, 0.70; 95%CI: 0.47 to 1.04)[50]. Another RCT from China enrolled 237 patients, but failed to demonstrate a significant difference in the time to clinical improvement with remdesivir in severe patients [21.0 d in remdesivir group *vs* 23.0 d in the control group, HR 1.23 (95%CI: 0.87 to 1.75)][51]. Nevertheless, the results should be interpreted with caution as the power of this study was limited by failure to complete full enrolment due to control of the outbreak in Wuhan.

Several studies have compared the efficacy and safety of 5 d *vs* 10 d of remdesivir treatment in patients with COVID-19[52,53]. Goldman *et al*[52] enrolled 397 COVID-19 patients with evidence of pneumonia and reduced oxygen levels but not requiring mechanical ventilation or extracorporeal membrane oxygenation. Similar clinical improvement was observed in the 5-d group and 10-d group based on assessment on day 14 ($P = 0.14$). The most common GI/hepatic adverse events were nausea (10% in the 5-d group *vs* 9% in the 10-d group), increased ALT (6% *vs* 8%), and constipation (7% in both groups)[52]. Spinner *et al*[53] randomized 596 patients with moderate COVID-19 to a 10-d course of remdesivir, a 5-d course of remdesivir, or standard care in a 1:1:1 ratio. At 11 d after starting treatment, those randomized to the 5-d course of remdesivir had a statistically significant difference in clinical status compared with standard care[53]. However, those receiving the 10-d course of remdesivir did not have a statistically significant difference in clinical outcome compared with standard care. Common side effects included nausea, hypokalemia, and headache. Elevated liver enzymes were observed in one-third of patients, and were of grade ≥ 3 severity in 2% of patients[53].

GI/hepatic adverse events were similar in the treatment and control arms of the two RCTs described above[50,51]. One patient receiving remdesivir developed a hemorrhage of the lower digestive tract and three patients discontinued treatment as a

Table 1 Gastrointestinal adverse events in key studies investigating treatments for coronavirus disease 2019

Ref.	Dosage	*n*	Age, yr	Gender, male (%)	Incidence of adverse events in treatment *vs* control arm, *n* (%)						
					Diarrhea	Vomiting	Abdominal pain	Constipation	Increased AST	Increased ALT	Drug termination due to AE
Lopinavir/ritonavir											
Cao *et al*[31]	400/100 mg twice a day for 14 d	Tx 99; control 100	Median 58 (IQR 49-68)	120 (60.3)	4 (4.2) *vs* 0	6 (6.3) *vs* 0	4 (4.2) *vs* 2 (2.1)	NA	2 (2.1) *vs* 5 (5.1)	1 (1.1) *vs* 4 (4.0)	14%
Li *et al*[32]	200/50 mg, twice a day for 7-14 d	Tx 34; control 17	mean ± SD, 49.4 ± 14.7	40 (46.5)	9/34 (26.5) *vs* 0	NA	NA	NA	NA	1/21 (4.8) *vs* 0	1/34 (2.94)
Remdesivir											
Beigel *et al* [50]	200 mg daily on day 1, followed by 100 mg daily on day 2-10	Tx 538; control 521	mean ± SD, 58.9 ± 15.0	684 (64.3)	NA	NA	NA	NA	15 (2.8) *vs* 20 (3.8)	8 (1.5) *vs* 9 (1.7)	49 (9.1)
Wang *et al* [51]	200 mg daily on day 1, followed by 100 mg daily on day 2-10	Tx 158; control 79	Median (IQR) 65 (56-71)	89 (56)	5 (3) *vs* 2 (3)	4 (3) *vs* 2 (3%)	NA	21 (14) *vs* 12 (15)	7 (5) *vs* 9 (12)	NA	18 (12)
Spinner *et al* [53]	200 mg daily on day 1, followed by 100 mg daily on day 2-5 or day 2-10	193; 193; 200	Median (IQR) 56 (45-66)	118 (61), 114 (60)	5% *vs* 6% *vs* 7%	NA	NA	NA	32 *vs* 32 *vs* 33	32 *vs* 34 *vs* 39	31 (7.8)
Hydroxychloroquine											
Cavalcanti *et al*[70]	400 mg daily	Tx 221; control 227	mean ± SD, 50.3 ± 14.6	388 (55.3)	NA	0 *vs* 1 (0.6)	NA	NA	17 (8.5) *vs* 6 (3.4)	NA	NA
Boulware *et al*[71]	800 mg once, followed by 600 mg	Tx 414; control 407	Median (IQR) 41 (33-51)	196 (47.3)	81 (23.2) *vs* 15 (4.3) for diarrhoea or abdominal pain or vomiting	81 (23.2) *vs* 15 (4.3) for diarrhoea or abdominal pain or vomiting	81 (23.2) *vs* 15 (4.3) for diarrhoea or abdominal pain or vomiting	NA	NA	NA	17 (4.1)
Favipiravir											
Chen *et al* [80]	1600 mg twice a day on day 1, followed by 600 mg twice daily on day 2-10	Tx 116; control 120	NA	59 (50.86)	NA	NA	NA	NA	10 (8.62)	NA	Nil
Nitazoxanide											
Rocco *et al* [82]	500 mg 3 times per day	Tx 194; control 198	18-77	101 (52)	57 (29.4) *vs* 49 (24.7)	9 (4.6) *vs* 3 (1.5)	10 (5.2) *vs* 5 (2.5)	NA	NA	NA	Nil
Tocilizumab											

Stone *et al* [120]	Tocilizumab 8 mg/kg IV inf not to exceed 800 mg	Tx 161; control 82	Median (IQR) 61.6 (46.4-69.7)	96 (60)	NA	NA	NA	NA	6 (3.7) *vs* 3 (3.7) for grade 3 or 4	8 (5.0) *vs* 4 (4.9) for grade 3 or 4	NA

AE: Adverse event; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; inf: Infusion; IQR: Interquartile range; IV: Intravenous; NA: Not available; Tx: Treatment.

result of liver enzyme elevation in the study by Wang *et al*[51]. No serious grade 3 or 4 liver dysfunction was reported in either arm[51].

GI and hepatic adverse events have also been reported in case series of patients receiving remdesivir. In a remdesivir compassionate use program (n = 53), 12 patients (23%) developed elevated hepatic enzymes, and 5 (9%) had diarrhea[54]. Two patients (3.8%) discontinued remdesivir prematurely because of elevated aminotransferases [54]. In another case series in 35 patients who received compassionate remdesivir treatment in Italy, hepatotoxicity was the most frequent adverse event, with a grade 3 to 4 increase in transaminase levels observed in 42.8% of the patients[55]. In the first 12 COVID-19 patients in United States, all 3 patients who received remdesivir experienced transient transaminitis and GI symptoms including nausea, vomiting, gastroparesis or rectal bleeding[56]. Another case series of critically ill patients receiving remdesivir in Italy reported that three of these four patients had elevated ALT and AST levels, ranging from 5 times to 8 times the upper limit of normal[57].

Hepatic adverse events are not unexpected with nucleoside analogues; these agents can cause direct hepatotoxicity by inducing mitochondrial dysfunction and/or idiosyncratic hepatotoxicity *via* an acute hypersensitivity reaction or the production of toxic intermediates[58]. Asymptomatic grade 1 or 2 ALT elevations were observed in healthy individuals who received remdesivir in phase 1 studies[59]. Pharmacokinetic studies in patients with hepatic impairment were limited, but remdesivir should be used with caution in patients with existing liver disease, and only if the potential benefit outweighs the risk[60]. Regular monitoring of liver function should be performed if possible[61].

Hydroxychloroquine/chloroquine ± azithromycin

Hydroxychloroquine/chloroquine are drugs commonly used in the management of rheumatoid arthritis, systemic lupus erythematosus and malaria. SARS-CoV-2 enters cells by binding to the ACE-2 receptor. Chloroquine may inhibit terminal glycosylation, thus preventing the virus from binding to the ACE-2 receptor[62]. Hydroxychloroquine prevents SARS-CoV-2 from binding to gangliosides which in turn prevents the virion from engaging with the ACE-2 receptor[63].

The use of hydroxychloroquine/chloroquine in the treatment of COVID-19 is controversial[64-71]. A multicenter, RCT was conducted in 504 hospitalized patients with COVID-19 who were receiving either no supplemental oxygen or a maximum of 4 L/min of supplemental oxygen. Patients were randomly assigned in a 1:1:1 ratio to receive standard care, standard care plus hydroxychloroquine 400 mg twice daily, or

standard care plus hydroxychloroquine 400 mg twice daily and azithromycin 500 mg once daily for 7 d[70]. Active treatment had no effect on patients' clinical status at 15 d compared with standard care. The proportional odds of having a higher score on the seven-point ordinal scale at 15 d was not increased by either hydroxychloroquine alone [odds ratio (OR) 1.21; 95%CI: 0.69 to 2.11; P = 1.00] or hydroxychloroquine plus azithromycin (OR, 0.99; 95%CI: 0.57 to 1.73; P = 1.00). In addition, a higher proportion of patients receiving hydroxychloroquine alone (8.5%) or with azithromycin (10.9%) developed elevated liver enzymes compared those who did not receive either agent (3.4%)[70]. Further randomized studies are needed to clarify the efficacy of hydroxychloroquine or chloroquine in the treatment of COVID-19.

These drugs also have a number of side effects. Apart from the well-known arrhythmogenic cardiotoxicity of the drugs, the most common adverse events of hydroxychloroquine and chloroquine are GI, including GI upset, nausea, vomiting, diarrhea, abdominal cramps, and a metallic taste[72-74]. In a study evaluating the use of chloroquine, nearly 24% of patients suffered from nausea or abdominal cramps and 17% reported diarrhea as side effects[75]. Up to 50% of patients receiving hydroxychloroquine in another study reported some GI side effects; the frequency was dose-dependent with GI events occurring more commonly with loading doses of 800 mg or higher[76].

Chloroquine and hydroxychloroquine should be administered with food to reduce nausea and vomiting. At the same time, chloroquine can be crushed and mixed with flavored syrups to mask the bitter taste. It is also recommended to avoid taking antacids within 4 h of chloroquine because of a potential for chelation and reduced bioavailability, but this drug interaction does not occur with hydroxychloroquine.

Azithromycin is a semisynthetic macrolide antibiotic that is commonly prescribed to treat infections with Gram-positive, Gram-negative and atypical pathogens. It has been used for the treatment of COVID-19 in combination with hydroxychloroquine or chloroquine and has produced synergistic effects in the context of combination therapy[77]. Azithromycin may cause GI side effects such as nausea and vomiting.

Ribavirin

Ribavirin is a guanine derivative used for the treatment of respiratory syncytial virus and HCV infections. It has been used in combination with other agents for the treatment of COVID-19[78]. In a prospective study of patients with mild to moderate COVID-19, the combination of interferon-beta, oral LPV/r and ribavirin produced a significantly shorter median time from start of study treatment to negative nasopharyngeal swab compared with LPV/r alone[78]. Patients in the combination group also had earlier relief of symptoms compared with the control group (4 d *vs* 8 d, P < 0.0001). This study suggests that combination therapy is more potent than single-agent antiviral therapy against COVID-19[78].

The common side effects observed in the combination therapy group included diarrhea (40%), fever (37%), nausea (35%) and elevated ALT levels (13%)[78]. Since CYP enzymes are not involved in the metabolism and elimination of ribavirin, there is minimal potential for drug-drug interactions.

Favipiravir

Favipiravir is an RdRp inhibitor[79]. Once inside cells, favipiravir is converted into an active phosphoribosylated form, which acts as a substrate for viral RNA polymerase, and then inhibits RNA polymerase activity. It is a broad-spectrum antiviral drug approved in Japan for the treatment of influenza. It has also been used for the treatment of Ebola and Lassa virus infection.

Chen *et al*[80] conducted a prospective, randomized, open-label multicenter clinical trial involving 240 adult patients with COVID-19 comparing the efficacy and safety of favipiravir *vs* umifenovir. The clinical recovery rate on day 7 was better in the favipiravir arm than in the umifenovir arm (71.43% *vs* 55.86%, P = 0.01). Favipiravir significantly shortened the latency to relief for pyrexia and cough compared with umifenovir, and dyspnea was significantly (P = 0.017) less common in the favipiravir group than in the umifenovir group. Deranged LFT is a common side effect of favipiravir and was found in 8.6% of patients.

Cai *et al*[81] conducted an open-label study in 80 patients with mild to moderate COVID-19 and assessed the effects of favipiravir in comparison with LPV/r for the treatment of COVID-19. Favipiravir was shown to have shorter viral clearance time (median 4 d *vs* 11 d). In addition, a higher proportion of patients in the favipiravir than the LPV/r groups showed improvement in chest imaging (91.43% *vs* 62.22%; P = 0.004), particularly in the group with viral clearance within 7 d of starting treatment. Multivariable Cox regression showed that favipiravir was significantly (P = 0.026)

associated with faster viral clearance[81].

The most common side effects of favipiravir were liver enzyme abnormalities, GI symptoms like diarrhea, and serum uric acid elevations. We would be cautious about prescribing favipiravir in patients with abnormal LFT results.

Nitazoxanide

Nitazoxanide is an antiparasitic prodrug with antiviral properties that is approved by the U.S. Food and Drug Administration (FDA). The effects of nitazoxanide against COVID-19 were examined in a multicenter, randomized, double-blind, placebo-controlled trial recruiting 392 patients presenting up to 3 d after onset of symptoms including fever, dry cough, and/or fatigue. The patients were randomized in a 1:1 ratio to receive either nitazoxanide 500 mg 3 times/d or matching placebo for 5 d after the diagnosis of SARS-CoV2 infection was made by reverse transcription polymerase chain reaction (RT-PCR) on a nasopharyngeal sample[82].

Although there was no difference between the nitazoxanide and placebo groups in the resolution of symptoms at the 5-d study visit, a significantly higher proportion of patients in the nitazoxanide group (29.9%) returned a negative PCR result for SARS-CoV-2 compared with the placebo group (18.2%; $P = 0.009$). There was also significantly greater reduction in viral load between the start and end of therapy in patients receiving nitazoxanide (55%) compared with placebo (45%; $P = 0.013$). GI side effects included nausea (14.4%), vomiting (4.6%), diarrhea (29.4%), and abdominal pain (5.2%) were reported in patients receiving nitazoxanide in the study[82].

Ivermectin

Ivermectin is an antiparasitic drug and was found to have a broad range of antiviral activity against many RNA and DNA viruses *in vitro*. It was also shown to be highly effective *in vitro* against SARS-CoV-2[83].

It was shown that the combined use of ivermectin, nitazoxanide and ribavirin plus zinc supplement achieved better clearance of the SARS-COV2 from the nasopharynx in a shorter time than symptomatic therapy in a non-RCT[84]. The viral clearance rates on the 7th day were 0% and 58.1%, respectively, in the groups receiving supportive treatment and combined antiviral therapy, and were 13.7% and 73.1%, respectively, on the 15th day. The corresponding cumulative viral clearance rates on the 15th day were 13.7% and 88.7%, respectively. Overall, 11.3% of patients had elevation of LFTs and 22.6% of developed GI upset during the study period.

Rajter *et al*[85] performed a retrospective study of 280 COVID-19 patients to assess the efficacy of ivermectin, in which 173 had been treated with ivermectin and 107 had not. Most patients in both groups also received hydroxychloroquine, azithromycin, or both. Mortality was significantly lower in the ivermectin group (13.3% *vs* 24.5%; $P < 0.05$). Mortality was also lower among ivermectin-treated patients with severe pulmonary involvement (38.8% *vs* 80.7%; $P = 0.001$). Eleven percent of phas a broad range of antiviral activity against many RNA and DNA viruses *in vitro* has a broad range of antiviral activity against many RNA and DNA viruses *in vitro*. Ivermectin has a broad range of antiviral activity against many RNA and DNA viruses *in vitro*.

Molnupiravir

Molnupiravir is an oral, direct-acting antiviral agent which was shown to be highly effective in reducing nasopharyngeal SARS-CoV-2 infectious virus and viral RNA. It is well absorbed after oral administration. Fischer *et al*[86] randomized 202 patients to molnupiravir (200, 400 or 800 mg) or placebo twice-daily for 5 d. Antiviral activity was assessed as time to undetectable levels of viral RNA by RT-PCR and time to elimination of infectious virus isolation from nasopharyngeal swabs.

The results showed a significant reduction in virus isolation in participants receiving 800 mg molnupiravir (1.9%) *vs* placebo (16.7%) at day 3 ($P = 0.02$). Virus was not isolated from any patient receiving 400 mg or 800 mg molnupiravir while 11.1% of patients receiving placebo had virus isolated at day 5 ($P = 0.03$).

There was decrease in the time to viral RNA clearance in patients given 800 mg molnupiravir compared with placebo (14 d *vs* 27 d, $P = 0.001$). There was also a higher rate of overall clearance in patients receiving molnupiravir. The side effects of molnupiravir include headache, insomnia, and increased ALT. We would be cautious using molnupiravir in patient with hepatic dysfunction.

Immunomodulatory agents

Cytokine storm is an important pathogenic process in COVID-19 patients[87]. SARS-CoV-2 binds to the toll-like receptor, activating the nuclear factor (NF)-κB pathway

and pro-inflammatory cytokines[88]. Cytokines are signalling molecules that recruit immune cells to the site of inflammation, induce vascular leakage and exudation, and stimulate the generation of free radicals and proteases[89]. Pro-inflammatory cytokines induce alveolar injury and reduced alveolar fluid clearance resulted in ARDS[90]. Compared with mild or moderate cases, patients with severe COVID-19 have higher levels of circulating IL-2, IL-6, IL-7, IL-10, interferon gamma, granulocyte colony stimulating factor, interferon-inducible protein 10, monocyte chemoattractant peptide , macrophage inflammatory protein-1A, and tumor necrosis factor (TNF)-α[7, 91-93]. This raises the possibility of using immunomodulatory agents to control the inflammatory response, and thereby improve the prognosis of COVID-19[94].

Corticosteroids

Corticosteroids inhibit NF-κB signalling and various pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, TNF-α, and IL-17. It also reduces the proliferation, activation, differentiation, and survival of T cells and macrophages[95]. Steroids may play a protective role in the respiratory and digestive systems by activating ACE-2 and suppressing the cytokine storm, in particular reducing IL-6 levels, in patients with severe or critical COVID-19[96]. Corticosteroids were used in early reports from Wuhan, China, where they were used in an attempt to reduce inflammation-induced lung injury[90].

Dexamethasone is the first treatment that has been shown to reduce mortality in severely ill COVID-19 patients[97,98]. The randomized evaluation of COVID-19 therapy (RECOVERY) trial compared 2104 patients receiving oral or intravenous dexamethasone (at a dose of 6 mg once daily) for up to 10 d with 4321 patients receiving usual care alone. The 28-d mortality rate was lower in the group receiving dexamethasone compared with usual care group in patients who were receiving invasive mechanical ventilation (29.3% *vs* 41.4%; rate ratio, 0.64; 95%CI: 0.51 to 0.81) or receiving oxygen without invasive mechanical ventilation (23.3% *vs* 26.2%; rate ratio, 0.82; 95%CI: 0.72 to 0.94). No survival benefit was seen among those who were receiving no respiratory support at randomization. Dexamethasone also reduced mortality in patients with symptoms for more than 7 d but not in those with more recent symptom onset[97].

The positive impact of steroids was confirmed in a prospective meta-analysis of seven clinical trials involving 1703 critically ill patients with COVID-19 conducted in 12 countries[99]. The meta-analysis showed that the use of systemic corticosteroids reduced all-cause 28-d mortality compared with usual care or placebo. The number of deaths was 222 in those receiving corticosteroids compared to 425 deaths in the usual care or placebo group. Dexamethasone could significantly suppress the odds of all-cause mortality.

The preliminary report of the RECOVERY study did not describe side effects. Previously reported side effects of steroids include hyperglycemia, hypokalemia, delayed viral clearance, risk of secondary bacterial infection, psychosis and avascular osteonecrosis[100-104]. Corticosteroids may induce various GI adverse events such as gastritis, peptic ulcer formation and GI bleeding, with the risk of bleeding significantly increased by concomitant non-steroidal anti-inflammatory drug use[105,106]. Direct SARS-CoV-2 invasion of the GI tract, causing erosion and ulcers in severe patients, may increase the risk further[1]. Prophylactic proton pump inhibitors should be considered in patients who receive dexamethasone[107].

Steroids increase the risk of acute pancreatitis by an unknown mechanism[108]. Steroids activate triglyceride synthesis and accumulation, increase fatty acid uptake and inhibit fatty acid beta-oxidation in the liver, while they also increase lipolysis, lipogenesis and the secretion of non-esterified fatty acids and adipokines in adipose tissue, which results in hepatic steatosis[109]. Diabetes and obesity are associated with the development of non-alcoholic fatty liver disease[110]. These metabolic risk factors may result in deleterious effects on host immunity, and are closely related to disease severity and mortality in patients with COVID-19[111-115]. Regular monitoring of liver function and glucose level is recommended for this high-risk group of patients receiving dexamethasone.

Tocilizumab

COVID-19 can trigger aggressive an inflammatory response resulting in cytokine release syndrome (CRS), which is associated with an unfavorable prognosis[116]. A meta-analysis of 6 studies including 1302 patients demonstrated 2.9-fold higher levels of IL-6 in patients with complicated COVID-19 compared with patients with non-complicated disease[117]. IL-6 is an important cytokine responsible for an inflammatory storm that leads to impaired oxygen diffusion in the lungs[7]. Tocilizumab is a

recombinant humanized monoclonal antibody against the IL-6 receptor and reduces the effects of CRS. This led to speculation that it could be used in the treatment of COVID-19, especially in severe patients with high IL-6 levels.

A retrospective, observational cohort study was carried out to investigate mortality in 544 patients with severe COVID-19 requiring support in the ICU; 179 patients received tocilizumab and 365 patients received standard care. There was an improvement in median overall survival from time of hospital admission in patients receiving tocilizumab when compared with the standard care cohort (20% *vs* 7%; $P < 0.001$)[118].

Another multicenter retrospective cohort study investigated outcomes in 4485 adults with COVID-19 admitted to ICU in 68 hospitals. Among critically ill patients, the risk of in-hospital mortality was lower in patients treated with tocilizumab in the first 2 d of ICU admission compared with patients whose early treatment did not include tocilizumab (HR, 0.71; 95%CI: 0.56 to 0.92)[119].

However, similar favorable results were not seen in a RCT involving 243 patients with hyperinflammatory states. Tocilizumab was not shown to be effective enough to prevent intubation or death in moderately ill, hospitalized COVID-19 patients in this trial[120]. Further research in RCTs is needed.

Reports have emerged of liver injury with an increase in transaminase levels associated with tocilizumab use in COVID-19 patients[121], and increases in liver enzyme levels were seen in 5% of patients in one of the cohort studies described above and in 1% of patients in the RCT[118,120]. In the cohort study by Gupta *et al*[119], 16.6% of patients receiving tocilizumab developed an AST of more than 250 U/L and 8.5% developed an ALT level of more than 500 U/L. Tocilizumab can interfere with serum concentrations of CYP3A4 substrates. It should be used with caution and liver function regularly monitored, especially when used in combination with another hepatotoxic drug or in patients receiving multiple concomitant medications.

Baricitinib

Baricitinib is a selective inhibitor of Janus kinase (JAK) 1 and 2, and orally administered. It was originally developed for the treatment of rheumatoid arthritis. Inhibition of JAK blocks intracellular signal transmission from cytokine or growth factor receptors and leads to reduced hematopoiesis[17]. This inhibition of signal transmission prevents phosphorylation and then activation of signal transducers and activators of transcription.

Baricitinib was used in combination with remdesivir in a RCT involving 1033 patients with COVID-19. The rationale for combining these two therapies is that clinical outcomes would be improved by reducing the immune response and preventing a hyperinflammatory state[122]. The combination was found to be significantly better than remdesivir alone in reducing recovery time and accelerating clinical improvement in patients with COVID-19. This effect was more marked in patients receiving high-flow oxygen or non-invasive ventilation. The time to recovery was 10 d in patients who received combination treatment compared with 18 d in patients who received remdesivir alone. The 28-d mortality was 5.1% in the combination group and 7.8% in the control group (HR for death, 0.65; 95%CI: 0.39 to 1.09).

The combination was associated with fewer serious adverse events. Transaminases increased in 1.2% of patients receiving combination therapy and 2% of patients receiving remdesivir, and bilirubin increased in 0.4% and 1.6%, respectively. Regular monitoring of liver function is recommended, especially when used in combination with remdesivir.

A summary of the side effects of the potential treatments for COVID-19 is shown in Table 2.

COVID-19 VACCINES AND LIVER AND GI DISEASES

Vaccination is an important method to protect the population from COVID-19 and is likely to be especially important in high-risk individuals, such as those with pre-existing health conditions. A minimum vaccine efficacy of 50% is necessary to get regulatory approval from the World Health Organization (WHO). Patients with chronic diseases have a higher mortality when they get infected with COVID-19. Therefore, this group of patients will benefit more from the vaccination. However, the phase 1-3 studies of the COVID-19 vaccines mainly recruited healthy individuals, so data are limited in patients with chronic diseases. The decision to be vaccinated may

Table 2 Gastrointestinal and hepatic side effects of potential treatments for coronavirus disease 2019

Drug name	Gastrointestinal and hepatic side effects
Remdesivir	Elevation of liver enzymes
Lopinavir-ritonavir	Nausea, vomiting, abdominal pain, gastroenteritis
Hydroxychloroquine/chloroquine	Nausea, vomiting, abdominal pain, diarrhea
Steroids	Epigastric pain, peptic ulcer, risk of HBV reactivation
Interferon	Diarrhea, nausea, elevated alanine aminotransferase level
Ribavirin	Elevated liver enzyme levels
Umifenovir	Nausea, vomiting and deranged liver function
Bromhexine	Deranged liver function
Favipiravir	Diarrhoea, liver enzyme abnormalities
Nitazoxanide	Nausea, vomiting, diarrhoea and abdominal pain
Imervectin	Elevation of liver enzymes
Molnupiravir	Elevated alanine aminotransferase
Tocilizumab	Liver dysfunction
Baricitinib	Nausea, liver dysfunction
Azithromycin	Nausea, vomiting

also depend on the stability of the patient's chronic illness and the prevalence of COVID-19 in the relevant country or region.

TYPES OF VACCINES

Different technologies were applied to the development of the vaccines including mRNA, viral vectors, inactivated viruses, recombinant DNA, protein subunits and live attenuated viruses.

The BNT162b2 mRNA vaccine (manufactured by Pfizer BioNTec) and the mRNA-1273 mRNA vaccine (manufactured by Moderna-NIH) was developed based on mRNAs that encode variants of the SARS-CoV-2 spike glycoprotein and are encapsulated into lipid nanoparticles[123-125]. The ChAdOx1 nCoV-19 vaccine (manufactured by AstraZeneca) uses an adenoviral vector and is approved by the WHO is currently being used in Europe, the United States and many other countries [126]. Another WHO-approved COVID-19 vaccine is Ad26.COV2.S, developed by Janssen (Johnson & Johnson); this is a single-dose viral vector vaccine based on a human adenovirus that has been modified to contain the gene for making the spike protein of the SARS-CoV-2 virus[127]. However, the use of this vaccine was stopped by the WHO because of the risk of thrombotic complications.

The two mRNA vaccines described above got the earliest approval from the WHO and are now being used, but these vaccines must be stored in very low temperature freezers. Common acute side effects of the vaccines include myalgia, fatigue, low-grade fever, headache, nausea and redness or soreness at the injection site. There do not appear to be many GI and hepatic side effects.

BNT162b2 was chosen by Pfizer/BioNTec as the most promising of two potential mRNA vaccine candidates based on safety and immunogenicity data from phase I studies in younger and older adults[123]. A two-dose regimen of BNT162b2 confirmed a 95% protection rate against COVID-19 in persons 16 years of age or older. The side effect profile was characterized mainly by fatigue, mild to moderate pain at the injection site, and headache[124].

A phase III study of the mRNA-1273 vaccine was carried out in 30420 healthy individuals aged 18 or above randomly assigned in a 1:1 ratio to receive either vaccine or placebo. It showed an efficacy of 94.1% at preventing COVID-19 illness, including severe disease[125]. There were no major safety concerns apart from transient local and systemic reactions.

The third approved vaccine is ChAdOx1 nCoV-19 vaccine (AZD1222) which was developed at Oxford University. It consists of a replication-deficient chimpanzee adenoviral vector ChAdOx1 which contains the SARS-CoV-2 structural surface glycoprotein antigen (spike protein; nCoV-19) gene. After receiving two standard doses of vaccine, the efficacy of the vaccine was 62.1% *vs* 1.6% of 4455 participants in the control group[126].

Recently, however, safety concerns have emerged about the thrombotic risk associated with the vaccine. A pathogenic PF4-dependent syndrome, which was unrelated to the use of heparin, was identified after the administration of the vaccine [128]. Clinicians should pay particular attention to individuals with thrombotic risk factors.

The fifth vaccine is an inactivated vaccine developed by Sinovac Life Sciences and is being used in some countries. CoronaVac was well tolerated and induced humoral responses against SARS-CoV-2, and it was approved for emergency use in China and some other countries and regions. Efficacy and safety were demonstrated in two phase I/II double-blind, placebo-controlled RCTs in healthy adults aged 18-59 years and 60 years or older[129,130]. A phase III, randomized, multicenter, double-blind, placebo-controlled clinical study is being carried out to assess the efficacy and safety of the adsorbed vaccine COVID-19 (inactivated) produced by Sinovac in two age groups: 18 years to 59 years and 60 years or more[131].

Another vaccine, Sinopharm, which is an inactivated vaccine developed in China, has been approved and used in some countries and regions. It showed promising results in phase I/II trials[132]. The phase III trial data will provide more information on the safety, efficacy and immunogenicity of the vaccine. A summary of the available COVID-19 vaccines is shown in Table 3. There are ongoing studies for these and other vaccines and more choices will become available over time.

COVID-19 VACCINES AND CLD

Patients with CLD, liver cirrhosis, hepatobiliary malignancies, and candidates for liver transplantation are at higher risk of COVID-19 infections. At the same time, these groups of patients have a lower immune response to vaccines.

The benefits and risks of vaccination for patients with chronic disease or immunocompromised patients should be weighed individually, taking into account the incidence of the infection in the country or community, the vaccine formulation, the type of immunosuppressive therapy (*e.g.*, chemotherapy, transplantation) the patient is receiving, and the extent of their immunosuppression.

There is a reduction of immune memory against and immune responses to certain vaccines as patients age and their CLD progresses[133]. Moreover, patients with alcohol-associated liver disease, CLD and cirrhosis may have an impaired immune response to vaccination. At the same time, they are more susceptible to infections and infection-related complications[134].

Patients with immunosuppressive conditions or liver diseases were usually excluded from the studies of the COVID-19 vaccines. A post-marketing study in a nationwide mass vaccination setting in Israel suggests that the BNT162b2 mRNA vaccine is effective for a wide range of COVID-19-related outcomes, a finding consistent with that of the randomized trial[135]. All persons who were newly vaccinated were matched to unvaccinated controls in a 1:1 ratio according to demographic and clinical characteristics. Each study group included 596618 persons, and the vaccinated population included 9699 (1.6%) patients with liver disease and 435 (0.1%) patients with solid organ transplantation[135].

There are currently limited published data on specific patient subgroups. Investigators have performed subgroup analyses, each time restricting the matching process to persons with a specific condition of interest, in order to maximize the sample size [136]. The results on the subgroup with CLD are not yet known.

Patients with CLD infected with SARS-CoV-2 infection have higher risk of adverse outcome than the general population. There are on-going trials in patients with liver diseases worldwide and the results are pending[137].

In view of the high rate of complications and decompensation caused by COVID 19 in CLD, we recommend SARS-CoV-2 vaccination in patients with CLD, and in candidates for liver transplantation, with prioritization of patients with risk factors for severe COVID-19.

In general, professional bodies like the European Association for the Study of the Liver and the American Association for the Study of Liver Disease recommend

Table 3 Summary of the ata for the currently used coronavirus disease 2019 vaccines

Vaccine	Mechanism	Number of participants	Efficacy
mRNA-1273 (Moderna)[125]	RNA (embedded in lipid nanoparticles)encodes a variant of the SARS-CoV-2 spike protein	30420 participants (randomized 1:1 vaccine *vs* placebo)	Efficacy 94.1% (11 vaccinated *vs* 185 controls with COVID-19)
BNT162b2 (BioNTech and Pfizer)[124]	RNA (embedded in lipid nanoparticles) encodes a variant of the SARS-CoV-2 spike protein	43548 participants (randomized 1:1 vaccine *vs* placebo)	Efficacy 95% (9 vaccinated *vs* 169 controls with COVID-19)
ChAdOx1 nCoV-19 (AZD122; AstraZenenca and University of Oxford)[126]	Replication-deficient chimpanzee adenovirus vector, containing the full-length codon-optimized coding sequence of SARS-CoV-2 spike protein	23848 participants (randomized 1:1 vaccine *vs* placebo)	Efficacy 70.4% [30 (0.5%) of 5807 vaccine recipients *vs* 101 (1.7%) of 5829 controls with COVID-19]
CoronaVac (Sinovac Life Sciences, Beijing, China) [129,131]	Inactivated vaccine candidate against COVID-19	600 participants	Seroconversion was seen in 114 (97%) of 117 in the 3 μg group, 118 (100%) of 118 in the 6 μg group, and none (0%) of 59 in the placebo group
Sinopharm vaccine[132]	Inactivated vaccine candidate against COVID-19	448 participants	Neutralizing antibodies were detected in 100% of recipients

COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

COVID-19 vaccination for patients with CLD as the benefits likely outweigh the risks [138,139].

Rituximab may be used for the treatment of CLD such as autoimmune hepatitis and its efficacy is shown in a recent retrospective study[140]. There is usually a blunted vaccine response after vaccination in patients with lymphoma[141-144] or autoimmune disorders[145-148] treated with rituximab. B cells are required for the development of humoral immune responses to neoantigens. Therefore, depletion of B cells following rituximab will likely reduce the humoral immune responses to the COVID-19 vaccine. Both T cell-dependent and -independent responses are also significantly impaired for at least 6 mo after rituximab treatment[148].

Assuming that immunological response to the COVID-19 vaccine correlates with disease protection, it is recommended that vaccination be performed at least 6 mo after rituximab infusion.

EFFICACY AND SAFETY OF VACCINES IN SOLID ORGAN TRANSPLANT RECIPIENTS

Solid organ transplant (SOT) recipients are on immunosuppression to prevent graft rejection, so they are at a higher risk of infection and infective complications. Vaccination is useful to prevent infections and the associated complications in transplant recipients.

COVID-19 vaccination is recommended for all SOT recipients including liver transplant recipients, and vaccination can be given 3-6 mo after SOT. Since the current approved vaccines do not contain live or attenuated virus, they are likely to be safe in immunosuppressed patients[139,149].

The immunogenicity of vaccines in SOT recipients is lower than in immunocompetent individuals because of the immunosuppressive therapy and the underlying chronic disease. Therefore, vaccination against COVID-19 is recommended for family members and healthcare professionals caring for these patients to reduce exposure to SARS-CoV-2[138].

COVID-19 VACCINE AND IBD

IBD is an umbrella term for the immune-mediated inflammatory conditions of Crohn's disease and ulcerative colitis.

IBD patients may receive immunosuppressive drugs such as high-dose corticosteroids, immunomodulators (thiopurines, methotrexate, and calcineurin inhibitors), anticytokine therapies (including anti-TNF and anti-IL-12p40 biologics), anti-integrin

therapies (vedolizumab), and small-molecule inhibitors of signalling (tofacitinib), which could leave them susceptible to infection.

Immunosuppressive drugs may reduce the humoral response to vaccines and thus their effectiveness, which could have major implications for the safety of immunosuppressed patients in the COVID-19 era. The risks associated with current COVID-19 vaccines are low, and guidelines recommend vaccination for patients with IBD[150, 151].

COVID-19 vaccination is also advocated for IBD patients younger than 16 years. Although pediatric patients may experience milder illness if they get infected by SARS-CoV-2[152,153], they can be the source of ongoing outbreaks and transmission [154]. The cessation of the COVID-19 pandemic relies on maximal community uptake of the COVID-19 vaccine in order to achieve herd immunity. On May 10, 2021, the U.S. FDA expanded the Emergency Use Authorization for the BNT162b2 mRNA vaccine to include people aged 12 years to 15 years[155]. This is based on the results of an RCT enrolling 2260 adolescents (12-15-year-old) who were randomized 1:1 to receive the BNT162b2 or placebo[156]. In 7 d after the second dose of BNT162b2, there were zero new case of COVID-19, translating into 100% vaccine efficacy, while there were 16 confirmed cases in the placebo group. Vaccinated adolescents 12- to 15-year-old had higher geometric mean titers of SARS-CoV-2 neutralizing antibodies (1239.5 *vs* 705.1) compared with recipients aged 16 years to 25 years. A favorable safety and side effect profile, similar to other age groups, was also demonstrated in the 12- to 15-year-old recipients of BNT162b2[156].

The use of COVID-19 vaccines is not recommended in pregnant women and there are no safety data of the vaccines in these women to date.

Another point to consider is that patients with IBD are at risk of thromboembolic complications, and COVID-19 increases the risk of thromboembolic events. Studies have shown that prophylactic anticoagulation can reduce the 30-d mortality risk in patients with COVID-19[157].

RECOMMENDATIONS

COVID-19 is a pandemic infection with a high burden of morbidity and mortality. Various drugs are under investigation for the treatment of the disease, but many are associated with GI and hepatic side effects. Caution and careful monitoring should be exercised when prescribing these therapies in patients with GI symptoms like diarrhea and vomiting. As liver impairment is a common observation among patients with COVID-19, we recommend that all patients with COVID-19 and liver impairment undergo investigations for potential causes of liver disease, including viral hepatitis serology, particularly in areas where HBV is prevalent.

Furthermore, increasing rates of liver dysfunction have been correlated with the severity of COVID-19[158]. We need to maintain a high index of suspicion as hepatotoxic drug effects may be difficult to detect in this condition.

High-dose corticosteroids and tocilizumab have been used for the treatment of patients with severe COVID-19. There is a risk of HBV reactivation, hepatitis flare, and even acute liver failure in patients with chronic HBV infection receiving this regimen. Screening for HBsAg is recommended, and antiviral prophylaxis with nucleoside analogs should be given to patients with COVID-19 who are positive for HBsAg during steroid therapy.

COVID-19 vaccines have been rapidly developed. Patients with CLD or IBD and liver transplant recipients are encouraged to receive vaccination. The benefits will outweigh the risks.

Vaccination against COVID-19 is also recommended for family members and healthcare professionals caring for these patients to reduce exposure to SARS-CoV-2. The vaccination against COVID-19 is encouraged for all individuals at risk of SARS-CoV-2 infection, including those with underlying chronic diseases. Recommendations by professional bodies, governments and health authorities will be important driver of COVID-19 vaccination[159].

CONCLUSION

Extensive research has been performed to identify potential treatments for SARS-CoV-2 infection. GI symptoms and liver dysfunction in COVID-19 patients could be due to disease manifestations or treatment side effects, which physicians should take into

consideration when choosing the best therapeutic strategy. The development of effective and safe vaccines is the light at the end of the tunnel to end the pandemic and should be encouraged, including for patients with CLD, IBD, liver transplant recipients their family members, and healthcare professionals.

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REVIEW

Genotype E: The neglected genotype of hepatitis B virus

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Abstract

Hepatitis B virus (HBV) (sub)genotypes A1, D3 and E circulate in sub-Saharan Africa, the region with one of the highest incidences of HBV-associated hepatocellular carcinoma globally. Although genotype E was identified more than 20 years ago, and is the most widespread genotype in Africa, it has not been extensively studied. The current knowledge status and gaps in its origin and evolution, natural history of infection, disease progression, response to antiviral therapy and vaccination are discussed. Genotype E is an African genotype, with unique molecular characteristics that is found mainly in Western and Central Africa and rarely outside Africa except in individuals of African descent. The low prevalence of this genotype in the African descendant populations in the New World, phylogeographic analyses, the low genetic diversity and evidence of remnants of genotype E in ancient HBV samples suggests the relatively recent re-introduction into the population. There is scarcity of information on the clinical and virological characteristics of genotype E-infected patients, disease progression and outcomes and efficacy of anti-HBV drugs. Individuals infected with genotype E have been characterised with high hepatitis B e antigen-positivity and high viral load with a lower end of treatment response to interferon-alpha. A minority of genotype E-infected participants have been included in studies in which treatment response was monitored. Of concern is that current guidelines do not consider patients infected with genotype E. Thus, there is an urgent need for further large-scale investigations into genotype E, the neglected genotype of HBV.

Key Words: Hepatitis B virus; Genotype E; Evolution; Clinical significance; Antiviral therapy; Vaccination

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Core Tip: Although genotype E was identified more than 20 years ago, and is the most widespread genotype in Africa, it has not been extensively studied. The current knowledge status and gaps in its origin and evolution, natural history of infection, disease progression, response to antiviral therapy and vaccination discussed in this review highlight the urgent need for further more in-depth and large-scale investigations into genotype E, the neglected genotype of hepatitis B virus.

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INTRODUCTION

Hepatitis B virus (HBV), a common cause of liver disease, is the prototype member of the family *Hepadnaviridae*. Despite the availability of vaccines, HBV infection remains a public health concern causing high morbidity and mortality rates, as a result of the serious clinical consequences of cirrhosis and hepatocellular carcinoma (HCC)[1]. It is estimated that a third of the world's population is or has been infected with HBV at some point in their lives[1]. As a result of its unusual mechanism of replication by reverse transcription through an RNA intermediate, and lack of proof reading ability of its viral polymerase[2], HBV displays sequence heterogeneity, which leads to the existence of at least 9 genotypes. Four genotypes, A to D, were recognized initially, with genotypes E to I being recognized subsequently[3]. A putative 10th genotype J, has been proposed[4]. All genotypes, except E and G, are further subdivided into subgenotypes. Most HBV genotypes and, in some cases subgenotypes have a distinct geographical distribution. HBV genotypes A and D have global distributions while genotypes B and C are predominantly found in East and Southeast Asia. Genotype E is found in West and Central Africa, genotypes F and H are found among various population groups, including indigenous peoples in Central and South America[5,6], while genotype G is found in the Americas and Europe[6]. Genotype I was reported in Vietnam and Laos[6], with the most recent putative genotype J identified in a Japanese patient living in Borneo island[4].

GENOTYPE E IN AFRICA AND ITS ORIGINS

Together with south-east Asia, Africa is one of the two regions in the world where HBV remains endemic. West Africa is the only major region in the world where HBV is still hyperendemic[5] — [> 8% of hepatitis B surface antigen (HBsAg) chronic carriers in the general population] and there is a correspondingly high incidence of HCC[7]. Genotype E was first described in 1992 from a HBsAg-positive Cameroonian blood donor[8]. It predominates in sub-Saharan Africa (SSA) accounting for 97% of individual infections and 17.6% of all HBV infections globally[9-11]. It is found almost exclusively throughout the vast expanses of the Western and Central Africa crescent including Angola, Liberia, Senegal[12,13], Ivory Coast[14], the Gambia, Nigeria[15], Mali, Burkina Faso, Togo, Guinea, Benin, Democratic Republic of Congo, Cameroon [16] and Namibia. The prevalence of genotype E decreases in proportions towards Eastern Africa, where, with the exception of Madagascar (genotype E), mainly genotype A has been found[5,9,11].

Genotype E has been found only in Africa, with some rare exceptions on other continents mainly in persons with a link to Africa[17,18]. Nonetheless, two cases, where no link to Africa could be established, have been documented, one in India[19] and another in Colombia[20]. Genotype A, on the other hand, circulates on every continent, including Africa, where it has the highest genetic diversity of 4% over the complete genome compared to 3% outside Africa[21]. Despite its high genetic diversity in Africa, genotype A is rarely found in West Africa. The dispersal routes of genotype A have previously been described to coincide with the slave trade leading to the dispersal of this genotype to the Americas and the Indian subcontinent[19,21-23]. Despite the forced migrations of slaves from West Africa to the New world[3,17], only

sporadic cases of genotype E have been reported in the Americas[17,24], Northern Europe[25] including Belgium[26] and the Netherlands[27]. This may suggest that genotype E was not in circulation before and during the slave trade (9th to 19th century) and has only been introduced into the West African population after the end of the slave trade in the late 1800s[23].

The conspicuously low genetic diversity of genotype E ranging between 1.2% and 1.95%[11,16,23,28,29] further supports a short natural history in Africa[16] and relatively recent introduction into the general population[16,30]. Various times from the most recent common ancestor (tMRCA) of genotype E have been calculated using Bayesian inference, with a median tMRCA of 130 years[30] whereas in Nigeria, a more recent tMRCA was estimated to be year 1948 [95% higher posterior density (HPD): 1924-1966] (73 years), with an increase in the genotype E-infected population over the last approximately 40 years to 50 years[31]. A recent study focusing on ancient HBV estimated a median MRCA to be year 1016 (95% HPD: 712-1358)[32]. These times differ from the estimated tMRCA of 6000 years[33]. Differences in the calculation of the nucleotide substitution rate of HBV are responsible for the variance of the estimated age of genotype E. Our recent study describing the phylogeography of full genomes of genotype E showed localized transmission, and limited movements within West and Central Africa. The study showed West Africa to be the most probable origin of the genotype E epidemic, with strains dispersing to the European region from there, whereas the strains dispersed to the Americas originated in Central Africa[29].

Studies on HBV-infected mummies from the 16th century revealed a very close relationship between the ancient and modern HBV genomes dating 400-500 years[34,35]. Furthermore, studies conducted by Krause-Kyora *et al*[36] reported ancient HBV sequences in the Neolithic age, while studies by Mühlemann *et al*[32] reported archeological ancient HBV and predicted recombination breakpoints in the polymerase gene leading to the formation of genotype A with similar recombination events involved in the creation of genotypes E and G[32,36-38] in the Bronze age[32]. Concurring with Mühlemann *et al*[32]'s study, Krause showed recombination events over time and similarity between the earliest ancient HBV sequences of the Neolithic era and modern HBV genotypes E and G[36]. By comparing the sequences from the above two studies, Datta *et al*[39] was able to confirm the previous findings of the presence of remnants of genotype E in ancient sequences from the Neolithic and Bronze age[32,36,39].

At first glance, the widespread prevalence and extensive geographic distribution of genotype E[17,28,29] may be difficult to reconcile with the long natural history of genotype A in Africa. However, isolation of genotype E in indigenous isolated tribes of Africa; Pygmies[37] and Khoi San (Kramvis unpublished data), believed to be direct descendants of earliest human lineages[6,37,40], and the recent discovery of the ancient HBV sequences in the Neolithic and Bronze era from skeletal remains of humans with remnants of genotype E[32,36,39], may support the theory that genotype E pre-existed but has been re-introduced into the population thus replacing genotype A. Similarly, the presence of recombinant sequences similar to extant genotypes D (subgenotype D6) and E, which are presently endemic in certain regions of Africa[6], together with the co-existence of genotypes E/A/D in SSA, including Sudan and Cameroon, also support the aforesaid possibility[37,41,42]. Possible mechanisms of introduction and routes of transmission include mass vaccination programmes carried out in Western Africa and a high frequency of hepatitis B e antigen (HBeAg)-positivity in mothers infected with genotype E [mother to child transmission (MTCT)][43,44] leading to chronicity due to HBe/HBcAg-specific T helper cell tolerance *in utero*[44]. In constrast to genotype E, the two subgenotypes of A, A1 and A3, circulating in Africa, are characterized by early loss of HBeAg seroconversion and a high frequency of HBeAg-negativity[10].

Genotype E, closely related to human strains, has also been isolated from captive and wild born chimpanzees originating from West and Central Africa[12,41,45]. The direction of transmission was not established[17] although, it was suggested that the practice of injecting human serum into chimpanzees after their capture in Africa was the most probable explanation[41,42,46]. Thus, chimpanzees may be a possible source of separate primate to human transmission events of HBV in West Africa[41,42,46]. Moreover, a closer relationship between the Neolithic and the African non-human primate strains compared to other human strains suggests African origin of extinct HBV genotypes and reciprocal cross-species transmission in the past[38,47] supporting preceding suppositions[48].

MOLECULAR STRUCTURE OF GENOTYPE E

Genotype E is the most prevalent genotype of HBV in Africa estimated to have infected close to 20% of chronic HBV carriers globally. However, due to limited studies and the lack of surveillance data in Africa, this estimate may be higher[17]. Genotype E is the second shortest genotype after D with a complete genome length of 3212 bp (Figure 1). It has a unique three-nucleotide deletion in the preS1 that can differentiate it from other genotypes (Figure 1) and a signature pattern of amino acids in the preS1. In addition, genotype E has a putative additional start codon in the preS1, which may lead to an elongated middle hepatitis B surface protein (317 amino acids in length instead of 281 amino acids)[11]. This elongated middle HBsAg has not been detected to date. The amino acids of the preS1, preS2 and S genes are well conserved, with signature motifs $Leu^{3}SerTrpThrValProLeuGluTrp^{11}$ in the preS1 specific to genotype E [11]. Additional signature amino acids are also found at Thr^{18}, Arg^{38}, His^{44}, Thr^{52}, Met^{83}, Lys^{85} and Thr^{108} in the preS1. All genotype E strains have a His at amino acid position 15 of the preS1 but no known unique signature motifs in the pre-S2 region. Arg^{122}, Lys^{160} and Leu^{127} residues are a characteristic of the S gene in this genotype and encodes for a unique serological subtype *ayw*4[11,12]. Although the reactivity to different diagnostic assays has been determined for genotypes A to D[49], it has not been tested for genotype E. The L209V substitution in the HBsAg was described as a unique feature among all genotype E sequences deposited in GenBank to date[50]. The spacer region of the polymerase (POL) has eight amino acids unique to genotype E: Met^{64}, Glu^{16}, His^{21}, Arg^{52}, Asp^{55}, Lys^{88}, Asn^{110} and His^{111}. Within the reverse transcriptase, Met^{164} is the only unique amino acid substitution in this genotype[11]. This introduces a start codon that theoretically could be translated into a protein of 344 amino acids. Although genotype E has the T1858 mutation in the precore (preC) region it does not frequently develop the G1896A mutation[44,51], which has been shown to stabilize the encapsidation signal (ε) converting the wobble to a stable Watson-Crick T-A pair[52]. This introduces a stop codon in the HBeAg precursor leading to no expression of the mature HBeAg[10,44,51]. As a result of its unique molecular structure, genotype E has a restriction map that differentiates it from other genotypes of HBV (Figure 1).

VARIANTS AND MUTANTS OF GENOTYPE E

Variants can play a critical role in HBV epidemics. From the limited studies on genotype E, a number of variants and mutants that can hypothetically affect detection, vaccination response and pathogenicity of HBV, have been described. Within the 'a' determinant of HBsAg, the vaccine and immune escape mutations R48T, P120T and G145R have been reported in genotype E HBV isolated from infected individuals[3, 53]. The preS2 F22L mutation, associated with cirrhosis, and a risk factor for the development of HCC, was found in genotype E isolates from Sudanese HCC patients [54].

Variants can also be generated through recombination[38] within an individual co-infected, with more than one genotype, resulting in drug resistant or diverse HBV strains. Recombinants can only occur when the various genotypes co-circulate in a population. Genotype E presents high chances of recombination, with A/E and D/E recombinants found in Ghana, A/E recombinant has been reported in Cameroon[37], Guinea, Burkina Faso and Nigeria[31] while D/E recombinant has been found in Gabon, Sudan, South Africa, Niger and Guinea[55,56].

Table 1, summarizes the different recombination events of genotype E with either D or A, mostly reported within Africa with different breakpoints within the HBV genome[37,54-61].

The F22L mutation and various deletions in the preS2 and the 1753V and 1762T/1764A mutations in the basic core promoter (BCP), are mostly found in HBV strains isolated from HCC patients[62] than in those from non-HCC controls[54,63]. Deletions in the core region have been reported in HBsAg-positive genotype E asymptomatic blood donors in Guinea. Another study conducted by Yousif *et al*[54] found preS2 deletion mutations in HBV from patients infected with either genotypes D or E in Sudan. The preS deletions in genotype E were found in the HBV isolated from HCC patients, while genotype D deletion mutants were detected in non-HCC patients [54]. The significance of this difference remains to be determined. On the other hand subgenotype A1, which is mostly found in SSA[5], has been shown to have a higher carcinogenic potential compared to other (sub)genotypes[64]. A meta-analysis study associated the preS deletion mutants with a 3.77-fold increased risk of HCC[65].

Table 1 Recombination events of genotype E with breakpoints across the genome

Parental genotype	Region	Genome position (from the *EcoR*I site)	Country
D/E	*preS1*		Niger, Ghana, Gabon, and Sudan[53-58]
D/E	*preC/C*		Ireland[59] and South Africa[60]
D/E	*Pol*	978, 1230	Sudan[56]
	X	1643	
	C/Pol overlapping region	2384	
	Pol	2756	
	preS1/Pol overlapping region	3000	
D/E	*X/preC* overlapping region	1649, 1932	Niger[58]
	C/Pol overlapping region	2392, 2385	
	Pol	2831, 2836	
	Pol/preS1 overlapping region	3075, 3083	
D/E	*X*	1651	Ghana[57]
	C/Pol overlapping region	2406	
	Pol	2823	
	Pol/preS1 overlapping region	3081	
E/D	*preS*	85-505	Niger[58]
	S-Pol overlapping region	796-1306	
A/E	*C*		Ghana[57]
A/E	*Pol*	874-1062	Cameroon[37]
	X		
E/A	*Pol*	908-1026	
	X-C		
A/E	*preC/C*		Guinea[57] and France[61]
E/A	*X*		

The *precore/core* (preC/C) encodes the e antigen (HBeAg) and core protein (HBcAg); *Pol* for polymerase (reverse transcriptase), *preS1* encodes the large surface protein and *X* is a transcriptional transactivator protein.

Furthermore, a prospective study revealed the predictive value of a combination of the preS and BCP mutants in the development of HCC and pro-oncogenic role of mutated envelope proteins through their intracellular accumulation[66]. These mutations may be used as biomarkers for screening high-risk individuals in resource limited regions such as SSA, who may potentially develop HCC[67].

TRANSMISSION OF GENOTYPE E

The prevalence of chronic HBV infection varies widely according to geographic area and is closely linked with the predominant routes of HBV transmission. In regions of Africa, where genotype E prevails, transmission can occur horizontally or vertically *in utero*, intrapartum or *via* breast-feeding[68] from mother to child[69]. However, about 50% of the infection in children cannot be accounted for by MTCT and in many endemic regions, prior to the introduction of neonatal vaccination, the prevalence peaked among children aged between 7 years to 14 years[70]. In the pre-vaccine era, most chronic carriers were infected horizontally in SSA and only 10% were infected through MTCT compared to 40% in Asia[71,72]. Horizontal transmission can occur early in life mainly from HBeAg-positive family members/household contacts, playmates or by unsafe medical interventions. Very few studies have been carried out

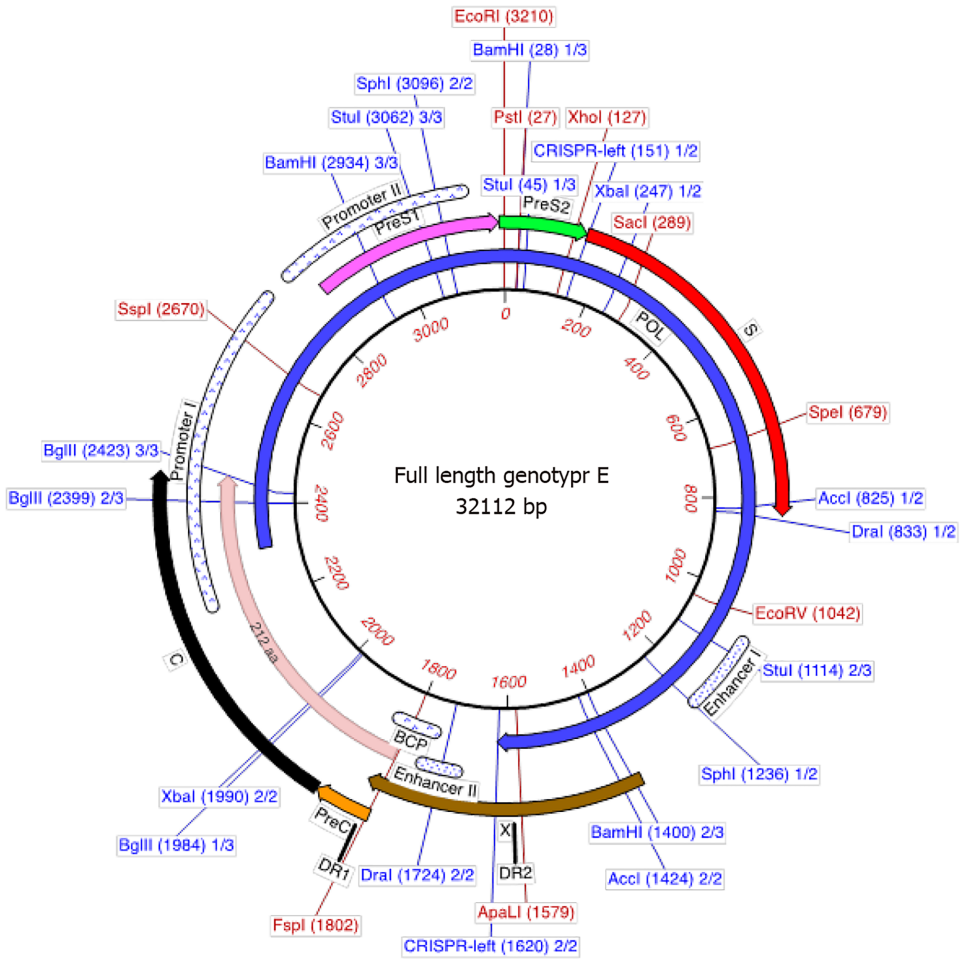

Figure 1 Organizational structure of hepatitis B virus genotype E genome. The hepatitis B virus genome consists of a partially double stranded DNA with the complete minus strand and the incomplete strand. The four open reading frames are shown: *precore*/*core* (*preC*/*C*) that encodes the e antigen (HBeAg) and core protein (HBcAg); *POL* for polymerase (reverse transcriptase), *preS1*/*preS2*/*S* for surface proteins (three forms of HBsAg, small, middle and large) and *X* for a transcriptional trans-activator protein. The promoters, enhancers and the unique restriction enzymes are shown.

in terms of identifying routes of transmission for genotype E. In the Gambia, MTCT is responsible for 16% of chronic infections and increases the risk of persistent viral replication and severe liver disease[73]. Strong evidence from a phylogenetic analysis showed intrafamilial transmission of HBV[73]. A study conducted in Ghana also concluded that the HBV is predominantly transmitted through horizontal transmission in childhood with intrafamilial, rather than interfamilial environment being the primary place of transmission[74]. However, a study conducted in Nigeria in two semi-isolated rural communities suggested that HBV transmission between siblings was not the major route of transmission with a complex pattern of transmission among the residents of the two communities[31]. So it appears that other factors may be at play in the transmission of genotype E in various communities. As has been shown in Burkina Faso, co-infection with human immunodeficiency virus (HIV), which leads to an increase in HBV viral load and frequency of HBeAg-positivity, can increase the risk of HBV transmission by as much as 2.5-fold[75,76]. Traditional cultural practices such as scarification and tattooing have been shown to be responsible for the transmission of HBV[77].

NATURAL HISTORY OF HBV GENOTYPE E INFECTION

Genotypes and subgenotypes can influence the natural history of infection. Comparing different (sub)genotypes is often difficult because the (sub)genotypes do not circulate in the same populations. The majority of the studies have compared genotypes B and C as well as A and D and have shown different clinical manifestations and the serious outcomes of disease [cirrhosis (LC) and HCC][78-81]. The natural history of infection

in individuals infected with genotype E has not been extensively studied, and has mostly been derived from anecdotal evidence. Genotype E has clinically been characterized, with high viral loads and the patients infected with this genotype are more likely to be HBeAg-positive than the patients infected with genotype D[5,10,53,54,56]. A higher HBeAg-positivity of this genotype has been shown to confer tolerance, with a milder clinical manifestation[10]. This could be the reason for the higher prevalence of genotype E in Sudanese blood donors, whereas genotype D is more prevalent in those patients with liver disease[28,54,56]. In addition, infection with genotype E has previously been linked to higher chronicity rates than other genotypes[10,54,56].

Table 2, which was compiled from limited data comparing genotype E to D in Sudan (Yousif *et al*[53,54]) and studies in the Gambia (Shimakawa *et al*[72]), summarizes the clinical manifestation of genotype E relative to other genotypes[53,54,72,82]. As is evident from this table most aspects of clinical characteristics of genotype E have not been formally studied.

In their study, Yousif *et al*[54] observed that genotype E infected liver disease patients and blood donors[56] had a higher frequency of HBeAg-positivity and higher viral loads compared to patients infected with genotype D (Table 2)[53,54]. Both genotype D and E have the 1858T, and thus can develop the G1896A mutation, however, what is puzzling is that G1896A is positively associated with genotype D and negatively associated with genotype E[51].

This lack of association may be the reason for the high frequency of HBeAg-positivity in individuals infected with genotype E compared to genotype D. A study focusing on chronic hepatitis B (CHB) and HCC in Burkina Faso showed patients infected with genotype E had lower viral loads, lower frequency of HBeAg-positivity and higher prevalence of cirrhosis than those infected with genotype C or C/E recombinants. With the majority of HCC, infected with genotype E (78%), HCC-associated risk factors were old age, male with high HBV viral load when comparing CHB in HCC patients to non-HCC patients[83]. Another longitudinal study conducted in Gambia showed that a majority of the genotyped CHB carriers were infected with genotype E[72]. Although the mean viral load and alanine aminotransferase levels were higher in carriers with HBsAg-positive mothers, a majority (47%) had undetectable viral loads with 22% of all chronic HBV infections having viral loads ranging between 50 and 200 IU/mL. HBV viral load has been used to predict progression from cirrhosis to HCC[84]. From this study, the rate at which the HBV DNA cleared was faster when compared to age progression making it difficult to predict HCC[72]. What should be noted from this study is that, the samples that were assayed for viral loads were from a different time frame (2012-2013), while the genotyped samples were from 2003. Successful genotyping would require viral loads high enough to allow amplification of the DNA and thus higher viral loads may be a factor that biases genotyping making it hard to draw any conclusion on the infecting genotype for the chronic carriers who had undetectable or low HBV DNA.

African regions in which genotype E is endemic are characterized by a higher incidence of HCC[85] and epidemiological studies have suggested the carcinogenic potential of genotype E[86]. Although the mechanisms underlying this oncogenic potential have not yet been clarified for genotype E, they could be related to immune escape phenomena[87], as well as to other possible cofounders that may be involved, such as HIV co-infection, dietary iron overload or aflatoxin consumption[85,88,89].

HBV-HIV CO-INFECTION AND OCCULT INFECTION

Globally, an estimated 10% of the 37 million HIV infected individuals are co-infected with HBV[90]. HBV/HIV co-infection in SSA accounts for 36% (2-4 million) with the highest rates reported in West- and Southern Africa[90]. Epidemiological and virological characteristics of HIV-infected individuals in West Africa showed an average of 13% prevalence of HBsAg-positivity, ranging between 1.1% in blood donors and 35.7% in pregnant women attending antenatal care[76,91-93], while 4.75% of HBV-HIV infected individuals were HBeAg-positive with the prevalence ranging between 3.2% and 7.2% in adults and anti-retroviral (ART) naïve adults, respectively[94,95]. An average HBV exposure rate of 74% (64%-81.7%) in ART naïve and adults initiating ART[90,94,96,97] has been documented. A high rate of morbidity has been reported in HBV/HIV co-infected individuals, while the progression of CHB to HCC is more rapid in genotype E HIV-positive individuals than in those with HBV alone[98]. In a study of Senegalese children, 47% who were HBV genotype E-HIV co-infected had elevated levels of drug resistance mutations (L180M, M204V/I, and S202N) to both

Table 2 Comparison of the virological and clinical characteristics of genotype E with other genotypes

	Genotypes							
	E	A	B	C	D	F	G	H
HBV DNA level	Increased	Decreased	Decreased	Increased	Not studied	Not studied	Not studied	Not studied
Frequency of precore G1896A mutation	Increased[1]	Decreased	Increased	Decreased	Increased	Not studied	Not studied	Not studied
Frequency of basic core promoter T1762A/A1764G mutation	Not studied	Increased	Decreased	Increased	Decreased	Not studied	Not studied	Not studied
Frequency of preS deletion mutation	Not studied	Increased	Decreased	Increased	Not studied	Not studied	Not studied	Not studied
Tendency of chronicity								
High		+		+				
Low			+		+			
Not studied	+					+	+	+
HBeAg positivity								
High	+			+				
Low		+	+		+			
Not studied						+	+	+
HBeAg seroconversion								
Early		+	+					
Late				+	+			
Not studied	+					+	+	+
HBsAg seroconversion								
More		+	+					
Less				+	+			
Not studied	+					+	+	+

[1]Relative to D3.

+: Classification of category; preS: Surface protein; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen. Adapted from Yousif *et al*[53,54], Shimakawa *et al*[72] and Schaefer *et al*[82].

HIV and HBV, significant levels of HBsAg escape mutations, HBV DNA persistence and HIV virologic failure[99]. This suggests that the use of the Tenofovir Disoproxil Fumarate regimen in the management of HBV, HIV and HBV-HIV co-infection is ideal in the SSA setting.

Occult HBV infection (OBI) is defined as the presence of replication-competent HBV DNA (*i.e.*, episomal HBV covalently closed circular DNA) in the liver and/or HBV DNA in the blood of people who test negative for HBsAg by currently available assays)[100]. OBI is frequent in HIV-infected individuals and has been described in individuals infected with genotype E, with a prevalence 10% and 15% in HIV-positive patients from the Ivory Coast and Sudan, respectively[97,101].

Biomarkers are very important in assessing risk factors for the development of serious clinical manifestations. As is evident from the above observations the same risk biomarkers may not be applicable to all (sub)genotypes and cannot be extrapolated from studies on other genotypes. Therefore, it is important that biomarkers are studied exclusively in genotype E.

TREATMENT AND RESPONSE TO ANTIVIRAL THERAPY

Current antiviral therapies, which include nucleos(t)ide analogues (NA) and interferon-alpha (IFN-α) reduce but do not eliminate the risk of liver cancer. As

curative therapies are developed, it will be important to monitor patients for progression to liver cancer, even if they have been cured of CHB infection. HBV genotype may influence the efficacy of the antiviral therapy but most studies that analyzed the role of HBV genotype in the treatment with NA mostly focused on genotypes A, B, C and D. Lamivudine (LAM) is the earliest used NA in the world and the association between HBV genotype and LAM has been demonstrated both in terms of response and the development of resistance mutations. Various response rates have been observed for various studies with genotype A being more likely to develop resistance mutations[102,103]. Studies have shown that HBeAg-positive patients infected with genotype B have a higher response rate to IFN-α than those infected with genotype C, while patients infected with genotype A have a higher response rate to IFN-α than those infected with genotype D[104].

There is a scarcity of information on the clinical and virological characteristics of genotype E-infected patients as well as on the efficacy of anti-HBV drugs[86]. However, a few studies have described genotype E's response to treatment[86,105-108] in a variety of scenarios: Treatment-naïve CHB patients initiating treatment with NA [entecavir (ETV) or tenofovir][86], HBV-HIV co-infected patients[109], rescued after LAM failure[110], adefovir phase III clinical trials[111]; a follow-up study of HBsAg decline in ETV-responding patients[107] and response to IFN[106,112]. As is evident from the above list, only one study looked at tenofovir the drug recommended by the World Health Organization (WHO), American Association for the Study of Liver Diseases, and the European Association for the Study of the Liver for antiviral therapy.

The phase III clinical trial of adefovir dipivoxil conducted by Westland *et al*[111] included a total of 6 genotype E patients and reported antiviral efficacy in patients on a 48-wk therapy regardless of the HBV genotype. Studies by Boglione *et al*[107] and Cuenca-Gómez *et al*[86] focused on genotype E treatment-naïve, CHB patients of SSA origin, on ETV or tenofovir antiviral therapy. A higher rate of HBsAg loss in patients infected with genotype E compared to genotypes A or D was observed. In addition, a high response rate to NA was reported with undetectable viral load and loss of HBeAg in a median time of 31.8 mo with no cases of HCC[86].

Two different treatment regimens were compared in CHB patients infected with genotype E, who had migrated to Italy. In the one arm, CHB patients with low viral loads, where given pegIFN for 24 wk, whereas in the second arm, CHB patients with high viral loads were treated sequentially with ETV for 12 wk and thereafter pegIFN for 24 wk. Those treated with monotherapy did not respond as well as those on dual therapy[106]. In a follow-up study, genotype E CHB patients were treated with pegIFN for varying lengths of time 48-, 72- and 96-wk. Prolonged treatment was beneficial and recommended for individuals infected with genotype E[106,108]. Thus, from these limited studies it is evident that genotype E infected individuals are unresponsive to conventional pegIFN treatment. However, in concurring with the Boglione *et al*[107] and Cuenca-Gómez *et al*[86] studies, a retrospective study conducted in Europe by Erhardt *et al*[105], focusing on HBV genotypes E-H the response to IFN-α or NAs (LAM, adefovir, ETV) therapy concluded that genotype E infected patients treated with IFN-α had lower end of treatment response but overall sustained virological response, while the patients on NAs had viral suppression within 48 wk[105]. It should be noted that the conclusion was reached with only 5 treatment-naïve genotype E mono-infected patients[103].

Taken together, the current international treatment guidelines do not consider patients with genotype E CHB. Thus, better management strategies for HBV infected patients are recommended taking into account the genotype in question. In order to deliver proper medical care, improve knowledge on the response to treatment, and the development of resistance of relatively under-studied genotypes like E, it is critical to issue proper and specific recommendations that could differ from those issued for other genotypes. Moreover, all gathered information on response to treatment of genotype E in Africa is useful, especially considering that the development of immune escape mutations[87] can have an epidemiological impact in other parts of the world with the dispersal of these strains *via* increased migration from Africa. As new finite cure strategies are developed it is important that the clinical trials include CHB patients infected with genotype E.

RESPONSE TO VACCINATION

The risk of developing chronic infection is about 90% following perinatal infection up to 6 mo but decreases to about 20%-60% between the ages of 6 mo to 5 years[68,73].

Thus, prevention of HBV infection by vaccination is very important and is most successful when it targets infants, and when prevention begins with administration of the first dose of HBV vaccine soon after birth. The HBV vaccine is about 80%-100% effective in managing HBV infection or clinical hepatitis following completion of the dose. However, inoculation will not help those chronically infected[1]. The two commonly used efficacious vaccines are either plasma-derived vaccines prepared from purified HBsAg obtained from chronic HBV patients or recombinant vaccines from synthesized HBsAg[113]. As of 2020, more than 190 WHO member states immunized infants against HBV as part of their routine vaccination schedule, and 84% of children received HBV vaccines[1]. Even with the vaccine roll out, the burden of HBV infections in SSA remains of concern attributed to the delay in the implementation, lack of birth doses and low coverage of the vaccine programme[114-117]. The high HBeAg positivity in mothers infected with genotype E is a risk factor for MTCT[118] (one in ten infants vaccinated at birth) suggesting that vertical/perinatal infection is still present in African countries[119-122]. Antenatal HBV screening is hardly performed in SSA (0%-20%)[123], with only 33% of countries having official guidelines[124]. HBV was first classified on the basis of the amino acid substitution on the HBsAg at positions 122, 127, 134 and 160. The serological subtypes contain the common 'a' determinant and one of each of the mutually exclusive determinants *d*/*y* and *w*/*r* [125]. Additional serological specificities, originally designated as subdeterminants of 'a' and subsequently as subdeterminants of *w*, have allowed the identification of ten serological subtypes *ayw*1, *ayw*2, *ayw*3, *ayw*4, *ayr*, *adw*2, *adw*3 *adw*4, *adrq*- and *adrq*+[6,8,126]. The humoral immune response following vaccination with HBV vaccines is largely directed against the common 'a' determinant, with a lesser response directed against the *d*/*y* and *r*/*w* subdeterminant epitopes[113,127].

All currently available genetically engineered HBV vaccines are produced with the subgenotype A2, serotype *adw*, which differs from the genotype E subtype *ayw*4. Available data show that current HBV-A2 vaccines are highly effective at preventing infections and clinical disease caused by all known HBV genotypes[128]. However, a study conducted on blood donors in the United States[129] questioned the ability of subgenotype A2-derived HBV vaccines to protect against non-A2 HBV (sub)genotypes. It was concluded that while breakthrough infections with non-A2 genotypes were recorded following vaccination, which only prevented clinical disease[128]. In addition, their findings suggested that the vaccine may be less effective for non-A2 infections. In view of the global variability in genotype distribution, any gap in the efficacy of A2 vaccines has potentially important implications for the ongoing protection of populations against HBV infection and its consequences[128]. Therefore, more studies need to focus on the response of genotype E to vaccination, especially considering that this is the genotype prevailing in the region of the world where the virus continues to be hyperendemic and all preventive measures should be optimized.

The emergence of HBV escape mutants may occur under medically induced immune pressure (in association with vaccine or hepatitis B immune globulin) or naturally induced immune pressure (as a result of CHB)[130]. These HBV mutants may carry multiple amino acid substitutions around- and within the HBsAg 'a' determinant, which can affect the binding of neutralizing antibodies (anti-HBsAg), with some of the former remaining undetectable by certain diagnostic tests, thus implying a potential risk in transfusion events[130]. The emergence of S escape mutants, raised concerns about the efficacy of the current vaccine on the African continent. To this day, very few studies have focused on the genotype E response to vaccination, although vaccination began over four decades ago.

CONCLUSION

In conclusion, genotype E has unique molecular and epidemiological characteristics. The natural history of genotype E has not been studied and very little is known about the virological breakthrough as a result of vaccination. Only a few studies that focused on the treatment of a limited number of genotype E infected patients exist, making it difficult to reach any firm conclusions. In addition, most of these studies have been conducted outside of Africa on a small number of individuals that had migrated from Africa, with only a minority of studies carried out on the African continent. Consequently, it is important that African CHB patients infected with genotype E are included in clinical trials focusing on new antiviral therapy, biomarkers and other possible preventive methods. There are multiple reasons for this. Western Africa, where genotype E prevails, is the only region in the world where HBV continues to be

hyperendemic. Although West Africa has a relatively long time span of vaccination against HBV, which began in the Gambia in the early 1980s, the infection is still being maintained in the community. There is a correspondingly high incidence of HBV-associated HCC, ranked fourth worldwide and in SSA, the second leading cancer for men and the third for women, with average age-standardised incidence rates of 18.9 and 8.0 per 100000 persons/year, respectively[85]. In this region, HCC presents in younger age groups and has a median survival rate of approximately 3-4 mo. Genotype E is being dispersed from high to low endemicity regions of the world as a result of migration and this may lead to changes in the natural history of HBV infection in countries of destination, where different genotypes predominate.

Toward achieving the WHO target for the worldwide elimination of viral hepatitis as a public health burden by 2030 there is an urgent need for more in-depth and large-scale investigations into genotype E, which has been under-represented in studies, resulting in the paucity of data on this neglected genotype.

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MINIREVIEWS

One stop shop approach for the diagnosis of liver hemangioma

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Abstract

Hepatic hemangioma is usually detected on a routine ultrasound examination because of silent clinical behaviour. The typical ultrasound appearance of hemangioma is easily recognizable and quickly guides the diagnosis without the need for further investigation. But there is also an entire spectrum of atypical and uncommon ultrasound features and our review comes to detail these particular aspects. An atypical aspect in standard ultrasound leads to the continuation of explorations with an imaging investigation with contrast substance [ultrasound/computed tomography/or magnetic resonance imaging (MRI)]. For a clinician who practices ultrasound and has an ultrasound system in the room, the easiest, fastest, non-invasive and cost-effective method is contrast enhanced ultrasound (CEUS). Approximately 85% of patients are correctly diagnosed with this method and the patient has the correct diagnosis in about 30 min without fear of malignancy and without waiting for a computer tomography (CT)/MRI appointment. In less than 15% of patients CEUS does not provide a conclusive appearance; thus, CT scan or MRI becomes mandatory and liver biopsy is rarely required. The aim of this updated review is to synthesize the typical and atypical ultrasound aspects of hepatic hemangioma in the adult patient and to propose a fast, non-invasive and cost-effective clinical-ultrasound algorithm for the diagnosis of hepatic hemangioma.

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Key Words: Hemangioma; Liver; Ultrasound; Contrast media; Diagnosis; Algorithm

Core Tip: Liver hemangiomas are benign tumors usually found on a routine ultrasound in an asymptomatic adult patient. A high-performance ultrasonographic system equipped with contrast-enhanced ultrasound software, allows the experienced examinator to orient the diagnosis quickly, cost-effectively and non-invasively in most cases. This article reviews the typical and atypical ultrasound features of hepatic hemangioma and proposes a diagnostic algorithm for liver hemangiomas in patients referred to the hepatologist.

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INTRODUCTION

After focal fatty sparing, hepatic hemangioma (HH) is the second most common benign solid lesion of the liver[1]. The rate of detection of HHs has increased as imaging methods have become more effectiveness and accessible. The prevalence depends on the method used for detection: 2%-4% for ultrasonography, up to 5% for computed tomography and up to 7% of cases in autopsy cases[2-5]. HH are more common in women than in men[4]. It can appear at any age but are detected more frequently between 30-50 years[6]. HHs are usually single, small in size, less than 3 cm, but can also be multiple and large in size up to 20 cm.

PATHOLOGY

HHs belong to the group of non-epithelial lesions, consisting of a blood-filled space, fed by hepatic arterial circulation. HH arises from a vascular malformation and increases in size mainly by dilating the vessels inside the tumour.

The pathogenesis of hemangioma is not entirely understood, the theory of congenital disorder[7] with possible hormonal dependence has been taken into account[4]. Macroscopically, HHs are well delineated, described as flat red-blue lesions. Hemangiomas are classified into three types: Cavernous, capillary and sclerosing hemangioma. Capillary hemangiomas are usually small, less than 3 cm, while cavernous hemangiomas reach sizes over 5 cm. Sclerotic hemangioma is small, completely fibrous, therefore it can occasionally be misdiagnosed as a malignant fibrous tumor[9,10]. Microscopically, hemangiomas consist of cavernous vascular spaces padded with a flattened endothelium divided by fibrous septa of varying thicknesses that are often incomplete. Currently, according to newer classification system of the International Society for the Study of Vascular Anomalies ISSVA, last updated in 2018, HH is a vascular tumor, considered as a slow flow venous malformation[11].

NATURAL COURSE

Small hemangiomas are usually asymptomatic, detected by chance on imaging evaluation. Multiple or bulky tumors can cause symptoms, as pain in abdominal right upper quadrant secondary to infarction, haemorrhage, torsion or distention of the Glisson's capsule. Other symptoms like fullness, nausea, vomiting and early satiety may result from compression of adjacent organs[12].

Liver function tests are usually normal. The natural history of hemangiomas is variable: Most of them remain stable, some may grow or involute. In the vast majority of cases does not require treatment or monitoring.

ULTRASOUND EXAMINATION IN HH

B-mode ultrasound

In recent years, ultrasound examination is the main method of detecting HH due to the fact that it is widely available, inexpensive, rapidly performed without exposing the patient to radiation. Because ultrasonography systems are becoming more and more efficient, smaller and smaller masses are detected, from 2-3 mm, especially if a linear probe with a frequency higher than 8 MHz is used (Figure 1).

The classic sonographic appearance of hemangioma is that of a homogeneous hyperechoic mass, measuring less than 3 cm in diameter with acoustic enhancement and sharp margins[13] (Figure 2). Sometimes it outlines a central hypoechoic area (Figure 3). HHs does not have a peritumoral halo and pushes the hepatic vessels without their invasion or thrombosis (Figure 4). The acoustic enhancement is due to the blood content. When located subdiaphragmatically it produces the artifact "in the mirror" (Figure 5). The hyperechoic appearance is related to the interfaces between vascular space and the fibrous stroma[13]. HH is usualy homogenous mass, but at dimension > 5 cm may show inhomogeneous echogenicity probably because of intratumorally changes, such as thrombosis or fibrosis[14] (Figure 6). No intra-tumoral vessels are seen at color Doppler exam due very slow intralesional flows, but power Doppler technique is more sensitive in detecting blood flow[13] (Figure 7). This aspect is found in most cases of HHs and corresponds histologically to the cavernous hemangioma[14]. Most typical-looking hemangiomas measure less than 3 cm[13].

Contrast enhanced ultrasound

Contrast enhanced ultrasound (CEUS) can be performed immediately after standard ultrasound exam while focal liver lesion (FLL) is found, in the same session, using a dedicated contrast software. Currently, four contrast agents are used in the imaging assessment of FLLs[15,16].

Traditionally CEUS reveals tissue perfusion in real time, in all arterial, portal and late phases but a new contrast agent (Sonazoid) allows the assessment of an additional postvascular phase (Kupffer)[17].

The aspect of the capture in the arterial phase orients on the tumor type while the presence or absence of the wash-out in the late phase differentiates the benign tumors from the malignant masses[15,16]. For the diagnosis of HH the arterial phase is the most important. The typical CEUS feature of a hemangioma, regardless of the injected contrast agent, is peripheral nodular enhancement in the arterial phase with progressive centripetal partial or complete fill-in[16] in portal venous phase and complete enhancement in late phase (Figures 8 and 9). In the postvascular phase (specific for Levovist) hemangioma is isoenhancement or slight hypoenhancement relative to surrounding liver parenchyma[18]. The described appearance is highly suggestive of hemangioma. When the two hallmarks of haemangioma, peripheral pools and centripetal progression, are present the diagnosis of HH is most likely, the specificity of the method approaching 100% in most studies[19,20].

Not all hemangiomas have typical enhancement, thus, the overall sensitivity of CEUS for diagnosis of hemangioma is lower than specificity, approximately 86% (95% confidence interval: 81%-92%) according to a meta-analysis including 612 cases from 20 studies[20]. As the years passed, the equipment evolved, and the examiners gained more experience. Recent multicenter European studies, each with over 1000 examined FLL, reveal that CEUS correctly diagnosed 85%-90% of hemangiomas[21-24] and if a computerized image analysis is added the diagnostic accuracy reaches 93.3%[25]. Moreover, there are studies that demonstrate CEUS to be approximately equal to the computed tomography (CT)-scan or magnetic resonance imaging (MRI) regarding to assessment of tumor differentiation and specification of newly discovered liver tumors in clinical practice, including for HH[26,27].

Because it is a proven method, WFUMB (World Federation for Ultrasound in Medicine and Biology) Guidelines for CEUS in the liver – update 2020 recommends CEUS as the first line imaging technique for the characterization of incidentally, indeterminate FLLs at ultrasound in patients with non-cirrhotic liver and no history or clinical suspicion of malignancy[15]. Similarly, the EASL (European Association for the Study of the Liver) Clinical Practice Guidelines on the management of benign liver

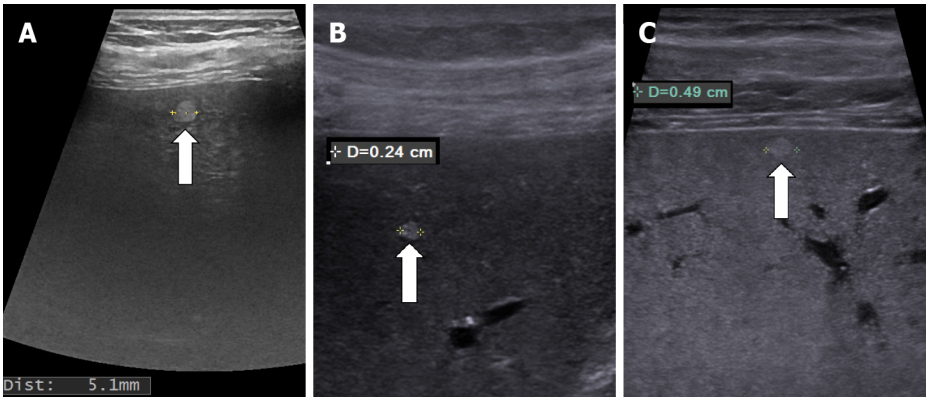

Figure 1 Very small (less than 5 mm), hyperechoic, well delimited hemangiomas showed by linear probe exam (arrows). A: Subcapsular hepatic hemangioma; B and C: Intraparenchymal hepatic hemangioma.

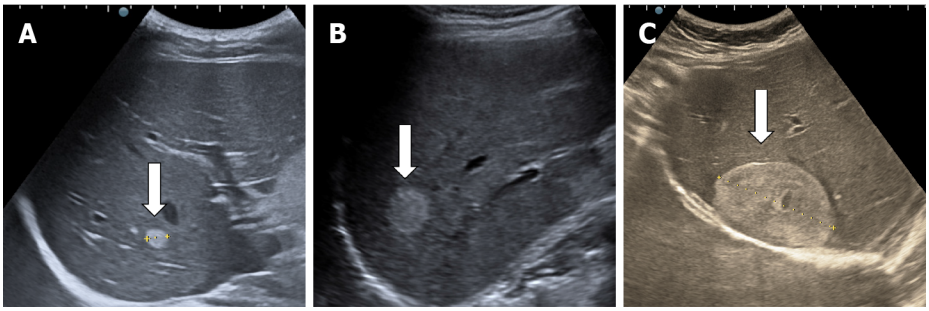

Figure 2 Typical hepatic hemangioma. Ultrasonography shows the hemangioma as a hyperechoic mass with sharp margins. A and B: Small hepatic hemangioma; C: Large hepatic hemangioma.

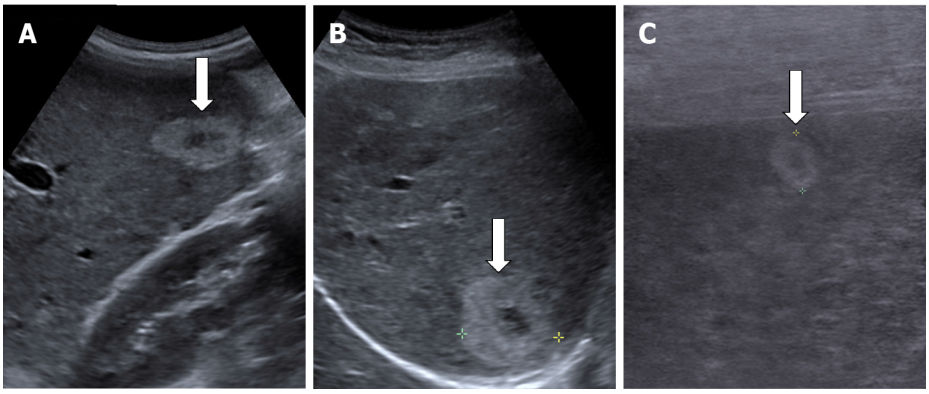

Figure 3 Examples of hyperechoic hepatic hemangioma with hypoechoic central area. A and B: Convex probe; C: Linear probe.

tumors recommends CEUS or another contrast imaging method (CT, MR) when in B-mode ultrasound the appearance is atypical, or when the lesion occurs in cancer patients or those with underlying liver disease[1].

The advantages of CEUS are related to the immediate availability in the ultrasound room where the lesion was detected, the real-time visualization of the tumor perfusion, non-ionizing technique and low financial costs[28,29]. Moreover, sonographic contrast agents have only a few contraindications and precautions, can be used regardless of renal and thyroid impairment and have excellent safety profiles[30].

There are few disadvantages of CEUS as compared to other imaging techniques: the dependence on the experience of the sonographer and providing only limited information in patients with high body mass index or bowel gas overlay. As a specific disadvantage for the diagnosis of hemangioma, CEUS with SonoVue cannot appreciate the very late phase of HH because the contrast substance is eliminated by breathing in about 5-6 min after injection.

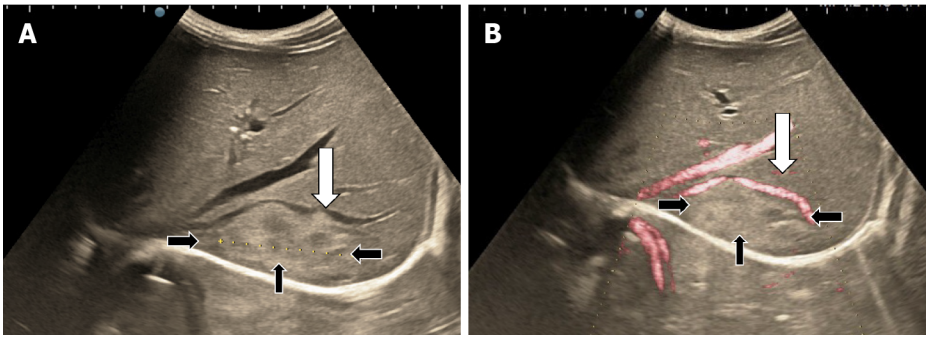

Figure 4 Subdiaphragmatic hepatic hemangioma (white arrows) that pushes the right hepatic vein (black arrows) without its invasion or thrombosis. A: B-mode ultrasound; B: Doppler ultrasound mode.

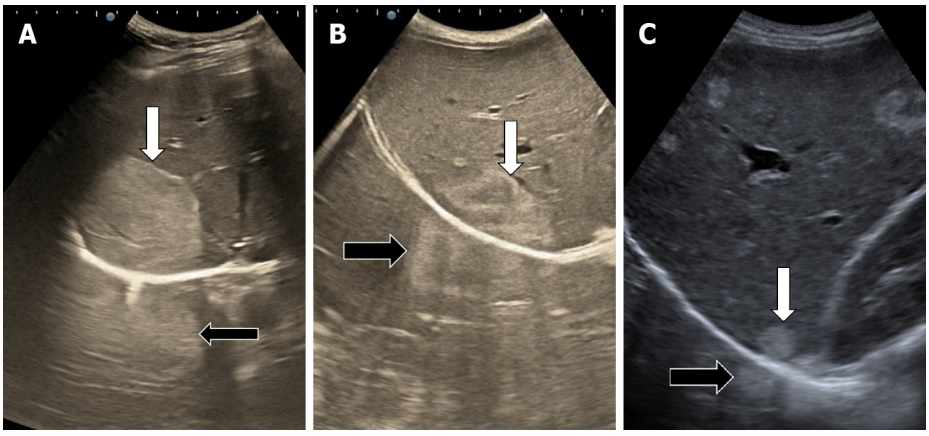

Figure 5 Examples of hepatic hemangioma located subdiaphragmatically (white arrows) with the artefact "in the mirror" (black arrows). A: Large, hyperechoic hepatic hemangioma; B: Inhomogeneous lesion; C: Small hepatic hemangioma.

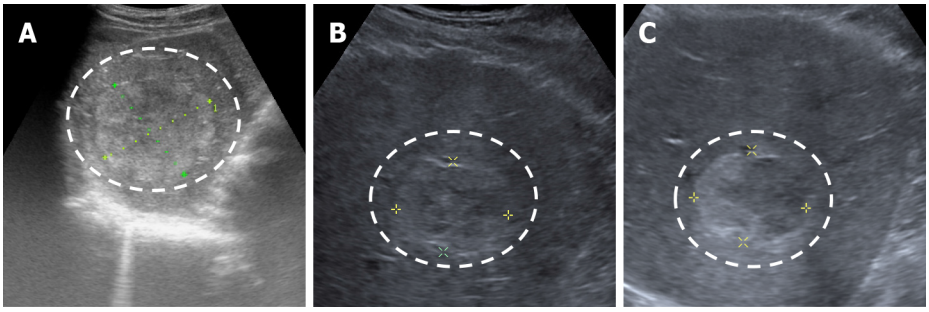

Figure 6 Illustration of hepatic hemangioma with inhomogeneous echogenicity. A-C: Hepatic hemangioma with intratumorally changes, such as fibrosis (A) or thrombosis (B and C).

In some cases, the phenomenon of pseudo-washout in the late phase observed due to hyperinsonation may induce differential diagnosis issues with malignant lesions but the typical appearance of the arterial phase is enough in clinical practice for a correct diagnosis of hemangioma (Figure 10).

HH VARIANTS

Flashfilling hemangioma

The diagnosis of HH is relatively easy if typical peripheral nodular enhancement with subsequent central fill-in is present. In about 16% of all hemangiomas, however, there is a rapid, uniform and intense homogeneous enhancement in the arterial phase, more

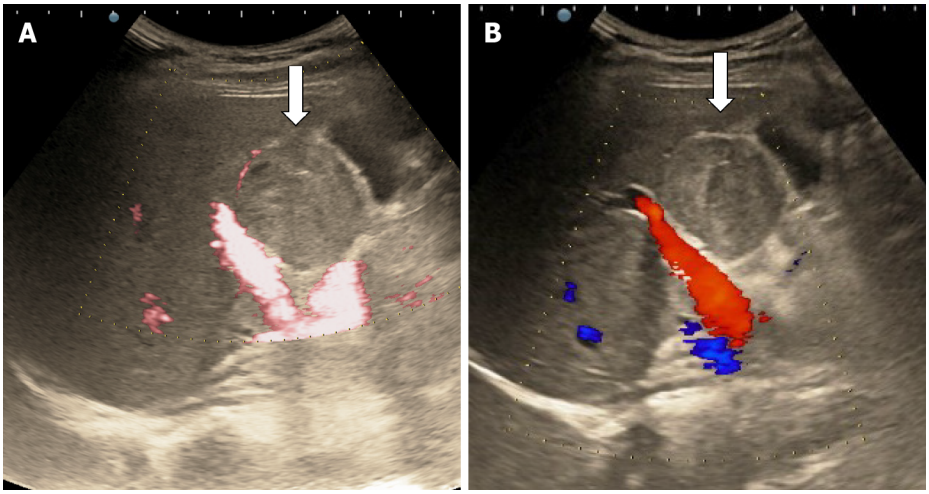

Figure 7 Doppler mode ultrasound for hepatic hemangioma. A and B: No intralesional vessels are seen at power (A) or color Doppler (B) exam due very slow intralesional flows.

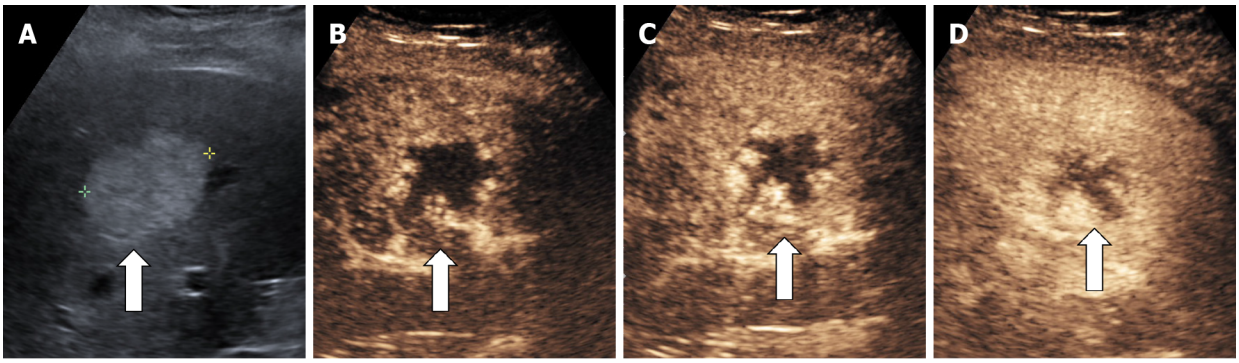

Figure 8 Typical hepatic hemangioma in B-mode ultrasound. A: Hyperechoic mass with sharp margins; B-D: After contrast agent administration the mass shows peripheral nodular enhancement in arterial phase (B and C) with partial centripetal filling in the late phase (D).

often in small hemangiomas (42% are under 1 cm in size)[13,31]. The homogeneous enhancement persists into the portal and late phases (Figure 11).

The mechanism of the enhancement is not clearly understood. The large proportion of small-sized hemangiomas with this type of loading suggests that this pattern may be due to a difference between blood spaces: the smaller the lesion, the more rapid is the spread of contrast agent within[31-33].

Rapidly filling hemangiomas could be difficult to be differentiated from hepatocellular carcinoma (HCC) and hypervascular liver metastases because they exhibit hypervascularity during the hepatic arterial phase. In the late phase, HH remains isoenhanced while metastases and most HCC show a typical washout of contrast agent during the portal and delayed phases. Differentiation remains difficult between small and well-differentiated HH and HCC, which do not show wash-out in the late phase [34].

Hemangioma with echoic border

In some cases (up to 15% of cases) HH has an echoic border, which is seen as a thick echoic rind or a thin echoic rim (Figure 12)[35]. The central part of the lesion has low echogenicity due to previous hemorrhagic necrosis, scarring, or myxomatous changes. On CEUS this type of HH often shows the typical pattern of enhancement so that the diagnosis can be made easily (Figure 13)[32,36].

Sclerosed/sclerosing hemangioma

When the HH is predominantly fibrosed with near complete loss of the vascular spaces it is called 'sclerosed/hyalinized' while partially affected lesions are called 'sclerosing/hyalinizing' hemangiomas[13,32].

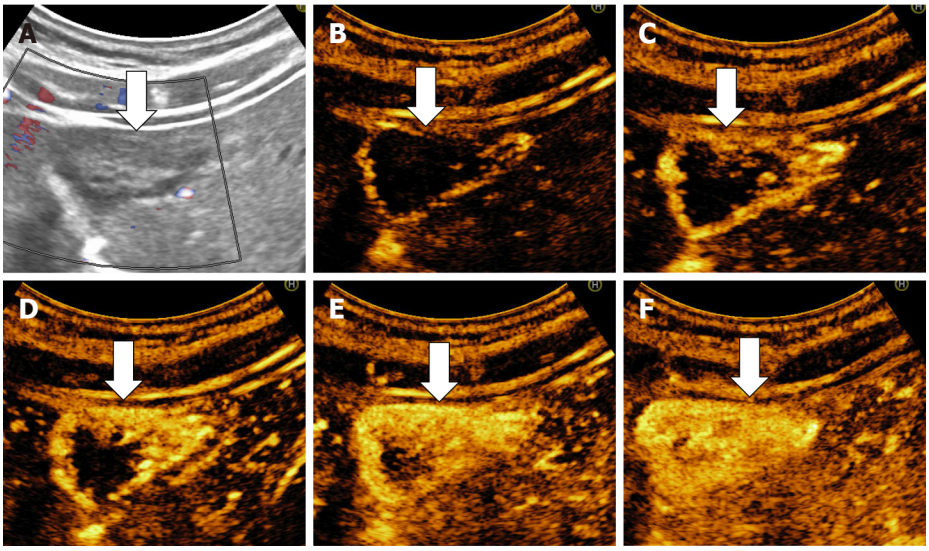

Figure 9 Example of hepatic hemangioma with inhomogeneous echogenicity. A: Gray scale ultrasound; B-E: On contrast enhanced ultrasound the hemangioma shows the typical peripheral nodular contrast enhancement (B and C) and centripetal fill-in (D and E); F: The mass shows strong homogenous enhancement in the late phase.

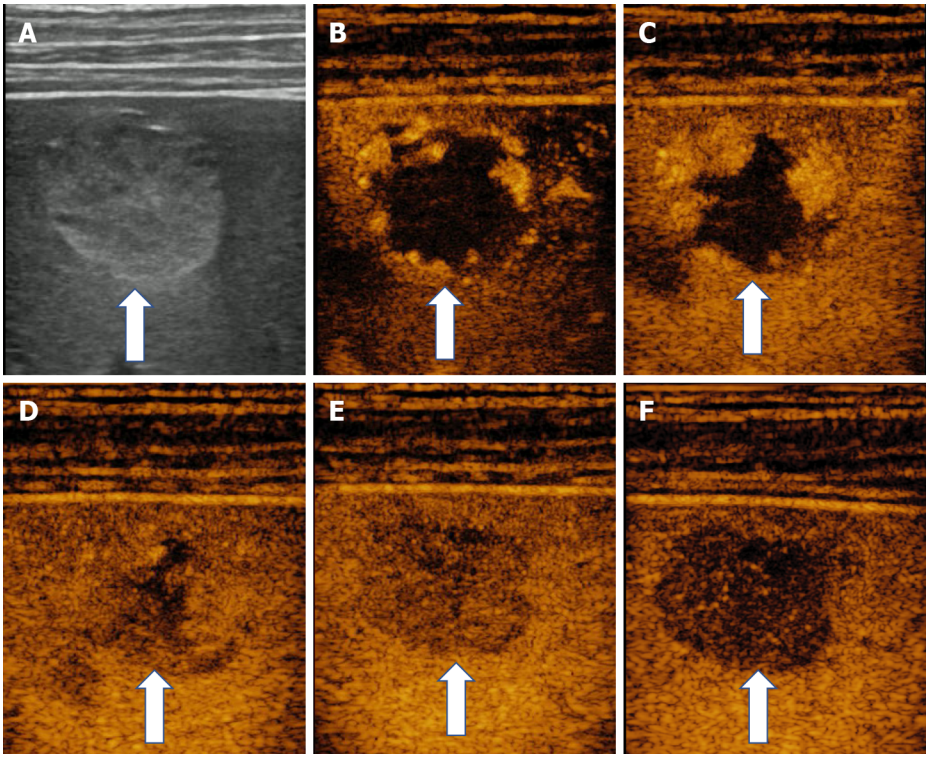

Figure 10 Ultrasound images using linear probe in a case of small, hyperechoic, subcapsular hepatic hemangioma. A: Gray scale ultrasound; B-E: A typical enhancement is showed in contrast enhanced ultrasound. Peripheral pools in arterial phase (B and C) and centripetal progression (D) followed by complete fill-in (E); F: In the late phase phenomenon of pseudo-washout is observed due to hyperinsonation determined by the proximity of the linear probe.

At ultrasound exam, sclerosed hemangioma are heterogeneous in echotexture with predominantly hypoechoic areas from sclerosis and geographic pattern [37]. When placed subcapsular HH causes capsular retraction. If the patient has been known for several years with HH and the images are evaluated dynamically, a reduction in size of the lesion over time can be observed[37].

In CEUS three patterns may be observed: no enhancement, persistent irregular ring enhancement and lack of early enhancement with slight peripheral enhancement in the late phase[33,38,39] (Figure 14). These enhancement patterns create differential diagnosis issues with the intrahepatic cholangiocarcinoma and liver metastasis[40]. In

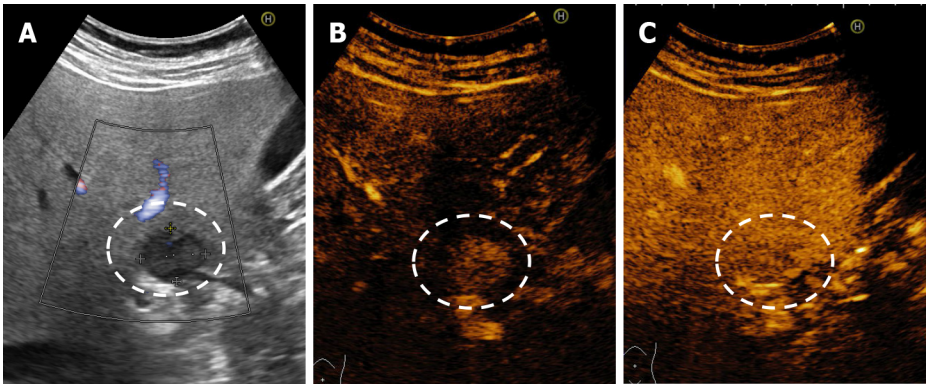

Figure 11 Example of a flashfilling hemangioma. A: On B-mode ultrasound a hypoechoic hemangioma is observed anterior of hepatic hilum; B and C: After injection of contrast agent, a rapid, uniform and intense homogeneous enhancement in the arterial phase (B) that persists into the late phases (C) is observed.

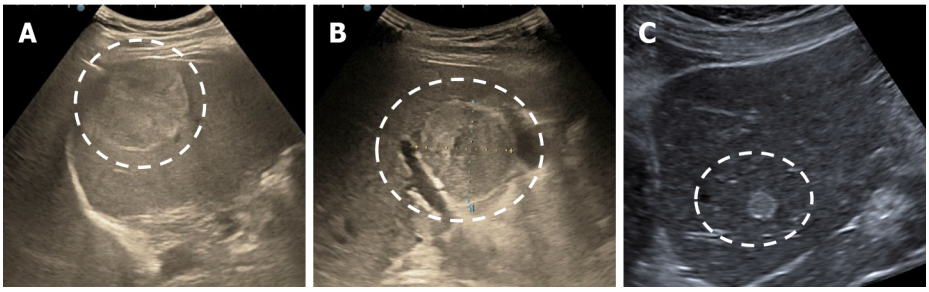

Figure 12 Illustration of hepatic hemangioma with echoic border. A-C: Hepatic hemangioma localized in the right (A and B) and left (C) liver lobe respectively.

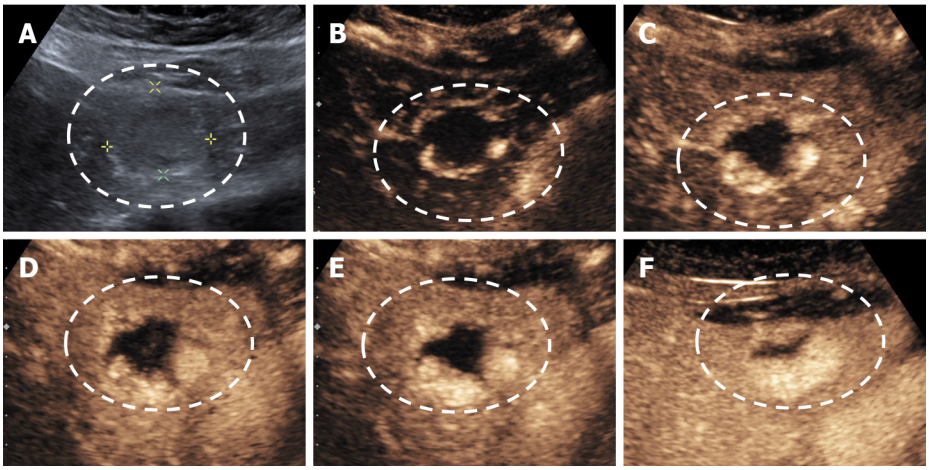

Figure 13 Example of hemangioma with echoic border. A: B-mode ultrasound; B-F: Typical pattern of enhancement: Peripheral nodular enhancement in arterial phase (B and C) centripetal filling (D and E) and incomplete enhancement in late phase (F).

a case report, reinjection of Sonasoid helped in the discriminate between the two entities[41].

Hemangioma with calcifications

In very rare cases, although the tissue is soft, HHs may have calcifications. It can appear in the marginal or central part of the lesion. There may be several spotted calcifications, which correspond to phleboliths or large coarse calcifications[13]. On post-contrast administration, calcified hemangiomas may appear poorly or no enhanced as the calcifications do not show enhancement[37] (Figure 15).

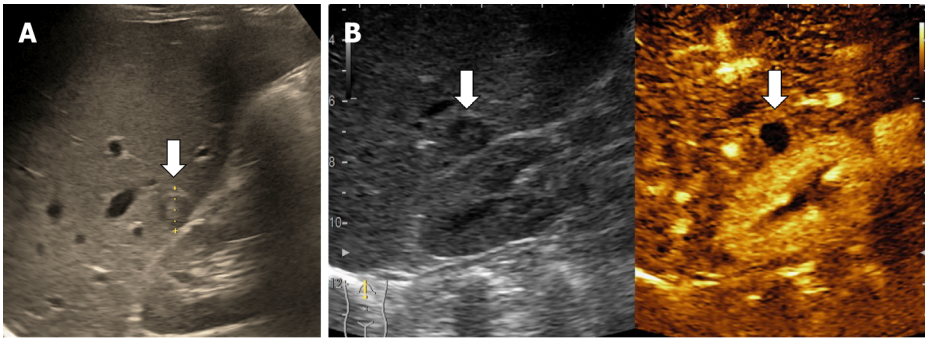

Figure 14 Sclerosed hemangioma in a 45-yr-old man detected on a routine ultrasound examination. A: B-mode ultrasound revealed a small hypoechoic lesion; B: In contrast enhanced ultrasound no enhancement is observed.

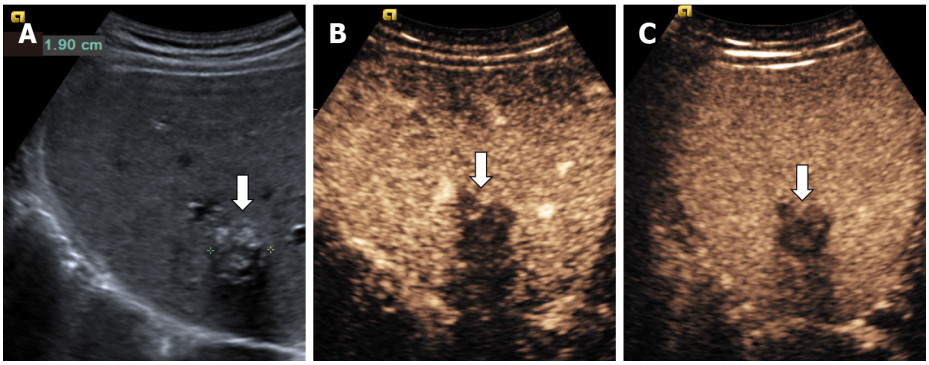

Figure 15 Hemangioma with calcifications in a 64-yr-old man detected on a routine ultrasound examination. A: On B-mode ultrasound several spotted calcifications are showed in the marginal and central part of the lesion and posterior acoustic shadow also; B and C: On post-contrast administration no enhanced is noticed in the portal (B) or the late phase (C).

Giant hemangioma

The majority of the authors define giant hemangiomas as lesions greater 12 cm in diameter[32,33,37]. On B-mode ultrasound, large hemangiomas often appear intense heterogeneous. After intravenous administration of contrast agent, the typical early, peripheral, globular enhancement is observed. However, during the venous and delayed phases, the progressive centripetal enhancement of the lesion is present but does not lead to complete filling[13,42] (Figure 16).

Cystic or multilocular hemangioma

Represents a very rare aspect of HH, cited in only few case reports[43-45]. On B-mode ultrasound appears as inhomogeneous lesion with a large central cavity that contains fluid and possible septa[13,46]. This type of hemangioma could originate from cystic degeneration caused by central thrombosis and hemorrhage[32]. The fluid cystic cavities appear anechoic on US or with hyperechoic material suggesting previous internal hemorrhage. In our experience, the typical early, peripheral, globular enhancement is observed, without centripetal progression of enhancement and the septa could have contrast enhancement as well (Figure 17). Although the appearance of B-mode ultrasound creates differential diagnosis issues with mucinous cystic neoplasm (biliary cystadenoma or cystadenocarcinoma)[47], epithelioid hemangioendothelioma[48] or angiosarcoma[49], CEUS directs the diagnosis to hemangioma.

Multiple hemangiomas and hemangiomatosis

HHs may be multiple in 10%-50% of cases[13]. In standard ultrasound multiple HH has hyperechoic, variable in size, well delimited (Figure 18). The presence of multiple FLLs in B-mode ultrasound has to be differentiated from liver metastases or other multiple malignancies.

Hemangiomatosis, also called diffuse hepatic hemangiomatosis (DHH), is a rare condition characterized by innumerable HHs distributed in the liver parenchyma[13]. In B-mode ultrasound the lesions appear frequently hyperechoic or hypoechoic and the boundary of the lesions is usually ill-defined as compared to multiple HH where

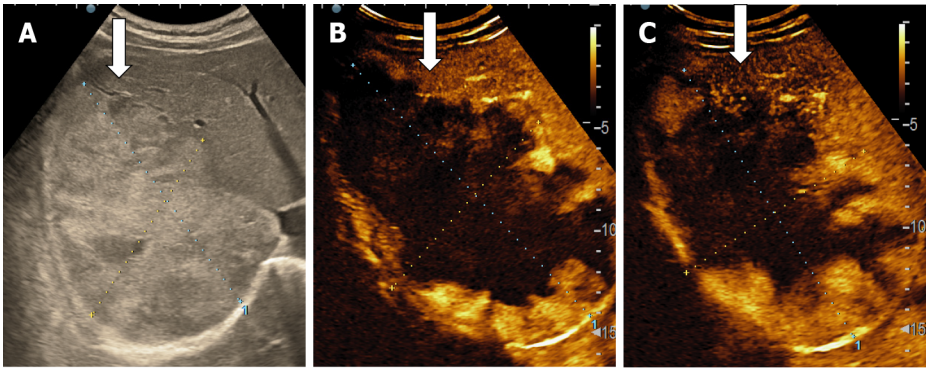

Figure 16 Oblique subcostal baseline image of the right liver lobe in a 45-yr-old woman. A: An intense heterogeneous, large hemangiomas (about 17 cm); B and C: After intravenous administration of contrast agent, the typical early, peripheral, globular enhancement (B) is observed followed by progressive centripetal incomplete enhancement of the lesion (C).

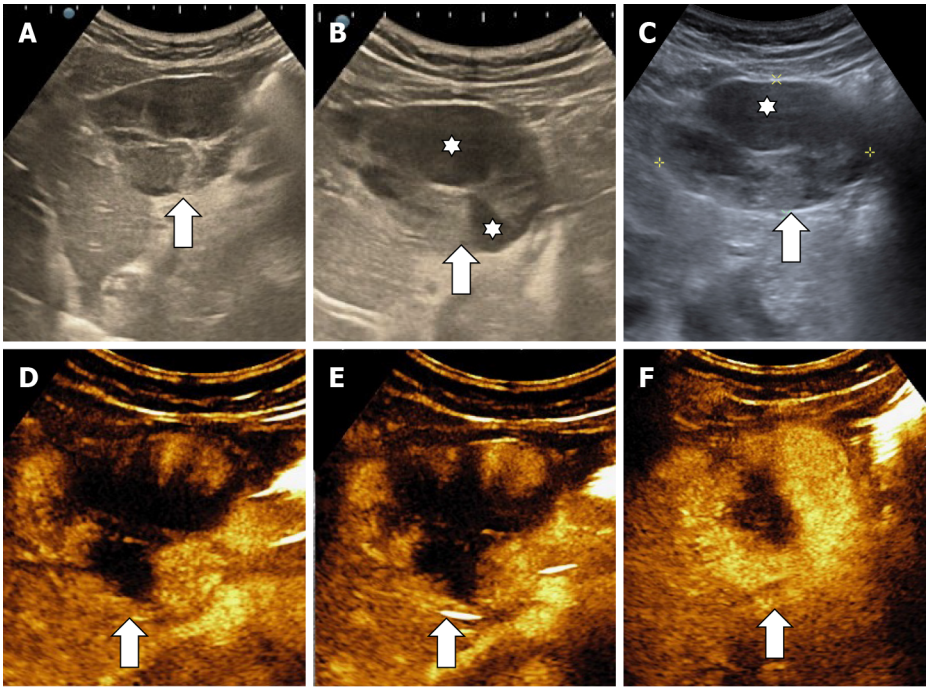

Figure 17 Multicystic hemangioma. A-C: B mode ultrasound shows an inhomogeneous lesion (A) with central cavity (stars) (B) that contains fluid and septa (C); D-F: In contrast enhanced ultrasound the mass shows a progressive (D and E) but partial filling (F) because of the presence of fluid-like cystic cavities that do not enhance.

the lesions are well delineated. DHH is more frequently seen in newborns where the entire liver is usually involved but uncommon cases of isolated DHH without extrahepatic involvement may be seen in the adult population (about 17 cases in the literature)[14,50].

Hepatic hemangiomatosis may present as two forms, a multinodular pattern consisting of multiple small discrete and coalescent nodules, and a diffuse pattern consisting of innumerable poorly defined lesions, with a tendency to confluence, replacing almost all of the liver[14]. To our knowledge, the appearance of DHH in contrast ultrasound has not yet been reported. In our experience, in DHH with multiple, small LFHs, the loading is of the "flashfilling" type (Figure 19).

HEMANGIOMA DEVELOPING IN ABNORMAL LIVER

Hemangioma in fatty liver

The incidence of liver steatosis has increased in recent years and HHs no longer have the typical ultrasound appearance in a hyperechoic liver. Most often they are

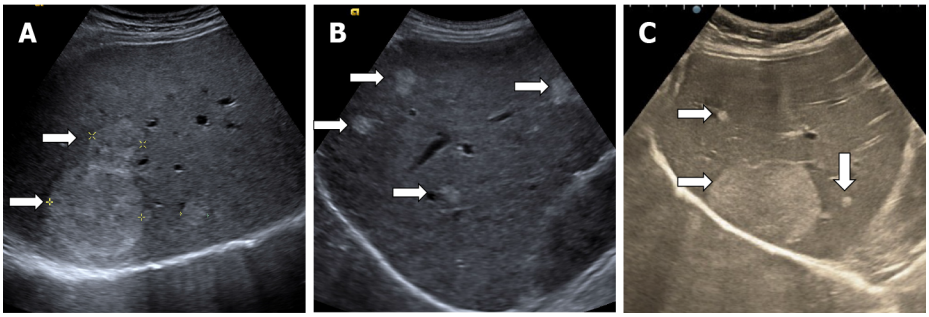

Figure 18 Illustration of multiple hepatic hemangioma in B mode ultrasound. A: Two hyperechoic lesions; B: Four small well delimited lesions; C: One large hepatic hemangioma besides two small hyperechoic lesions.

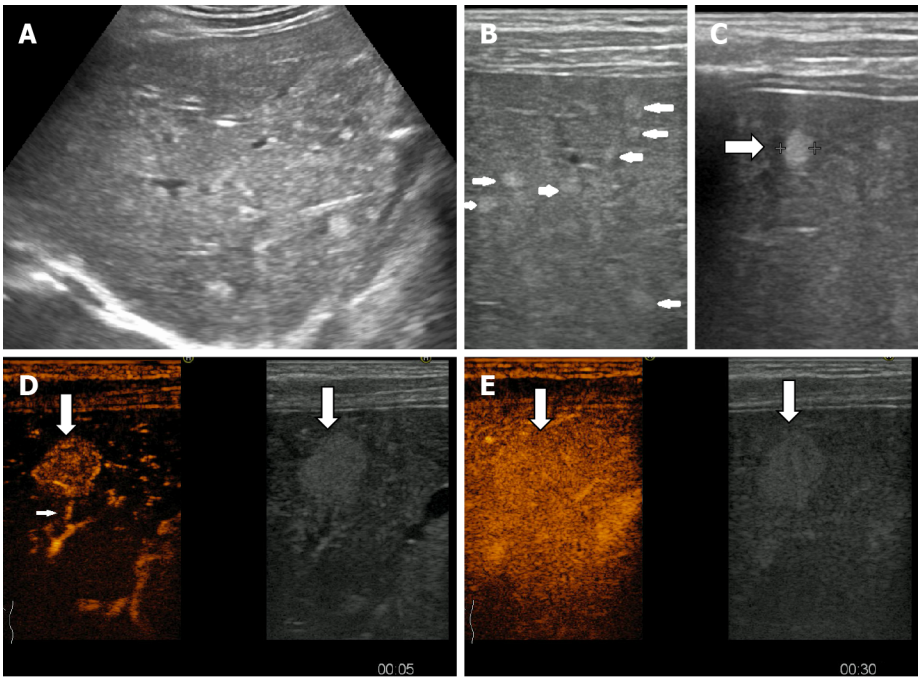

Figure 19 A multinodular pattern of hepatic hemangiomatosis on ultrasound. A: Small hyperechoic lesions are scattered throughout the right liver lobe; B and C: Multiple subcapsular infracentimetric hemangiomas on ultrasound exam using linear probe; D and E: On contrast enhanced ultrasound examination fast-filling hemangioma displaying early homogenous enhancement and visible afferent artery in the artherial phase (D), homogenous enhancement with surrounding parenchyma on early portal phase (E).

isoechoic, or hypoechoic relative to a hyperechoic, fatty liver[13]. In some cases, the area surrounding the hemangioma appears hypoechoic and resembles a halo, an appearance termed a "pseudohalo"[51] (Figure 20). Fortunately, in CEUS HH in fatty liver show a typical enhancement pattern of cavernous or flash-filling hemangioma[52-54] (Figure 21).

Hemangioma in cirrhosis

HHs in cirrhotic liver are uncommon compared to their incidence in non-cirrhotic liver [55]. It appears that the process of cirrhosis (necrosis and fibrosis) obliterates existing hemangiomas. In B-mode ultrasound, HH in cirrhotic liver had an atypical appearance, are often solitary and small in size[13,37,55] difficult to be differentiated from dysplastic nodules and HCC. In CEUS, the enhancement pattern of a cavernous hemangiomas (Figure 22) is enough for diagnosis but flash-filling enhancement of a HH is similar to the enhancement of an HCC in the arterial phase (Figure 23)[56]. Therefore, in the case of an FLL with a hyperenhancement appearance in the arterial phase, it is necessary to complete the imaging assessment.

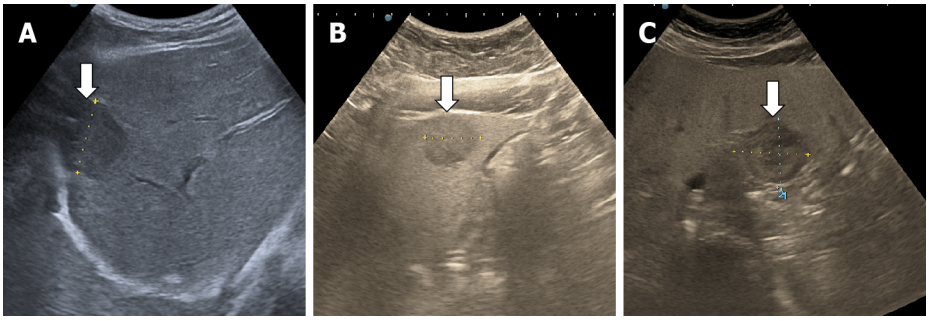

Figure 20 Examples of hypoechoic hemangioma relative to a hyperechoic, fatty liver. A and B: B mode ultrasound show a hypoechoic lesion with a subdiaphragmatic (A) and subcapsular position (B); C: Case of hepatic hemangioma in fatty liver with an area surrounding the lesion appears hypoechoic and resembles a halo, an appearance termed a " pseudohalo".

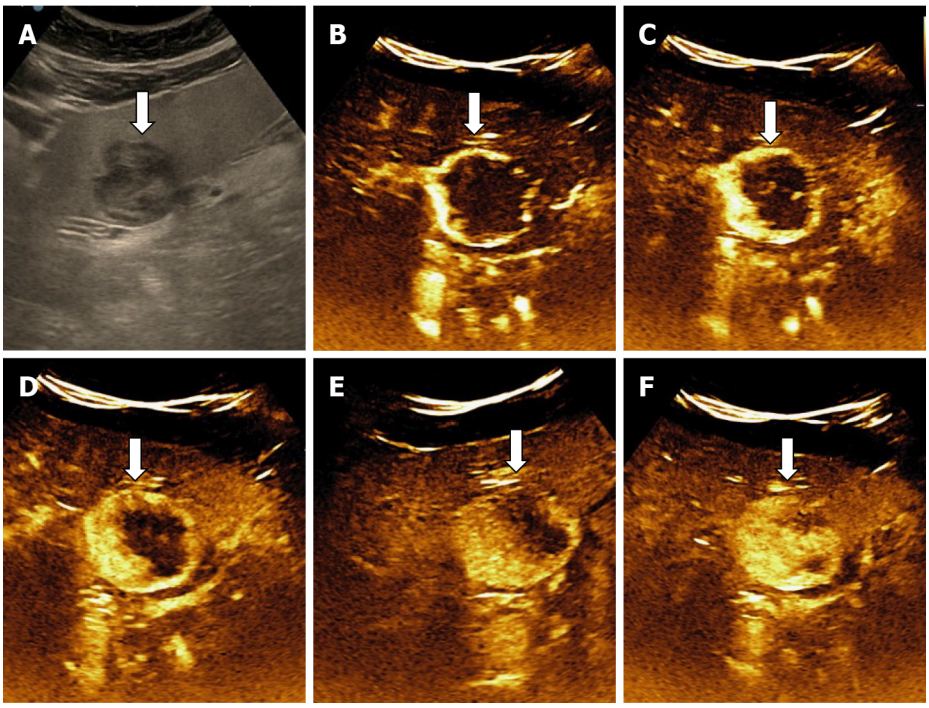

Figure 21 Hypoechoic hemangioma in 57-yr-old woman with liver steatosis. A: B mode ultrasound image; B-F: After intravenous administration of contrast agent, the typical early, peripheral, globular enhancement (B and C) is observed followed by progressive, centripetal (D and E) incomplete (F) enhancement of the lesion.

ONE STOP SHOP APPROACH

Ultrasound has been introduced into clinical practice for over 50 years. Contrast ultrasound after more than 15 years of use has been shown to provide more information than standard ultrasound in the diagnosis of liver tumors. In several countries, the hepatologist also practices ultrasonography. Thus, it has the possibility to complete on the spot the information obtained through anamnesis and clinical examination with imaging data. In an asymptomatic adult patient, without liver or oncological disease, the detection on standard ultrasound of a FLL below 3 cm with homogeneous hyperechoic appearance, sharp margin, posterior enhancement, absence of halo sign, without intra-tumoral vessels at colour Doppler directs the diagnosis to HH and does not require further investigation[1,16]. However, if ultrasound shows a lesion with features other than those described, measures over 3 cm or has been detected in oncology patients or those with underlying liver disease, contrast enhanced imaging (CEUS, CT or MRI) is required[1]. EFSUMB Guidelines for CEUS in the liver – update 2020 recommends CEUS as the first step[16]. CEUS can be performed immediately after standard ultrasound in the consulting room, without the need to assess renal function as needed in the administration of contrast agents for CT/MRI. Studies to date have shown that CEUS has similar performance to computed

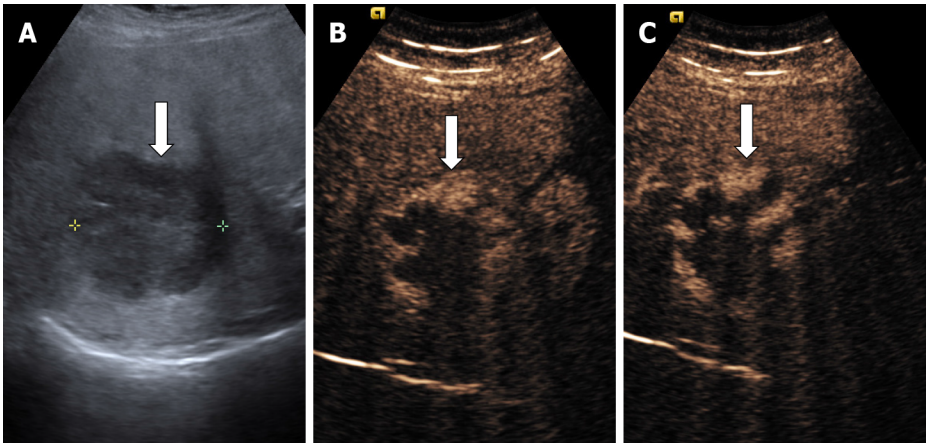

Figure 22 A case of cavernous hemangioma detected in a 64-yr-old man with liver cirrhosis. A: On B mode ultrasound is observed a hyperechoic inhomogeneous liver and a hypoechoic large lesion in the right liver lobe; B: On contrast enhanced ultrasound, the liver lesion shows a typical early, peripheral, globular enhancement; C: In the late phase incomplete enhancement is noticed.

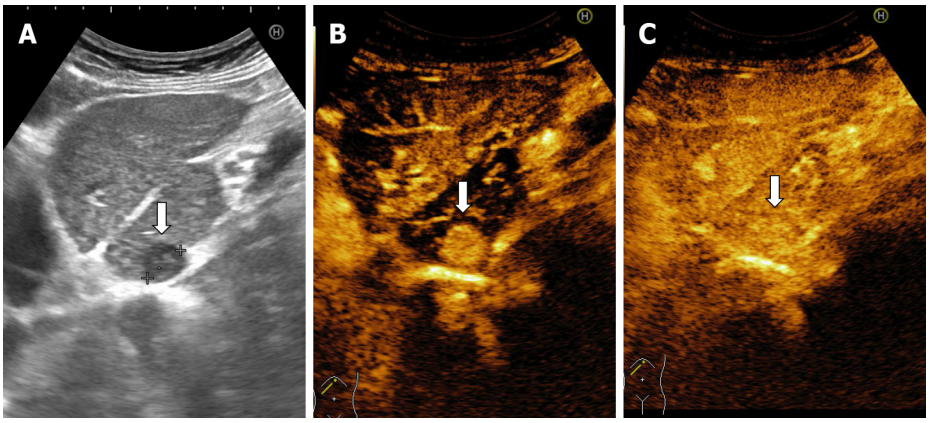

Figure 23 A difficult diagnosis in a case of a flash-filling hemangioma in a woman with hepatitis C liver cirrhosis. A: On B mode ultrasound is observed inhomogeneous liver structure and enlargement of caudate lobe. A small hypoechoic lesion is detected in the caudate lobe; B: Flash-filling enhancement in arterial phase is noticed that is similar to the enhancement of an hepatocellular carcinoma; C: Even in the late phase the liver lesion had the same enhancement comparative with liver, the segmental resection was performed. On histopathological exam the conclusion was: liver hemangioma.

tomography or MRI in the diagnosis of HH. The cost is lower[28,29,57,58], no irradiation and the contrast agent administered has lower toxic and allergic effects. A typical aspect of hemangioma in contrast ultrasound (peripheral and globular enhancement on arterial phase followed by a central enhancement on delayed phases) guides the diagnosis in a maximum of 30 min, stops further investigations and provides mental comfort to the patient. According to studies, this strategy includes approximately 85%-90% of patients[21-24]. If the appearance in the CEUS is not typical, the patient must be scheduled for further investigations. This diagnostic algorithm is applicable to the adult patient in countries where the hepatologist has an ultrasonography system equipped with CEUS software in the consulting room. CEUS saves time, is cost effective and non-invasive.

To our knowledge it is the first article to illustrate the typical and atypical aspects of HH in the adult patient by B-mode ultrasound along with CEUS. It is also for the first time when an algorithm for diagnosing HH is proposed in the consulting room, adapted according to the latest guidelines of EASL and WFUMB (Figure 24).

CONCLUSION

In conclusion, standard and contrast-enhanced ultrasound examination in a clinical context guides the diagnosis of HH in most patients.

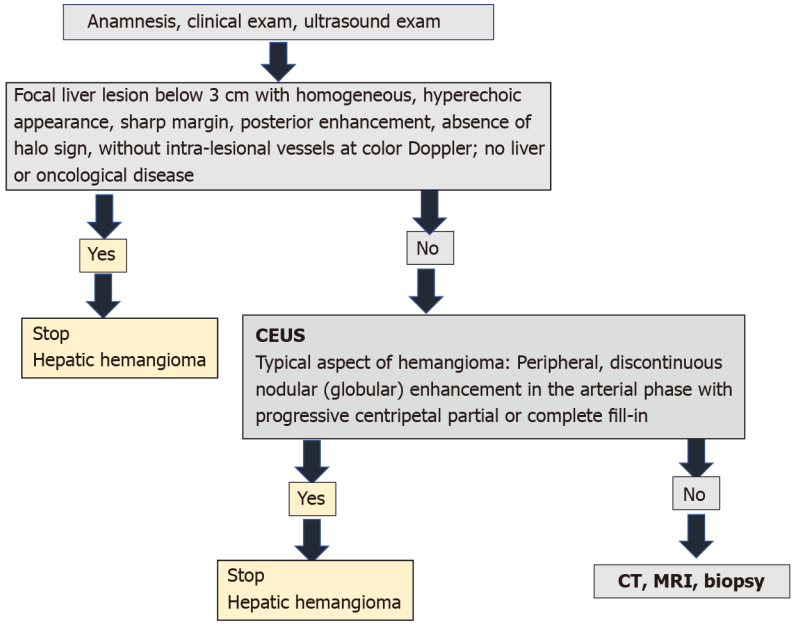

Figure 24 One stop shop approach for the diagnosis of liver hemangioma. Algorithm for diagnosing hepatic hemangioma in the consulting room, adapted according to the latest guidelines of European Association for the Study of the Liver and World Federation for Ultrasound in Medicine and Biology. CEUS: Contrast enhanced ultrasound; CT: Computed tomography; MRI: Magnetic resonance imaging.

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MINIREVIEWS

Liver function in COVID-19 infection

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Abstract

Coronavirus disease 2019 (COVID-19) disease affects multiple organs, including anomalies in liver function. In this review we summarize the knowledge about liver injury found during severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection with special attention paid to possible mechanisms of liver damage and abnormalities in liver function tests allowing for the evaluation of the severity of liver disease. Abnormalities in liver function observed in COVID-19 disease are associated with the age and sex of patients, severity of liver injury, presence of comorbidity and pre-treatment. The method of antiviral treatment can also impact on liver function, which manifests as increasing values in liver function tests. Therefore, analysis of variations in liver function tests is necessary in evaluating the progression of liver injury to severe disease.

Key Words: COVID-19; Pathogenesis of liver injury; Angiotensin-converting enzyme 2 receptor; Liver function tests; Severe COVID-19; Treatment effect

Core Tip: The frequency of abnormalities in liver function tests (LFTs) in coronavirus disease 2019 (COVID-19) infected patients increases with age and is observed in males more than females. A pre-existing history of liver disease and comorbidity increases LFT abnormality and the likelihood of severe liver damage in COVID-19 infection. Antiviral treatment and treatment of comorbid diseases intensifies the hepatotoxic effect on the liver, which often manifests itself in higher levels in LFTs.

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INTRODUCTION

Pulmonary disease is the primary clinical manifestation in patients with coronavirus disease 2019 (COVID-19) disease. There is increasing evidence of the involvement of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in multiple organs including the heart, kidneys, central nervous system and liver. In this paper we summarize data concerning liver injury in COVID-19 patients with special attention paid to the possible mechanisms of liver damage and laboratory tests to monitor liver injury during SARS-CoV-2 infection.

GENERAL CHARACTERISTICS

COVID-19 is an acute respiratory infectious disease caused by SARS-CoV-2[1,2]. The SARS-CoV-2 belongs to the *Coronaviridae* family of enveloped, single-stranded RNA viruses[3]. There is evidence that SARS-CoV-2 shares nearly 80% of its genomic sequence with SARS-CoV and about 50% with Middle East respiratory syndrome coronavirus[2,4]. COVID-19 is a viral infectious disease affecting all age groups (from infants to the elderly) resulting in a wide range of clinical manifestations[5-7]. The incubation period of COVID-19 tends to vary from 1 d to 14 d[8].

Multiple organ involvement

Furthermore, COVID-19 infection can present itself with differing degrees of severity, varying from asymptomatic and mild disease to viral pneumonia, in addition to various other extra-pulmonary manifestations, including for example heart, kidney, central nervous system or liver affection, with a risk of fatality[5-7]. Thus, the virus is capable of affecting any organ in the body, and in critically ill patients multiple organs are often affected. Mild cases of COVID-19 infection exhibit symptoms such as fever, dry cough, fatigue, vomiting, diarrhea, muscle weakness, and chest pain[5,7,8]. Patients may also suffer from headaches, as well as loss of smell and taste. While, in severe cases, respiratory distress and/or hypoxemia occur one week after the onset of the disease leading to deterioration into acute respiratory distress syndrome (ARDS), metabolic acidosis, septic shock, and in some cases, even death[5,7,8]. SARS-CoV-2 presents primarily as a lower tract respiratory infection transmitted *via* air droplets, but evidence of the multisystemic nature of COVID-19 is still significantly increasing [5,7,8]. The complications of COVID-19 are associated with several risk factors, namely, advancing age (> 65 years old), cardiovascular disease, hypertension, chronic respiratory disease, diabetes, and obesity[5,8]. The most common reported complication is ARDS, but other severe or even fatal complications are pneumonia, sepsis, metabolic acidosis, heart failure, and acute kidney injury[5,9-11].

Main pulmonary manifestations

Pulmonary affection is the most common serious COVID-19 manifestation[7]. There is evidence that the severity of pulmonary affection caused by SARS-CoV-2 ranges from lack of symptoms or mild pneumonia in 81% of cases, to severe cases associated with hypoxia - in 14% of cases; critical disease associated with shock, respiratory failure and multiple-organ failure - in 5% of cases; or death - in 2.3% of cases[7,12]. SARS-CoV-2 infection induces alveolar damage and interstitial inflammation. During the course of inflammation, the dendritic cells and alveolar macrophages phagocytose epithelial cells infected by SARS-CoV-2, whilst at the same time, the immune mechanisms with T cell responses are activated[7,13].

So, in patients with COVID-19 infection levels of proinflammatory cytokines and chemokines *e.g.*, interleukin 6 (IL-6), IL-1β, tumor necrosis factor, interferon γ, granulocyte stimulating factor are increased[7,8,14]. There is a suggestion that cytokine storms play a crucial role in the immunopathology of the COVID-19 infection.

Cardiac manifestations

Cardiac injury is a common characteristic of patients with COVID-19 infection. Furthermore, despite the fact that cardiovascular diseases might significantly worsen

the clinical outcome of COVID-19 patients, SARS-CoV-2 infection might also induce new cardiac complications[5,15]. Additionally, this cardiac damage might even occur without of any signs or symptoms of pneumonia and with an absence of other complications[5-7]. The major effects of SARS-CoV-2 infection on cardiomyocytes, include for example, acute myocardial injury, heart failure, impaired renal function, arrhythmias, cardiac arrest, myocarditis, sepsis, and septic shock[5,8,16]. The most frequently presented cardiac complication associated with COVID-19 infection is an acute myocardial injury with an estimated prevalence of 8%-12%[5,6,17]. Additionally, the most prevalent complications, with an estimated incidence of 16.7%, are brady- or tachyarrhythmias, also blood pressure abnormalities and dysfunction of the left ventricular[5,6,18]. Importantly, cardiac complications may occur long after viral clearance and recovery, because the inflammation can persist and evolve silently[6,7]. Confirmation of this thesis is exemplified by pulmonary fibrosis, avascular necrosis or dyslipidemia which have evolved over the long term in many survivors of SARS infection, which is closely related to COVID-19 caused by SARS-CoV-2[6]. There is evidence that about one-half of fatal cases show acute cardiac injury and heart failure [6]. These conditions are more probable in elderly patients, while in younger patients myocarditis is the more likely cause.

Although pulmonary disease is the primary clinical manifestation in patients with COVID-19, with cardiac and kidney injury also being common, as we mentioned above there is increasing evidence of its involvement in multiple organs. In this paper we summarize data concerning liver injury in COVID-19 patients.

POSSIBLE PATHOMECHANISMS OF LIVER INJURY

The alteration of hepatocyte damage biomarkers, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and bilirubin is a common laboratory finding in patients with COVID-19 infection. However, the pathomechanism of liver injury during infection is convoluted and not yet fully understood[8,19]. Is not clear if the liver damage is caused by the direct viral effect or if it perhaps reflects a more severe inflammatory response with hepatic injury[20,21]. The possible major pathomechanisms of liver damage are presented in Figure 1. It has been reported that the angiotensin-converting enzyme 2 (ACE2) was identified as the SARS-CoV binding site[19,20,22]. This data facilitated confirmation that SARS-CoV-2 may also directly enter the host cells through binding of its S protein to ACE2 on the surface of the host cell, although with a 10-20-fold higher affinity[2]. The ACE2 receptor expression is higher in many organs, such as lungs, heart, kidney, and it is widely expressed across a variety of cell types[8,22]. Hepatocytes and bile duct epithelial cells also express the ACE2 receptor[7,8,19]. Nevertheless, no significant altered histopathological features have been detected in such cells from COVID-19 patients[8,23]. Only single studies have claimed that the derangement of liver function is usually mild and there is not enough evidence that late-onset symptoms are related to increasing liver damage in patients with COVID-19 infection[2,19]. Additionally, recent data has suggested that SARS-CoV-2 may directly bind to ACE2 expressed in cholangiocytes, because there is evidence that ACE2 expression is displayed in 2.6% of hepatocytes and 59.7% of cholangiocytes[2,19]. Moreover, the alteration of cholangiocyte injury biomarkers, such as alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) has been observed in some cases, and consistent with biliary epithelial cell damage, and about 10% of patients with COVID-19 infection have an elevated total level of bilirubin[2,24]. There is evidence that specific expression of ACE2 in bile duct epithelial cells was about 20 times higher than in hepatocyte. Furthermore, the bile duct epithelial cells play a substantial role in immune response and liver regeneration. So, this data suggests that liver damage in COVID-19 infection results from bile duct cell injury rather than a direct viral effect in liver cells[19].

On the other hand, the liver is a vital organ for the metabolism of drugs. It is well known that patients suffering from certain viral infections caused for example, by the human immunodeficiency virus or hepatitis C virus are more prone to develop drug-induced liver injury, particularly when it is associated with highly active antiretroviral therapy[25-27]. Therefore, nowadays it is postulated that the same mechanism of liver injury could be present in COVID-19 as a result of the SARS-CoV-2 virus. Thus, hepatotoxicity during the course of the COVID-19 infection, may be initiated by the different types of antiviral drugs, antibiotics and steroids which are currently used to treat COVID-19 patients[25,28]. However, there is a lack of evidence for liver damage in chronic COVID-19 patients being completely drug-induced. A

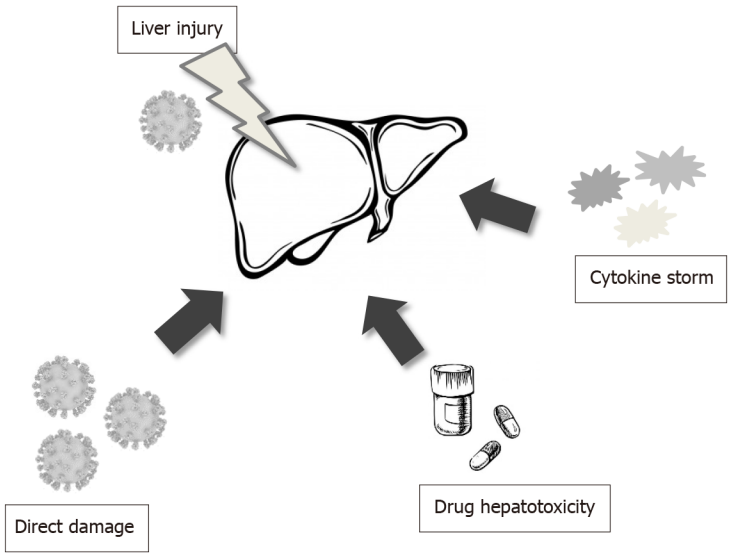

Figure 1 Possible pathomechanism of liver injury in patients with severe acute respiratory syndrome coronavirus 2 infection.

potential example of the relationship between the use of certain drugs and resulting liver damage is found in the study of Fan *et al*[29]. They reported that a high percentage of patients with abnormal values in liver function tests (LFTs) were treated with lopinavir and ritonavir during hospitalization. Similar results appeared in the study of Cai *et al*[30]. Moreover, they reported an almost four-fold increase in liver injury after lopinavir/ritonavir were used in the treatment of severe COVID-19 infection. This finding is consistent with some liver biopsy findings[31]. Certain studies have reported mild lobular and portal activity and moderate microvascular steatosis in patients who died from COVID-19[23]. Further evidence also showed minimal lymphocytic infiltration and mild sinusoidal dilatation in COVID-19 patients [24]. However, these alterations are nonspecific and may be caused by drug-induced liver injury, not excluding the possibility of hypoxemia or having come directly from the SARS-CoV-2 virus[19,23]. Considering these facts, it is very important that these patients be treated with drugs that can inhibit inflammatory response while at the same time protecting hepatic functions.

Another possible reason for liver damage in patients with COVID-19 infection may be dysregulation of the innate immune response[2,19,22]. There is evidence that inflammatory cytokine storms were found in chronically ill patients. The increased values of inflammatory indices, such as C-reactive protein (CRP), IL-6, neutrophils and lymphocytes can be observed in patients with COVID-19 infection, which suggests a relationship between liver damage and inflammatory response induced by severe COVID-19 infection.

ABNORMALITIES IN LABORATORY TESTS

There are many studies showing abnormal laboratory test results in patients with severe COVID-19 disease[32-35]. The first cases of COVID-19 patients from China with liver abnormality were documented by Chen *et al*[32]. Elevations in ALT, AST and lactate dehydrogenase (LDH) were present in 43 out of 99 patients, while most of these cases showed some mild abnormality, whilst one patient exhibited a large increase in test results (ALT of 7590 U/L and AST of 1445 U/L). Most of the participants were male, half of them with chronic diseases. LFTs not only showed abnormalities such as aminotransferases, but also noted were decreased haemoglobin, platelets, an increase of creatine kinase, LDH, ferritin, CRP and a decrease/increase in leucocytes[32].

Cai *et al*[30] conducted laboratory tests on a population of 417 patients with COVID-19 in Shenzhen hospital, China. Three hundred and eighteen patients were confirmed with abnormal liver test results, whilst another 90 had liver injury during hospitalization. The patients were qualified to the appropriate types. Abnormalities such as: hepatocellular type [elevated ALT and/or AST more than 3 × the upper limit unit of normal (ULN)], cholangiocyte type (raised ALP or GGT 2 × ULN) or mixed type (elevated ALT and/or AST more than 3 × the upper limit ULN and raised ALP or GGT

2 × ULN). The highest increase (3 × ULN) in liver enzymes such as ALT (23.4 % of patients), AST (14.8%), total bilirubin (TBIL) (11.5%) and GGT (24.4%) was noticed during the second week of hospitalization. Out of 318 cases, the mixed type dominated and there was a noted increase in all the above tests, except for ALP. In relation to the population of 90 patients, an increase was seen in ALT and GGT, while AST and TBIL were hardly visible. Mixed type patients or those with abnormal test results are at a greater risk of advanced to severe disease. Patients treated with lopinavir/ritonavir had much higher levels of TBIL and GGT, with an associated four-fold increase in the risk of liver damage[30].

A Study carried out on 292 patients in Italy led researchers to different conclusions than Cai *et al*[30]. In their opinion, LFTs are not associated with the patient's condition deteriorating to a severe form of pneumonia. Elevations in AST (18.5%), ALT (26.7%), GGT (36.2%), TBIL (10.6%) and ALP (9.2%) were inconsiderable[36]. Only ALP was not ruled out as a predictive factor, however, it may be associated with bad patient condition, systemic inflammatory response or SARS-CoV-2 tropism for the liver and ACE2 converting enzyme expression in cholangiocytes and hepatocytes. Although 250 patients were treated with lopinavir/ritonavir and 56 patients died, 82 deteriorated and 56 were admitted to intensive care, this was not in any way related to LFTs. Researchers recommended drawing conclusions carefully in the context of a complex multi-organ disease[36].

Wang *et al*[37] conducted an experiment on 156 people diagnosed with the SARS-CoV-2 virus from 2 chosen centers in China, in which they tested the correlation between the prognosis of patients and liver enzyme abnormalities, or lack of such abnormalities. Sixty-four of them had elevated AST and ALT which correlated with disease severity, higher alveolar-arterial oxygen partial pressure difference, growth of GGT, lower albumin and CD4+ T cells and B lymphocytes. The histological trial revealed severe liver apoptosis. Cytopathy in hepatocytes showed ultrastructural features such as endoplasmic reticulum dilatation, mitochondrial swelling and an impaired cell membrane. The above evidence shows that the virus has an influence on the increase in the value of liver enzymes. The most important observation was an association between a very high level of alveolar-arterial oxygen tension difference (A-aDO2) and elevated transaminases. According to this study, SARS-CoV-2 virus infection is a direct factor in liver disease[37].

Conclusions from a study carried out on 5771 adult patients from 10 hospitals in Wuhan indicated a need for monitoring hepatic parameters during hospitalization [38]. On admission to the hospital, chronically ill patients had AST levels significantly higher than ALT. Abnormalities in LFTs have been additionally associated with males, treatment, chronic liver disease, lymphocyte, neutrophil and platelet count. Abnormalities in LFTs, such as AST, ALT, TBIL, GGT, were related to mortality, however AST had the highest correlation. A significantly higher level of AST compared to ALT was also confirmed in the study of Guan *et al*[39] and Chu *et al*[40].

The medical records of 838 patients hospitalized in China indicated an increased level of AST and GGT[40]. Anomalies in LFTs (AST, GGT) were associated with organ injuries, hypoxia, inflammation and the use of antiviral drugs. The level of AST, ALT, GGT and total bilirubin displayed no significant difference between patients who were treated or not treated with umifenovir. By way of contrast, patients who underwent lopinavir/ritonavir treatment had higher levels of AST and GGT. Among the total number of COVID-19 patients, 48.8% showed normal liver function and 51.2% liver injury. Fan *et al*[29] observed abnormal liver function defined as increased LFTs in 57.8% of SARS-CoV-2 patients treated with lopinavir/ritonavir. Moreover, research in Italy suggested that remdesivir may be significant in the origin of hepatocellular injury [41]. Four out of five patients who switched from lopinavir/ritonavir to remdesivir had a reduced level of bilirubin, and significantly increased levels of AST and ALT.

In a study of 2115 people conducted in China, a more notable level of liver injury was uncovered in the group treated with lopinavir/ritonavir than in the untreated group[42]. Patients with COVID-19 and with pre-existing liver injury had more severe disease and a higher prevalence of mortality. However, the observed changes did not mimic the so-called 'cytokine storm' because the absolute lymphocyte count was lower and ESR was higher in the liver injury group than that of the non-liver injury group.

Hundt *et al*[43] observed abnormal liver tests at admission (AST 66.9%, ALT 41.6%, ALP 13.5%, and TBIL 4.3%) and peak of hospitalization (AST 83.4%, ALT 61.6%, ALP 22.7%, and TBIL 16.1%). Moreover, the type of treatment used (hydroxychloroquine, lopinavir/ritonavir, remdesivir, tocilizumab) was associated with abnormal liver transaminase elevations during hospitalization. The results of liver tests were associated with intensive care unit (ICU) admission, mechanical ventilation and death, as well as age, sex and comorbidities. Patients with severe COVID-19 showed an

increase in the total of bilirubin and regardless of severity, a significant rise in transaminases and decrease in albumin was observed[43]. Studies conducted on the Indian population also confirmed the link between laboratory test abnormalities and the severity of the disease[44]. Kumar *et al*[44] included 91 patients in their study, excluding those with pre-existing liver disease (hepatitis B and C, alcoholics, those on known hepatotoxic treatment). The analysis of patients divided into groups (I. asymptomatic, II. mild, III. moderate, IV. severe) showed that the level of transaminases was highest in group IV, ALP was highest in group III but for total bilirubin growth there was no difference between the groups. This study showed that AST and ALP are better tests for indicating the severity of liver damage in COVID-19 than ALT and TBIL.

LFT abnormality was confirmed in 17.6% of Chinese patients with the COVID-19 infection (a population of 159 patients)[45]. The authors concluded that frequency of LFT abnormality was greater in patients with chronic disease than those with mild/moderate illness, especially in older patients. In the another study (148 cases) abnormal liver function was noted in 37.2% of patients on admission and nearly half of those were over 50 years old, half of the 37.2% being men[44]. The patients with abnormal liver function had higher inflammatory indexes (CRP and procalcitonin). On admission, patients who received lopinavir/ritonavir treatment displayed a higher frequency of abnormal LFTs than those with normal liver function. The effect of antiviral treatment on liver function was observed in the study of Zampino *et al*[41]. Treatment of COVID-19 patients with remdesivir can cause hepatocellular injury with aminotransferase elevation, in contrast to the trend of bilirubin elevation with lopinavir/ritonavir treatment.

Abnormally raised liver enzymes were seen in about half of patients with COVID-19 disease[46]. AST and/or ALT > 3 × ULN, and/or ALP and/or GGT > 2 × ULN was seen in 53.5% of patients with hepatocellular injury. In addition, an association between LFTs and markers of inflammation (CRP and ferritin) was observed. Total protein and albumin, were significantly reduced in patients with abnormal liver enzymes and in patients with liver injury, in contrast to the total bilirubin level, which was significantly increased in these patients. Hepatocellular and cholestatic liver injury was more frequent in patients below the age of 50, whereas in patients over 50 years old, more common was the mixed type of liver injury.

Among a French cohort of 281 patients, 102 of them had increased liver enzymes (36.3%)[47]. The most common was an increase in GGT, followed by AST and ALT. Cases with elevated LFTs and CRP value were associated with higher rates of admission to ICU and mortality. Age, sex, diabetes and hypertension were not associated with disease severity. High levels of ALT or AST are associated with disease severity. The authors suggested that liver abnormalities are due to sepsis and tissue hypoxemia, which is documented by apoptotic injuries visualized in the histological examination (vesicular steatosis and watery degeneration). In summary, liver test abnormalities are associated with a poorer prognosis in patients with the coronavirus disease 2019[47].

A study conducted in Istanbul confirmed that liver test abnormalities, especially the AST/ALT ratio, was a good marker of mortality risk and the need for ICU admission [48]. A poorer prognosis rate was associated with higher levels of AST and ALT in the mixed pattern group followed by the hepatocellular injury group and the cholestatic injury group. Mortality in patients with abnormal AST and ALT was higher than that of patients with normal results. The patients with increased AST and ALT showed elevated levels of CRP, procalcitonin, ferritin, D-dimer, lactate and TBIL, which ultimately extended the hospitalization period[48]. The percentage of people in the ICU with elevated aminotransferases was higher than those with normal test results. Patients with ratio AST/ALT > 1 had a higher level of CRP, fibrinogen, LDH, APTT, d-dimer and lower levels of lymphocyte, albumin and GGT. This study showed that low albumin may be marker of severity in SARS-CoV-2 during the hospital admission. Abnormalities in LFTs are more common in men compared to women.

Comorbidities in people with liver diseases are a huge problem, which may have an impact on the severity of COVID-19. A prime example is obesity, in which a person is more prone to develop non-alcoholic fatty liver diseases (NAFLD)[49]. In adipose tissue, there may be a greater expression of ACE2, which increases the risk of severe COVID-19. Chronic liver disease also affects the severity of the disease. This may be related to low levels of blood platelets and lymphocytes[50]. A higher index of cytokines has also been reported, which may influence the progression of NAFLD[51]. In the course of liver cirrhosis, attention should be paid to the activation of cytokines, which leads to hepatocyte necrosis. A study population, from 9 hospitals in Lombardy showed higher mortality (17 out of 50 respondents died)[52]. There was a decrease in

albumin in patients and a significant increase in bilirubin, creatinine and prothrombin. Zou *et al*[53] detected elevated LFTs in 105 Wuhan patients with chronic HBV infection and coexisting SARS-CoV-2 (ALT 20.95%, AST 27.62%, TBIL and GGT 6.67%). These values changed during hospitalization, where 28.57% of the subjects developed acute or chronic liver failure[53]. Research carried out on 9 pregnant women showed lymphopenia (< 10×10^9 cells per L) in 5 of them, elevated CRP (> 10 mg/L) in 6 and 3 had raised AST and ALT[54]. One patient demonstrated a very high level of AST (1263 U/L) and ALT (2093 U/L).

Liver injury in severe COVID-19

The liver test abnormalities mentioned above are more frequently found in severe COVID-19 infection than in mild courses of the same infection. A few studies have demonstrated a relationship between liver test abnormalities, disease severity and mortality of patients with COVID-19[30,55]. A higher rate of LFT abnormalities was observed in severe COVID-19 infection. The higher liver test markers such as ALT, AST, GGT and total bilirubin were reported more in severe patients than in non-severe ones[56,57]. A large cohort study totalling 1099 patients, reported a much higher level of ALT and AST in severe patients (28% and 39%, respectively) than in non-severe patients (20% and 18%, respectively)[39]. So-called weighted mean difference for AST, ALT, total bilirubin and for albumin were associated with a significant increase in the severity of COVID-19 infection[58]. Among the 3381 patients included in the retrospective cohort study, 67.2% of them who were positive for SARS-CoV-2 had higher initial and peak of ALT than those who were negative[59]. Additionally, severe acute liver injury was significantly associated with elevated inflammatory markers including ferritin and IL-6. Besides ferritin and IL-6, other tests such as WBC count, lymphocyte count and platelet count were strong discriminators for severe disease[60].

There is a discrepancy between the frequency of liver test abnormalities and the liver injury in COVID-19 patients. For example, elevated liver damage markers were present in 76.3% of hospitalised patients but only 21.5% of them had liver injury[30]. This variance can be explained by pre-existing liver diseases, which contributed to the severity of liver injury during COVID-19 infection[61,62]. Finally, patients with severe liver injury are more likely to have a poorer prognosis[21]. On the other hand, pre-existing liver disease can increase the risk of COVID-19 infection[63].

CONCLUSION

Not all COVID-19 patients have liver injury and abnormalities in LFTs. However, after measuring the wide variations in these tests, the clinicians can come to some conclusions about the severity of the liver disease and improve the prognosis for patients with liver damage.

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MINIREVIEWS

Potential role of noninvasive biomarkers during liver fibrosis

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Abstract

Various types of liver disease exist, such as hepatitis and alcoholic liver disease. These liver diseases can result in scarring of liver tissue, cirrhosis, and finally liver failure. During liver fibrosis, there is an excess and disorganized accumulation of extracellular matrix (ECM) components which cause the loss of normal liver cell functions. For patients with chronic liver disease, fibrosis prediction is an essential part of the assessment and management. To diagnose liver fibrosis, several invasive and noninvasive markers have been proposed. However, the adoption of invasive markers remains limited due to their inherent characteristics and poor patient acceptance rate. In contrast, noninvasive markers can expedite the clinical decision through informed judgment about disease stage and prognosis. These noninvasive markers are classified into two types: Imaging techniques and serum biomarkers. However, the diagnostic values of biomarkers associated with liver fibrosis have also been analyzed. For example, the serum levels of ECM proteins can react to either matrix accumulation or degradation. During virus-host interactions, several regulatory steps take place to control gene expression, such as the change in cellular microRNA expression profiles. MicroRNAs are a class of non-coding RNAs (18-20 long nucleotides) that function by post-transcriptional regulation of gene expression. Although various noninvasive markers have been suggested in recent years, certain limitations have restricted their clinical applications. Understanding the potential of non-invasive biomarkers as a therapeutic option to treat liver fibrosis is still in progress.

Key Words: Liver fibrosis; Non-invasive biomarkers; Viral hepatitis; MicroRNA; Cirrhosis; Fibroscan

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Core Tip: Liver disease is quite common these days. Hepatitis, alcoholic liver disease, and non-alcoholic fatty liver disease can lead to liver cirrhosis. Liver fibrosis assessment is a crucial step for diagnosis and treatment purposes. Various markers have been proposed, including both invasive and non-invasive markers. Liver biopsy is the gold standard method but due to its invasiveness, it is not preferred these days. Non-invasive methods include serum biomarkers and imaging techniques. Combinational panels along with microRNAs are also used for the identification of liver fibrosis. Besides their cost-effectiveness, these panels are more dependable when compared with an individual biomarker.

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INTRODUCTION

The liver is the main organ of our body. The functions of the liver include synthetic functions, metabolic functions, and most importantly the detoxification and excretion of toxic substances. The synthetic functions include the synthesis of cholesterol, triglycerides, plasma proteins, and lipoproteins. The metabolic functions include the metabolism of carbohydrates, lipids, and proteins. Ammonia is converted to urea in the liver. Any injury to liver cells will lead to the alteration in these functions. Various types of liver disease exist, such as acute and chronic hepatitis, alcoholic liver disease, and non-alcoholic fatty liver disease (NAFLD). Hepatitis is essentially the inflammation of the liver, a condition that can be self-limiting, although it can progress to other adverse situations, including fibrosis, cirrhosis, or even liver cancer. There are various causes of this condition, and the most implicated ones include infections, certain drugs, toxic substances, and autoimmune diseases. Mainly, there are five different types of hepatitis, namely, A, B, C, D, and E. Alcoholic liver disease occurs due to excessive consumption of alcohol. All these diseases lead to injury of the liver parenchyma which is studied based on their stages. The stage and degree of liver disease are fundamental in the diagnosis, prognosis, treatment, as well as follow-up of all hepatic diseases.

STAGES OF LIVER DISEASE

The progression of liver disease passes through various stages, as depicted in Figure 1. The figure also shows the factors promoting liver cell injury and thereafter the progression of the disease. The stages of liver disease are discussed below.

Inflammation stage

There are many types of liver failure, but despite the type, the progression towards full-blown disease is the same. The first stage is associated with inflammation and typically denotes the immune system's reaction to the offending agents like toxins. In this case, the hepatitis C virus (HCV) would be responsible[1]. In the process of inflammation, the liver becomes tender and greatly enlarged. Before inflammation, massive viral infection leads to an increase in the production of inflammatory cytokines, and chemokine levels are also shown to increase (they are the inflammatory biomarkers).

Fibrosis

The second stage is associated with fibrosis, which is stimulated by chronic inflammation. Fibrosis usually occurs as a result of the liver's healing process, and it happens continuously with the regeneration of the liver's damaged areas. Fibrosis is a way that wound healing takes place with a balance between fibrogenesis and fibrinolysis[2]. The process of inflammation causes quiescent hepatic stellate cells (HSCs) to be

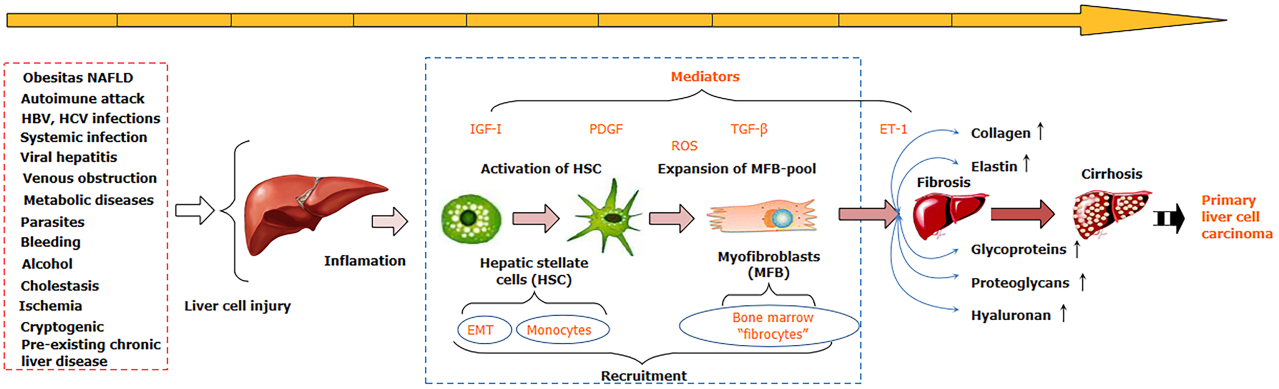

Figure 1 Factors promoting liver cell injury leading to fibrosis, cirrhosis, and carcinoma. NAFLD: Non-alcoholic fatty liver disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PDGF: Platelet growth factor; IGF: Insulin-like growth factor; TGF: Tissue growth factor; ROS: Reactive oxygen species; ET-1: Endothelin-1; EMT: Epithelial-mesenchymal transition.

activated, which then differentiate and form myofibroblasts[3].

Myofibroblasts are important in fibrogenesis and are responsible for producing several components of the extracellular matrix (ECM), which then replace the damaged tissues. When the ECM is deposited excessively, it leads to scar formation, which can be altered by fibrolysis[4]. The process of fibrosis is dynamic, and it is bound to be reversed upon the resolution of the HCV infection[5]. The chronic damage that stimulates fibrogenesis and insufficient fibrolysis is linked to a reduction of the reversibility potential.

Cirrhosis stage

Cirrhosis is the point where the liver is completely scarred and is beyond the self-healing ability. The development of cirrhosis is long due and could even take decades, meaning that interventions can be started in the initial stages before getting to this point. After several injurious exposure or inflammatory responses by the different mediators, HSCs undergo a transition from the quiescent to the activated state. The damaged hepatocytes lead to the release of reactive oxygen species, and apoptosis could occur[6].

Cirrhosis occurs in two stages: Compensated cirrhosis and decompensated cirrhosis (end-stage liver disease). During the compensated cirrhosis, there is liver damage, but it is not severe enough to hinder some of the cells' functioning. At this stage, one can be asymptomatic, although portal hypertension may be present[7]. The chronicity of the infection could induce G1 arrest and then impair the functioning of hepatic cells, limiting regeneration.

Recent studies have determined that shortening of the liver's telomeres and their senescence results in fibrotic tissue formation in the cirrhosis stage of liver disease. During the cirrhosis stage, some clinical features become apparent: Increased propensity to bleeding, possible development of insulin resistance, sensitivity to some medications, skin itch, and water build-up leading to edema. It is also possible for the build-up of toxins in the brain, affecting memory and other mental functions.

End-stage liver disease (decompensated cirrhosis)

This is the stage where the liver has completely failed, and neither can the cells heal; it can be both acute and chronic[8]. In HCV infection, it is a chronic occurrence. This is also called decompensated cirrhosis, and it follows inflammation of the hepatocytes, which leads to fibrosis and then disruption of the liver structure and function. During this stage, there is the development of complications like jaundice, variceal bleeding, ascites, and hepatic encephalopathy.

Clinical evidence has revealed that the median survival age for decompensated cirrhosis is about 2 years, and it is a common predictor of death in patients with cirrhosis. It has also been shown that decompensation can improve once the offending agent has been eliminated[8]. Failure to remove the offending agent, therefore, means that liver transplant is the only remaining solution.

ASSESSMENT OF LIVER FIBROSIS

For assessment of liver fibrosis, various methods have been proposed, including both invasive and non-invasive methods (Figure 2). However, in clinical practice, finding the most effective and the best method for evaluating liver impairment in patients remains a major challenge. This is mainly because the prognosis and effective treatment are dependent on the assessment of liver damage as well as the extent of liver fibrosis in patients. Historically, all these parameters were provided through liver biopsy. Liver biopsy is among the oldest, effective, and most accurate assessment methods of evaluating liver histology and the progression of liver damage. The comparison of the main features of both invasive and non-invasive methods is shown in Table 1.

INVASIVE METHOD (LIVER BIOPSY)

As discussed by Shrivastava *et al*[9], liver biopsy is a process that is considered by many experts in determining the best therapeutic approaches for patients. This is also the best approach in dealing with hepatitis C especially when it comes to chronic hepatitis. It is an invasive procedure for liver assessment[10]. Consequently, liver biopsy as an assessment method of liver damage in hepatitis C patients brings forth several risks as well as sampling errors. Sampling errors in liver biopsy occur due to suboptimal biopsy size. Due to the increased risks of liver biopsy and sampling errors among other pitfalls of this assessment method, different markers have been developed. Research shows that during the pathological progression of liver fibrosis, especially in patients with hepatitis C, there is an excessive buildup of the matrix. The serum levels of different biomarkers tend to change[9]. According to the authors, there are physical and biological non-invasive approaches that are based on serum biomarkers that have been proposed.

Scoring system for liver fibrosis

The scoring system of liver fibrosis assessment based on three methods, *i.e.*, International Association of Study of Liver (IASL), Batts-Ludwig, and METAVIR scores are depicted in Table 2[11].

Limitations of liver biopsy

There are several limitations of liver biopsy that have led to the development and replacement of the assessment method with non-invasive biomarkers as an assessment method of liver damage and liver fibrosis in patients with hepatitis. One of the limitations of liver biopsy is that this method does not efficiently reflect the different fibrotic changes that may be occurring in the entire liver. This is mainly because any optimally sized liver biopsy contains a small number of complete portal tracks that reflect a small volume of the liver[12]. Besides, the process of hepatic fibrosis is not liners. As a result, to cover hepatic fibrosis in the entire liver, biopsies have to be conducted on different areas of the liver. Besides, research shows that liver biopsies may miss cirrhosis in patients with hepatitis C. This is mainly because liver biopsy cannot differentiate between early and progressed cirrhosis. Consequently, liver biopsy cannot be relied upon as an ideal and accurate prognostic predictor[12].

Research shows that there are several risks of complications that tend to arise from liver biopsy[13]. Most of these complications, however, carry symptoms such as injury to the biliary system, mild abdominal pain, and severe hemorrhage. The occurrence of such complications as a result of liver biopsy may increase hospitalization. There is variability in the interpretation of pathologists which is yet another limitation of liver biopsy. Research shows that biopsy cannot be conducted in hepatitis patients with diabetes, ascites, metabolic syndrome, and coagulopathy. Although liver biopsy has been considered as a keystone for the diagnosis of liver damage in patients with liver diseases such as hepatitis C, the invasive procedure has significant limitations mainly due to surgical complications and sampling heterogeneity.

NON-INVASIVE TECHNIQUES FOR LIVER DAMAGE ASSESSMENT

There are various methods in which non-invasive biomarkers are used to assess the damages in the liver. A conclusion reveals that through these assessments, experts can

Table 1 Comparison of characteristics of invasive and non-invasive methods

No.	Feature	Invasive	Non-invasive
1	Invasiveness	Yes	No
2	Sampling error	Yes	No
3	Cost-effective	No	Yes
4	Patient-friendly	No	Yes
5	Hospitalization required	Yes	No

Table 2 Scoring systems for liver fibrosis

Stage	IASL	Batts-Ludwig	METAVIR
No fibrosis	No fibrosis	Stage 0	F0
Fibrosis portal expansion	Mild fibrosis	Stage 1	F1
Few bridges or septa	Moderate fibrosis	Stage 2	F2
Numerous bridges or septa	Severe fibrosis	Stage 3	F3
Cirrhosis	Cirrhosis	Stage 4	F4

IASL: International Association for the Study of the Liver.

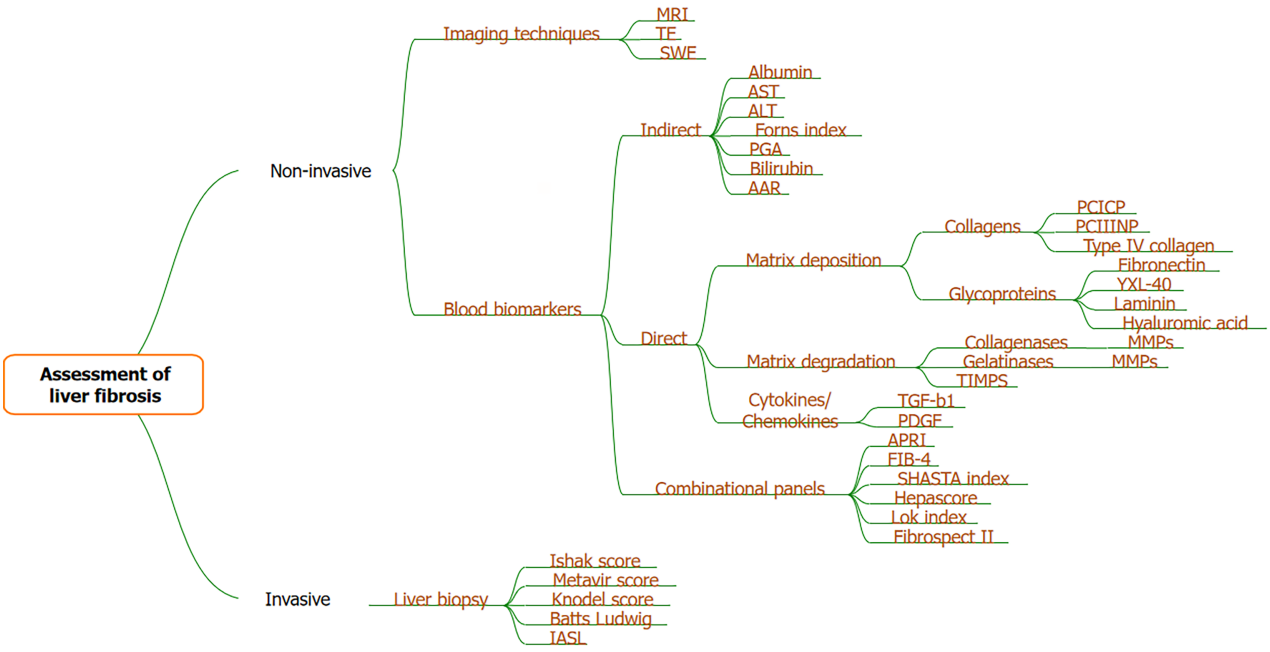

Figure 2 Various methods for assessment of liver fibrosis. MRI: Magnetic resonance imaging; TE: Transient elastography; SWE: Shear wave elastography; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AAR: Aspartate aminotransferase aspartate aminotransferase/alanine aminotransferase ratio; TGF-β: Transforming growth factor β; PDGF: Platelet growth factor; APRI: Aspartate aminotransferase to platelet count ratio; FIB-4: Fibrosis-4; PCICP: Procollagen type 1; PCIIINP: Procollagen type 3; MMP: Matrix metalloproteinase.

understand more about liver disease and analyze the various approaches which can be relied upon in managing the condition of the patient[13]. These methods are distinctively classified into two, the natural or physical approach and the biological approach. The physical approach is majorly used with various imaging techniques while the biological method is based on the popular serum biomarkers[14]. The two methods are quite distinct in the way that the conditions are valued and assessed but they are both based on conceptions and rationales that are quite different.

PHYSICAL APPROACH

There are many types of physical approaches that experts rely on in assessing liver conditions. These physical approaches include Doppler analysis, computed tomography, acoustic radiation force impulse imaging, transient elastography (TE), ultrasonography, magnetic resonance imaging, and real-time elastography. Menessy *et al*[13] also discuss that most of these methods are based on scanning and imaging techniques by which the experts analyze the liver and the condition of the systematic process. There are some of these methods that are widely considered more than others. There are the ones that are quite fast enough for experts while there are the slow ones. Some provide a distinct value of images or scans that can be relied on comfortably.

Transient elastography

TE is the most appropriate approach due to its speed. Fallatah[15] discusses that on top of that, the approach is quite reproducible and at the same time does not depend on operators. The approach is also quite common among many hepatitis experts since it provides and measures the stiffness of the liver and compares the same stiffness and its elasticity. With such considerations, it is quite easy to analyze the conditions of hepatitis and also conduct the corresponding analysis of its physical properties, which is highly genuine. The technique is also considered for its ability to predict the issues around severe fibrosis and also its accuracy in identifying cases of liver cirrhosis that are underlying the hepatitis condition. There are, however, issues of the method's examination of fibrosis which are mostly associated with this disease. In some cases, the approach is unable to provide information that is quite sufficient for experts to diagnose cases of significant fibrosis especially with the main consideration being the hepatitis C condition. This means that the technique does not provide distinct stages and processes for the analysis of the condition, and that there should be experts to analyze and interpret the information provided through the technique despite the results from the basic approach being straightforward. This means that an expert, who has been aware of and dealt with the clinical background of the patient, especially with his or her case of hepatitis C, should be at the center of measurements and results [15].

When compared with the METAVIR score of liver biopsy, the sensitivity and specificity of the cut-off value of TE are shown in Table 3[16].

Shear wave elastography

This has been a recently developed method for measuring liver elasticity. It has been considered that it is a reliable non-invasive tool for monitoring liver stiffness in HCV patients with an accuracy of 97.6%. It is a novel, rapid, and noninvasive method for measuring liver stiffness. It determines liver stiffness by estimating the velocity of shear waves emitted in the liver tissue. Moreover, the velocity of this shear wave (*i.e.*, lateral wave) is calculated. The benefit of this mode of assessment is that the real-time images are seen with the help of a normal B-mode ultrasound probe[17].

The area under the receiver operating characteristic curve (AUROC) for F > 2 and F4 were found to be 0.87 and 0.93, respectively[18]. Shear wave elastography was 85% specific and 79% sensitive when compared with the METAVIR score by taking a cut-off value of 1.34 for the F2 stage of fibrosis[19,20].

BIOLOGICAL APPROACH

Many developments have been realized across all industries. Among these industries are the medicine and clinical areas. A new era of biotechnology and biomedicine has taken a central part in developing our clinical and medical worlds. Stasi and Milani[21] make consideration that over the years, the world of medicine has seen major developments with tremendous strides having been realized in both the biotechnology and biomedical world[13]. This has brought up a new generation of medical approaches that are characterized by rapid, novel, and non-invasive approaches. These approaches have brought up some challenging ideas of the previous settings of medicine with major changes being recognized in the invasive diagnostic and therapeutic approaches. Some characteristics need to be fulfilled by the non-invasive methods, with most of these being the factors of accessibility, simplicity, high accuracy, and being liver-specific, satisfactorily validated, and easily interpretable[14].

Class I biomarkers (direct) to assess liver fibrosis are the remnants of liver matrix components. These are formed by HSCs during ECM remodeling. These markers

Table 3 Correlation of transient elastography cutoffs with METAVIRscore

METAVIR score	Cutoff TE score (kPa)	Sensitivity	Specificity	NPV	PPV
F ≥ 2 (F0-F1 *vs* F2-4)	7.1	0.67	0.89	0.48	0.95
F ≥ 3 (F0-F1-F2 *vs* F3, 4)	9.5	0.73	0.91	0.81	0.87
F ≥ 4 (F0-F1-F2-F3 *vs* F4)	12.5	0.87	0.91	0.95	0.77

TE: Transient elastography; NPV: Negative predictive value; PPV: Positive predictive value.

directly reflect either deposition or removal of ECM[22].

Whereas indirect (class II) markers include routine investigations such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum bilirubin, gamma-glutamyltransferase (GGT), haptoglobin, and α_2-macroglobulin. These markers are not specific for assessing intermediate stages of fibrosis[23].

Combinational panels by computing indirect markers have also been studied. These include fibrosis-4 (FIB-4), APRI (AST to platelet count ratio), SHASTA index, Fibroscore, Hepascore, and Lok index.

Class I biomarkers (direct)

Over the years, there have been major demands to understand the pathophysiology of the liver better. This has prompted and enabled many scientists and experts in this field to establish major research while investigating the major developments in the area. Class 1 biomarkers are therefore types of non-invasive biomarkers that mimic the liver metabolism and its ECM. It has been considered that though majorly associated with the fibrosis stages, these biomarkers are also associated with the fibrogenic cells and the changes that are majorly seen in the same[10]. It has been discussed that besides measuring and assessing the conditions of the liver concerning the hepatitis C condition, these biomarkers have another clinical usefulness in which they assess the rate at which other underlying issues progress besides staging the liver fibrosis[24]. As revealed by Stasi and Milani[21] with such assessments done by the biomarkers, the same data and measurements from the assessment are turned or else translated into prognostic information that is quite effective. This is then made as a tool in which responses are evaluated. In the long run, they also help in monitoring the efficiency of the associated ant fibrotic drugs. This is where the data that is provided in these circumstances gets to be used as variables for the performance and availability measurements. The direct markers are classified as below.

Direct markers linked with matrix deposition: Collagens and glycoproteins

Collagens: These direct markers are found in the connective tissues and have three types. Pro-collagen is the precursor of the collagen which is cleaved by two different enzymes at amino (type 3) and carboxyl (type1) terminal ends to form collagens[25]. The collagens formed are procollagen type 1 (PCICP) and procollagen type 3 (PCIIINP). PCICP is the main component of connective tissue[25]. The upper limit of normal values is 202 and 170 μg in males and females, respectively[26-28]. It is increased in cirrhosis progression. PCIINP is increased with fibrotic stage and correlates well with bilirubin levels in cirrhosis cases[29-31]. The only drawback of this marker is that it increases in other medical conditions also. Also, the efficacy is decreased as compared to hyaluronic acid (HA)[27,31]. Type IV collagen is the third collagen serving as a direct marker. It acts as a surrogate marker to assess liver fibrosis [32]. Its levels are manifold increased in liver diseases and correlate well with fibrosis [33,34]. An area under the curve (AUC) of 0.82 with a negative predictive value (NPV) of 83.6% was found with a cut-off value of greater than 5.0 ng/mL in NAFLD[34].

Glycoproteins: HA is an example of a direct serum marker used in the diagnosis of liver damage in patients[35]. It is integrated and dispersed all over the extracellular space. This process is done by the HSCs. The damaged liver tends to provide HA in high quantities. As a result, this marker is used to predict the level of liver damage based on elevated serum levels. This is because the levels of HA correlate with liver fibrosis[36]. Research shows that the HA serum direct marker is more accurate than most non-invasive indices. However, this method of diagnosis works best when combined with other liver markers. NPV was 98%-100% in cirrhosis[35-38]. Also, HA levels start decreasing with the treatment of liver disease[39-41]. Laminin is a

glycoprotein that is non-collagenous and is formed by the HSCs[10]. In a patient with liver fibrosis, elevated levels of laminin correlate well with the degree of the fibrosis. However, its diagnostic value is not of much significance when compared with HA. The cut-off value of 1.45 was proposed by Sebastiani[32] for detecting fibrosis and cirrhosis. It is 77% accurate for detecting fibrosis in HCV cases. YKL-40 is another diagnostic tool used to assess liver damage in patients with hepatitis C. It is a mammalian homologue of bacterial chitinases which are involved in the remodeling or degradation of ECM[21]. The levels of YKL-40 correlate with the severity of fibrosis. Fibronectin (FN) is a high molecular weight glycoprotein of the ECM which binds to integrins (receptor proteins). It is synthesized by various cells but mainly by hepatocytes. In blood, FN exists in two major forms, *i.e.*, cellular FN (cFN) and plasma FN (pFN)[42].

Direct markers that are associated with matrix degradation: Collagenases, gelatinases, and tissue inhibitors of matrix metallo proteinases

Collagenases: Metalloproteinase-1 (MMP-1) is found to be inversely correlated with necrosis as well as fibrosis[43].

Gelatinases: Two matrix metalloproteinases MMP-2 and MMP-9 have been found. They are also known as gelatinases, *i.e.*, gelatinase A and B, respectively. Previously, MMP-2 was found to have no significant association with liver fibrosis stage[44,45]. But later Boeker *et al*[44] found an accuracy of 92% for detecting cirrhosis in HCV patients. It is increased by 2.4 folds in HCV patients as compared to controls. MMP-9 is inversely correlated with histological severity in hepatitis. Its levels start decreasing as cirrhosis progresses[46,47].

Tissue inhibitors of matrix metallo proteinases: They interact with MMP functioning and further lead to ECM degeneration inhibition. It shows a positive correlation with fibrosis stage[45-48].

Cytokines/chemokines in liver fibrosis

These include transforming growth factor (TGF)-β1, TGF-α, and platelet growth factor (PDGF). TGF-β1 correlates well with fibrosis in HCV-infected patients. The value of < 75 ng/mL is considered to be normal[49,50]. TGF-α is found to be more correlated with fibrotic stage in hepatocellular carcinoma (HCC)[51]. PDGF levels are associated with liver fibrosis and a cut-off value of 40.50 ng/L is an indicator for inflammation and fibrosis[52].

Class II biomarkers (indirect)

Back in the day, the first approach that majorly assessed the conditions of the liver and issues like hepatitis C and liver fibrosis included hematological tests and routine biochemical tests which are classified as non-invasive biomarkers. Class II biomarkers are also referred to as indirect biomarkers. They are mostly based on common functional alterations in the liver and the evaluations that are attached to the same[13]. These alterations, however, do not reflect the turnover and changes associated with the fibrogenic cells. For the class II biomarkers, the basis of the measurements and evaluation is algorithmic and single elaboration. These are mainly based on the alterations that have been observed in the liver and its functions.

AST/ALT ratio: The AST/ALT ratio (AAR) index is an example of an indirect serum marker used in the diagnosis of liver damage in patients with hepatitis C. However, it is important to note that when the stages of fibrosis are not advanced, the performance of the AAR index is low[13]. Haukeland *et al*[53] validated this test in different liver diseases. The ratio of more than 1 predicts liver cirrhosis[54,55].

APRI: It provides a quick estimate for predicting severe fibrosis or cirrhosis[56]. This is among the most validated noninvasive biomarkers[13]. APRI was calculated as [AST level/AST (upper limit of normal)]/[platelet count (10^9/L)] × 100. It was originally developed by Wai *et al*[57] in 2003. The AUC was 0.8 and 0.89 for fibrosis and cirrhosis, respectively. Loaeza-del-Castillo *et al*[56] found that it is not a diagnostic marker in autoimmune hepatitis.

BARD score: This is the combination of AAR and body mass index (BMI) and other measures of diabetic patients. NPVs of 96% and 81.3% were found[58].

ALT: Due to its high sensitivity as well as specificity, it is used as a better indicator of liver disease[59].

Forns index: It involves parameters like age, platelet count, cholesterol, and GGT[60]. Forns index was calculated as [7.811 - 3.131 × ln(platelet count)] + [0.781 × ln(GGT in IU/L)] + [3.467 × ln(age) - 0.014 × cholesterol in mg%]. It differentiates mild fibrosis from severe fibrosis.

PGA and PGAA index: PGA is used to assess fibrosis in alcoholics[61]. A combination of prothrombin index, GGT, and apolipoprotein A is used in calculating PGA. It is considered 65% accurate in detecting liver fibrosis. Furthermore, a2 macroglobulin was added and PGAA was invented. It has a 70% accuracy in detecting fibrosis[62].

FIB-4: It is a simple, fast, and cheap test that gives immediate results[23]. It is a validated test used for detecting hepatitis B and C. The AUC of 0.85 and 0.81 for detecting severe fibrosis was found in HCV and HBV, respectively[63,64]. IB-4 was calculated as [Age (years) × AST (U/L)]/[Platelet count × √ALT (U/L)]

Fibroindex: It is a simple scoring system[65]. It showed an AUC of 0.83 for fibrosis detection. Also, a cutoff value of 2.25 was strongly associated with F2-F3 fibrosis stage with an NPV of 90%[65]. Fibroindex was calculated as [1.738 - 0.064 × platelet count ($104/mm^3$)] + [0.005 × AST (U/L)] + [0.463 × gamma globulin (g/dL)].

Fibrotest: It includes certain parameters like age, gender, haptoglobin, a2 macroglobulins, apolipoprotein A1, GGT, and serum bilirubin[66,67]. This is considered as a most validated marker for detecting liver fibrosis[68,69].

Acti test: A simple addition of ALT in Fibrotest was made which reflects liver fibrosis as well as necro-inflammatory activity[70,71]. Acti test is a parameter that was initially validated for patients with chronic hepatitis B and C. It was used in collaboration with the Fibrotest as an alternative to liver biopsy. The Acti test combines five components of the Fibrotest and ALT. The assessment is crucial for treatment prescription especially in patients with moderate or severe necro-inflammatory activity as well as cirrhotic patients.

Tests for NAFLD: Initially, the simplest test was developed by using age, BMI, platelet count, ALT: AST ratio, serum albumin, and glycemic status[72]. AUC was calculated as 0.88 with an NPV of 93%. Steato test was later proposed by combining fibrotest and Acti test[73]. A cut-off value was fixed at 0.7 with a 90% specificity.

MICRORNAS AND THEIR BIOSYNTHESIS

MicroRNAs (miRNAs) are also nowadays considered potential biomarkers in assessing liver fibrosis. They are small non-coding strands of RNA, responsible for the regulation of the expression of genes after the transcription process. They usually target and regulate the biological processes and then influence the complex programs of the expression of genes in several cellular processes[74]. Notably, miRNAs are deemed principal regulators that control main cell functions in several physiological and pathophysiological processes.

The biogenesis of miRNAs is made up of two cleavage pathways; after forming the mature miRNA, there is one nuclear and one cytoplasmic. The miRNA precursors are sorted into different pathways. However, the process is unclear but appears to be determined by the site where the miRNA originates, the sequence, and even the thermodynamic stability[75]. Regulatory functions of miRNAs occur through the silencing complex induced by RNA, specific for a particular miRNA.

MiRNAs are usually transcribed from the introns and exons of the genes responsible for protein-coding or the intergenic areas. The transcription of the miRNA genes is the basis of primary transcripts, which contain the hairpin structure that consists of a terminal loop and a double-stranded stem. Later, there is then cleavage of the stem-loop structure with the help of the RNase III-like enzymes that are known as Drosha and the binding partner DGCR8[76]. The result is the formation of the precursor miRNA (pre-miRNA).

There is then the transfer of pre-miRNA from the nucleus into the cytoplasm, and this is helped by exportin-5 and the accompanying co-factor Ran-GTP. The GTP is bound to the Ras-related nuclear protein. The cofactor is then processed into a structure that is duplex by the RNA polymerase II dicer. When an miRNA binds to its

target, it leads to the degradation of the target mRNA or the suppression of the mRNA translation[76]. Figure 3 depicts the entire process of miRNA biogenesis.

More than 1500 miRNAs have been determined in the human genome, which are involved in the cell processes, including the development, differentiation, and proliferation of cells, the process of death, the pathology, and defense against viruses.

MiRNAs are essential in the process of the pathogenesis of HCV infection through the control of the signaling pathway. In this regard, they play a role in the response of both the innate and adaptive immune systems. MiR-122 has been determined to be the most abundant miRNA in the normal liver parenchyma, and it accounts for more than 70% of the miRNAs found in the hepatocytes[77]. The miR-21 gene is located on chromosome 17, and it is highly conserved. Inside the cell, miRNA-21 is found in the cytosol and the extracellular exosome. At the organ level, miRNA is located in the bone marrow, lungs, kidney, peripheral blood, colon, intestines, and thyroid.

When miR-122 binds to a 5′-untranslated region (5′-UTR) of the genomic constituent of HCV RNA, which is critical for the replication of the virus, it then stimulates translation of the viral protein and then protects HCV RNA that is uncapped from the process of degradation. Over time, the upregulation of the miR-21 leads to the feedback of inhibition of type I interferon, which is mediated by the antiviral response. This then promotes viral replication[78]. Moreover, miR-21 is detected in the oncogenic miRNA and controls the process of cell cycle and tumorigenesis.

As indicated above, miR-21 is a contributor to the development of fibrogenesis in the muscles and various organs, including the liver. Clinical data has demonstrated that miR-21 is always upregulated in the liver of patients who have biliary atresia-induced liver fibrosis. MiR-21 can induce fibrosis through activation of HSCs and then collagen synthesis. The overexpression of miR-21 leads to the promotion of oxidation, and this then increases the production of collagen, which in return, activates angiotensin. MiR-21 can affect the expression of several proteins by binding to the 3′-UTR of specific mRNAs. This results in a complex interaction network as a result of downstream effects of the signaling pathways[76]. Various signaling pathways have been identified to be the basis of the pathophysiological fibrosis process, including the phosphoinositide 3-kinase, TGF-β/Smads, and the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase pathways[79].

Activation of angiotensin occurs through several pathways: Spry1/ERK/NF-κB, PTEN/Akt, programmed cell death 4/AP-1, and Smad7/Smad2/3/NADPH oxidase 4. In recent findings, research has been able to elucidate that a moiety that is deficient in the methionine choline diet of NASH is linked to liver damage[79]. MiR-21 then results in a decrease of steatosis, lipo-apoptosis, and inflammation with impairment of fibrosis. Recent findings have shown that antisense inhibition or the deletion of genes of miR-21 does not alter the HSC activation or fibrosis. MiR-21 is frequently upregulated in human beings with solid malignancies like breast, colon, pancreas, lung, and liver tumors[79]. MiR-21 has also been shown to be a survival factor in the course of liver injury and the development of HCC.

MiR-449a is found to be dysregulated in hepatitis C infection only. Its significance is not found in alcoholics and NAFLD. It regulates YKL-40 by targeting the NOTCH signaling pathway in HCV infection[80]. Also, the expression of miR-155 was significantly increased, which further led to tumorigenesis by modulating the Wnt signaling pathway[81].

NOVEL FINDINGS SUPPORTING IMPORTANCE OF NONINVASIVE MARKERS

According to Menessy *et al*[13], noninvasive markers are crucial. This is mainly because these procedures are effective in the evaluation of the stage of liver fibrosis in patients with hepatitis C whereby there are no clear indications for liver biopsy. Liver biopsy is not ideal for frequent development. Given the rapid development of new medications for the treatment of hepatitis C, there is an increased need for frequent evaluations of liver damage and liver fibrosis. Consequently, the use of non-invasive assessment methods for liver fibrosis in patients with hepatitis C is crucial.

For HCV infection, there are high chances of developing liver cirrhosis and liver fibrosis in some patients. This means that physicians examining a patient should be keen to verify the infections that are underlying in cases of the main condition which is hepatitis C. The presence of non-invasive biomarkers makes all these possible by establishing a process in which the necrotic processes and the inflammatory activities are considerably detected and analyzed. These biomarkers help in establishing a clear

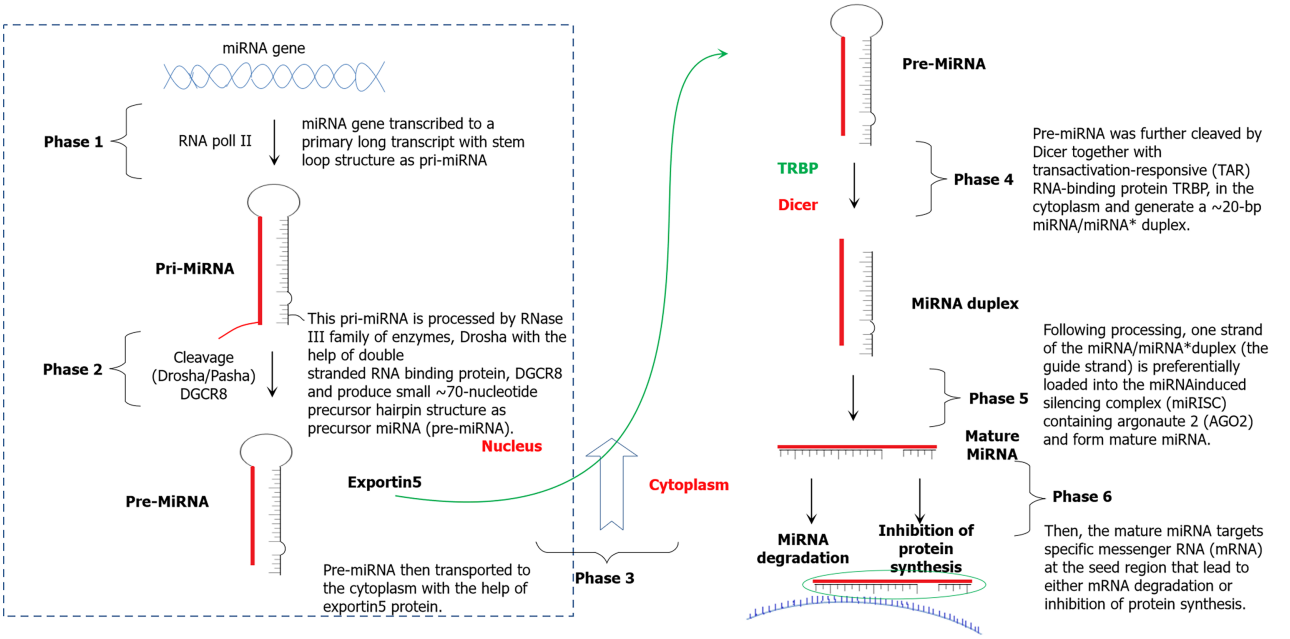

Figure 3 Process of microRNA biogenesis. miRNA: MicroRNA.

process of detecting the major changes in the liver as the patient deals with hepatitis C. The non-invasive biomarkers generally help in forecasting the main course that the HCV takes[13].

Similarly, Stasi and Milani[21] assert that non-invasive assessment methods for liver fibrosis tend to be readily available, simple, reliable, safe, inexpensive, and well-validated. As a result, they are effective in evaluating the progression of liver disease. Non-invasive biomarkers offer numerous advantages over liver biopsies. Some of these advantages include the absence of adverse effects and reduced risks of sampling errors. These bring about objectiveness when it comes to the interpretation of the results. Noninvasive biomarkers lack any reported ceiling effect hence effective as compared to liver biopsy Noninvasive assessment methods are appropriate as they allow for repeated assessment.

Various researchers argue that by definition, noninvasive biomarkers, however, cannot outperform liver biopsy even though they tend to be more accurate in the assessment of liver fibrosis. This is because of the method as well as its limitations. Some of its limitations are unreliability and feasibility especially in obese patients or under limited operator experience. The procedure is also contradicted during ascites, pregnancy, and implanted cardiac pacemaker patients. Besides, the knowledge of noninvasive biomarkers is still incomplete. This poses a challenge to clinical practice since it greatly hinders the development of accurate treatment and noninvasive diagnostic means with adequate sensitivity for liver fibrosis[24].

Similarly, Oksuz *et al*[82] affirm that for the assessment of necroinflammatory histological activity, few biomarkers have been proposed. Fallatah[15] argues that improving the accuracy of noninvasive biomarkers is essential for a correct diagnosis of liver damage in patients. This can be done using serum-based algorithms as sequential and simultaneous procedures. In a study, the comparison of TE to liver fibrosis was done[83]. The authors found that TE performed better in predicting all stages of fibrosis as well as severe fibrosis. Fibroscan values showed a good correlation with the levels of fibrosis markers. Also, the Fibroscan value of 15KpA was a significant separation limit for differentiating advanced fibrosis stages (F3 and F4). They suggested that these Fibroscan values are clinically useful to predict fibrosis stages in chronic hepatitis patients[84]. Other researchers correlated Fibroscan with fibrosis degree in liver biopsy and stated that it can be used as a noninvasive tool to diagnose moderate fibrosis[85]. Recently, there has been increased interest in detecting liver fibrosis through the application of non-invasive techniques. The APRI is the most useful score to predict fibrosis[56]. Attallah *et al*[86] found that FN discriminant scores based on FN, APRI, and albumin can be used to predict liver fibrosis (Table 4).

Table 4 Sensitivity and specificity of non-invasive biomarkers in liver fibrosis

Marker	Parameters involved	Disease	AUROC for liver fibrosis	Sensitivity	Specificity	Ref.
AST/ALT ratio	AST and ALT	NAFLD; HCV	0.83; -	74; 47	78; 96	McPherson *et al*[87]; Park *et al*[88]
BARD score	BMI, AST, ALT, DM	NAFLD	0.76	74	66	Sun *et al*[89]
APRI	AST, platelet count	NAFLD	0.67	27	89	McPherson *et al*[87]
ALT	ALT	HCV	0.716-0.815	-	-	Pradat *et al*[59]
Forns index	Age, platelet count, GGT, cholesterol	HCV	0.81-0.86	94	51	Forns *et al*[60]
PGA and PGAA	Prothrombin time, GGT, apolipoprotein A1, α2 macroglobulin	Acute liver disease	0.84-0.86	-	-	Nguyen-Khac *et al*[90]
FIB-4	Platelet count, AST, ALT, age	HCV; NAFLD	0.74-0.77; 0.85	67; 84	71; 69	Sebastiani[23]; Sun *et al*[89]
Fibro test	Haptoglobin, apolipoprotein A1, α2 macroglobulin, GGT, bilirubin, age, and gender	HBV; HCV; ALD	0.84; 0.87; 0.83	61; 75; -	80; 85; -	Salkic *et al*[91]; Imbert-Bismut *et al*[66]; Naveau *et al*[62]
Hepascore	GGT, bilirubin, HA, α2 macroglobulin, age, and gender	HCV	0.82	-	-	Naveau *et al*[62], Adams *et al*[92]
SHASTA index	HA, AST, and albumin	HCV	0.87	50	94	Kelleher *et al*[93]
Fibrospect II	α2 macroglobulin, HA, and TIMP-1	HCV	0.82-0.83	77-83	66-73	Patel *et al*[94]

AUROC: Area under receiver operating curve; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NAFLD: Non-alcoholic fatty liver disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; BMI: Body mass index; DM: Diabetes mellitus; APRI: Aspartate aminotransferase to platelet count ratio; FIB-4: Fibrosis-4; GGT: Gamma-glutamyltransferase; HA: Hyaluronic acid; TIMP-1: Tissue inhibitors of metalloproteinases-1.

PROS AND CONS OF NON-INVASIVE BIOMARKERS

Various authors had made the remarks that non-invasive biomarkers can be used instead of liver biopsy because its acceptance has faced some key resistance from different sectors[14]. Some of the factors that bring the cases of resistance are attached to the paucity of well-designed studies and literature that discuss the non-invasive methods extensively giving a view of both sides. There are also issues with the validation of some of the non-invasive biomarkers and proposals for some of them in terms of the lack of validated data. With the ones that their proposals have been provided, some changes in terms of assessing the severity and the growth rate have not been discussed and analyzed extensively[12]. As per Menessy *et al*[13] for others, there has not been enough time to validate them in terms of testing and analysis in their use when it comes to the cases of hepatitis C[9]. What is needed in most of these cases is the specific etiology validation, especially for most of these non-invasive biomarkers. In these cases, each etiology should be considered to deal with the issues of the specific pathogenesis, associated comorbidities, and natural history.

In the clinical practice related to the hepatitis condition, there should be a careful evaluation of all risk factors that are attached to failure and errors that can be associated with the specific non-invasive tools or biomarkers. A careful evaluation is needed to interpret the result and measurements adequately[21]. For the liver biopsy, a key concern for most experts is to note the role that these non-invasive biomarkers play in achieving the right clinical practice. With these biomarkers, most of these experts can create a cost-effective and attractive approach that is quite better and advantageous than the liver biopsy.

It has been revealed that the biomarkers are substantially less invasive, which provides a different experience for the clinical experts[9]. Besides the same advantage, other significant factors make them better than the biopsy. First, they practically have no or fewer sampling errors which enable a sufficient and efficient approach in the analysis and assessments. On the other hand, they also have very few complications that are related to health and clinical advancements. Shrivastava *et al*[9] make a point that the observer-related variability is also very small, which explains the high considerations from different experts. Lastly, the measurements and assessments may be

performed and considered repeatedly even from different labs, and the instruments and the equipment for this process do not need to be complicated. This means that they can allow for the dynamic monitoring of the health condition and other issues related to liver damage. This underlines the huge role that biomarkers play in assessing and proposing the conditions of the liver which is the main body part affected by the disease.

CONCLUSION

We agree with the above discussions that the use of two or more noninvasive biomarker methods will increase the accuracy of an individual to be assessed for fibrosis. In such case, the choice of the algorithm to be used in the combination in clinical practice should be based on some specific considerations. Considerations that must be made include what is locally available, what is not related to the patient's co-morbidities, what is recently validated, and the method that the physician feels comfortable to use. We have found that a combinational panel of noninvasive biomarkers is cheap and simple as compared to the use of individual biomarkers and liver biopsy. Finally, we would suggest that one or more direct biomarkers along with one imaging technique can be used for the assessment of liver fibrosis.

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MINIREVIEWS

Imaging evaluation of the liver in oncology patients: A comparison of techniques

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Abstract

The liver is commonly affected by metastatic disease. Therefore, it is essential to detect and characterize liver metastases, assuming that patient management and prognosis rely on it. The imaging techniques that allow non-invasive assessment of liver metastases include ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET)/CT, and PET/MRI. In this paper, we review the imaging findings of liver metastases, focusing on each imaging modality's advantages and potential limitations. We also assess the importance of different imaging modalities for the management, follow-up, and therapy response of liver metastases. To date, both CT and MRI are the most appropriate imaging methods for initial lesion detection, follow-up, and assessment of treatment response. Multiparametric MRI is frequently used as a problem-solving technique for liver lesions and has evolved substantially over the past decade, including hardware and software developments and specific intravenous contrast agents. Several studies have shown that MRI performs better in small-sized metastases and moderate to severe liver steatosis cases. Although state-of-the-art MRI shows a greater sensitivity for detecting and characterizing liver metastases, CT remains the chosen method. We also present the controversial subject of the "economic implication" to use CT over MRI.

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Core Tip: Several imaging methods are clinically available to evaluate and characterize liver metastases. Both computed tomography and magnetic resonance imaging (MRI) are currently the techniques that show the highest diagnostic performance and are also the most suitable for assessing therapy response and follow-up. Several studies have shown that MRI has a higher sensitivity for detecting and characterizing liver metastases; therefore, it may be the ideal imaging method for treatment planning before and after neoadjuvant chemotherapy. The traditional paradigm for ordering imaging studies emphasizes diagnostic accuracy, which is why we believe that MRI should be favored when available, the first-line imaging for detecting liver metastases, and pre- and post-treatment follow-up.

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INTRODUCTION

The liver is one of the most common organs involved with metastatic disease. Secondary lesions are about 18-40 × more common than primary liver tumors[1,2]. Liver metastases are most often secondary to colorectal carcinoma (CRC) (40%), stomach (20%), pancreas (20%), lung (10%), and breast cancer (10%)[3]. Other less frequent primary tumors include neuroendocrine tumors (NETs), gastrointestinal stromal tumors (GISTs), and renal cell carcinomas[3].

The spectrum of presentation is broad. Liver metastases frequently present as multifocal and separate lesions; however, they can also be solitary or less frequently manifest as confluent masses[4]. The solitary mass form of presentation is most often associated with colon cancer. Meanwhile, breast cancer metastases may infrequently diffusely involve the liver in a pseudocirrhosis pattern (mimicking cirrhosis), particularly following chemotherapy[3].

Solid liver metastases are typically supplied by arterial blood flow; hence they can be classified as hypovascular or hypervascular[1]. The main group of hypovascular metastases includes CRC, gastric, breast, and lung cancer[5]. On the other hand, hypervascular liver metastases are more commonly seen in renal cell carcinoma (especially clear-cell type), NETs, melanoma, thyroid carcinoma, and GISTs. Breast cancer liver metastases may appear hypovascular and hypervascular. Additionally, liver metastases may be cystic, arising from cystic primaries, such as ovarian carcinoma or mucinous cystadenocarcinoma of the GI and pancreas. These may also arise from GIST, leiomyosarcoma, malignant melanoma, carcinoid, and pheochromocytoma[1]. Calcification may be present in mucinous adenocarcinomas from the gastrointestinal tract or the ovary and in breast, lung, renal, and medullary thyroid carcinoma[6,7].

In the current perspective of oncologic liver surgery or local ablation, imaging shows a vital role in the detection, characterization, and determination of metastases' exact location, on a per-patient and per-lesion basis, even in patients with stage IV disease. Surgery and a variety of interventional radiologic techniques are also performed in selected patients with oligometastatic disease.

Stage IV CRC is defined as distant metastasis that either is confined to one organ or site (stage IVa) or affects more than one organ or site or the peritoneum (stage IVb). The past decade has seen a paradigm shift in stage IV or metastatic CRC (mCRC) management, leading to a significant increase in overall survival for these patients, from less than 6 mo to nearly 2 years[6]. Much of this success is credited to the increased utilization of hepatectomy in patients with oligometastatic liver disease, the

development of newer chemotherapy regimens, and the identification of new molecular targets and their inhibitors. Imaging plays an essential role in the workup of patients with mCRC by helping enumerate the number and sites of metastases, determine resectability, assess response to systemic and liver-directed therapies, and detect drug toxicities and disease recurrences.

This paper aims to briefly review each imaging technique and subsequently evaluate them in assessing liver metastases, including detection, characterization, diagnosis, and treatment response evaluation.

IMAGING TECHNIQUES

Ultrasonography

Ultrasonography (US) is a safe, accessible, and inexpensive technique. Nevertheless, it has considerable limitations, including dependency on operator expertise, patient's body habitus, cooperation, and bowel gas interposition[8]. The lower performance of this technique is also explained by limited spatial resolution, and for this reason, small (< 3-5 mm), isoechoic, and deep-seated metastases can be missed[1,8]. The conventional US's general sensitivity for detecting liver metastases is approximately 69% (sensitivity of 50%-76% in series with a true gold standard – intraoperative US or resection)[1,9]. This sensitivity is probably lower in patients with subdiaphragmatic lesions, chronic hepatic disease, and severe hepatic steatosis, which may be induced by chemotherapy. Moreover, the ambiguity in segmental localization leads to a lack of reproducibility compared to computed tomography (CT) and magnetic resonance imaging (MRI).

The appearance of metastases on ultrasound is diverse, but most appear rounded with sharp or smooth margins. They show variable echogenicity (hypo-, iso, or hyperechoic relative to the surrounding parenchyma), with the hypoechoic pattern being the most common (65%)[7]. Sometimes a hypoechoic halo is noted (40%), especially if the lesion is iso- or hyperechoic (Figure 1)[7]. Hepatic metastases of CRC are typically well-defined, solid, hypoechoic lesions and hypovascular on Doppler ultrasound, and occasionally present a peripheral halo ("target" or "bulls-eye" appearance)[8,9]. This broad spectrum of appearance makes the distinction between benign and malignant lesions difficult, reducing its specificity[8].

Contrast-enhanced ultrasound (CEUS) has improved the sensitivity for the detection of liver metastases. A study by Kong *et al*[10], including 240 patients with liver metastases, showed that diffuse homogeneous hyperenhancement followed by rapid washout was the most common pattern on CEUS (55.4% and 96.2%, respectively).

Regarding CEUS, reports differ, mainly because they depend more on operator expertise and other technical factors. Bernatik *et al*[11] found that CEUS detected 97% of the lesions diagnosed by CT[8,11]. Piscaglia *et al*[12] examined 109 patients with colorectal and gastric cancer. They showed that CEUS improves sensitivity in the detection of liver metastases to 95.4% when compared to conventional US (76.9%) and CT (90.8%)[12]. Cantisani *et al*[8,13] showed that CEUS improved US sensitivity from 67.4%-71.6% to 93.4%-95.8%. On the other hand, Vialle *et al*[14] reported that the CEUS sensitivity was inferior to CT in detecting hepatic metastases from colorectal cancer (CEUS 64.5% *vs* CT 80.4%). Moreover, since metastatic liver disease frequently shows multiple lesions, the per-lesion evaluation would need multiple doses of ultrasound contrast agent[7].

The accuracy for the detection of hepatic lesions may differ with the US mode. Two-dimensional (2D) CEUS shows limitations in evaluating liver metastases since it is more prone to sampling errors, such as imaging caption of a single section and plane-to-plane perfusion variation. On the other hand, three-dimensional (3D) CEUS imaging techniques can image the tumor as a whole, provide spatial information, and allow volumetric images. El Kaffas *et al*[15] showed that 3D dynamic CEUS is superior to 2D dynamic CEUS imaging by reducing the sampling errors from heterogeneous tumor perfusion. Other studies have shown no significant differences between the two modes concerning sensitivity[16]. Nevertheless, the perception of the feeding arteries is improved with the 3D CEUS, which might be helpful for the treatment of hypervascular liver metastases[16].

Computed tomography

Cross-section imaging techniques, including CT, and positron emission tomography (PET)/CT, have advanced considerably, leading to early and accurate liver metastasis

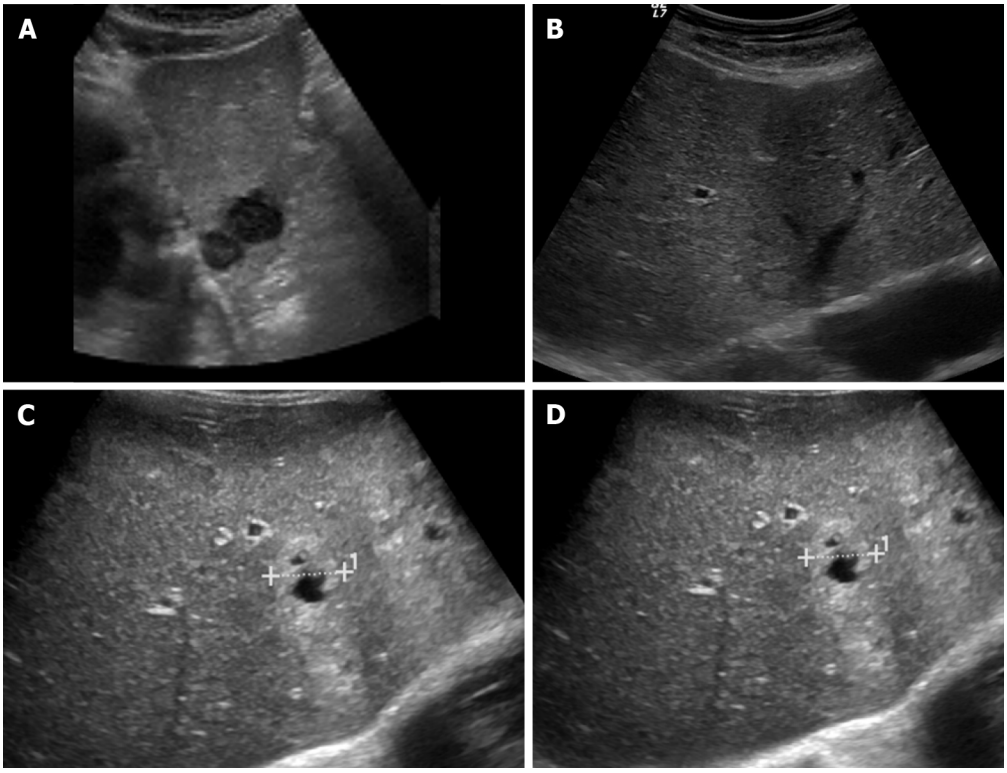

Figure 1 Ultrasound images showing variable echogenicity of liver metastases. A: Two hypoechoic lesions in the left liver lobe consistent with metastases in a patient with lung cancer; B: Isoechoic liver metastasis from lung cancer demonstrating a hypoechoic halo; C: Occult primary tumor with hepatic metastases, predominantly solid and hyperechoic; D: Occult primary tumor with hepatic metastases, showing central necrosis.

detection[17]. Multidetector CT is a reliable technique for detecting liver metastases and preoperative staging, allowing volumetric acquisition with high-quality multiplanar reformatted images, liver volume calculation, and 3D reconstructions preoperative tumor resection planning[3]. CT is fast and accessible, allows high-quality liver imaging and entire abdomen and chest coverage, and depicts extrahepatic disease[18]. CT shows a specificity of 77.3% and sensitivity up to 73.5% for the detection of liver metastases[19].

Liver metastases usually appear as hypo or iso-dense nodules on unenhanced CT. These nodules tend to be well-defined, but they can also be irregular, depending on size[6]. Necrosis and cystic transformation may be present, appearing as a central area of low attenuation. Besides, at times liver metastases may also show high attenuation due to hemorrhagic content[3].

Dynamic imaging is crucial, and its concept, perception, and evaluation are similar between CT and MRI (Figure 2). Most liver metastases are hypovascular and are best detected during the portal venous phase (PVP), which begins approximately 60-80 s after the initial injection. In this phase, the liver parenchyma enhances through the dominant blood supply by the portal vein. Hypovascular metastases appear as hypodense/hypoattenuating lesions compared to the background liver parenchyma (Figure 3)[1]. They usually show a peripheral rim enhancement in the late arterial phase (LAP), which fades centrally in the venous phase ("target appearance")[5,6]. On the other hand, hypervascular metastases enhance earlier in the LAP, which is demonstrated by contrast in the portal vein and absence in the hepatic veins. These lesions may fade and become isodense with the remaining liver parenchyma or show variable degrees of washout in the PVP and delayed acquisitions[5,6,20].

The PVP is considered the most critical phase, with a sensitivity of 91.5% for detecting hypovascular metastases[21]. However, the optimal number and choice of acquisition phases are still under debate, given the potential risks of higher radiation doses[1]. Honda *et al*[22] showed that adding a LAP improved liver metastases' detectability, particularly in lesions smaller than 10 mm. However, other studies, such as that from Ferlay *et al*[23], concluded that for evaluating CRC liver metastases, the addition of the LAP and delayed phases did not improve the performance compared to the PVP alone.

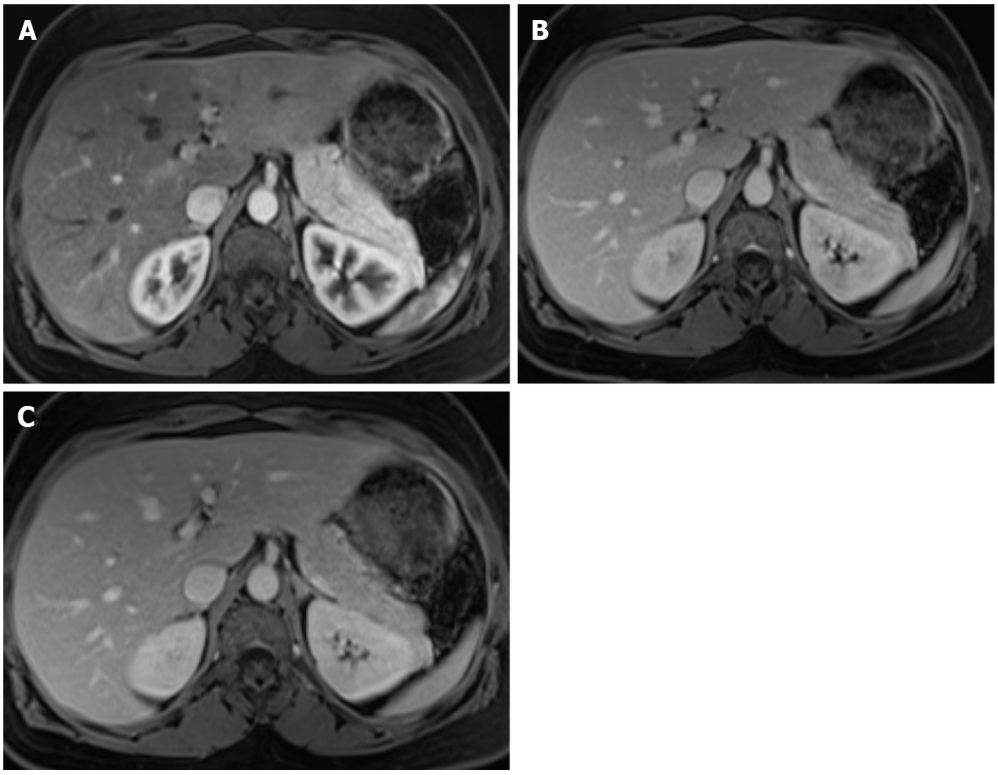

Figure 2 Dynamic phases of enhancement. A: Late hepatic arterial phase. It is characterized by contrast in hepatic arteries and portal veins, not in hepatic veins. It is helpful for hypervascular lesions and perfusional abnormalities. Note that the normal pancreas enhances greater than the liver; B: Portal venous phase. It is recognized by the contrast in the hepatic and portal veins. It is useful mainly for hypovascular lesion detection; C: Interstitial or delayed phase. It is helpful for lesion characterization, especially for late enhancement perception.

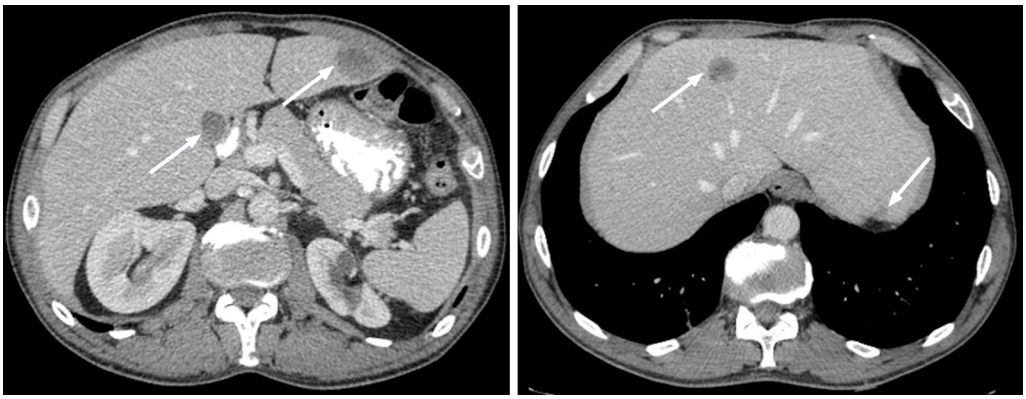

Figure 3 Metastatic lesions from lung cancer. Axial contrast-enhanced computed tomography in the portal-venous phase shows multiple hypodense and hypovascular lesions (arrows) consistent with metastatic lesions from lung cancer.

For hypervascular metastases, non-contrast-enhanced CT (NE-CT) only adds a small incremental value to contrast-enhanced CT (CE-CT) for their detection and characterization based on existing evidence. It seems that it is not worth adding further radiation exposure and the increased number of images for interpretation associated with NE-CT acquisition[24]. Still, NE-CT may be helpful as calcifications are present in up to 11% of liver metastases at initial presentation[25,26].

CT is the workhorse for abdominal imaging staging; however, liver metastases may be missed. The detection rate of lesions by CT declines as its diameter decreases, with a detection rate estimated at 72% for lesions measuring 10-20 mm and 16% for lesions smaller than 10 mm[19]. Benoist *et al*[27] showed that the rate of missed liver lesions after chemotherapy could be as high as 83%.

A recent study demonstrated that some liver metastases without sufficient contrast enhancement were more likely to be overlooked, as were subcapsular lesions, in case of liver steatosis or in cases of examination indication other than assessing malignant

tumors[17].

It has been shown that imaging during the exact correct vascular phase of contrast and an adequate iodine concentration (300-400 mg/mL) is essential for improving the detectability of hypoattenuating metastases[28]. However, it is known that higher contrast concentration may harm renal impaired patients and may also lead to contrast-induced nephropathy. As most patients will frequently need repeated examinations and extended follow-up periods, radiation exposure should also be kept in consideration, representing one of the most critical limitations of CT.

Dual-energy CT (DE-CT) scanners are getting progressively more available. It involves the acquisition of two or more CT measurements with distinct energy spectra. Using the differential attenuation of tissues and materials at different X-ray energies, DE-CT allows the distinction of tissues and materials beyond what is possible with conventional CT[29].

A study comparing DE-CT-driven low-keV virtual monoenergetic imaging to standard linearly blended images concluded that low-keV images improved quantitative size measurements and diagnostic accuracy of CRC liver metastases[30]. Also, this new technique improves the CT accuracy in differentiating liver abscesses from liver metastases in the context of hypovascular metastases, a common clinical dilemma. This technique may increase hypervascular and hypovascular liver lesions' conspicuity, improving CT performance in detecting metastases, especially in cases of concomitant liver steatosis[31].

Magnetic resonance imaging

Multiparametric MRI is frequently used as a problem-solving technique in the evaluation of liver lesions. MRI has evolved substantially over the past decade, including hardware and software developments and specific intravenous contrast agents[3]. Technological improvements also potentially allow better quality imaging in non-cooperative patients, one of the main challenges in MRI. Therefore, when reviewing this imaging technique's performance, one should be aware of these recent advances in the field of MRI, preferring the recent literature.

MRI allows anatomic and morphologic evaluation, as well as functional imaging. The diagnostic sensitivity in detecting hepatic metastases is approximately 87% and has increased with the introduction of diffusion-weighted imaging (WI) in routine protocols and the development of hepatocyte-specific contrast agents, reaching a sensitivity of 95%[21,26]. This technique significantly improves the diagnostic efficacy and accuracy in the approach of liver metastases. Several studies reported the superiority in detecting liver lesions compared to CT, especially if they are small[32, 33].

Contrary to CT, non-enhanced sequences in MRI are essential for the detection and characterization of liver metastases. Frequently, metastases are hypo- to isointense on T1-WI sequences and mildly hyperintense on T2-WI[1]. However, some liver metastases, such as those derived from NETs and sarcomas, may show moderately high signal on T2-WI. Moreover, cystic and necrotic metastases (such as from ovary tumors, NETs, melanoma, and sarcomas) may show moderately to markedly high T2 signal intensity[3]. Liver metastases may occasionally present intralesional hemorrhage, fat, or glycogen deposition and appear hyperintense on T1-WI. Also, melanoma and mucinous adenocarcinoma metastases may show high signal on T1-WI due to their high melanocytic and mucin content, respectively (Figure 4). Occasionally, they may appear as a target sign on T2-WI sequences, characterized by hyperintense central necrosis delimited by a lesser intense rim of viable tumor. On T1-WI, a hypointense rim surrounding a center of even lower signal intensity is known as the doughnut sign (Figure 5)[1,6].

Diffusion-WI (DWI)-MRI allows the interrogation of lesions' cellularity, taking advantage of water molecules' movement. Tissues with high cellularity (tumor, fibrosis, abscess, and cytotoxic edema) show restricted diffusion[1]. Diffusion may be quantified by the apparent diffusion coefficient (ADC), and low ADC values correspond to restriction. ADC values are reported to vary between 0.94-2.87; however, there may be an overlap between the ADC values for primary malignant hepatocellular lesions, such as hepatocellular carcinoma and benign hepatocellular lesions[34]. In clinical practice, the evaluation of DWI relies on subjective appreciation. DWI may also pose disadvantages due to the inherent low spatial resolution, low signal-to-noise ratio, and predisposition to artifacts, especially for subcapsular/subdiaphragmatic lesions.

Kim *et al*[35] reported a higher sensitivity for DWI when compared to CT (79% *vs* 50%) in the detection of small liver metastases (< 1 cm) (Figure 6). Other studies concluded that DWI is more sensitive than unenhanced T2-WI (88%-91% *vs* 45%-62%),

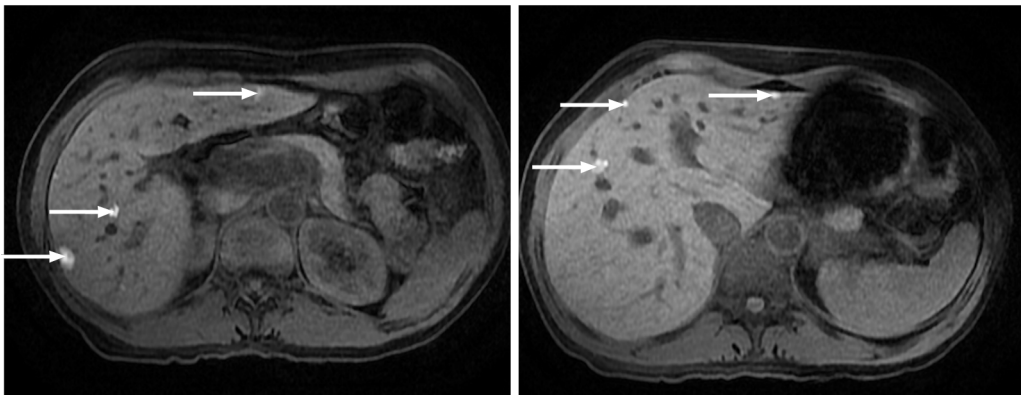

Figure 4 Multiple liver metastases from melanoma. Hepatic metastases showing a characteristic high signal on fat saturated T1-weighted imaging due to their melanocytic content (arrows).

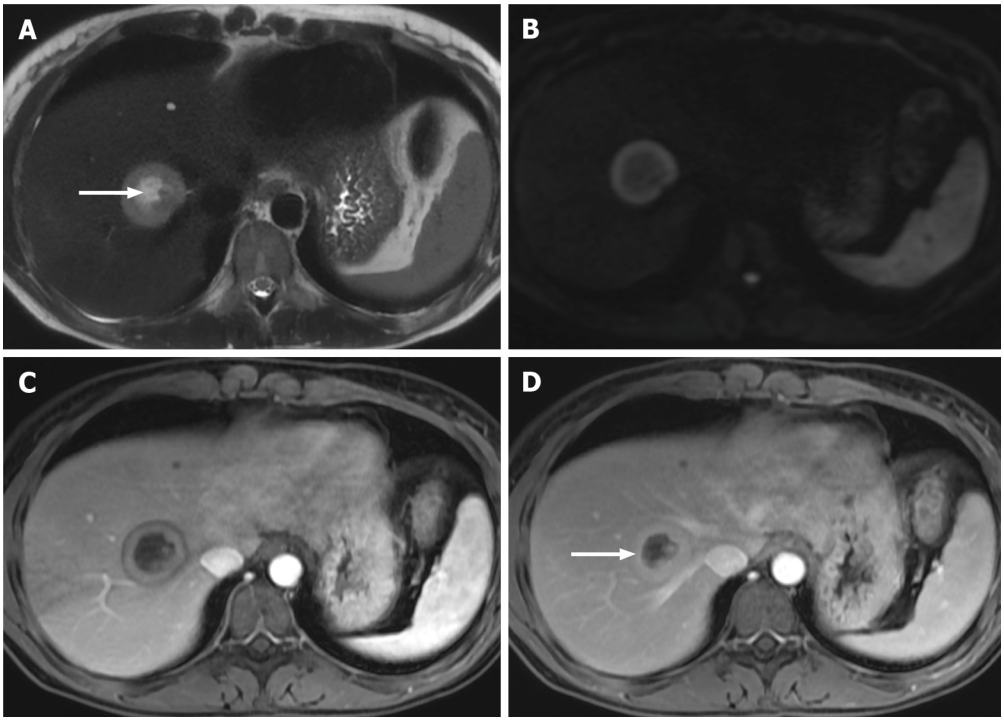

Figure 5 Right lobe liver metastasis from breast cancer. A: Axial T2-weighted imaging (WI) of the metastatic lesion shows a target sign characterized as a hyperintense center (arrow) - necrosis - marginated by a lesser intense rim of viable tumor; B: Diffusion WI shows viable tumor characterized by an increased signal; C: Axial fat sat (FS) contrast-enhanced magnetic resonance imaging (CE-MRI) T1-WI in the arterial phase shows a characteristic doughnut sign; D: Axial FS CE-MRI T1-WI in the interstitial phase reveals a mild progressive enhancement of the peripheral tumor (arrow).

and the difference is even more obvious when only small metastases are considered (85% *vs* 35%)[36,37].

For the characterization of liver metastases, it is crucial to combine pre- and post-contrast sequences as mentioned above. After entering the liver *via* the portal vein and hepatic artery, the extracellular gadolinium-based contrast agent (GBCA) is distributed through the extracellular interstitial space[1]. The desired effect is tissue enhancement on T1-WI, which is achieved by shortening the T1 and T2 relaxation times of adjacent hydrogen protons. The suggested dose for liver imaging is 0.1 mmol/kg, administered through a bolus injection at 2-3 mL/s[38]. Compared to iodine-based contrast agents (used on CT), a greater sensitivity and greater perception of enhancement are observed with GBCAs. GBCAs are considered safe, primarily because they are not nephrotoxic at the recommended doses and show fewer acute reactions than iodinated contrast agents. Although some centers still refrain from using GBCAs in renal impaired patients, one should know that class II contrast agents are rarely associated with nephrogenic systemic fibrosis. A risk-benefit analysis for every individual is required [39,40].

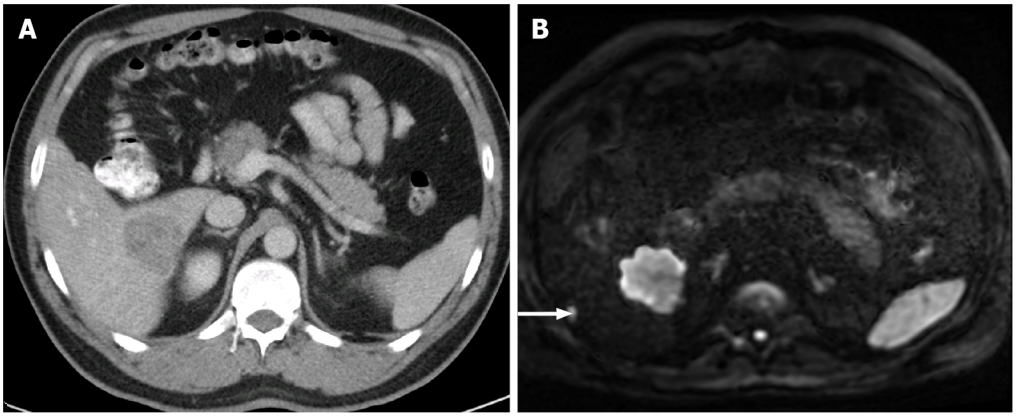

Figure 6 A 85-year-old man with a large hypovascular metastasis in the right lobe from pancreatic carcinoma proposed for tumorectomy.
A: Axial contrast-enhanced computed tomography (CE-CT) in the portal-venous phase shows a large hypodense and hypovascular metastasis; B: The patient underwent magnetic resonance imaging. An additional subcapsular small metastasis was depicted in diffusion-weighted imaging (DWI) (arrow). This example illustrates the higher sensitivity for lesion detection of DWI compared to CT.

As observed with CT, the characteristics of liver metastases vary with the primary tumor. Hypervascular metastases show a hyperintense signal in the LAP, and hypovascular metastases appear hypointense in the PVP (Figure 7). Hypovascular metastases tend to show a thin peripheral rim type enhancement in the LAP and PVP, with progressive central enhancement in interstitial phases (Figure 8)[3]. In the LAP, hypervascular metastases may show homogeneous enhancement (if smaller than 2 cm) or heterogeneous enhancement (if larger than 2 cm), demonstrating variable degrees of washout or in delayed phases (Figure 9). Isovascular metastases may be seen in breast cancer and avascular metastases on cystic metastases, such as ovarian cancer, and may demonstrate septal or wall enhancement (Figure 10). Chemotherapy-treated metastases may appear isovascular or avascular.

After being distributed in the vascular and extra-vascular space during the LAP, PVP, and delayed phases, hepatocyte-specific contrast agents are incorporated by functioning hepatocytes. The available hepatocyte-specific MRI contrast agents are gadobenate dimeglumine (Gd-BOPTA; MultiHance), with a recommended dose of 0.1 mmol/kg, and gadoxetic acid (Gb-EOB-DTPA; Primovist/Eovist), with a recommended dose of 0.025 mmol/kg[38]. The hepatobiliary phase is acquired after 90-150 min for MultiHance and 15-20 min for Primovist. These temporal differences for the hepatocyte phases are related to the degree of biliary excretion, estimated at 3%-5% for MultiHance and 50% for Primovist[1]. The kidneys excrete the remaining.

The normal functioning hepatocytes uptake the hepatocyte-specific MRI contrast agents and excrete them into the biliary system due to cellular membrane transporters. The contrast agent is responsible for shortening the T1 relaxation, which results in higher signal intensity of the healthy liver parenchyma on T1-WI in the hepatobiliary phase[1]. In the later (hepatobiliary) phase, there is also a subsequent excretion into the biliary canaliculi, allowing imaging of the biliary pathways. Therefore, the hepatobiliary phase is easily recognized because the normal liver parenchyma and bile ducts appear enhanced[41]. Non-hepatocellular lesions, as well as lesions with impaired hepatocytes, appear hypointense. In short, as liver metastases lack functioning hepatocytes and biliary ducts, they appear hypointense in the hepatobiliary phase. Allergic reactions are infrequent and comparable with those of extracellular GBCAs.

In a recent meta-analysis, Zhang *et al*[42] showed that the sensitivity of gadobenate (MultiHance) for detecting liver metastases on a per-lesion basis for pre-contrast and combined dynamic, delayed hepatobiliary phase imaging was 77.8%, 88.1%, and 95.1%, respectively. These results are comparable to those reported for gadoxetate (Primovist/Eovist).

Resembling only the MRI's specificities, a meta-analysis published in 2016 showed that the sensitivity of DWI and gadoxetic acid-enhanced MRI (GA-MRI) was 87.1% and 90.6%, respectively. When both sequences were combined, the sensitivity for detecting liver metastases on a per-lesion basis was the highest (95.5%)[43].

Therefore, MRI plays a crucial role in evaluating liver metastases and is considered the ideal imaging method for detection and follow-up in many university hospitals.

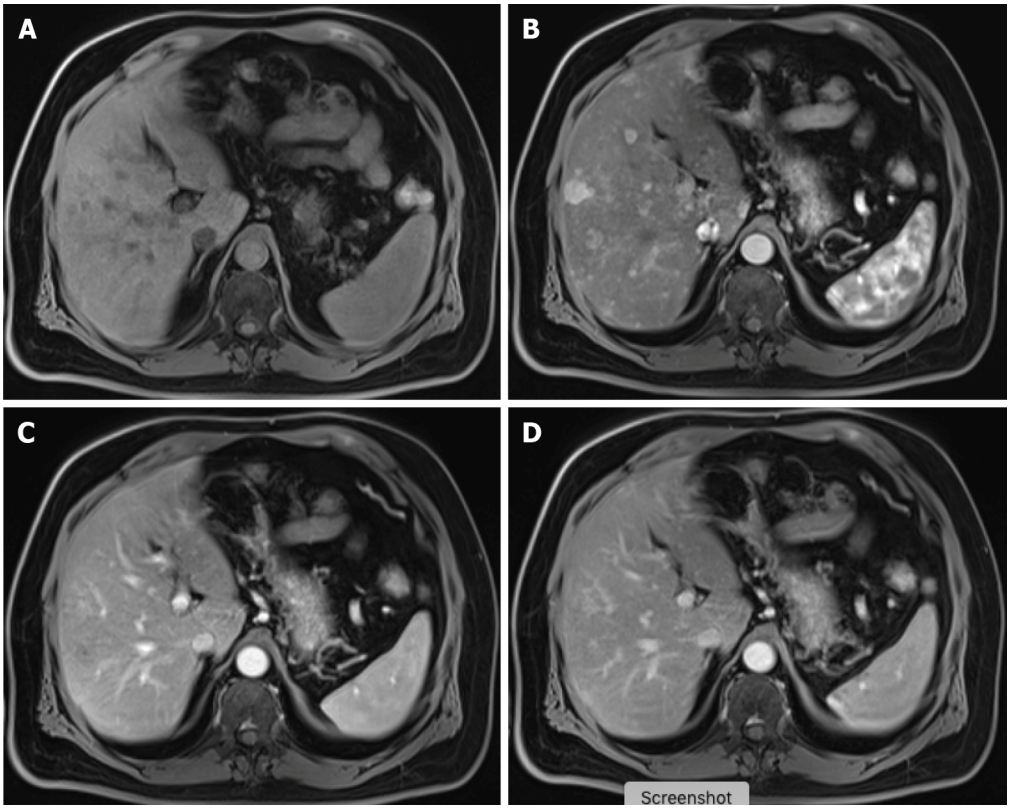

Figure 7 Carcinoid tumor with countless hypervascular liver metastases. A: Axial fat saturated (FS) non-contrast-enhanced magnetic resonance imaging (CE-MRI) T1-weighted imaging (WI) with barely imperceptible hypointense lesions; B: Axial FS CE-MRI T1-WI in the arterial phase detecting multiple hyperenhancing lesions compatible with hypervascular liver metastases; C: Axial FS CE-MRI T1-WI in the portal-venous phase shows fast fading of the lesions previously depicted; D: In the axial FS CE-MRI T1-WI in the delayed phase, the lesions become barely imperceptible. The arterial phase is crucial for the detection of hypervascular metastases.

Positron emission tomography/computed tomography

Liver metastases may have significant fluorine-18-labeled fluorodeoxyglucose (FDG) uptake. Previous investigations mentioned the impact of FDG-PET on the detection of such lesions (Figure 11). A meta-analysis published by Maffione *et al*[44] suggests that FDG-PET/CT is highly accurate in detecting liver metastases on a patient-based analysis, besides showing an added value in identifying extrahepatic disease. However, conventional PET proved to be less sensitive than MRI and CT in detecting CRLM, both on a patient-based (93% *vs* 100% *vs* 98%, respectively) and lesion-based analysis (66% *vs* 89% *vs* 79%, respectively). In addition to the detection of extrahepatic disease, PET/CT has the advantage of assessing treatment response (*i.e.*, chemotherapy) of liver metastases, demonstrated by a decrease in FDG uptake[1]. However, false negatives may arise immediately after completing a chemotherapy cycle due to residual metabolic inhibition. For this reason, PET/CT is not recommended to be performed earlier than 4 wk after finishing chemotherapy, and a negative result must not be fully trusted[45].

Positron emission tomography/magnetic resonance imaging

PET/MRI is a more recent technique that combines the advantages of metabolic imaging (FDG-PET) with MRI sensitivity to assess liver metastases. PET/MRI is a helpful diagnostic technique in detecting small hepatic lesions and may improve the evaluation of treatment response after radiation and chemotherapy. Beiderwellen *et al* [46] demonstrated that PET/MRI has a higher diagnostic accuracy for detecting liver metastases than PET/CT or multidetector CT. However, according to Lake *et al*[47], there is no significant difference in the diagnostic performance between PET/MRI and Gd-EOB-DTPA MRI. Moreover, PET/MRI also shows an incremental value for detecting additional extrahepatic metastases[47].

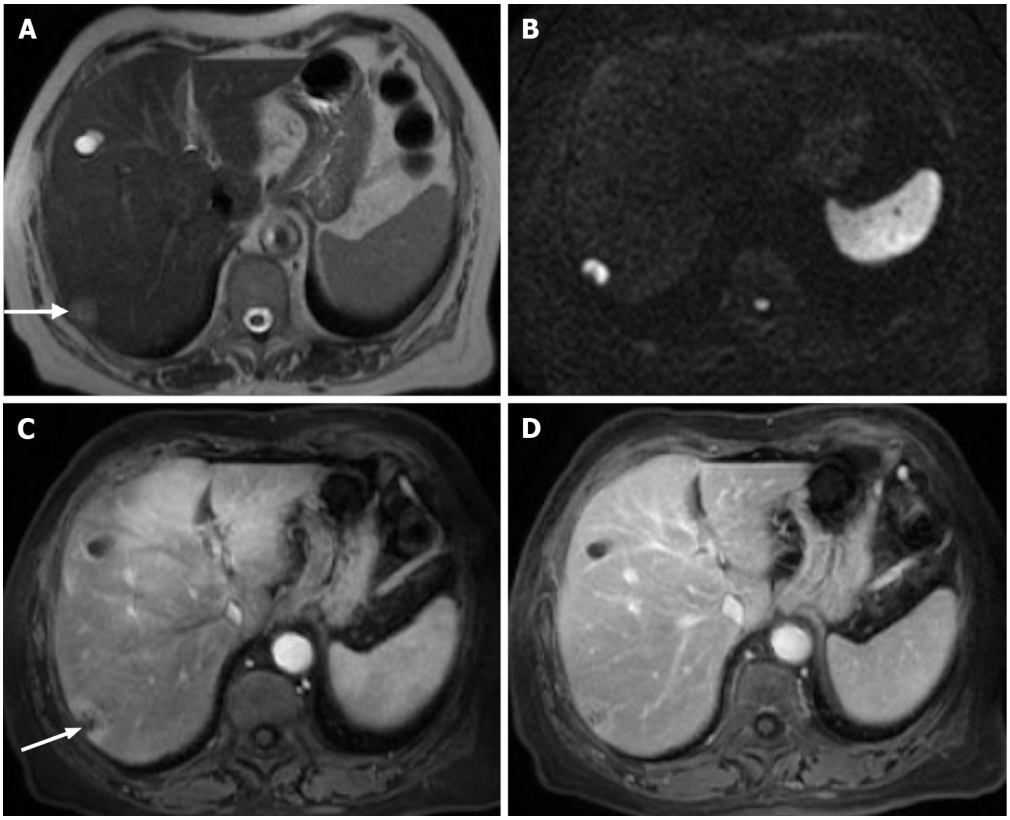

Figure 8 Pancreatic cancer liver metastasis is seen in the subcapsular region of segment VII. A: Axial T2- weighted imaging (WI) shows the pancreatic liver metastasis as a mildly hyperintense lesion (arrow); B: Note the very high signal intensity on high *b* value diffusion-weighted imaging; C: Axial fat saturated (FS) contrast-enhanced magnetic resonance imaging (CE-MRI) T1-WI in the arterial phase. Despite being hypovascular, it is common to find perilesional hyperenhancement in pancreatic cancer subcapsular metastases (arrow); D: Axial FS CE-MRI T1-WI interstitial phase - progressive central enhancement is appreciated in the interstitial phase.

DECIDING BETWEEN TECHNIQUES

It is crucial to detect hepatic metastases as accurately as possible in a per-patient and per-lesion manner to improve patient's clinical evolution, prognosis, and treatment planning. CT, MRI, and FDG-PET are historically the most accurate and precise imaging techniques for this purpose[45]. Below, we refer to various studies comparing these techniques, which will help choose the best option for evaluating liver metastases. Table 1 summarizes the *pros and cons* of cross sectional techniques.

Several studies reported that CE-MRI is more sensitive and specific than CE-CT for detecting liver metastases, mainly due to high intrinsic soft-tissue contrast, technical versatility, sensitivity to blood flow, and contrast enhancement and biochemical information[6]. Vreugdenburg *et al*[32] confirmed in their systematic meta-analysis that in terms of per-lesion diagnostic accuracy, GA-MRI is superior to CE-CT (sensitivity 86.9%-100% *vs* 51.8%-84.6% and specificity 80.2%-98% *vs* 77.2%-98%). This difference is more evident in lesions smaller than 10 mm, in which GA-MRI is notably more sensitive but less specific. Based on the reported sensitivity, an equivocal result will happen more frequently with CE-CT, which leads to a modest impact on patient prognosis and management. In 2017, similar results were reported by Choi *et al*[48], who compared MRI, CT, and PET/CT for the detection of CRC liver metastases, showing a sensitivity of 93.1% *vs* 82.1% *vs* 74.1% and specificity of 87.3%, 73.5%, and 93.9%, respectively (Figure 12). MRI showed a better accuracy than CT in detecting CRC liver metastases and presented an incremental value when added to CT alone to detect additional metastases[48]. In this study, the authors reported that neoadjuvant chemotherapy decreases the sensitivity of both CT and MRI; however, it does not significantly affect the sensitivity of PET/CT[48].

The superiority of MRI is self-evident in small metastases. It is supported by various studies, including that by Schulz *et al*[49], where they reported that the detection of CRLM should rely on MRI. Overall sensitivity/specificity for MRI, CT, and PET was 90%/87%, 68%/94%, and 61%/99%, respectively; and the sensitivity/specificity for lesions smaller than 10 mm for MRI, CT, and PET was 74%/88%, 16%/96% and

Table 1 Deciding between different imaging methods for liver metastasis diagnosis based on articles' analysis

Imaging methods	Critical details
MRI	*Pros:*
	Most accurate method, and superior to CT and PET-CT for the detection of liver metastases:
	Especially useful for smaller lesions (< 1 cm), characterization of hypervascular metastases, and in the setting of liver steatosis
	High grade of confidence in the distinction between malignant and benign lesions
	Anatomic and morphologic evaluation.
	Non-enhancing sequences play an important role
	Therapy response assessment
	Absence of ionizing radiation
	Less allergic reactions
	May be the most cost-effective option:
	Higher detection rate > more curative approach > avoids additional imaging examinations
	Cons:
	Lower availability
	Non-cooperative patients may result in suboptimal study
	Limited for pacemaker carriers
	Limited use if Glomerular filtration rate < 15 mL/min
CT	*Pros*:
	Low cost
	Higher availability
	Higher sensitivity compared to ultrasonography
	Whole-body evaluation
	Therapy response assessment
	Cons:
	Ionizing radiation
	Lower sensitivity for the detection of smaller metastases or in the setting of liver steatosis compared to MRI
	Low confidence in the distinction between malignant and benign lesions
	Not adequate for renal impaired patients
PET-CT	*Pros*:
	Accurate detection of extrahepatic disease
	Therapy response assessment
	Cons:
	False negatives after a chemotherapy cycle
	Lower sensitivity for small liver metastases
	Lower availability
	Highest ionizing radiation dose

MRI: Magnetic resonance imaging; CT: Computed tomography; PET-CT: Positron emission tomography-computed tomography.

9%/98%, respectively[49].

With the introduction of surgical removal of metastatic liver nodules, the overall survival rate has increased. Therefore, it is crucial to ensure the best imaging method to detect them, mainly the smaller ones, which can be easily missed. Ko *et al*[50]

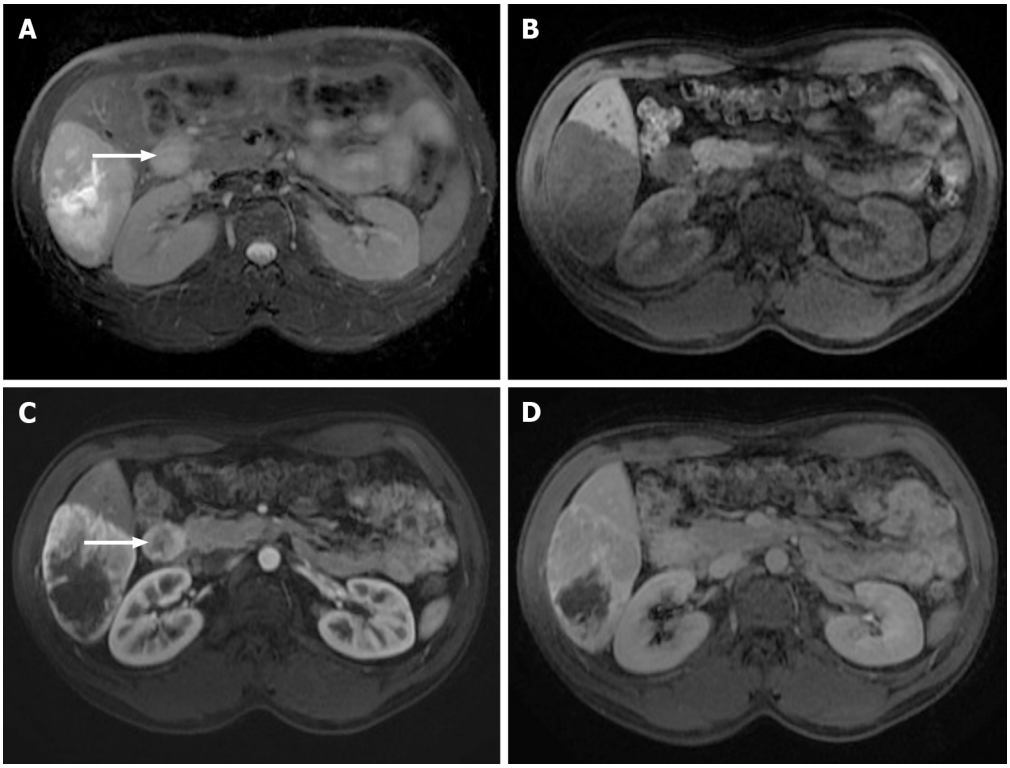

Figure 9 Images show a large liver metastasis from a duodenal neuroendocrine tumor. A: In the axial fat saturated (FS) T2-weighted imaging (WI), the liver metastasis is characterized by hyperintense central necrosis delimited by a lesser intense viable tumor. Note the duodenal neuroendocrine tumor (arrow); B: Axial FS non-contrast-enhanced magnetic resonance imaging (CE-MRI) T1-WI shows large hypointense liver metastasis; C: Axial FS CE-MRI T1-WI in the arterial phase demonstrates viable tumor with avid heterogeneous enhancement. The primary lesion is also hypervascular and depicted in the 2nd portion of the duodenum (arrow); D: Axial FS CE-MRI T1-WI in the interstitial phase reveals fading of the lesion.

showed that the sensitivity of CT was 8%, 55%, 91%, and 100% for nodules of 1-5 mm, 6-10 mm, 11-15 mm, and > 20 mm, respectively. Consequently, it appears obvious that in metastases that are "too small to characterize," CT has a limited role, particularly for those smaller than 5 mm[50]. However, GA-MRI and CE-CT seem equivalent for detecting lesions larger than 10 mm[21,26].

Maegerlein *et al*[51] confirmed that MRI was significantly superior (sensitivity of 87.4%) compared to PET/CT (sensitivity of 68.2%).

For metastases in a fatty liver background, the sensitivity of MRI is approximately 85%-88% (*vs* 65%-68.3% for CE-CT)[18,52]. In these conditions, Kulemann *et al*[18] found that MRI detects 66% of lesions up to 10 mm, while CT detects only 11%. Therefore, they determined that MRI is superior to CT in detecting CRLM in liver steatosis, especially the smaller ones[18,52].

MRI also showed to be significantly better than CE-CT in the detection and characterization of hypervascular liver metastases. For instance, according to Seemann *et al*[53], MRI presented a sensitivity of 98.2%, and CT showed a sensitivity of only 37.1% for detecting carcinoid metastases.

Nowadays, debate continues over whether MRI should be a first-line imaging technique for suspected liver metastases. The current European Society for Medical Oncology (ESMO) guidelines for rectal cancer diagnosis and follow-up (2017) consider that MRI is the imaging method of choice for loco-regional staging. However, CT is preferred for distant metastases[54]. Still, these recommendations are relatively poor (level V), and curiously that manuscript does not make any reference to the use of hepatospecific contrast agents[55]. The American College of Radiology in 2017 also stated that "the available evidence supports that both MRI and CT detect liver lesions with high accuracy."

The updated NCCN guidelines (March 2019) for colon and rectal cancer suggest chest, abdominal, and pelvic CT for metastatic disease's initial workup[21,24,56]. However, if surgical resection of hepatic metastases is considered, contrast-enhanced MRI (extracellular or hepatospecific contrast agent) is preferred over CT to assess their number and distribution[56]. Also, PET-CT may be pondered in selected cases with surgical curable M1 disease[21,26].

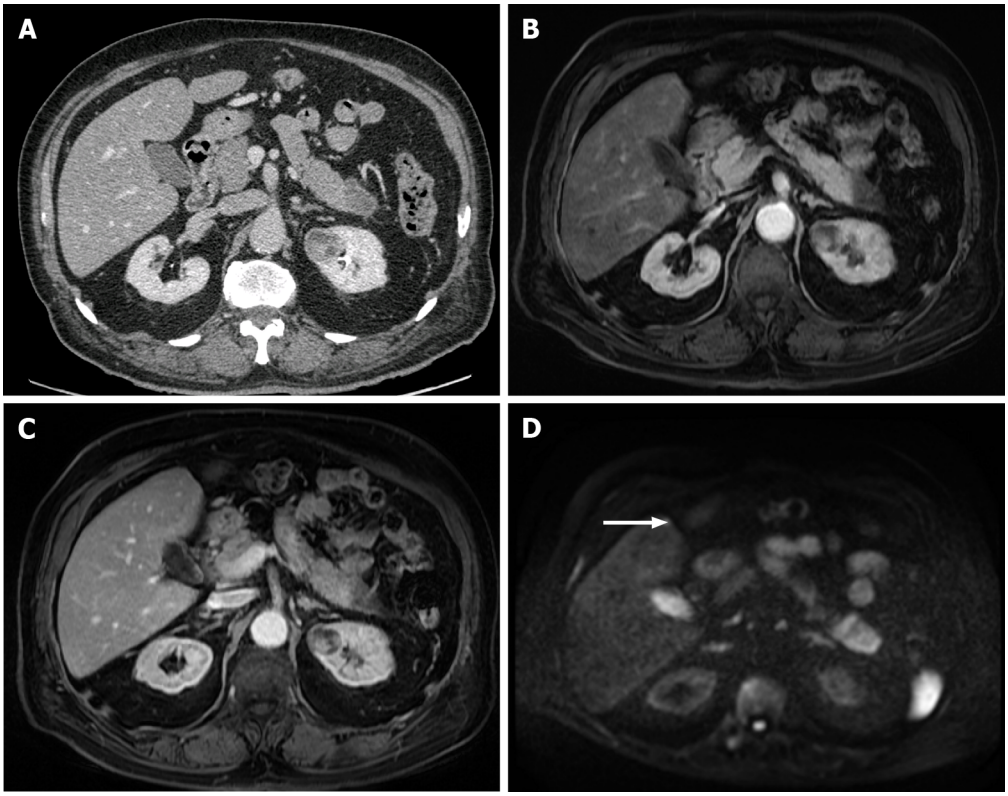

Figure 10 A 40-year-old woman with breast cancer showing a subcapsular millimetric iso-vascular metastasis only depicted in the diffusion-weighted imaging. Contrast-enhanced computed tomography (CE-CT) and dynamic magnetic resonance imaging (MRI) sequences could not detect the lesion. A: Axial CE-CT in the portal-venous phase; B: Axial fat saturated (FS) CE-MRI T1-weighted imaging (WI) in the arterial phase; C: Axial FS CE-MRI T1-WI in the portal-venous phase; D: Diffusion-weighted imaging showing a small lesion with high signal intensity on high *b* value corresponding to liver metastasis (arrow).

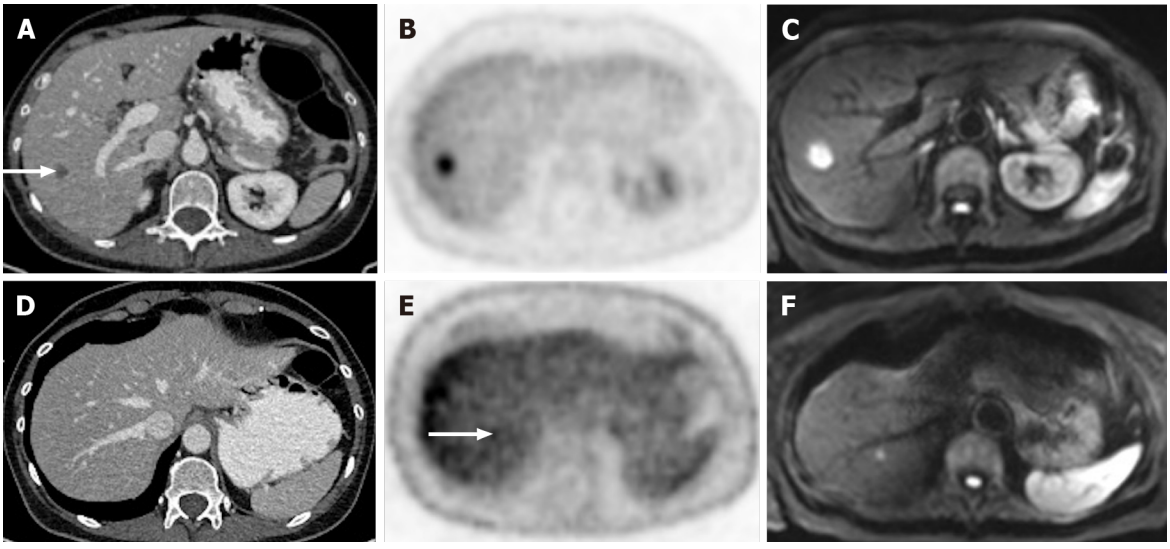

Figure 11 A 65-year-old woman with colorectal carcinoma shows liver metastasis in segment VII. A: Axial contrast-enhanced computed tomography (CE-CT) reveals a hypodense lesion corresponding to liver metastasis (arrow); B: Fluorodeoxyglucose (FDG) positron emission tomography (PET)-CT confirms metastatic origin; D: Axial CE-CT shows no apparent lesion; E: FDG PET-CT shows an additional barely visible nodule not seen in CT (arrow); C and F: Diffusion-weighted imaging confirmed that both lesions were secondary.

Many clinicians use the "economic implication" to use CT instead of MRI[55]. Patients often are referred to CT rather than MRI because of the perceived impression that money is being saved in the healthcare system. Zech *et al*[57] compared the three imaging techniques (GA-MRI, CE-MRI, and CE-CT), considering the diagnostic workup and surgery costs for patients with CRLM. The countries analyzed included Austria, Germany, Italy, Sweden, Switzerland, and Thailand and all of them showed

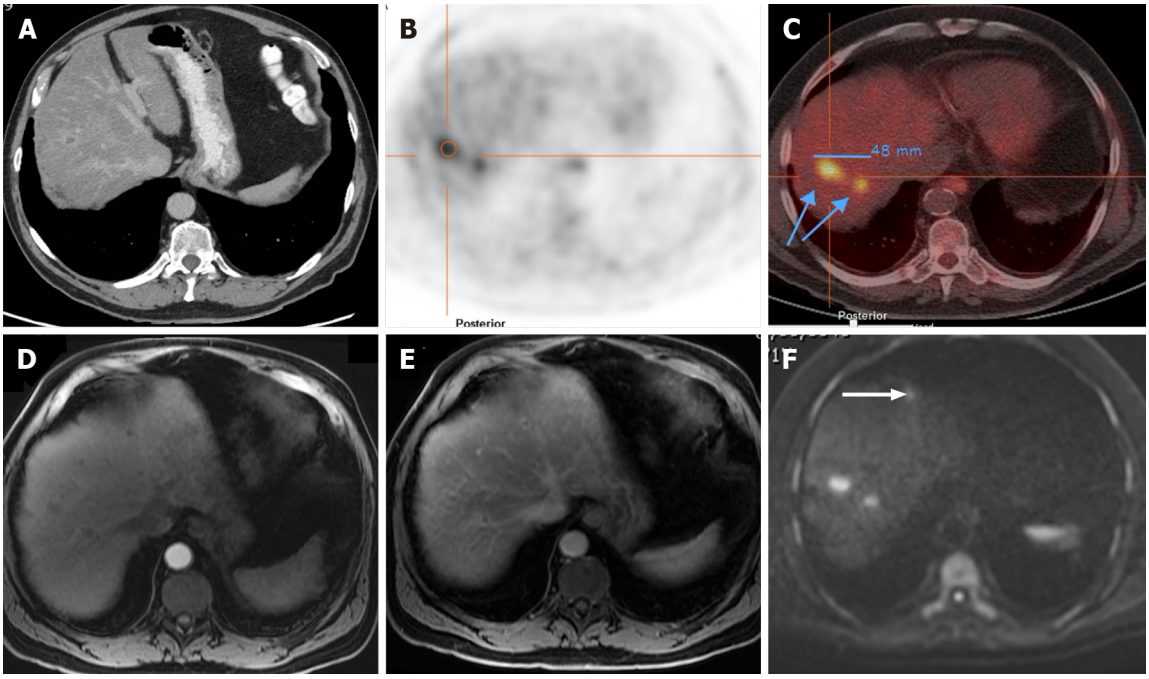

Figure 12 A 71-year-old man with colorectal carcinoma presenting with liver metastases. A: Axial contrast-enhanced computed tomography (CE-CT) in the portal-venous phase shows barely identified non-specific liver micronodules; B and C: Fluorodeoxyglucose positron emission tomography (PET)-CT shows two hypermetabolic lesions in the right lobe, consistent with viable neoplastic tissue; D: Axial fat saturated (FS) CE- magnetic resonance imaging (MRI) T1-weighted imaging (WI) in the arterial phase shows hypovascular liver lesions; E: Axial FS CE-MRI T1-WI in the venous phase confirms the liver metastases, showing hypointense nodules with venous ring enhancement; F: In the diffusion-weighted imaging study, these lesions are more conspicuous. Also, an additional metastasis (arrow) that was not detected either by CE-CT, CE-MRI, or PET-CT is shown.

an overall lower cost with GA-MRI compared to the other techniques[57]. The reason is that no patient needed any additional imaging technique to achieve a decision concerning the treatment in the group that used GA-MRI as the initial imaging method. However, in the group of patients submitted to extracellular CE-MRI and CE-CT as an initial approach, approximately 18.1% and 39.7%, respectively, performed an additional examination. Furthermore, it was also noted that the costs of surgery were higher in the GA-MRI group since more liver metastases were detected and consequently needed surgery for a curative approach.

According to these data, we concur that GA-MRI shows a superior sensitivity in detecting hepatic metastases, which leads to a more curative approach, avoids additional imaging examinations, and can be the most cost-effective option. Sadly, these studies did not significantly affect the current clinical guidelines, especially the latest consensus of ESMO, where MRI is still considered a second-line method[45,54].

In addition, according to a recent study, laparoscopic liver ultrasound might improve liver staging for CRLM compared to liver-specific contrast-enhanced MRI (sensitivity of 93.1% *vs* 85.6%)[58].

IMAGING TECHNIQUES FOR FOLLOW-UP

Approximately 80% of CRLM are unresectable at initial presentation, and chemotherapy is the treatment of choice (Figure 13). Some studies have reported that some of these lesions might respond to chemotherapy and become resectable, showing better long-term results than "conversion chemotherapy"[59]. As above-mentioned, these patients submitted to neoadjuvant chemotherapy may then appear with liver steatosis, especially after irinotecan and 5-FU or with sinusoidal obstruction (oxaliplatin), which may limit CT liver evaluation[21,60].

In follow-up studies of CRLM, CT may be used to evaluate response to systemic chemotherapy. In contrast, MRI (with hepatospecific contrast agent and DWI sequences) can be used to assess metastases after neoadjuvant chemotherapy, to assess resectability, and to estimate "disappearing" or "vanishing" metastases (DLM) (Figure 14)[21]. This term corresponds to complete radiologic response – treated metastases that are too small to be detected at follow-up imaging studies – ranging

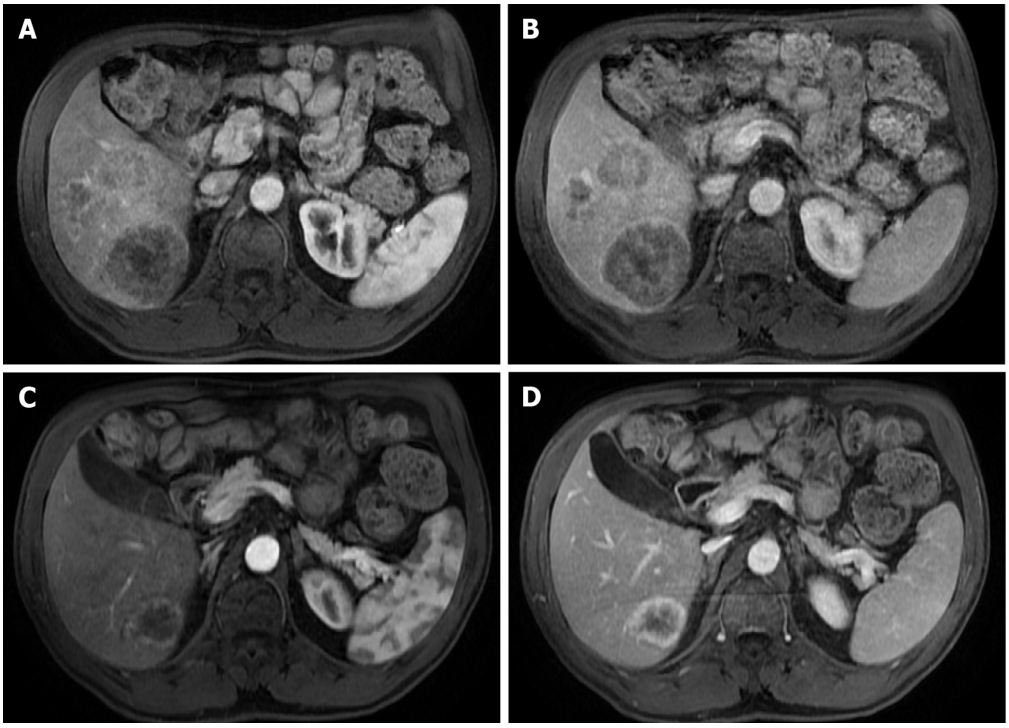

Figure 13 A 71-year-old man with unresectable CRLM. A and C: Axial fat saturated (FS) contrast-enhanced magnetic resonance imaging (CE-MRI) T1-weighted imaging (WI) in the arterial phase; B and D: Axial FS CE-MRI T1-WI in the portal-venous phase. Initial presentation of three heterogeneous hepatic lesions corresponding to unresectable CRLM before treatment (A and B). After chemotherapy (C and D), the patient presented partial response, with the disappearance of two lesions and reduced size of the larger lesion, which still presents viable peripheral tumor.

from 7%-24% in CRLM[21,61].

Barimani *et al*[61] showed that the combination of CE-CT, MRI, and intraoperative ultrasound (IOUS) showed promising results in detecting DLM in CRLM. Furthermore, it was suggested that when DLM remains undetectable by MRI and IOUS, it is a valid option to leave DLM *in situ* as an alternative approach to surgical resection.

According to Jhaveri *et al*[62], GA-MRI is superior to CE-CT for the detection of small CRLM (< 1 cm) in both categories of non-treated patients and those who underwent neoadjuvant chemotherapy.

In 2017, a study by Park *et al*[63] also concluded that MRI has a higher positive predictive value for the absence of tumors after chemotherapy than CT (78% *vs* 35.2%, respectively).

The RECIST criteria were developed to reach a standardized pattern of tumor response evaluation[64]. These criteria show limitations and appear inadequate for patients treated with immune checkpoint inhibitors due to the "pseudoprogression" phenomenon. Pseudoprogression may occur when molecular target agents diminish the tumor attenuation and enhancement to a lesser degree when compared to the surrounding liver, making the preexisting lesion now visible and mimicking disease progression. To assess this limitation, iRECIST criteria, based on RECIST-based measurements and immune-related response patterns, have been developed[55]. However, iRECIST criteria still need validation.

RECIST evaluation concerning CRLM often fails to identify clinically meaningful responses to bevacizumab-containing therapy. In this matter, Liu *et al*[65] created a developed-RECIST (D-RECIST) by combining CE-MRI and DWI-MRI. They showed that responders employing D-RECIST had a longer median disease-free survival than non-responders and that defined responses provided important prognostic information. It was concluded that D-RECIST might serve as a better response evaluation than RECIST in CRLM treated with bevacizumab-chemotherapy.

Some morphologic and dynamic features of liver metastases in MRI may predict the response before therapy[21,66]. For instance, a study showed that tumors with lower ADC values correlate with a better response to chemotherapy, while others report a poorer survival[67].

Besides chemotherapy, ablative therapies such as microwave ablation, transarterial chemoembolization, and radioembolization lead to a low-density lesion on CT and

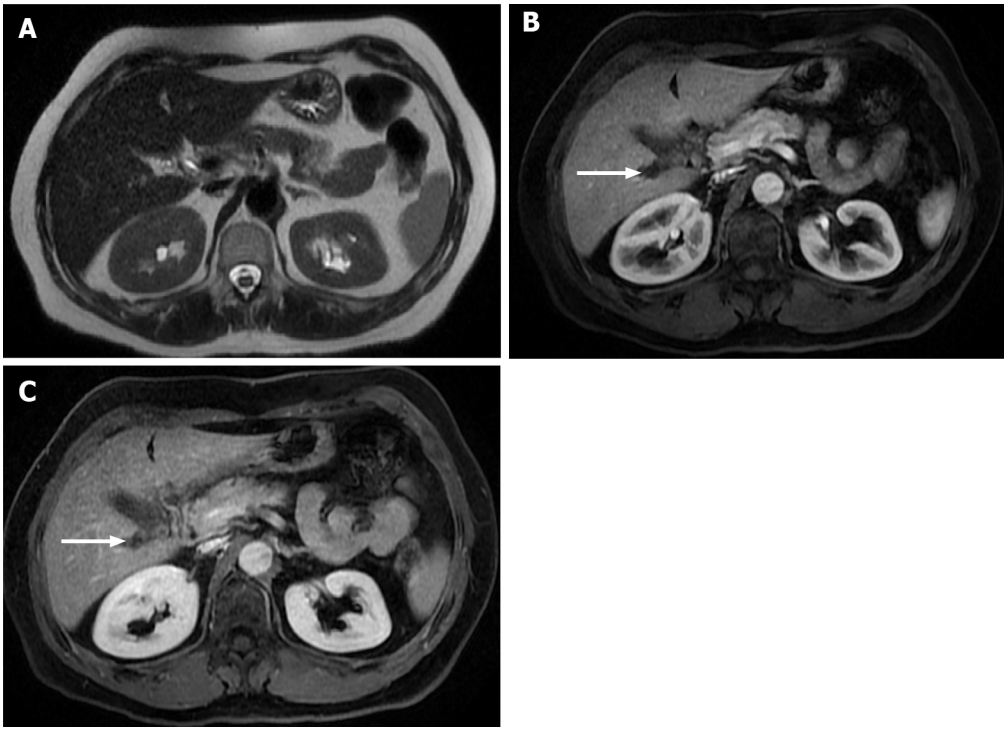

Figure 14 Follow-up of a 66-years-old woman with previous breast cancer liver metastases submitted to chemotherapy showing complete response in 2015. A: Axial T2-weighted imaging (WI) shows the liver metastasis characterized by an isointense lesion; B and C: Axial fat sat contrast-enhanced magnetic resonance imaging T1-WI in the arterial (B) and interstitial (C) phases present the liver metastasis without noticeable enhancement in the post-contrast dynamic study (arrow, B and C), which is consistent with treated metastasis (no viable tumor). To date, after 6 years, the patient is free of recurrent disease.

high T1 signal / low T2 signal on MRI due to coagulative necrosis[3]. These areas tend to shrink progressively with time. The existence of thick linear peripheral enhancement surrounding the lesion or nodular enhancement may suggest recurrence. Partial response is suggestive by a decrease in enhancement, and a complete response/successful embolization is shown by the absence of enhancement on CT/MRI and low T2 signal[3].

CONCLUSION

The liver is one of the most common organs involved with metastatic disease. Both CT and MRI are currently the techniques that show the highest diagnostic performance and are also the most suitable for assessing therapy response and follow-up. Studies have shown that MRI plays a crucial role and has a higher sensitivity in evaluating liver metastases. Therefore, it may be the ideal imaging method for treatment planning before and after neoadjuvant chemotherapy and is also considered the best technique for detection and follow-up in many university hospitals.

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MINIREVIEWS

Liver manifestations and complications in inflammatory bowel disease: A review

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Abstract

Hepatobiliary manifestations are common in inflammatory bowel disease (IBD), with 30% of patients presenting abnormal liver tests and 5% developing chronic liver disease. They range from asymptomatic elevated liver tests to life-threatening disease and usually follow an independent course from IBD. The pathogenesis of liver manifestations or complications and IBD can be closely related by sharing a common auto-immune background (in primary sclerosing cholangitis, IgG4-related cholangitis, and autoimmune hepatitis), intestinal inflammation (in portal vein thrombosis and granulomatous hepatitis), metabolic impairment (in non-alcoholic fatty liver disease or cholelithiasis), or drug toxicity (in drug induced liver injury or hepatitis B virus infection reactivation). Their evaluation should prompt a full diagnostic workup to identify and readily treat all complications, improving management and outcome.

Key Words: Hepatobiliary manifestations; Inflammatory bowel disease; Drug induced liver injury; Primary sclerosing cholangitis; Viral hepatitis; Crohn's disease; Ulcerative colitis

Core Tip: Hepatobiliary manifestations are common in inflammatory bowel disease (IBD), ranging from incidental findings in asymptomatic patients to life-threatening liver failure. Their pathogenesis can be intrinsically linked to IBD (auto-immune background or metabolic abnormalities) or to its medication. Early recognition of these manifestations as well as a full diagnostic workup are mandatory to improve management and prognosis. In this review, we describe all hepatobiliary manifestations

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INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic and recurrent gastrointestinal inflammatory conditions that result from the interaction of genetic, environmental, and immune factors. IBD is mainly divided into Crohn's disease (CD) and ulcerative colitis (UC), affecting equally men and women, with peak incidence between 20 and 30 and also from 50 to 60 years of age[1].

Extra-intestinal manifestations are described in up to 50% of patients, including arthropathy, metabolic bone disease, ocular, dermatological, hepatobiliary, neurologic, cardiovascular, pulmonary, and urological complications[2].

Hepatobiliary alterations are one of the most common extra-intestinal manifestations of IBD; up to 30% of patients have abnormal liver tests and 5% will develop chronic liver disease[3,4]. A wide diversity of hepatobiliary complications has been reported, ranging from incidental findings in asymptomatic patients to severe and life-threatening liver failure[5].

The pathogenesis of liver disease in IBD is not totally understood but multiple pathways may link them (Table 1)[2,5,6].

Inflammatory bowel disease related diseases

Diseases that share a common auto-immune background include primary sclerosing cholangitis (PSC), IgG4-related cholangitis, primary biliary cholangitis (PBC), auto-immune hepatitis, and overlap syndromes.

Diseases associated with intestinal inflammation include portal vein thrombosis, Budd-Chiari syndrome, granulomatous hepatitis, and liver abscesses.

Diseases associated with malabsorption or metabolic impairment are cholelithiasis, amyloidosis, and non-alcoholic fatty liver disease (NAFLD).

Inflammatory bowel disease related medications

Disorders associated with IBD treatment include direct hepatotoxicity with medications such as 5-aminosalicylic acid (5-ASA) compounds, methotrexate, azathioprine, or anti-TNF agents or hepatitis B reactivation due to immunosuppressants.

They can occur at any time during the natural history of disease and typically follow an independent course from the underlying intestinal disease activity. Granulomatous hepatitis, hepatic abscesses, cholelithiasis, and amyloidosis are more commonly observed in CD and PSC and auto-immune hepatitis in UC[6,7].

Moreover, these patients may present unrelated liver disease, making abnormal liver tests in IBD a challenging differential diagnosis.

Early recognition of these manifestations is of paramount importance to avoid liver injury and improve management of both diseases (Figure 1).

The aim of this paper is to review the hepatobiliary manifestations and complications found in IBD patients.

DISEASES SHARING A COMMON AUTO-IMMUNE BACKGROUND WITH INFLAMMATORY BOWEL DISEASE

Primary sclerosing cholangitis

PSC is a chronic and progressive bile duct disorder, characterized by multifocal intrahepatic and/or extrahepatic strictures and dilatations, that may result in cirrhosis and end-stage liver disease. The diagnosis is usually made by combination of clinical (jaundice, abdominal pain, and itching but it may also be asymptomatic), biochemical

Table 1 Inflammatory bowel disease related diseases and inflammatory bowel disease medication related diseases

IBD related diseases		IBD medication related diseases	
Ulcerative colitis	**Crohn's disease**	**Ulcerative colitis**	**Crohn's disease**
Primary sclerosing cholangitis	Granulomatous hepatitis	Drug-induced liver injury	Drug-induced liver injury
Auto-immune hepatitis	Liver abscesses	HBV reactivation	HBV reactivation
Overlap syndromes	Cholelithiasis		
Primary biliary cholangitis	Hepatic amyloidosis		
Portal vein thrombosis			
NAFLD	NAFLD		

IBD: Inflammatory bowel disease; HBV: Hepatitis B Virus; NAFLD: Non-alcoholic fatty liver disease.

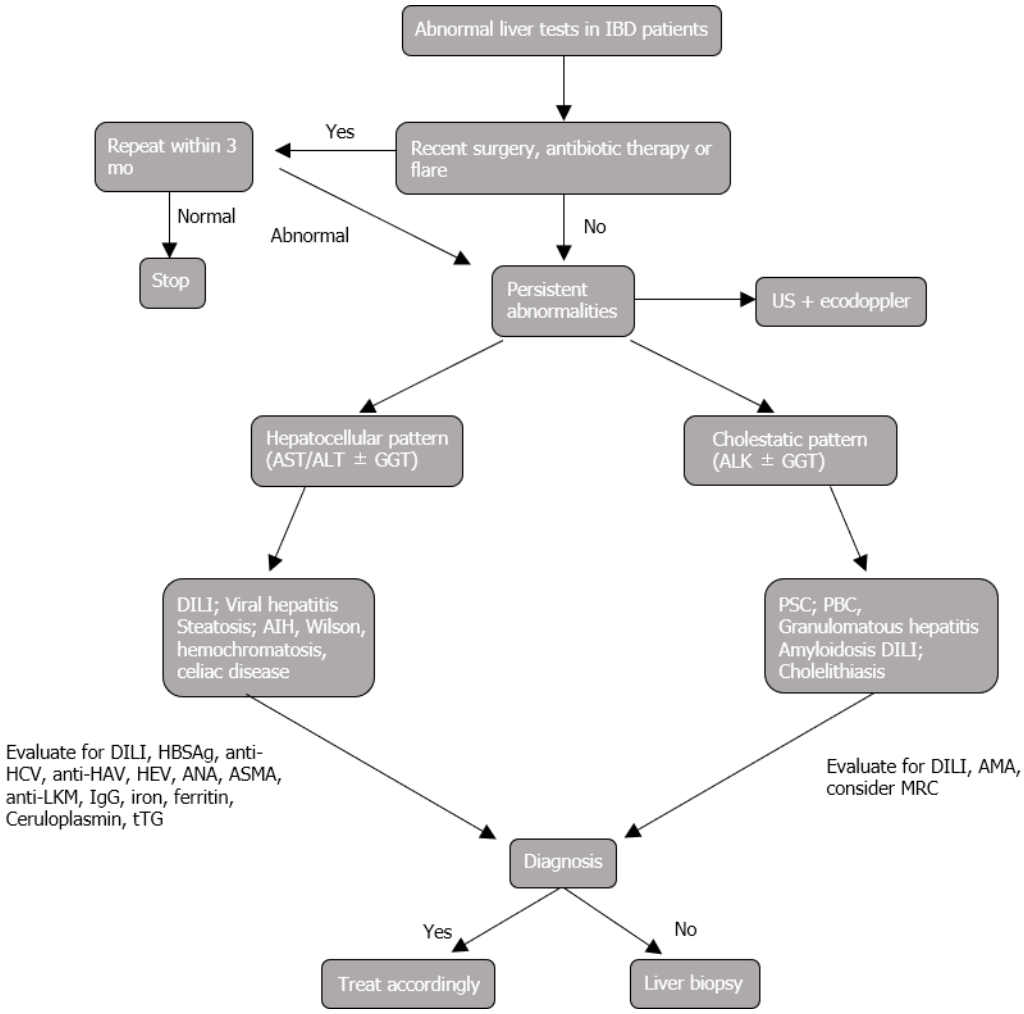

Figure 1 Management of abnormal liver tests. IBD: Inflammatory bowel disease; US: Ultrasonography; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cholangitis; DILI: Drug induced liver injury; AMA: Anti-mitochondrial antibody; MRC: Magnetic resonance cholangiography.

(elevated cholestatic liver enzymes - alkaline phosphatase and/or GGT) and imagiological [magnetic resonance cholangiography (MRCP)] findings. The mean age at diagnosis is 30 to 40 years old and it has a male predominance[8,9].

PSC is closely linked to IBD, which occurs in 70% of patients, with a UC predominance (75%). On the other hand, only up to 3% of CD and 2%-8% of UC patients develop PSC[10]. Therefore, the presence of unexplained cholestasis should prompt an immediate investigation by MRCP in those with IBD and patients with PSC should

routinely undergo colonoscopy with biopsies, even in the absence of symptoms. If the index colonoscopy is negative, it should be repeated every 3 to 5 years[10,11]. The two disorders can occur at different times, but IBD diagnosis usually precedes that of PSC [12].

IBD in the setting of PSC is associated with a different clinical course, typically presenting extensive disease, rectal sparing (6% to 66% *vs* 2% to 25% in IBD without PSC), backwash ileitis (5% to 46% *vs* 3% to 24% in UC without PSC), and mild intestinal activity, as well as more frequent right colonic involvement[10,13]. Marelli *et al*[14] showed an inverse relationship between PSC severity and IBD activity. On the other hand, the effect of IBD in PSC prognosis is less established - higher rates of combined intrahepatic and extrahepatic involvement have been reported, although long-term outcomes of PSC do not seem to be changed[10,15,16].

PSC-IBD patients also present a greater risk of colorectal dysplasia and cancer, which supports the current recommendation of annual surveillance colonoscopy in this subset of patients. Although there are no specific recommendations, colectomy is suggested in case of indefinite or low-grade dysplasia, due to a high risk of colorectal cancer[10,17,18]. Similarly, prolonged duration of IBD was associated with an increased risk of cholangiocarcinoma, with a 33% higher risk per 10 years[19].

Small-duct primary sclerosing cholangitis

Small-duct PSC is very similar to large-duct PSC (close biochemical and histopathological findings) but presents a normal cholangiogram. The diagnosis requires liver biopsy and some patients may later develop the classic PSC (12%-23%)[6,20]. Almost all patients have IBD, mainly UC, and it affects females at greater rates than males. Small-duct PSC has a better prognosis and a negligible risk of cholangiocarcinoma[9, 21].

IgG4-associated cholangitis

IgG4-associated cholangitis, considered a secondary sclerosing cholangitis, is characterized by elevated serum levels of IgG4, dense infiltration of IgG4-positive plasma cells and lymphocytes, and fibrosis and obliterative phlebitis in the bile duct wall, being frequently associated with autoimmune pancreatitis[22]. The link between IgG4-associated cholangitis and IBD has been reported, but it is far less common than in PSC. Differential diagnosis is vital due to its responsiveness to corticosteroids[9].

Primary biliary cholangitis

PBC is an autoimmune liver disease that presents with chronic cholestasis and histological findings of nonsuppurative destructive cholangitis. The diagnosis is usually made by detection of anti-mitochondrial antibodies[23]. There are only few reports of PBC in patients with IBD, affecting mainly UC males and those at younger age[24,25].

Autoimmune hepatitis

Autoimmune hepatitis (AIH) is a rare and heterogeneous disease, affecting mostly middle-aged women. It is characterized by abnormal liver tests, hypergammaglobulinemia, circulating autoantibodies [mainly antinuclear antibody (ANA), smooth muscle antibody, and anti-liver-kidney muscle antibody], and interface hepatitis on liver histology[26].

A relationship between AIH and IBD has already been established in a study that demonstrated the presence of UC in 16% of patients with AIH[3,27].

More relevant is the fact that coexistent AIH and IBD can have a different course from either process alone - patients with UC and concurrent AIH are more likely to relapse, need proctocolectomy, have more extensive disease, and present right colon lesions[3,28]. Likewise, liver disease may also have distinct progression, developing at younger age, being more likely to be refractory to treatment, and determining higher risk of death and liver transplantation[3].

Overlap syndromes

Patients with AIH may also present features of other immune-mediated liver diseases. In patients with UC, AIH-PSC is the most common overlap syndrome, described in up to 10% of PSC patients with UC[3,29]. However, cases of overlap syndrome in CD have also been described[30]. AIH-PSC is more common in children and young adults, PSC features usually develop later, and it has a better prognosis than PSC alone[31,32].

DISEASES ASSOCIATED WITH INTESTINAL INFLAMMATION

Portal vein thrombosis and Budd-Chiari syndrome

IBD is associated with a pro-inflammatory hypercoagulable state that increases the risk of portal and mesenteric vein thrombosis, with an estimated incidence of 1% to 2% [33]. Several risk factors have been identified: elevated platelet count, high fibrinogen, high factors V and VIII levels, and acquired prothrombotic factors - surgery, extent of colon disease, immobilization, inflammation, corticosteroids, and smoking[6,31]. Portal vein thrombosis has been more frequently described in UC patients after proctocolectomy and Budd-Chiari syndrome has an eight-fold risk during acute flares[31,34,35]. Anticoagulation is the mainstay of treatment, even in cases with previous gastrointestinal bleeding. Pharmacological thromboprophylaxis is recommended during hospitalizations and suggested in cases of active disease after hospital discharge and after surgery[2].

Granulomatous hepatitis

Granulomatous hepatitis is a rare complication of IBD, with a prevalence lower than 1%, mainly affecting CD patients[31]. Clinical suspicion is raised by elevated alkaline phosphatase and it is diagnosed by identification of granulomas in liver biopsy. It is mainly asymptomatic and follows a benign course, rarely requiring treatment (corticosteroids and immunosuppressants)[6,31]. It has also been associated with sulfasalazine use but differential diagnosis includes infections (tuberculosis) and malignancies[6,36].

Liver abscesses

Liver abscesses are a rare complication of IBD, but can also be its first manifestation (mainly in CD)[31]. They can result either from direct extension of an intra-abdominal abscess or from portal pyemia secondary to increased intestinal permeability[6]. They are often multiple and more frequently located in the right lobe, presenting with fever, abdominal pain, jaundice, diarrhea, and hepatosplenomegaly, as well as elevated inflammatory markers and alkaline phosphatase[31,37].

In contrast with liver abscesses in the general population, isolated *Streptococcus* species are the most common isolated pathogens[9,37].

The treatment of choice is prolonged intravenous antibiotics, with percutaneous drainage in case of a large abscess or refractory disease[31,38].

DISEASES ASSOCIATED WITH MALABSORPTION OR METABOLIC IMPAIRMENT

Cholelithiasis

Cholelithiasis is a known complication of IBD, with CD patients presenting a two-fold risk of developing gallstones. On the contrary, UC is not associated with an increased risk of cholelithiasis[39]. The incidence of cholelithiasis in patients with ileal involvement or resection ranges from 13% to 34%. It is associated with malabsorption of bile salts, resulting in disruption and increased entero-hepatic circulation, which predisposes to formation of gallstones[40]. Many risk factors have been described, such as ileo-colonic localization, disease duration (> 15 years), extent of ileal resection (> 30 cm), longer hospital stay, higher number of hospitalizations (> 3), multiple total parenteral nutrition treatments, lifetime surgeries, and number of clinical recurrences (> 3)[39,40]. Complications of cholelithiasis may be an indication for cholecystectomy but systematic cholecystectomy following ileal resection is not recommended[31,40,41].

Hepatic amyloidosis

Hepatic amyloidosis is a rare complication of IBD, more frequent in CD (0.9%) than in UC (0.07%)[42]. There is a male predominance and prominent colonic involvement. It results from amyloid deposition due to chronic inflammation, presenting as asymptomatic disease or hepatomegaly. Treatment is focused on lowering systemic inflammation by controlling it in the gut[6,31,43].

Non-alcoholic fatty liver disease

NAFLD is one of the most common liver diseases with a prevalence of 25% worldwide [44]. IBD patients seem to have a higher susceptibility to NAFLD and its prevalence

reaches almost 40%[45,46].

The main risk factor for NAFLD in the general population is metabolic syndrome but IBD patients develop NAFLD with fewer metabolic risk factors. In turn, IBD-associated factors that increase the risk of NAFLD include small bowel surgery, disease activity and duration, parenteral nutrition, and use of high doses of corticosteroids[47]. The influence of anti-TNF therapy on NAFLD risk is controversial: Some studies reported the development of biopsy-proven NAFLD in patients under anti-TNF therapy while others suggested a protective effect of these treatments[48,49].

There are no current guidelines for screening or assessing for NAFLD in patients with IBD.

IBD RELATED MEDICATIONS - DRUG INDUCED LIVER INJURY

Most drugs used for IBD treatment have been reported to cause acute and/or chronic liver injury, although the incidence of serious complications is low. The mechanism of hepatotoxicity is complex and multifactorial; thus, causality may be difficult to establish[31,50,51].

Sulfasalazine and 5-aminosalicylic acid compounds

Sulfasalazine and 5-ASA compounds are used in mild-to-moderate UC. Sulfasalazine was the first aminosalicylate used for the treatment of IBD and can induce liver injury by several mechanisms[31]: (1) Hypersensitivity reaction that usually occurs within 2 mo of therapy initiation. A study revealed an incidence of 0.4% and symptoms include fever, rash, hepatomegaly, lymphadenophaty, atypical lymphocytosis, and eosinophilia. In most cases, stopping the medication is sufficient. In more severe cases, antipyretics, antihistamines, or corticosteroids may be considered[51-53]; (2) Sulfasalazine-induced granulomatous hepatitis, with elevated alkaline phosphatase and bilirubin and noncaseating granulomas on histology[51]; and (3) Cholestatic liver injury and, in rare cases, development of vanishing bile duct syndrome[54]. Mesalamine (5-ASA) is also associated with liver enzyme abnormalities in up to 2% of patients but, in most cases, it is not clinical significant[55].

Thiopurines

Azathioprine and its principal metabolite, 6-mercaptopurine, are immunomodulators used for maintenance or achievement of remission in patients with IBD.

Azathioprine is metabolized in mercaptopurine and then thiopurine methyltransferase (TPMT) will be responsible for its conversion to 6-methylmercaptopurine. Genetic polymorphisms of *TPMT* determine the level of enzyme activity and should be routinely tested before initiation of these medications. In cases of absent or low activity, thiopurines should be avoided due to high risk of toxicity, whereas in intermediate activity, a dose reduction should be applied[51,56].

The annual incidence of hepatotoxicity can reach 13% in prospective studies, although most resolve spontaneously or with dose adjustment, and need for discontinuation is rare (< 4%)[31,50,57].

Most cases of liver injury result in transient elevations of AST and ALT, but there are different types of hepatotoxicity[31,51,58-61]: (1) Allergic reaction, usually within the first month of treatment, which is not dose-dependent and should prompt immediate halt; (2) Non-allergic reactions, mainly associated with TPMT activity and dose-dependent, that can cause infections, bone marrow suppression, or hepatitis. Allopurinol has been suggested to alter metabolite levels and reduce hepatotoxicity; (3) Cholestatic liver injury, usually within the first 3 mo of therapy, requiring discontinuation; and (4) Hepatic endothelial injury that may present within 3 mo up to more than 4 years after therapy initiation. It can include sinusoidal dilatation, sinusoidal obstruction syndrome, peliosis, or nodular regenerative hyperplasia (NRH). NRH occurs due to endothelial injury and/or obliterative portal venopathy, with an estimated incidence of 0.8%, and can cause non-cirrhotic portal hypertension. It is dose-dependent and should prompt drug discontinuation.

Liver tests should be checked before starting thiopurines and repeated at weeks 2, 4 and 8, and every 3 mo thereafter. In the absence of previous liver disease, the prognosis of thiopurines-induced liver injury is good[51,56].

Methotrexate

Methotrexate is an immunosupressive and anti-proliferative agent used in the event of adverse effects or lack of efficacy of thiopurines for maintenance of clinical remission

in CD[6].

Myelossupression and liver toxicity are the most common side effects, with presence of abnormal aminotransferases levels in 24% of cases[62]. This liver injury is mainly associated with alcohol consumption, while folic acid supplementation seems to be protective[6].

There are also some reports of liver fibrosis and cirrhosis development, despite being more common in rheumatologic conditions, due to higher weekly dose use[6].

Most patients with liver injury due to methotrexate will have their liver function tests back to normal while on therapy and dose adjustment or discontinuation is rarely needed[62]. Regular liver function tests are recommended but liver biopsy is not routinely performed. Transient elastography is emerging as an interesting non-invasive tool to follow these patients[31,63].

Anti-TNF agents - infliximab and adalimumab

Infliximab and adalimumab are anti-TNF agents used for induction and maintenance of remission in moderate to severe CD and UC.

The main adverse effects are myelosuppression, opportunistic infections (namely tuberculosis), neurological diseases, and liver injury. There are reports of ALT increase in 39% of patients, although most (76%) of them were self-limited[64].

An auto-immune pattern of liver injury induced by anti-TNF agents with serological evidence (ANAs) has also been reported, which generally has a good prognosis as soon as the drug is stopped[51,65]. Cases of cholestatic liver injury and acute liver failure requiring liver transplant are very rare[66].

Liver functions tests should be checked in all patients before treatment institution [51].

Vedolizumab

Vedolizumab is an $\alpha_4\beta_7$ integrin inhibitor used in moderate to severe CD and UC.

In the premarketing trials, significant (≥ 3 ULN) elevations occurred in less than 2% of patients, similarly to those in the placebo arm[31]. Cholestatic and hepatocellular liver injuries have already been described in the post marketing analysis, which improved after drug discontinuation[67].

Naturally available anti-inflammatory compounds

Although less studied, there are several natural compounds that are tested for the treatment of IBD.

Curcumin, the main active compound of the plant *Curcuma longa*, has been shown to have anti-inflammatory, anti-oxidant, and antibacterial activities[68]. Kesharwani *et al* [69] showed that curcumin might have an important role in inhibiting IBD severity and colitis associated cancer. In addition, it has a good safety profile and is extremely well tolerated, besides some reports of its hepatoprotective effect[68,70-72].

Viral hepatitis and inflammatory bowel disease

Previous studies have suggested a higher prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections in patients with IBD, due to blood transfusions and/or endoscopic procedures, which has not been demonstrated in more recent data [40,73,74].

HBV reactivation is one of the main concerns during IBD treatment, given the risk of fulminant hepatic failure and death[75]. Reactivation of HBV has already been described with high dose corticosteroids, thiopurines, and infliximab, though almost exclusively with concomitant use of other immunosuppressants[76-80]. Therefore, it is generally accepted that all patients with IBD should be screened for HBV exposure, preferably at diagnosis, which includes HBsAg and anti-HBs and anti-HBc antibodies [76,81]. According to the European Crohn's and Colitis Organisation (ECCO), IBD patients should follow these preventive measures[81]: Seronegative patients (HBsAg and anti-HBc negative) should be vaccinated and assessed for subsequent serological immune status; seropositive patients (HBsAg positive) should receive prophylactic treatment with nucleotide/nucleoside analogues for the time of treatment and at least 12 mo after stopping immunosuppressants; and HBsAg negative and anti-HBc positive patients should be monitored by HBV DNA quantification every 2-3 mo, since risk of HBV occult infection reactivation is low.

Regarding HCV infection, immunosuppressive therapy does not seem to have a detrimental effect on its course. Nevertheless, there are some reports of worsening liver function in the setting of concomitant HBV or HIV infection. Thus, the latest ECCO guidelines recommend systematic screening for HCV infection[81].

CONCLUSION

Hepatobiliary disease is one of the most common extra-intestinal manifestations in IBD patients, ranging from asymptomatic mild elevations of liver chemistries to life-threatening conditions.

Monitoring liver tests at regular intervals is crucial and must be routinely part of IBD management.

Abnormal liver tests in IBD patients may appear in the context of drug induced liver injury, common and easy to manage diseases such as NAFLD or cholelithiasis, as well as chronic and more complex diseases such as PSC or auto-immune hepatitis. As so, it should always prompt a structured and complete work-up and even benefit from a multidisciplinary approach, in order to improve patient management and outcomes.

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MINIREVIEWS

Dengue hemorrhagic fever and the liver

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Abstract

Dengue hemorrhagic fever (DHF) is one of the most rapidly emerging infections of tropical and subtropical regions worldwide. It affects more rural and urban areas due to many factors, including climate change. Although most people with dengue viral infection are asymptomatic, approximately 25% experience a self-limited febrile illness with mild to moderate biochemical abnormalities. Severe dengue diseases develop in a small proportion of these patients, and the common organ involvement is the liver. The hepatocellular injury was found in 60%-90% of DHF patients manifested as hepatomegaly, jaundice, elevated aminotransferase enzymes, and critical condition as an acute liver failure (ALF). Even the incidence of ALF in DHF is very low (0.31%-1.1%), but it is associated with a relatively high mortality rate (20%-68.3%). The pathophysiology of liver injury in DHF included the direct cytopathic effect of the DENV causing hepatocytes apoptosis, immune-mediated hepatocyte injury induced hepatitis, and cytokine storm. Hepatic hypoperfusion is another contributing factor in dengue shock syndrome. The reduction of morbidity and mortality in DHF with liver involvement is dependent on the early detection of warning signs before the development of ALF.

Key Words: Dengue hemorrhagic fever; Dengue viral infection; Liver involvement; Liver injury; Acute liver failure; Hepatocyte apoptosis; Cytokine storm; Severe dengue disease

Core Tip: The liver is the most common organ involvement in dengue hemorrhagic fever (DHF) patients with ranges from mild subclinical biochemical changes to severe liver disease as an acute liver failure (ALF). However, the low incidence of ALF in

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DHF with liver injury is associated with a high fatality rate. The hepatocyte injury is caused by direct viral cytopathic, immune-mediated, and poor hepatic perfusion. Early detection of severe hepatocellular injury development may reduce the morbidity and mortality in DHF patients with liver involvement.

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INTRODUCTION

Dengue virus (DENV) is a mosquito-borne flavivirus that consists of four serotypes (1–4) circulating in endemic areas. Most DENV infections are asymptomatic. However, the clinical manifestation of DENV infections could be dengue fever (DF), dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS). Dengue is one of the most rapidly evolving vector-borne infections, affecting 129 countries, 70% of the actual burden is in Asia, causing nearly 390 million affected patients each year, of which 96 million manifests clinically. The number of dengue cases reported to World Health Organization increased over eightfold during the last two decades, from 505430 cases in 2000 to over 2.4 million in 2010 and 4.2 million in 2019[1]. It is predicted that the transmission of dengue will be more strengthened in dengue-endemic countries, and due to climate change and increases in international traveling, the infection may spread to countries in Europe and the US that are currently not significantly affected by DENV[2,3]. Liver injury associated with DENV infection was first reported in 1967 [4]. The liver is one of the common organs involved in dengue infection. Hepatic complications were found in 60%-90% of infected patients included hepatomegaly, jaundice, elevated aspartate aminotransferase (AST), elevated alanine aminotransferase (ALT), and acute liver failure (ALF). All four serotypes have been associated with dengue-related liver injury, but DENV-1 and DENV-3 have more significant injuries[5]. Abnormal liver function in DENV infections resulted from the direct viral effect on hepatocytes or a dysregulated immunologic injury against the virus[6]. Moreover, underlying chronic diseases common among adults in several tropical and sub-tropical countries potentially compound the effects of acute dengue-related liver injury. However, the evidence to date is still conflicting and needs to be elucidated. We review the current evidence on liver injury in DHF patients and discuss the association between clinical manifestations, laboratory findings, pathological findings, and molecular evidence with the pathophysiology of a derangement of the liver in DHF.

GENOMIC ORGANIZATION OF THE DENGUE VIRUS

DENV genome is a linear, single-stranded, positive-sense RNA which translated as a single open reading frame. It was bordered by 50 and 30 untranslated regions on each side. DENV particle was a spherical 50 nm virion. The ssRNA genome was encapsulated by multiple copies of the capsid (C) protein to form a nucleocapsid core. This core is covered by a lipid bilayer forming an outer glycoprotein envelop (E) protective casing. When DENV enters the host cell, the positive ssRNA genome is released from the capsid and translated to a polyprotein of 3400 amino acids. The polyprotein is subsequently cleaved by viral and host proteases to 10 kinds of protein. These proteins are three structural proteins [C, E, pre-membrane (prM)] and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5)[7,8]. The structural proteins are essential in virion assembly, release, maturation, and infectivity. In comparison, viral replication and eluding a host cell's immune response are the NS proteins' primary functions. DENV has four serotypes (DEN 1-4), each sharing 60%-70% amino acid sequence homology.

DENGUE HEMORRHAGIC FEVER AND LIVER INVOLVEMENT

Clinical manifestations and laboratory findings

The spectrum of symptoms in DHF patients is very diverse, ranging from mild to severe dengue disease (SDD). DENV infection (DVI) has an incubation period of 3-14 d with the same symptom as a common cold and gastroenteritis. The patients usually have an abrupt fever, retro-orbital pain, headache, muscle ache, arthralgia, nausea, vomiting, diarrhea, and rashes. Less than 5% of DVI patients progress to severe life-threatening manifestations, particularly those previously infected with different serotypes. DHF has 3 distinct phases comprise of febrile, critical, and recovery. The patient has a biphasic fever commonly over 40°C with retro-orbital pain and headache ranging 2-7 d for the febrile phase. Fifty to eighty percent of the patients exhibit rashes or petechiae. The critical phase is characterized by plasma leakage with or without bleeding, which starts abruptly after defervescence. During this phase, an increase in capillary permeability with the rising of hematocrit can occur[9,10]. Moreover, the accumulation of fluids in the abdominal cavities and thoracic could be detected, leading to hypovolemic shock resulting in multiple organ dysfunctions, metabolic acidosis, disseminated intravascular coagulation (DIC), and severe bleeding. The mortality rate of SDD is relatively high at 20%, while early and appropriate treatment with intravenous fluid can decrease mortality to less than 1%. The recovery phase lasts for a few days with rash and a fluid overload, affecting the brain as a reduced level of consciousness or seizures[11,12].

Hepatic injury in DVI is more common in DHF than DF. Moreover, it is more severe in children patients, especially in previous dengue infection (primary infection), high hematocrit values, low platelet counts, and vascular leakage[13-15]. The clinical manifestations of DHF with hepatic involvement were from mild biochemical changes without symptoms to ALF. It manifests as right subcostal pain, hepatomegaly with tenderness, elevated aminotransferase enzymes, hyper-bilirubinemia, hypoalbuminemia, or ALF. The prevalence of liver involvement in DHF has many variations across different investigators (Table 1). This variation probably from the difference in DENV serotypes, case definition, age group, host susceptibilities, pre-existing diseases, especially chronic liver diseases (CLD). The most common symptoms associated with liver involvement in DHF are anorexia, nausea, vomiting, and abdominal pain[16-19,23,25-27,29]. The most common physical sign is hepatomegaly, with a wide range from several studies between 10.0 to 80.8% of the patients. The smaller number of DHF patients are clinically jaundiced (3.6%-48%)[16,21,26,28,29,31]. The hepatomegaly demonstrated an increased risk for SDD with an odds ratio of 4.75 (95%CI: 1.76-12.57)[32].

The elevation of AST and ALT is the commonest finding of DHF with liver involvement[16-31]. The elevated AST is usually modest and greater than ALT. The greater elevation in AST than ALT is partly due to AST release from muscles damaged. Mean AST and ALT concentrations ranged from 2-fold to 5-fold rises, which demonstrated mild hepatitis with self-limited. The 10-fold elevation of AST and ALT was reported in 4%-15% of the patients associated with SDD and may deteriorate to be ALF[33,34]. The physical sign of hepatomegaly with hepatic tenderness did not predict the rising of AST and ALT[16]. The highest level of AST and ALT occurs approximately day 7 of fever and should return to the normal level within 21 d of illness. The elevation of AST and ALT appears to correlate with SDD[30,35]. Hypoalbuminemia has been reported in broad ranges from 35.3%-76.0% in several studies due to the population heterogeneity and the disease severity[16,20,27-29]. The meta-analysis conducted by Huy and colleagues revealed that hypoalbuminemia was significantly associated with DSS[35]. Abnormal coagulation has been found in many studies with 34.0%-42.5% of prolonged prothrombin time (PT) and partial thromboplastin time (PTT)[16,21,26]. Notably, consumptive coagulopathy may also contribute to DSS.

Pathological findings

Pathological studies in humans DHF are uncommon and limited as the liver biopsy is invasive and hazardous. The human hepatocytes are an essential site for replication of DENV[36]. In 2014, Aye and colleagues reported an autopsy study of 13 patients who died of severe DHF. They found that the liver had significant levels of DENV RNA and histopathological changes consisting of microvesicular and macrovesicular steatosis, Councilman bodies, hepatocellular necrosis, and lack of inflammatory cell infiltrates[37]. In the liver, DENV infection occurred in hepatocytes and Kupffer cells but not in endothelial cells. Other studies reported the same pathological findings[34,38,39]. Recently, Win and colleagues reported that the prominent findings of the ultrastructure features of human liver specimens from patients who died of DHF were

Table 1 Clinical and laboratory findings of Dengue hemorrhagic fever with liver involvement

Investigators	No. of patients	Hepatomegaly (%)	Elevated AST (%)	Elevated ALT (%)	Hyper-bilirubinemia (%)	Low albumin (%)
Bandyopadhyay *et al* [16]	110	79.1	92.7	78.2	4.5	66.4
Kittitrakul *et al*[17]	127	34.6	88.2	69.3	N/A	N/A
Saha *et al*[18]	570	28.6	N/A	N/A	N/A	N/A
Roy *et al*[19]	120	80.8	94.2	89.2	N/A	N/A
Nascimento *et al*[20]	68	N/A	83.8	73.5	N/A	35.3
Karoli *et al*[21]	138	N/A	N/A	92.0	48.0	N/A
Lee *et al*[22]	690	N/A	86.0	46.0	N/A	N/A
Jagadishkumar *et al*[23]	110	79.0	93.6	78.2	N/A	N/A
Parkash *et al*[24]	699	N/A	95.0	86.0	N/A	N/A
Trung *et al*[25]	644	34.8	97.0	97.0	N/A	N/A
Wong and Shen [26]	127	11.8	90.6	71.7	13.4	N/A
Uehara *et al*[27]	41	10.0	80.5	61.0	N/A	48.4
Itha *et al*[28]	45	N/A	96.0	96.0	30.0	76.0
Fernando *et al*[29]	55	36.4	90.1	81.8	3.6	72.7
Souza *et al*[30]	1585	N/A	63.4	45.0	N/A	N/A
Kuo *et al*[31]	270	N/A	93.3	82.2	7.2	N/A

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; N/A: Not applicable.

extensive cellular damage and steatosis. Moreover, no virus-induced endoplasmic replicating structures have been identified in the hepatocytes. They postulated that DENV in the hepatocytes and Kupffer cells might not be the key contributor to hepatic steatosis[40]. Hepatic steatosis was the significant pathologic finding in acute alcoholic and non-alcoholic steatohepatitis[41]. The hypotheses on the mechanism of hepatic steatosis were the breakdown of the intestinal barrier, allowing bacterial pathogens to reach the liver (microbial translocation). Recent studies demonstrated that elevated lipopolysaccharide (LPS) levels during DVIs correlated with disease severity, primarily when determined in plasma leakage[42,43].

DHF AND ACUTE LIVER FAILURE

ALF is a rare condition in DHF patients. Kye Mon and colleagues conducted a retrospective cohort study to evaluate the incidence and clinical outcome in 1926 patients with DHF. They reported the 0.31% incidence of ALF associated with DHF. It was most common among young adults with the median duration from onset of fever to ALF development was 7.5 d. The patients with the severe stage of dengue had a higher risk of developing ALF. They concluded that although the development of ALF is relatively rare in patients with DHF, it is associated with a high mortality rate (66.7%) (Table 2)[44]. In 2010, Trung and colleagues conducted a study to evaluate the liver involvement associated with DVI in 644 adults and found that ALF was 0.77% with a 20.0% mortality rate. They concluded that clinically severe liver involvement was infrequent but usually resulted in severe clinical outcomes[25]. In 2016, Laoprasopwattana and colleagues reported the study of clinical course and outcomes of liver functions in children with dengue viral infection-caused ALF. They found that 41 patients (1.1%) of 3630 DHF children had ALF. The fatality rate of DVI-caused ALF in this study was 28 of 41 (68.3%) compared with 2 of 197 (1.0%) in severe dengue patients without ALF. They concluded that the DHF patients with ALF had the major cause from the profound shock, which induced microcirculatory abnormality in the liver cells[45]. In 2020, Devarbhavi and colleagues conducted the study to determine the incidence and clinical outcome in 10108 DHF patients. They found that 36 patients

Table 2 The incidence and mortality rate of acute liver failure in Dengue hemorrhagic fever patients with liver involvement

Investigators	Countries	Study population	Incidence rate (%)	Mortality rate (%)
Teerasarntipan *et al*[46]	Thailand	2311 adults	0.71	58.82
Devarbhavi *et al*[34]	Qatar	10108 adults	0.35	58.30
Laoprasopwattana *et al*[45]	Thailand	3630 children	1.10	68.30
Trung *et al*[25]	Vietnam	644 adults	0.77	20.00
Kye Mon *et al*[44]	Thailand	1926 age ≥ 15 yr	0.31	66.70

(0.35%) developed ALF with a 58.3% mortality rate. They concluded that dengue hepatitis progressing to ALF is rare and were seen in only 0.35%. However, the development of ALF is associated with a very high mortality rate. Lactate levels, pH, and model for end-stage liver disease (MELD) score at admission were the only predictors of mortality[34]. Recently, Teerasarntipan and colleagues conducted a retrospective study of 2311 serologically confirmed adult dengue patients to evaluate ALF and fatality rate incidence. They found that ALF incidence in their study was 17 of 2396 DHF patients (0.71%). The mortality rate of ALF was 10 of 17 SDD patients (58.82%). They concluded that the MELD score is the best predictor of ALF in dengue-induced severe hepatitis (DISH) patients[46].

PATHOPHYSIOLOGY OF LIVER DAMAGE IN DHF

The mechanism of hepatocellular injury in DHF is poorly understood. Several findings include the direct cytopathic effect of the DENV causing hepatocytes apoptosis, immune-mediated hepatocyte injury by CD4 lymphocyte induced hepatitis, and cytokine storm. Poor hepatic perfusion is also a potential contributing factor in SDD patients.

Direct cytopathic effect

There have been very few studies reporting the presence of DENV in hepatocytes of DHF patients. Moreover, the association between DENV replication and hepatocellular damage has never been concluded. In 1989, Rosen and colleagues firstly demonstrated the recovery of DENV from 5 of 17 livers of children who died from DHF[47]. In 1995, Kangwanpong and colleagues detected DENV RNA in hepatocytes located in the mid-zonal region of the DHF patients' liver by in situ PCR method[48]. In 1999, Couvelard and colleagues confirmed that DENV RNA was found in liver specimens of DHF patient. They concluded that nested PCR was the most sensitive method to identify the DENV RNA in clinical specimens[49]. Furthermore, Huerre and colleagues identified dengue antigens in formalin-fixed paraffin-embedded human liver by immunohistochemical analysis in 2001[50]. Several studies could demonstrate the cytopathic effects of DENV, which induced hepatocytes apoptosis[51-54]. Therefore, the exact effect of DENV in direct cytopathic effect and caused hepatocytes apoptosis is be confirmed. Although hepatocyte apoptosis could contribute to liver injury in DHF patients, it probably has a beneficial effect in inhibiting DENV replication and spread.

Immune mediated hepatocyte injury and cytokine storm

Macrophages and Kupffer cells recognize DENV particles and release cytokines and chemokines, which activated the inflammatory cells and act as antigen-presenting cells. Furthermore, Th1 cells released pro-inflammatory cytokines, which induce parenchymal cell damage and vascular vasodilatation. Moreover, NK cells induced TNF-related apoptosis-inducing ligand (TRAIL) expression and contribute to hepatocytes apoptosis[55,56]. Cells involved in the immune response for DVI include CD8+ cells, NK cells, and Th1 cells. The different immune cells caused hepatocyte damage at different stages of the disease. CD8+ cells are attracted to hepatocytes by regulated inactivation, and normal T cell expressed and secreted have been shown to recognize the $NS4B_{99-17}$ epitope expressed on infected hepatocytes[57]. NK cell infiltration correlated with a rise in cleaved caspase 3 in liver tissue, meaning that it could induce hepatocytes apoptosis. Although the exact mechanisms of NK cell-mediated apoptosis are not well understood, up-regulation of TRAIL maybe a significant role

[56]. During a secondary DVI, memory T cells from the previous infection were rapidly stimulated, leading to a potent inflammatory response. However, the cross-reactive memory T cells have less specificity to the new DENV strain. Hence, the T cell activation would be insufficient to inhibit the virus but potent enough to cause immunopathogenesis[58]. Monocytes have been recognized as important targets of DVI and amplification, particularly in low concentrations of dengue-specific antibodies. The dramatic enhancement by dengue antibody of DENV replication in monocytes and other cells is known as antibody-dependent enhancement (ADE). During a secondary DVI, ADE contributes to severe manifestations caused by IgG antibodies from the primary infection. It fails to neutralize the different strains of DENV, but it could opsonize the viral particles and facilitate the viral uptake into the immune cells. DENV infection of monocytes stimulates the release of numerous immunological factors, some of which modulate the function of other cells, particularly vascular endothelial cells. TNF released by antibody-enhanced DENV-infected monocytes activates endothelial cells. Circulating TNF levels are altered in severely afflicted dengue patients, and TNF is a crucial factor in DENV-induced hemorrhage. This phenomenon could promote a severe inflammatory response with numerous cytokines released as cytokine storms[59,60].

Poor hepatic perfusion

ALF frequently occurs in SDD with shock. Poor hepatic perfusion has been considered a causative factor. However, extensive research regarding the role of microcirculatory injury resulting in hepatocyte ischemia has not been adequately studied[29,61].

In 2019, Kulkarni and colleagues conducted a study to compare the manifestations of DVI in 95 patients with and without the liver disease [group A (without liver disease) = 71, group B (chronic hepatitis) = 12, and group C (cirrhosis = 12)]. They found that one patient in group A had ALF with renal failure and shock. Another one in group A had DHF with multiorgan failure and ARDS. A total of 3 patients expired in group C compared to 1 in group A and none in group B. Moreover, patients in group C required prolonged hospital stay compared to those in group A and group B. They concluded that DVI could have varied manifestations, ranging from simple fever to acute-on-chronic liver failure (ACLF) and ALF[62]. In 2013, Jha *et al*[63] conducted a prospective study to evaluate the etiology, clinical profile, and in-hospital mortality of ACLF in 52 ACLF patients. They found 46.1% hepatitis virus infection and 36.5% bacterial infection were the most common acute infection. The other acute injuries were drugs, autoimmune disease, surgery, malaria, and dengue. The mortality rate was higher in patients with dual insults than single insult (66.6% *vs* 51.1%). They concluded that dual acute insult is not uncommon and may increase mortality in these patients. DVI may be associated with ACLF[63]. In 2019, Galante and colleagues reported the first case in the world of liver transplantation performed in a patient with severe ALF due to DF. Liver transplantation may be considered as a treatment option for patients presenting with acute ALF secondary to DVI[64].

CONCLUSION

The clinical manifestations, laboratory, and pathological findings suggest that liver involvement is very common in DHF. The extent of liver damage may range from asymptomatic with slightly elevated AST and ALT to ALF. Hepatic injury in DHF could be from the direct cytopathic effects of DENV and caused hepatocytes apoptosis. Moreover, the immune-mediated hepatocytes injury by CD4 lymphocyte induced hepatitis and cytokine storm are also crucial factors. Notably, poor hepatic perfusion in SDD with shock is another co-factor in hepatocellular damage. Host defense mechanisms may overcome DVI with a less virulent strain and low viral loads. Infection with a more virulent DENV serotype with high viral loads would lead to extensive hepatocyte damage. Although ALF is a rare condition in DHF patients, the mortality rate in these patients is very high. The early detection of warning signs before the development of ALF in DHF is a critical issue, reducing the fatality rate.

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MINIREVIEWS

Artificial Intelligence in hepatology, liver surgery and transplantation: Emerging applications and frontiers of research

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Abstract

The integration of artificial intelligence (AI) and augmented realities into the medical field is being attempted by various researchers across the globe. As a matter of fact, most of the advanced technologies utilized by medical providers today have been borrowed and extrapolated from other industries. The introduction of AI into the field of hepatology and liver surgery is relatively a recent phenomenon. The purpose of this narrative review is to highlight the different AI concepts which are currently being tried to improve the care of patients with liver diseases. We end with summarizing emerging trends and major challenges in the future development of AI in hepatology and liver surgery.

Key Words: Liver disease; Machine learning; Deep learning; Artificial neural networks; Transplantation; Hepatectomy

Core Tip: Much of the advanced technologies utilized by medical providers today have

quality classification
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Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
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INTRODUCTION

Artificial intelligence (AI) is gradually changing the way that medicine is being practiced across the world, with technological advancements in the field of imaging, navigation and robotic intervention. It is increasingly being used for risk stratification, genomics, imaging and diagnosis, precision medicine, and drug discovery. The introduction of AI in hepatology and liver surgery is more recent and it has a strong root in machine learning (ML)-based algorithms, imaging and navigation, with early techniques focused on feature detection and computer-assisted intervention for both pre-operative planning and intra-operative guidance. AI-based solutions can assist in timely detection of liver tumors, more precise diagnosis and predicting disease course as well as outcomes. Diseases affecting the liver are heterogeneous and complex in nature, caused by various etiological factors, such as genetics, sex, ethnicity, body mass index (commonly known as BMI), environmental exposures to toxins, and comorbid conditions like diabetes mellitus. AI-based approaches could be highly useful in analyzing these various types of complex data in hepatology practice and research.

Components of AI systems can be broadly classified into expert system, search algorithm, ML, and deep learning (DL)[1]. Among them, ML is the most commonly used term, which can be considered as a branch of AI in which computers learn from data, with emphasis on computational algorithms, and analyze tons of data within no time[1]. ML can be of supervised or unsupervised learning. Supervised learning can be defined as a kind of ML which helps in predicting a known outcome, based on inputs, in the presence of an expert 'supervisor'[2]. While unsupervised learning is another type of ML, which can discover naturally occurring patterns without a pre-defined outcome, in the absence of an expert 'supervisor'[2]. The artificial neural network (ANN) is a type of statistical system used to derive outputs, based on interactions of weighted inputs and outputs and it mimics the intricate architecture of neuronal networks in the brain[3]. One other subset of ML is DL, which uses automatic discovery of representations from raw data (representation learning) for detection or classification[4]. Convolutional neural network (CNN) is a kind of DL ANN which utilizes multiple building blocks, such as pooling layers and convolution layers, and performs feature extraction to yield final output[5]. CNNs can be considered as one of the most successful DL models, due to their exceptional capability for processing spatial information[6]. Another type of neural network, known as recurrent neural network, utilizes feedback connections and displays great accuracy in labelling and forecasting sequential data[7]. Radiomics is another method in AI that extracts innumerable features from radiographic images by using data-characterization algorithms[8]. These radiomic features have the potential to unearth many characteristics of a disease that fail to be appreciated by the naked eye examination of a clinician. Radiomics can be coupled with AI, as it is capable of handling a massive amount of data in contrast to the traditional statistical methods[9]. Almost all AI techniques require a large dataset comprising laboratory and radiological findings, and outcome data. In the future, AI will definitely be useful in supporting clinical decisions, minimizing medical errors, and forecasting clinical outcomes. In this article, we will review the emerging role of AI in the management liver diseases, liver surgery

and liver transplantation.

AI IN LIVER DISEASES

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a growing epidemic globally, in part attributable to the increasing incidence of obesity and insulin resistance resulting in liver accumulation of free fatty acids and triglycerides. NAFLD patients are at higher risk of liver-related as well as cardiovascular-related mortality, and it is rapidly becoming the chief indication for liver transplantation[10,11]. Besides, NAFLD has been identified as a major risk factor for hepatocellular carcinoma (HCC)[12]. ML has been explored extensively for pattern recognition in NAFLD (Table 1). Timely identification of patients with NAFLD is paramount to arrest the disease progression to cirrhosis and related complications. Liver biopsy remains the gold standard for definitive diagnosis but it is invasive and inappropriate for screening. The development of non-invasive advanced imaging, biochemical and genetic tests as well as AI techniques will undoubtedly offer clinicians a great deal of information in the near future that can be utilized for early diagnosis and targeted treatment options.

Imaging of liver with ultrasound (US) is considered as a keystone for the initial diagnosis of NAFLD as it is widely available and image acquisition is easy. Magnetic resonance imaging (MRI) with proton density fat fraction (PDFF) has been considered as the reference standard in the quantification of hepatic steatosis; however, this technique has its own limitations, like cost and limited availability[13]. Methods exist for sonographic diagnosis of NAFLD, but these are often qualitative. Han *et al*[14] attempted to develop and evaluate DL algorithms that use radiofrequency data for NAFLD assessment, with MRI-derived PDFF as the reference. The investigators analyzed data of 204 prospectively enrolled adult research participants. The image acquisition was conducted *via* a typical right intercostal approach, with a 1–4 MHz curved probe and time-gain compensation, with the addition of 10 radiofrequency frames acquired during a breath-hold in shallow expiration. They found that DL algorithms with radiofrequency US data are very precise for diagnosis of NAFLD and hepatic fat fraction quantification with fairly good correlation (Pearson r = 0.85) with MRI PDFF when other causes of steatosis are excluded[14]. In another study, Byra *et al*[15] used CNN to automatically detect the amount of fat in liver from US images and showed high accuracy [area under the curve (AUC) of 0.98] compared to gold-standard liver biopsy, thus showing that ML can help in overcoming the issue of inter-operator variability as well.

ML-based algorithms were also used for early identification of patients with high risk for development of hepatic steatosis. Perveen *et al*[16] used a systematic ML-based decision-tree method to analyze data from electronic medical records in four Canadian populations and accurately predicted risk of development and progression of NAFLD. A similar application of ML to predict and screen for NAFLD in a Chinese population was carried out by Ma *et al*[17] and showed high accuracy, sensitivity and specificity. In a comparison study of different ML-based algorithms, the investigators found that all ML-based algorithms were found to be more efficient than the hepatic steatosis index (commonly known as HSI; F-measure 0.524) and the Fatty Liver Index (commonly known as FLI; F-measure, 0.318) and the Bayesian network model performed the best of 11 ML-based algorithms in the classification of patients with NAFLD (F-measure, 0.655).

ML-based algorithms have been deployed to analyze images from liver biopsy by using 47 unique liver biopsy images with manual annotations, performed by two pathologists. Vanderbeck *et al*[18] devised a classification algorithm. By utilizing a color analysis protocol, the algorithm was able to find out key features in biopsy specimens (macrosteatosis, portal veins, sinusoids and bile ducts) with good precision and high recall (> 82%)[18]. Similarly, Gawrieh *et al*[19] developed an AI-based tool to accurately quantify hepatic fibrosis and architectural pattern in liver biopsy specimens. These examples show that various ML tools may be chosen for application in appropriate situations for a specific problem.

Viral hepatitis

Progression to cirrhosis is an important event to be monitored in patients with hepatitis B virus (HBV) as well as hepatitis C virus (HCV) infections. Rates of progression to cirrhosis vary dramatically across individuals and not all patients progress to cirrhosis. Accurate risk stratification is essential to avoid excess monitoring

Table 1 Review of articles where artificial intelligence has been studied in the context of non-alcoholic liver disease

Ref.	Dataset	Number	ML algorithms	Problem	Performance measures
Byra *et al*[15], 2018	Department of Internal Medicine, Hypertension and Vascular Diseases, Medical University of Warsaw, Poland	55	Deep CNN	Automatically diagnose the amount of fat in the liver from US images	AUROC, Delong statistical test, lasso regression method, Spearman correlation coefficient, Meng test
Perveen *et al*[16], 2018	CPCSSN	667907	Decision tree	Classification, NAFLD progression risk	Micro- and Macro-average of Precision, Recall and F-measure, MCC, AUROC
Ma *et al*[17], 2018	First Affiliated Hospital, College of Medicine, Zhejiang University, China	10508	Several, Weka open source software	Classification, feature selection	Accuracy, specificity, precision, recall (*i.e.* sensitivity), and the F-measure
Vanderbeck *et al*[18], 2014	Medical College of Wisconsin, Milwaukee, United States	59	SVM	Automated assessment of histological features of NAFLD	Precision rate, recall rate, and AUROC
Meffert *et al*[68], 2014	SHIP	4222	Boosting algorithm, discrimination and calibration plots	Scoring system for hepatic steatosis risk	Discrimination (AUROC) and calibration
Sowa *et al*[69], 2014	University Hospital Essen	82	Logistic regression, decision trees, SVM, RF	Distinguish NAFLD from ALD	Sensitivity, specificity, and accuracy
Kuppili *et al*[70], 2017	Instituto Superior Tecnico, University of Lisbon, Portugal	63	Extreme Learning Machine-SLFFNN	Stratification of FLD disease in US liver images	AUROC, reliability and stability analysis
Sorino *et al*[71], 2020	MICOL cohort	2970	SVM	Stratify NAFLD risk to reduce need for imaging	Accuracy, variance, calculated confidence limits (95%), the weight of each model (as a %) and the number of ultrasound examinations it could avoid
Wu *et al*[72], 2019	New Taipei City Municipal Hospital Banqiao Branch	577	ANN, NB, RF, LR	Diagnosis and risk stratification in NAFLD	Accuracy, sensitivity, specificity

ALD: Alcoholic liver disease; ANN: Artificial neural network; AUROC: Area under the receiver operating characteristic; CNN: Convolutional neural network; CPCSSN: Canadian Primary Care Sentinel Surveillance Network; FLD: Fatty liver disease; LR: Logistic regression; MCC: Matthews correlation coefficient; MICOL: Multi-centre Italian study on cholelithiasis; ML: Machine learning; NAFLD: Non-alcoholic fatty liver disease; NB: Naïve Bayes; RF: Random forest; SHIP: Study of Health in Pomerania; SLFFNN: Single-layer feed-forward neural network; SVM: Support vector machine; US: Ultrasound.

of slow progressors as well as for appropriate monitoring of rapid progressors, for timely treatment. Availability of highly accurate risk prediction models would facilitate proactive identification of patients in need of more intensive monitoring and management. ML methods were used for genetic analyses of various HCV strains and was then applied to recognize relevant genetic markers related to fibrosis progression in HCV[20]. Shousha *et al*[21] combined data-mining strategies and ML algorithms (NN algorithms) using IL28B genotype and biochemical markers to predict advanced fibrosis in HCV patients, yielding a higher performance than both aspartate aminotransferase-to-platelet ratio index (commonly known as APRI) and fibrosis-4 (commonly known as FIB-4).

Primary sclerosing cholangitis

ML has been useful in patients with primary sclerosing cholangitis (PSC) throughout the disease course, from diagnosis to prediction of liver decompensation risk and post-transplant survival. Ringe *et al*[22] showed that PSC-compatible cholangiographic changes on 3D-magnetic resonance cholangiopancreatography (commonly known as MRCP) can be detected by DL algorithms with high sensitivity (95%) and low mean absolute error (7%). The PSC Risk Estimation Tool (referred to as PREsTo), which was developed by Eaton *et al*[23] using a gradient boosting machine (commonly known as GBM) algorithm, has been validated in an international multicenter cohort to accurately predict risk of liver decompensation in these patients and has also been shown to be far more accurate than existing prediction systems. LT in PSC patients is a contentious issue in view of the association with inflammatory bowel disease and risk of colorectal neoplasia and cholangiocarcinoma. Due to limited organ availability, identifying individuals who are most likely to benefit from the procedure is of paramount importance in patient selection. Andres *et al*[24] analyzed data of 2769 PSC patients from the Scientific Registry of Transplant Recipients (referred to as SRTR) database using a novel multitime-point calibrated model for the prediction of individual survival after LT. The accuracy of the model in predicting long-term survival was shown to surpass the traditional Cox regression analysis, which completely fails at 10 years.

Liver space occupying lesions and underlying liver disease

The application of ML toward image recognition has evolved into facial recognition software programs which are commonly used in smartphones. Employing this feature in healthcare, Park *et al*[7] were able to create an algorithm based on recurrent neural network to accurately predict visual field examination, thereby aiding in the diagnosis of optic neuropathies. Others have utilized similar ML tools in detection of lung nodules and cerebral aneurysms[25]. Recently, such computer-aided diagnosis/detection has been used in hepatology as well. Hassan *et al*[26] used a stacked sparse auto encode system based on support vector machines to differentiate HCC, hemangioma and liver cysts from US images. This method was shown to have 97.2% accuracy, outperforming software based on other DL algorithms. A DL system was developed by Schmauch *et al*[27] to diagnose and categorize space occupying lesions in the liver into malignant or benign tumors. By means of a supervised training using a database of 367 US images together with the radiological reports, the resulting algorithm could detect and characterize the lesions with a mean receiver operating characteristic of 0.93 and 0.916, respectively[27]. Although this model needs validation, it could warn of possible malignant lesions and boost the diagnostic yield of US for liver lesions. Another study used the patient's clinical data along with MRI sequences to devise an automated classification system cataloguing such hepatic lesions as cyst, adenoma, hemangioma, HCC and metastasis, with acceptable sensitivity and specificity rates[28]. A retrospective study analyzed the yield of an ANN, composed of three layers, for classifications of liver lesions by means of contrast-enhanced CT into five groups (A, classic HCC; B, malignant tumors apart from HCC; C, indeterminate masses, dysplastic nodules or early HCC and benign masses other than cysts or hemangiomas; D, hemangiomas; E, cysts)[29]. They obtained a high accuracy for the classification of hepatic lesions after supervised training using data from more than 55000 images, particularly for the distinction between groups A-B and C-D[30].

Diagnosis of HCC is currently based on imaging, tumor markers and sometimes biopsy. However, several other routine tests, such as biomarkers of liver inflammation, liver function test and viral markers, can help in prediction of HCC risk. The contribution of each variable toward accurate HCC prediction could be identified by data mining analysis of large volumes of data of patients with HCC and this in turn could help in the formation of a prediction model. This was attempted by Sato *et al*[31] when they analyzed data from 4242 patients at the University of Tokyo's hospital liver clinic. The patients were divided into those who had HCC diagnosed at first presentation (who formed the HCC-positive group of 539 patients) and others who developed HCC in follow-up (who formed the HCC-negative group of 1043 patients) after eliminating those with insufficient data. The available data was analyzed, and the gradient boosting provided the highest predictive accuracy for the presence of HCC (87.34%) and produced an AUC of 0.940. By using a cut-off of 200 ng/mL for alpha-fetoprotein (AFP), 40 mAu/mL for Des-gamma carboxyprothrombin (DCP), and 15% for AFP-L3, the accuracies of AFP, DCP, and AFP-L3 for predicting HCC were 70.67% (AUC: 0.766), 74.91% (AUC: 0.644), and 71.05% (AUC: 0.683), respectively[31]. Furthermore, an innovative model devised by Książek *et al*[31], used patient information, such as

viral status, occurrence of comorbidities and laboratory results to forecast the development of HCC. This is based on 23 quantitative and 26 qualitative features and has attained an 88.5% accuracy for this prediction model. When analyzing large data sets, ML models have proven superior over the classical statistical regression models. This framework of identifying optimal classifiers is the path towards fine-tuning personalized medicine.

Another important arena in the management of HCC is risk stratification for recurrence, which has been facilitated by the ability to digitize pathology slides. Saillard *et al*[32] showed that DL algorithms based on digitized slides were more accurate in predicting survival of HCC patients after liver resection compared to scores formed using various clinical, biological and pathological factors. Another DL model by Chaudhary *et al*[33] used data from The Cancer Genome Atlas to identify a subgroup of HCC patients with inactivation mutations in *TP53* genes, frequent BIRC5 expressions and stemness markers (KRT19 and EPCAM), and a high proportion of activated Akt and Wnt signaling pathways associated with aggressive tumors[33].

After HCC resection, vascular microinvasion (VMI) is considered as one of the major predictive factors of recurrence. In a recent publication by Dong *et al*[34], radiomic algorithms based on US images were used to elaborate radiomic signatures with the potential to aid in the preoperative prediction of VMI and to classify patients with VMI into low risk (≤ 5 MVI in adjacent liver tissue and ≤ 1 cm from the tumor) and high-risk groups (> 5 MVI or MVI in liver tissue and > 1 cm from the tumor) with promising results. Moreover, researchers have validated CT-based ANN and deep CNN to predict survival of HCC patients[35,36]. Ji *et al*[35] designed a novel three-feature radiomic signature of the contrast-enhanced CT image, where performance was enhanced by combining it with clinical features [concordance-index (c-index): 0.63–0.69 *vs* 0.73–0.801]. Wang and colleagues[36] employed multiphase CT radiomics features along with clinical models to yield a combined model (AUC: 0.82).

Tsilimigras *et al*[37] attempted to identify the most important prognostic factors in the pre- and postoperative setting for each Barcelona Clinic Liver Cancer (BCLC) stage by using a ML method. The investigators used a Classification and Regression Tree (CART) model to analyze data drawn from an international multi-institutional database. The preoperative CART model selected AFP and Charlson comorbidity score as the first and second most important preoperative factors of overall survival among BCLC-0/A patients, whereas radiologic tumor burden score was the best predictor of overall survival among BCLC-B patients. The postoperative CART model showed the lymphovascular invasion as the best postoperative predictor of long-term survival among BCLC-0/A patients, whereas tumor burden score remained the best predictor of long-term outcomes among BCLC-B patients in the postoperative setting[37].

AI algorithms were also successfully employed to predict response to transarterial chemoembolization (commonly known as TACE) and radiofrequency ablation (commonly known as RFA)[38-42]. A fully automated ML algorithm was proposed by Morshid *et al*[38] using the clinical information and features of CT images and to forecast the response to the treatment by TACE. Using the combination of BCLC stage and quantitative imaging features, the investigators attained a prediction accuracy of 74.2% against using just the BCLC stage alone. Liu *et al*[41] validated three AI-based predictive models (one deep and two ML), using radiomic features of contrast-enhance US scans. In that study, the DL model was found to be superior to the two other methods in assigning patients in the validation cohort to either objective-response to TACE or non-response, with a decent accuracy (AUC: 0.93)[41]. Wu *et al*[42] developed an ANN-based on 15 clinical features to predict 1-year and 2-year disease-free survival of patients who underwent CT-guided percutaneous RFA in early stages of HCC. The accuracy of the model was better when predicting 1-year disease-free survival than 2-year disease-free survival, with an accuracy of 85.0% and 67.9%, respectively[42].

AI IN LIVER SURGERY

Surgery offers the best chance of cure for patients with liver tumors. However, surgical removal of liver tumors is challenging because of its complex anatomy and concerns about functional liver remnant. Accurate knowledge of liver anatomy is thus a key point for any successful hepatic resection or living donor LT (LDLT). Even a minor change in the surgical plan can have a dramatic impact on the surgical outcome. The anatomy is so complex that it is often difficult to reconstruct it mentally based on CT or MRI images alone. Over decades, intraoperative visualization of preoperative image data in hepatic surgery has been a hot research topic for computer scientists and

clinicians. The introduction of AI in liver surgery is more recent and it mainly focuses on imaging and navigation that make pre-operative planning and intra-operative guidance easier. 3D visualization techniques and 3D printing technology can significantly benefit the understanding and display of surgical anatomy. ML has been applied in various aspects of the 3D printing technique to improve the whole design and manufacturing workflow[43]. Virtual liver resection can be performed before actual surgery using 3D visualization techniques to assess the resectability of the lesion and calculate future liver remnant (FLR)[44]. In LDLT, 3D imaging can predict the requirement for vascular reconstruction based on the vascular anatomy of the donor liver, resulting in improved safety and outcome of LDLT[44]. The application of 3D printing technology in liver surgery has been evaluated in a few studies. In pediatric LDLT, 3D-printed liver models have been found useful in evaluating discrepancies in size between small pediatric recipients and adult liver grafts[45]. Nevertheless, there are still many issues (like cost and time of manufacturing) that must be addressed before 3D printing can become more accepted and widespread. ML could be exploited to solve these problems by streamlining the 3D modelling process through rapid medical image segmentation and improved patient selection and image acquisition [46].

Automated hepatic volumetry

It is widely accepted that accurate assessment of volume of FLR can reduce post-hepatectomy liver failure. Hepatocytes in the remnant liver after resection must overcome necrosis and regenerate sufficiently to preserve synthetic function which requires an adequate volume of functional FLR. Widely followed limits of FLR for safe resection range between 20% and 30% for normal liver and 30% and 40% in those with underlying liver disease. Several imaging modalities have been experimented in liver volume assessment, including even conventional US and 3D US[47,48]. However, contrast-enhanced CT scan is globally accepted for FLR assessment, pre-transplant LD evaluation and for assessment of response to FLR volume induction. The first described method of liver volume assessment based on manually tracing the entire liver was time-consuming but precise. Recently, semi-automatic and automatic segmentation techniques using mathematical model,s such as the ones reported by Suzuki *et al*[49] and Nakayama *et al*[50], have shown good accuracy. A CNN-based algorithm has been developed by Wang *et al*[51] to fully automate liver volume assessment from CT as well as MRI. A similar algorithm developed by Winkel *et al*[52] has shown good accuracy, speed and good agreement with manual segmentation. The criticism of fully automatic segmentation is that it often can be unsuccessful for some CT images that are low in contrast or have missing edges due to similar intensity of adjacent organs or machine artifact.

Surgical navigation systems

Surgical navigation systems have been playing a crucial role in neurosurgery and spinal surgery for many years; yet, they have not become established as standard in liver surgery. This is largely due to the technical challenge of navigating a moving organ. The surgical navigation system must be able to measure the intraoperative alterations in position and shape of the liver due to respiration and surgical manipulation, in order to adapt the preoperative navigation data to the current situation. Techniques like augmented virtuality (referred to as AV), augmented reality (referred to as AR) and mixed reality can be used to synchronize 3D reconstructed images with real-time surgery and can offer a safe and reliable surgical navigation method. Accurate surgical navigation can better guide laparoscopic surgeons to perform hepatectomy and improve the safety of surgery. In a preliminary trial, Phutane *et al* [53] demonstrated that AR-based hepatectomy for HCC could help detect intrahepatic tumors, decide the transection plane, and locate the hepatic veins, which can result in improved safety of operation by reducing bleeding and duration of surgery. The laparoscopic hepatectomy navigation system (LHNS) is a multimodal assistant system presented by Zhang *et al*[54] which consists of a fusion model of CT-based 3D models with indocyanine green (commonly known as ICG) fluorescence images. LHNS was used for real-time visualization of the relationship between liver lesions and intrahepatic anatomical structures. Using LHNS, the optimal cutting plane for the liver resection can be planned preoperatively. The system consisted of preoperative model segmentation, intraoperative laparoscopic stereo surface reconstruction, intraoperative laparoscopic posture tracking modules and intraoperative registration. Authors retrospectively compared the clinical outcomes of patients who underwent the laparoscopic hepatectomy using the LHNS (LHNS group) with patients who underwent the procedure without LHNS guidance (non-LHNS group). They found that the LHNS

group had significantly less blood loss, less intraoperative blood transfusion rate and a shorter postoperative hospital stay than the non-LHNS group. There was no significant difference in operative time and the overall complication rate between the two groups. The LHNS system was also helpful to clearly delineate the liver transection line in most cases[54]. Ntourakis *et al*[55] reported in a pilot study that AR helped in detecting missing lesions after chemotherapy for CRLM and obtaining a margin negative resection status without any local recurrence at a median follow-up of 22 mo. Application of AR in robotic hepatectomy can enhance the ability of the surgeon to achieve a safe tumor resection with adequate peritumoral margin[56,57].

AI to predict postoperative morbidity

AI algorithms are also being used to predict postoperative morbidity and recurrence of tumor after surgery. Post-hepatectomy liver failure is a worrisome complication after major liver resection for HCC and is the chief cause of postoperative mortality. Early identification and timely intervention are vital to avoid the mortality associated with it. Mai *et al*[58] attempted to validate an ANN model to forecast severe post-hepatectomy liver failure in patients with HCC who underwent partial hepatectomy (353 patients). They found that the predictive performance of the ANN model for severe post-hepatectomy liver failure surpassed the traditional logistic regression model and normally used scoring systems[58].

AI IN LIVER TRANSPLANTATION

Liver transplantation is a complex process that involves analysis of numerous variables related to both donor and recipient and expert decisions that are essential for long-term graft and patient survival. The high number of variables involved often makes the decision-making process difficult. In such a circumstance, ML techniques play an important role, with the ability to build accurate models for liver graft survival.

Organ allocation and donor-recipient matching

In a liver transplantation program, the major bottleneck in delivery of care now is organ availability. The United Network for Organ Sharing (commonly known as UNOS) survey has identified about a 20% drop-out of patients listed for liver transplantation[59]. Attempts to reduce this dropout rate by utilization of extended criteria donors (older donors, donors with fatty liver, donation after cardiac death donors) have resulted in inferior post-transplant outcomes and decreased utilization due to an increase in discarded grafts. This problem is expected to worsen in the coming years as growth in the general population is projected to overtake growth in the donor pool, thus potentially exacerbating the organ shortage and further increasing the waiting time for transplant. Such insights demonstrate the precious nature of each liver graft and the paramount importance of appropriate organ allocation to reduce waiting list mortality as well as to promote efficient utilization of available organs. A first attempt at guiding organ allocation using donor information was the quantitative donor risk index by Feng *et al*[60], which used a Cox regression model to predict graft failure using donor characteristics alone. The widely validated model for end-stage liver disease (MELD) score, which is the keystone of current allocation policy in the United States and worldwide, is based on the "sickest-first" principle, utilizing recipient information alone. Undoubtedly, a method which utilizes donor as well as recipient characteristics for appropriate pairing would ideally reduce waiting list mortality and organ wastage with good post-transplant survival. Many strategies, including ML, are being tried to reduce the discrepancy between the number of potential liver graft recipients and the number of organs available. This was attempted by Pérez-Ortiz *et al*[61] using ordinal regression and the support vector machine to arrive at a model that could be used in conjunction with the MELD score to allocate the organ to one of the first patients on the waiting list (according to MELD score) who would have a higher survival possibility. This can circumvent flaws in MELD score-based allocation and also eliminates futile transplants. The Optimized Prediction of Mortality (commonly known as OPOM) model developed by Bertsimas *et al*[62] employing ML optimal classification tree model in comparison with MELD-based allocation using Liver Simulated Allocation Model (commonly known as LSAM) has been shown to reduce waiting list mortality on average by 417.96 deaths every year. OPOM has been found to adhere more accurately to the "sickest-first" principle and utilizes more variables than the MELD and MELD-Na scores. Another neural

network-derived algorithm is the MPENSGA 2 developed by Cruz-Ramírez *et al*[63] which seeks to complement MELD-based allocation and improve its efficiency.

In 2014, a donor–recipient matching model was presented by Briceño *et al*[64] which can make the clinical decision-making easier in liver transplantation. The investigators used two ANN models: One was to enhance the probability of graft survival, and the other was to reduce the probability of graft loss. They analyzed variables of 64 donors and recipients from a set of 1003 LTs from a multicenter study. The chief aim was to devise an innovative decision-making system that can optimize the principles of fairness, efficiency and equity in allocating liver graft. They found that ANN models were significantly more accurate than already validated scores of graft survival [MELD, Delta MELD, donor-risk index (DRI), Survival Outcomes Following Liver Transplant (SOFT), the preallocation (P)-SOFT and balance-of-risk (BAR)][64]. Wingfield *et al*[65], from the United Kingdom, published the first ever systematic review of AI computing techniques being used in liver transplantation to predict individual patient graft survival. They concluded that AI techniques can provide high accuracy in predicting graft survival based on donors and recipient variables; additionally, compared with the standard techniques, AI methods had the benefits of being dynamic and able to be trained and validated within every population. Table 2 provides a concise review of recently published studies where AI-based algorithms have been applied to liver transplantation.

Challenges and prospects

It is evident from the above-mentioned studies that ML is going to be a powerful weapon in the armamentarium of the hepatologist and liver surgeon, with applications ranging from screening to postoperative follow-up. Given the recent advances in AI and the lack of any precedence, the Hippocratic philosophy of 'do no harm' should be at the forefront of any decision to integrate it into the clinical practice. There are some ethical and legal issues to be addressed before widespread adoption of AI into clinical practice. Data privacy and cyber security are the main ethical concerns. Next is the issue of accountability. For example, if a ML tool gives a wrong diagnosis or incorrectly assesses the hepatic volume, resulting in post-hepatectomy liver failure, whom should be held responsible?

AI is going to be a major player in organ allocation, donor-recipient matching, and even in optimizing immunosuppressant doses[66,67]. AI can be employed *via* smartphones to remotely monitor patient health. However, like any other evolving technology, AI is not without shortcomings. The ability of ML to analyze large volumes of data is responsible for its most important handicap. Quality of the output is inexorably linked to the quality of input data. This is the case with conventional biostatistical methods as well. Hence, high-quality data collection is essential for the development of AI systems as data sets are the lifeblood of algorithms and statistical modelling on which AI systems are trained. So, it is the duty of all physicians to come forward to help drive these innovations rather than passively waiting for the technology to become useful in their practice. Hepatologists and liver surgeons should seek opportunities to partner with data scientists to capture novel forms of clinical data and help generate meaningful interpretations of that data. Moreover, the accuracy of any AI system can be affected by factors such as study design, data integration strategy, selection of ML model and the relevance of the selected ML model to the particular study setting. Hence, physicians must have clearly defined, clinically relevant questions that require AI technology as the analysis tool. Early work in ML has focused on individual areas, such as radiomics or genomics, but future work should be aimed more towards amalgamating these to form a comprehensive care plan of the patient.

CONCLUSION

To conclude, as the incorporation of AI into the management of liver diseases seems inevitable, training of clinicians in interpreting and applying it into the routine practice is of paramount importance. If appropriately designed and implemented, AI has the potential to revolutionize the way hepatology and liver surgery is taught and practiced, with the promise of a future optimized for high-quality patient care.

Table 2 Review of recently published studies where artificial intelligence-based algorithms have been applied to liver transplantation

Ref.	Dataset	Number	ML algorithms	Problem	Performance measures
Bertsimas *et al* [62], 2019	STAR dataset	-	OCT	Predict 3 mo waitlist mortality-OPOM	ROC curve
Cruz-Ramírez *et al* [63], 2013	Spanish multi-center study	-	Radial basis function NN	Improve donor-recipient matching using rule-based allocation—MPENSGA 2 algorithm	Accuracy, minimum sensitivity, ROC curve, RMSE, Cohen's kappa
Briceño *et al*[64], 2014	Spanish multi-center study	1003	Neural Net Evolutionary Programming	Improve equity in donor-recipient matching	Multiple regression analysis, simple logistic regression analysis, ROC curve
Ayllón *et al*[73], 2018	King's College Hospital,United Kingdom + MADR-E, Spain	1437	ANN	Classification, end-point (3 mo, 1 yr)	ROC curve
Wadhwani *et al* [74], 2019	UNOS	1482	RF	Classification, end-point (3 yr)	Chi-square test, *t*-test, Wilcoxon rank sum test
Dorado-Moreno *et al*[75], 2017	King's College Hospital, United Kingdom + MADR-E, Spain	1492	Ordinal ANN	Ordinal classification, fourclasses	MAE and the MZE, accuracy, GMS, AMAE
Guijo-Rubio *et al* [76], 2019	UNOS	39095	Cox, SVM, GB	Survival time	C-index, ROC curve, concordance index ipcw
Lee *et al*[77], 2018	Seoul National University Hospital	1211	Several ML methods compared, GBM found to be best	Prediction of AKI after liver transplant	ROC curve, accuracy
Lau *et al*[78], 2017	Austin Hospital, Melbourne, Australia	180	RF, ANN, logistic regression	Predict 30-d risk of graft failure	ROC curve

AKI: Acute kidney injury; AMAE: Average mean absolute error; ANN: Artificial neural network; c-index: Concordance index; GB: Gradient boosting; GBM: Gradient boosting machine; GMS: Geometric mean of the sensitivities; MADR-E: Model for Allocation of Donor and Recipient in España; MAE: Mean absolute error; MPENSG-A: Memetic Pareto evolutionary non-dominated sorting genetic algorithm; ML: Machine learning; MZE: Mean zero-one error; NN: Neural network; OCT: Optimal classification tree; OPOM: Optimized prediction of mortality; RF: Random forest; RMSE: Root mean squared error; ROC: Receiver operating characteristic; STAR: Standard Transplant Analysis and Research; SVM: Support vector machine; UNOS: United Network for Organ Sharing.

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MINIREVIEWS

De novo and recurrence of metabolic dysfunction-associated fatty liver disease after liver transplantation

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Abstract

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a new acronym adopted from the consensus of international experts. Given the increasing prevalence of MAFLD in pre-transplant settings, *de novo* and recurrent graft steatosis/MAFLD are common in post-transplant settings. The impact of graft steatosis on long-term outcomes is unclear. The current knowledge of incidence rate, risk factors, diagnosis, long-term outcomes, and management of graft steatosis (both *de novo* and recurrent) is discussed in this review.

Key Words: Metabolic dysfunction-associated fatty liver disease; Metabolic dysfunction-associated steatohepatitis; *De novo*; Recurrent; Graft steatosis; Fibrosis; Survival

Core Tip: Metabolic dysfunction-associated fatty liver disease (MAFLD) is common after liver transplantation. Post transplant metabolic dysfunction, obesity and consequences of immunosuppressant contribute to the development of either *de novo* or recurrent graft steatosis. Post liver transplant MAFLD impact on cardiovascular outcome without significant impact on graft and patient survival. Weight control and tailoring of immunosuppression are the main strategies to prevent post liver transplant MAFLD.

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INTRODUCTION

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a new acronym adopted from the consensus of international experts. MAFLD is defined by the evidence of hepatic steatosis and one of the following criteria: Overweight or obesity, presence of type 2 diabetes mellitus (DM), or evidence of metabolic dysfunction[1,2] Given the increasing prevalence of obesity, nonalcoholic fatty liver disease (NAFLD) has become one of the leading causes of liver transplantation in the United States[3]. The utilization of immunosuppressants in post liver transplant (LT) patients significantly impacts metabolic dysfunction through the development of insulin resistance (IR), DM, hypertension, obesity, and hyperlipidemia[4-7]. Either *de novo* or recurrent graft steatosis can occur after liver transplantation[8]. Most of the studies showed an association between metabolic dysfunction and the occurrence of either *de novo* or recurrent graft steatosis[9-12]. Therefore, the graft steatosis can be referred to as post LT MAFLD. The ongoing injury from graft steatosis can progress to the different stages of hepatic fibrosis and eventually cirrhosis which may develop further complications. In this review, we are going to discuss epidemiology, risk factors or predictors, diagnostic techniques, natural history, outcomes, and management of *de novo* and recurrent graft steatosis.

EPIDEMIOLOGY

Hepatic steatosis has been recognized as the hepatic manifestation of metabolic syndrome (MetS). LT resolves the complications of cirrhosis due to metabolic-associated steatohepatitis (MASH), but the metabolic risks persist and often can get aggravated by exposure to immunosuppressive therapy after LT[13]. Therefore, it is not surprising to expect a higher rate of recurrent graft steatosis after LT compared to that of *de novo* graft steatosis due to the underlying MetS and IR that initially led to cirrhosis[14]. Recurrent or *de novo* graft steatosis after LT poses potential threats to the viability and survival of allografts, and therefore it is critical to characterize and identify the prevalence of recurrent and *de novo* graft steatosis after LT, and identify the risk factors for post-LT MAFLD to improve the overall clinical outcomes in the transplant recipients.

The true incidence of recurrent and *de novo* graft steatosis after LT remains uncertain as previously published studies were from single-center, retrospective studies with heterogeneous definitions of the diseases and methodologies[11,15]. Despite these limitations, we aim to describe the rates of recurrence and occurrence of steatosis in allografts, mainly abstracted from systematic reviews and meta-analyses by Saeed *et al* [11] and Losurdo *et al*[12]. In the review by Saeed *et al*[11] 17 studies representing 2378 patients primarily from North American and Europe were included, and they were categorized into three groups based on the nature of included studies: Recurrent, *de novo*, and combined graft steatosis among LT recipients at 1, 3, and ≥ 5-year follow-ups after LT. The estimated incidence rates of recurrent graft steatosis are 59% (range: 8%-100%), 57% (24%-100%), 82.1% (59%-100%) at 1, 3, and ≥ 5-year after LT respectively while those of recurrent steatohepatitis are 53% (24%-82%), 57.4% (31%-100%), and 38% (4%-71%)[11]. Recurrent graft steatosis was very common after LT, recurring in more than half of the recipients as early as 1 year after LT[11]. The studies assessing both recurrent and *de novo* graft steatosis and steatohepatitis reported 1, 3, and ≥ 5 year incidence rates as 42% (30%-65%), 34% (23%-52%), and 33% (26%-33%) for graft steatosis while 10% (5%-15%), 11% (6%-17%), and 19% (10%-27%) for steatohepatitis [11]. One of the largest studies with 275 subjects assessing recurrent graft steatosis and steatohepatitis has reported the recurrence of graft steatosis in 31% of patients and the recurrence of graft steatohepatitis in 4% of patients after LT[16].

The study by Dumortier *et al*[17] reported *de novo* graft steatosis in 31% and graft steatohepatitis in 3.8% of 421 recipients at 3.3 years after LT. In the systematic review

and meta-analysis by Saeed *et al*[11], incidence rates for *de novo* graft steatosis at 1, 3, and ≥ 5 years after LT were 67%, 40%, and 78% while 13%, 16%, and 17% for *de novo* graft steatohepatitis. These incidence rates were varied depending on the different follow-up periods, but *de novo* graft steatosis was overall very common in post-transplant patients[11]. Also, these incidence rates noted in the review by Saeed *et al* [11] were higher compared to another systematic review and meta-analysis by Losurdo *et al*[12], which reported summarized weighted prevalence of *de novo* graft steatosis as 26% [95% Confidence interval (CI): 20%-31%] and *de novo* graft steatohepatitis as 2% (95%CI: 0-3%). Larger, prospective future studies with clear, consistent inclusion and diagnosis criteria are warranted to better characterize the incidence of recurrent and *de novo* MAFLD and MASH, but existing studies consistently demonstrated very high rates of recurrence and occurrence of graft steatosis among LT recipients.

RISK FACTORS/PREDICTORS

The development of graft steatosis after LT is related to different factors: Recipient, environmental, genetic, and immunosuppressive factors[13]. A retrospective study by El Altrache *et al*[18] reported the association of recurrent graft steatosis with the occurrence of metabolic abnormalities after LT. Similarly, another study by Dureja *et al* [19] described the risk factors for the development of recurrent graft steatosis including an increased body mass index (BMI), post-transplant hypertriglyceridemia, steroid use, MetS, and insulin use. A retrospective study by Galvin *et al*[20], identified risk factors for *de novo* graft steatosis in a post-LT cohort included diabetes, weight gain, BMI, hepatitis C virus (HCV) infection, sirolimus-based immunosuppressant therapy. If none of these factors existed, *de novo* graft steatosis occurred in only 5.4% of patients, but if all 5 factors were present, it would occur in 100% of patients[20]. All these risk factors are associated with IR, and therefore it was suggested that IR might be at the root of the development of *de novo* graft steatosis[20] In a study by Vallin *et al* [10] in comparing recurrent and *de novo* graft steatosis, the prevalence of DM was significantly higher in the recurrent graft steatosis group compared to the *de novo* graft steatosis group (100% *vs* 37.5%, $P < 0.01$)

Among patients with pre-transplant NAFLD, hepatic and peripheral IR leads to insufficient inhibition of hepatic gluconeogenesis, increased lipid accumulation, and reduced glycogen synthesis[21]. Increased circulating free fatty acids from the above-mentioned process further promote inflammation and endoplasmic reticulum stress, which aggravates IR more, leading to a vicious cycle[22]. The immunosuppressive regimen used after LT also plays a critical role in MetS as corticosteroids decrease peripheral glucose absorption, increase hepatic glucose production, and therefore increases the risk of developing post-LT diabetes[13]. Calcineurin inhibitors (CNIs) that are often used as a part of immunosuppressive therapy also are diabetogenic in nature[23]. The chronic use of sirolimus, which inhibits mammalian target of rapamycin (mTOR) multiprotein complexes, has also been shown to lead to hepatic IR [24].

Despite these proposed risk factors for developing graft steatosis after LT, there were inconsistencies among previous studies, likely related to the relatively small sample sizes, and therefore further studies with larger sample sizes are required to better elucidate the heterogeneous findings[25]. In the multivariate analysis with 9 related studies, the most consistent predictors of post-LT graft steatosis and steatohepatitis were post-LT BMI, hyperlipidemia, and history of alcohol use[11]. However, a subsequent meta-analysis showed that post-LT BMI was the only risk factor with a significant impact, a summarized odds ratio of 1.27 (1.19-1.35, $P < 0.001$)[11]. Pre-transplant variables did not have a consistent independent impact on the risk of post-LT graft steatosis and steatohepatitis in the meta-analysis, and immunosuppressive regimens did not show consistent effects[11]. Although post-LT BMI was identified as the consistent predictor, given inconsistent findings of pre-LT variables as a significant risk factor for post-LT graft steatosis and steatohepatitis, immunosuppressive regimen, and hyperlipidemia as risk factors, targeting post-LT obesity may not be sufficient for effective risk factor reduction.

In another meta-analysis assessing *de novo* graft steatosis and steatohepatitis in liver-transplanted patients, alcoholic and cryptogenic cirrhosis was related to the highest prevalence of *de novo* graft steatosis, 37%, and 35% respectively[12]. Ethanol consumption can cause excessive reactive oxygen species, hepatic lipid peroxidation [26], and cryptogenic cirrhosis is often thought to be "burnt-out" steatohepatitis, and

underlying steatohepatitis may be under-recognized. Therefore, such association of the highest prevalence of *de novo* graft steatosis in alcoholic and cryptogenic cirrhosis aligns with existing literature findings[12].

Dumortier *et al*[17] reported steatosis in donors as an important predictor of *de novo* NAFLD, and therefore the interaction between donor and recipient genetics may also affect disease recurrence[13]. Previous genomic studies have reported genetic variation in the patatin-like phospholipase domain as conferring susceptibility for the risk of fibrosis and steatosis[27]. The clinical implication of utilizing steatotic graft is uncertain, and therefore it is not clear if graft steatosis itself is a risk factor for post-LT graft steatosis[28]. Detecting recurrent or *de novo* graft steatosis/steatohepatitis is critical for better clinical outcomes in transplant recipients, and therefore further studies assessing optimal follow-up methodology such as specific diagnostic modalities and timing of follow-ups are warranted to quality care in this vulnerable population. Overall risk factors are summarized in Figure 1.

DIAGNOSIS

Liver biopsy is the gold standard to diagnose hepatic steatosis, hepatic fibrosis, and cirrhosis[29]. Although it has limitations of invasiveness, a small risk of complications, and potential sampling errors[30,31], liver biopsy is shown to be a safe and adequate diagnostic tool in post LT patients. It provides an ability to exclude or detect the presence and/or severity of the coexisting chronic liver disease[29,32]. The approach to diagnose graft steatosis and fibrosis is summarized in Figure 1.

Steatosis

The sensitivity of ultrasound to detect hepatic steatosis is poor when the liver occupies less than 20% of steatosis[33]. Computed tomography-based liver to spleen attenuation ratio can identify only if hepatic macrovesicular steatosis is more than 30%[34]. Biomarker panels such as the fatty liver index and the hepatic steatosis index can enhance the result of ultrasound in identifying hepatic steatosis[35,36]. However, there is limited literature regarding the roles of biomarkers in diagnosing hepatic steatosis in post-transplant settings. Transient elastography (TE) with controlled attenuation parameter (CAP) can predict the degree of hepatic steatosis in pre-transplant settings [37,38]. One study showed detecting graft steatosis with CAP in post LT patients but there is no histologic validation in the study[39]. Magnetic resonance imaging (MRI) based techniques such as MR spectroscopy and MRI-proton density fat fraction (MRI-PDFF) has been shown to accurately detect different degrees of hepatic steatosis[37, 38]. Further studies of MRI-based techniques in diagnosis post-transplant graft steatosis are warranted.

Fibrosis

Both ultrasound and computed tomography are unable to detect different stages of hepatic fibrosis unless the patients have the late stage of cirrhosis with portal hypertension[40]. Ultrasound based shear wave elastography (SWE), using acoustic radiation force impulse (ARFI) techniques, detect fibrosis in fatty liver patients. Studies showed point SWE and two-dimensional SWE accurately detect advanced fibrosis with good sensitivity and specificity in pre-LT setting[38]. Liver stiffness measured by TE also provides good performance in identifying advanced fibrosis. However, obesity, significant ascites, postprandial state, and significant hepatic inflammation or congestion can influence the interpretation. MR elastography (MRE) has also provided a useful and accurate way to identify advanced hepatic fibrosis[37, 38]. Noninvasive serum biomarker especially NAFLD fibrosis score (NFS), aspartate aminotransferase (AST) to platelet ratio index (APRI), and FIB4-score, AST, alanine aminotransferase (ALT) ratio (AAR), BARD, and fibrospect test have been shown to provide good performances in identifying advanced fibrosis in pretransplant NAFLD patients. However, the accuracy of MRE is outperformed compared to that of simple serum biomarkers to predict advanced fibrosis[41]. The major limitations of MRI-based techniques are availability, technical complexity, high cost, and contraindication in claustrophobic patients[37].

In post LT patients, quantifying the degree of liver stiffness or graft fibrosis is challenging. It can be due to preservation injury, fibrosis present before the transplantation. Fibrosis can be heterogeneous across the graft[42]. The acute cellular rejection or any inflammatory conditions overestimates liver stiffness measurement [43]. Given thrombocytopenia persists after liver transplantation despite the resolution

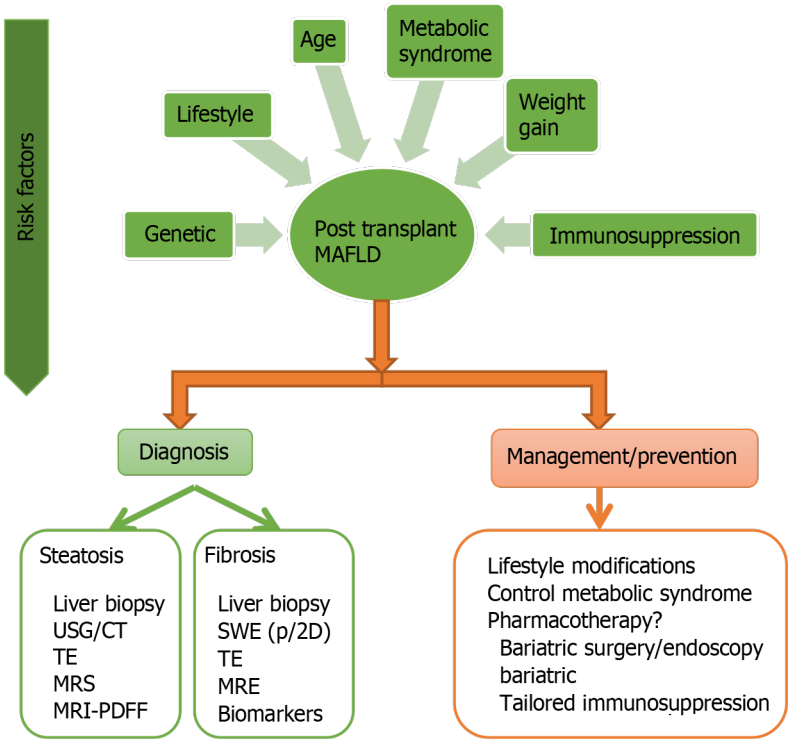

Figure 1 Overview of approach and management of post liver transplant metabolic dysfunction-associated fatty liver disease patients.
USG: Ultrasound; CT: Computed tomography; TE: Transient elastography; MRS: Magnetic resonance spectroscopy; MRI-PDFF: Magnetic resonance imaging-proton density fat fraction; SWE: Shear wave elastography; MRE: Magnetic resonance elastography.

of portal hypertension, serum biomarkers such as APRI or FIB4 that rely on platelet count may overestimate fibrosis[42]. There are a few studies regarding different noninvasive fibrosis tests (NITs) in post LT patients to identify recurrent fibrosis in different types of liver disease conditions. The meta-analysis suggests TE performs better than APRI and FIB4-score to diagnose significant fibrosis. The summary odds ratio was significantly higher for TE (21.27, 95%CI: 14.10-31.77, $P = 1 \times 10^{-30}$) compared to APRI (9.02, 94%CI: 5.79-14.07; $P = 1 \times 10^{-30}$) and FIB-4 (7.08, 95%CI: 4.00-12.55; $P = 1.93 \times 10^{-11}$). However, the majority of the studies are HCV patients[44]. Liver stiffness measured by TE at 3-mo post LT also predicts survival in LT recipients[45]. In a prospective study using ARFI to correlate histologic fibrosis score in 58 post-LT patients of mixed etiologies, the result demonstrated that SWE accurately detect advanced fibrosis (F ≥ 3) and cirrhosis (F4) with AUROC of 93 % and 80%, respectively. However, authors did not provide data on graft steatosis in these populations[46]. In a study of 32 post LT patients, the accuracy of both MRE and fibrospect test is high (AUROC of 0.87 and 0.84, respectively) in detecting fibrosis due to recurrent HCV[47]. In another study of 31 patients who underwent living donor liver transplantation with recurrent HCV infection to compare the accuracy of MRE, TE, and serum biomarkers (APRI and fibro α score to identify advanced fibrosis defined by Metavir stage ≥ 3, it showed MRE and fibro α score can accurately diagnose advanced fibrosis with AUROC of 0.708 and 0.833, respectively. The correlation of TE and APRI was not statistically significant to detect advanced fibrosis[48]. In a pooled analysis of MRE in LT recipients, AUROCs of MRE in detecting advanced fibrosis (stage ≥ 3) using a cut-off of 4.10 kPa and cirrhosis using a cut-off of 5.91 kPa were 0.83 and 0.96 respectively, suggesting high diagnostic accuracy[49].

However, there is limited literature in identifying different stages of hepatic fibrosis with NITs in post LT patients with either *de novo* or recurrent graft steatosis. A study by Galvin *et al*[20] of 430 post LT patients who developed *de novo* graft steatosis showed that the modest accuracy of FIB-4 and NFS to identify advanced fibrosis (F3-4) with AUROCs of 0.75 and 0.74, respectively. AAR with the optimal threshold of > 1.625 was found to have high specificity and accuracy with AUROC of 0.99 to identify cirrhosis (F4). However, only 9 (6%) of patients in the cohort had cirrhosis[20].

More studies are necessary to explore the accuracy of NITs in the diagnosis and assessment of steatosis and fibrosis in the post LT patients with either *de novo* or recurrent MAFLD.

NATURAL HISTORY AND LIVER OUTCOMES

Time-dependent relationships of either *de novo* or recurrent graft steatosis in the post LT patients were found in a few studies. Recurrent graft steatosis was diagnosed by TE in 87.5% of 56 post LT patients at a median time of 75 mo from liver transplantation. Advanced fibrosis was found in 26.8% whereas clinically compensated cirrhosis was found in 5.4% of patients. Recurrent graft steatosis was diagnosed by liver biopsy in 88.2% of 34 post LT patients at a median time of 47 mo from liver transplantation. Recurrent graft steatohepatitis was found in 41.2% of patients and bridging fibrosis was also found in 20.6% of patients who underwent liver biopsy[50]. Another study also showed that a time-dependent increase in the risk of recurrent graft steatosis approached 100% by 5 years compared to approximately 25% incidence of *de novo* graft steatosis in weight-matched controls who were being transplanted for primary biliary cirrhosis/primary sclerosing cholangitis or alcoholic liver cirrhosis[51]. *De novo* graft steatosis was found in 36.11% of 252 post LT patients after 5 years of liver transplantation in a study by Tejedor-Tejada *et al*[52]. Among the patients with *de novo* graft steatosis, significant fibrosis (F ≥ 2) was found in 85.6% with NFS, 81.9% with FIB4, 57.9% with APRI, 61.7% with AAR, and 83% with BARD after 5 years post LT. Similarly, 33.3% of 430 post LT liver biopsies from all causes were found to have *de novo* graft steatosis or steatohepatitis at a median of 3 years after liver transplantation. The significant risk factor for the development of significant fibrosis is age (OR 1.092, 95%CI: 1.02-1.17) on logistic regression analysis. The annual progression of fibrosis in patients with *de novo* graft steatosis was estimated to be 0.4 (interquartile range: 0.2-0.7) per year based on an approximation of fibrosis stage in relation to the number of years after liver transplantation. Insulin use is the only modifiable factor associated with the development of significant fibrosis (F ≥ 2)[20]. In a study by Vallin *et al*[10] that compared the natural history of *de novo* graft steatosis to recurrent graft steatosis, *de novo* graft steatosis was found in 67% and recurrent graft steatosis was found in 100% after 1 year. The prevalence of *de novo* graft steatosis increased to 69% after 3 years and 78% after 5 years. Steatosis disappeared in 22.5% of patients with *de novo* graft steatosis but none of the patients with recurrent graft steatosis disappeared graft steatosis. Recurrent graft steatosis developed advanced fibrosis (stage ≥ 3) in 71.4% of patients whereas *de novo* graft steatosis developed advanced fibrosis in only 12.5% of patients after 5 years post LT. Similarly, more frequent graft steatohepatitis was found in the recurrent graft steatosis group compared to the *de novo* graft steatosis group (71.4% *vs* 17.2%, $P < 0.01$).

Studies have shown worse outcomes in patients being transplanted from steatohepatitis with HCC as well as patients being re-transplanted for graft steatohepatitis[53,54]. *De novo* neoplasms were generally increased in patients with *de novo* graft steatosis compared to controls[52]. However, there is no literature showed an increase in the incidence of recurrent HCC in post LT patients with either *de novo* or recurrent graft steatosis.

PATIENT AND GRAFT SURVIVAL

In a large *de novo* graft steatosis cohort studied by Galvin *et al*[20], there is no significant difference in the short term (1 year) or long-term survival up to 15 years of patients with *de novo* graft steatosis ($n = 143$) compared to those without graft steatosis ($n = 287$) (log-rank 0.54). In another study by Narayanan *et al*[9], neither graft steatosis nor steatohepatitis (regardless of *de novo* or recurrent) was associated with patient mortality at 1 year after adjusting other patient characteristics ($P = 0.25$). De novo steatosis did not statistically significant impact patient survival (time-dependent HR 1.36, 95%CI: 0.99-1.87, $P = 0.057$) or graft survival (time-dependent HR 1.26, 95%CI: 0.92-1.72, $P = 0.15$) after excluding patients with pretransplant hepatic steatosis. Graft survival was not affected by time-dependent graft steatosis nor pre-transplant steatohepatitis. None of the cohorts required re-transplantation due to recurrent steatohepatitis. The study did not show any significant difference in death and fibrosis progression between patients with biopsy-proven *de novo vs* recurrent steatohepatitis [9]. In a study of 252 post LT patients by Tejedor-Tejada *et al*[52], there is no significant difference in the medium and long-term survival between patients with *de novo* graft steatosis and controls[52].

EXTRAHEPATIC OUTCOMES

MAFLD, by definition, is associated with obesity, IR, dyslipidemia, and hypertension, and those conditions have an important impact on transplanted patient outcomes. MAFLD and MetS are intertwined, and this is evident in post-transplant patients that develop MAFLD, either *de novo* or recurrent. In recurrent MAFLD, the MetS risk factors that exist before transplant will persist. In *de novo* MALFD, those risk factors are triggered by immunosuppression (IS) or rapid weight gain after transplant. In both cases, patients carry the same metabolic profile: IR, dyslipidemia, hypertension, and obesity. Indeed, one-third of patients develop DM and obesity in 3 years post-transplant[55]. Another common element between *de novo* and recurrent MAFLD is the use of IS after transplant. Steroids, CNIs are known to cause hypertension, hyperglycemia. mTOR inhibitors often triggers hyperlipidemia in post-transplant patients.

The evidence shows that transplanted patients with recurrent graft steatosis have an increased rate of DM, dyslipidemia, and weight gain[56]. There is reciprocity between MAFLD and MetS. Transplanted patients with *de novo* graft steatosis are five times more likely to be obese and two times more likely to have DM[57]. On the other hand, Sprinzl *et al*[58] reported that almost one-third of patients who underwent a LT in his cohort developed MetS, linked to graft steatosis. Indeed, obesity and dyslipidemia were predictors for the development of *de novo* graft steatosis within one year post LT [58].

The most common cause of death in the population with steatohepatitis are cardiovascular (CV) disease and malignancies[9]. It is easy to extrapolate that the CV and malignancies are also a significant cause of mobility and mortality in post-transplant patients who develop MASH, either *de novo* or recurrent. CV events included myocardial infarction, angina, ischemic stroke, sudden death, and peripheral artery disease. Extrahepatic malignancy included urology, head and neck, skin, lung, hematological, gynecological, gastrointestinal, and brain cancer. Bhati *et al*[50] showed that mortality was attributed to cancer in 25%, infections in 25%, and CV complications in 21% in post LT patients with recurrent graft steatosis[50]. Gitto *et al*[57] demonstrated that post LT patients with *de novo* graft steatosis had an increased risk for CV disease and extrahepatic cancers. Specific factors associated with CV disease in the post-transplant setting are age > 55 years old, male sex, DM, and kidney failure [59]. In a study by Tejedor-Tejada *et al*[52], CV events were found more frequently in patients with post LT *de novo* graft steatosis than controls (23.08% *vs* 19.88%). Similarly, *de novo* malignancies were found more in *de novo* graft steatosis group compared to control (24.18% *vs* 19.25%)[52]

MANAGEMENT

There is very scarce data about post LT *de novo* and recurrent MAFLD management, but recommendations can be drawn from the treatment of MAFLD in the general population. In general, prevention of MetS and gaining weight is the best approach in post-transplant patients. Overall management is summarized in Table 1 and Figure 1.

Lifestyle modifications

Lifestyle modifications are the backbone of the treatment of MAFLD. This approach can target specific components of MetS and is the recommended first treatment for hepatic steatosis[29,60]. Fussner *et al*[61] showed that an increase in BMI was a concrete risk factor for MetS at one-year post-transplant. Hence, avoiding excessive weight gain in the immediate post-transplant setting can help decrease the incidence of MetS. Lifestyle modifications include various and multidisciplinary strategies like physical activity, personalized diet, and behavioral interventions to hold weight gain. Loss of 3%-5% of the body weight showed improved steatosis, and loss of 7%-10% of body weight improved steatohepatitis on a report by Vilar-Gomez *et al*[62]. Evidence shows that decreasing the caloric intake by 750-1000 kcal/d or by 30% resulted in improved IR and hepatic steatosis[63,64]. The literature also shows that high cholesterol diets can trigger steatohepatitis in a mice model[65]. Additionally, the European Association for the Study of the Liver (EASL) recommends avoiding fructose intake since it is associated with hepatic steatosis[60]. The American Association for the Study of Liver Diseases recommends abstinence of heavy alcohol drinking (more than four standard drinks on any day or more than 14 drinks per week in men or more than three drinks on any day or seven drinks per week in women)[29].

Table 1 Summary management strategies

Lifestyle modifications	Dietary modification
	Exercise/ physical activity
	Avoid heavy alcohol consumption
	Benefit with coffee consumption
Pharmacotherapy	No approved drug for MAFLD in post liver transplants patients
Bariatric treatment	Surgery
	Endoscopic
Tailored Immunosuppression	Early taper of steroids
	Decreasing CNIs as possible
	Avoid/cautious use of mTOR inhibitors

CNIs: Calcineurin inhibitors; MAFLD: Metabolic dysfunction-associated fatty liver disease.

In comparison, EASL recommends keeping the alcohol consumption below 30 g in men and 20 g in women since there is evidence of a decrease in the prevalence of hepatic steatosis with moderate alcohol[60]. Interestingly, coffee consumption has been associated with fibrosis risk reduction[66].

In terms of exercise, Kistler *et al*[67] reported that vigorous physical activity held fibrosis progression in hepatic steatosis. The combination of caloric restriction and exercise resulted in weight loss associated with histological improvement of steatohepatitis[62]. However, a trial of dietary counseling and exercise *vs* standard of care after liver transplantation reported only a moderate benefit; still, adhesion to the program was achieved on only 37% of the patients[68]. Therefore, the recommendation for post LT patients with MAFLD is weight loss through diet and exercise.

Pharmacotherapy

It is essential to acknowledge that there is no approved drug for the specific treatment of MAFLD. Nevertheless, there is a significant number of drugs under investigation for hepatic steatosis and steatohepatitis. Pharmacotherapy in patients with hepatic steatosis is used in two ways: to achieve control goals in diabetes, dyslipidemia, and hypertension and to target the progression of the hepatic steatosis. In both cases, caution with drug interaction in post-transplant patients is recommended[69]. MAFLD patients with MetS comorbidities need to have reasonable control of their sugars, lipids, and blood pressure, and they should be referred to a specialist in those areas if necessary. Although not recommended for the treatment of MAFLD *per se*, statins should not be held for those patients meeting lipid profile criteria for statin use[29,70]. The same can be said for diabetic agents; none of them are approved for MALFD treatment but may be used in diabetic patients with steatosis as some have shown some benefits such as pioglitazone and empagliflozin.

In the PIVENS trial, both pioglitazone and vitamin E improved biopsy-proven NASH, although the histological improvement with vitamin E was better[71]. Vitamin E should be used only in diabetic patients. Interestingly, pioglitazone was associated with weight gain. Liraglutide, a glucagon-like peptide-1, was associated in a randomized trial with the resolution of steatohepatitis, minor progression of fibrosis, and weight loss in patients with biopsy-proven NASH[72]. More recently, empagliflozin, a sodium-glucose cotransporter-2 inhibitor, has been shown to reduce steatosis and improve ALT in NAFLD diabetic patients[73]. Orlistat, a medication used for weight loss, has been associated with steatosis improvement, though this effect can be attributed to the weight loss in itself[74].

Metformin, ursodeoxycholic acid, and pentoxifylline have been tried with poor outcomes. Nevertheless, many other drugs as obeticholic acid and elafibranor, are under investigation with promising results. There is no clinical trial of an investigational drug in post LT patients with either *de novo* or recurrent MALFLD.

Bariatric surgery

Maintaining an adequate weight proves to be challenging. Although weight loss of > 7% was associated with improvement in steatohepatitis, only half of the patients

Table 2 Summary of clinical significances and outcomes of *de novo* and recurrent metabolic dysfunction-associated fatty liver disease in post liver transplant patients

	De novo MAFLD	Recurrent MAFLD
Risk factors/Predictors for post LT MAFLD	Post LT weight gain	Post LT weight gain
	HCV	Post-transplant hypertriglyceridemia
	Sirolimus-based immunosuppressant therapy	Steroid
	Insulin resistance/diabetes mellitus	Post LT Metabolic syndrome
		Insulin use
		Insulin resistance/ diabetes mellitus
Progression to steatohepatitis and advanced fibrosis	Less common	More common
Cardiovascular events	Common	Common
Patient and graft survival	No significant impact	No significant impact

LT: Liver transplant; HCV: Hepatitis C virus; MAFLD: Metabolic dysfunction-associated fatty liver disease.

achieved this goal[62]. Bariatric surgery improves long-term mortality from CV disease and cancer in the general population[75]. In a study with steatohepatitis patients who underwent bariatric surgery, 85% had resolution of steatohepatitis with improved fibrosis in 33% of the patients[76]. There are some case reports of bariatric surgery in transplanted patients; Al-Nowaylati *et al*[77] described improvement in weight, glycemia, and HDL in seven patients. Diwan *et al*[78] reported similar findings, but with a high rate of complications and mortality of 20%. Endoscopic bariatric approaches are also on the rise; those techniques demonstrate to be effective weight loss leading to improvement in steatohepatitis[79]. Endoscopy bariatric treatment can be a very feasible option in the post-transplant setting for patients with MAFLD.

Tailored IS

It is known that IS is a contributing factor in the development of MetS after LT. IS can exacerbate preexisting risk factors and contribute to recurrent MAFLD. Similarly, IS can create the conditions to develop *de novo* MAFLD in patients transplanted for other causes requiring higher IS, such as autoimmune hepatitis or rejection. Alas, IS is essential in the post-transplant period. Consequently, a tailored approach looking to reduce the risk factors for MetS and hence MAFLD should be used. Early taper of steroids and decreasing as possible CNIs by adding other agents can add to the glycemic control in transplanted patients with diabetes. Everolimus plus a low dose of tacrolimus has shown a moderate decrease in weight in post-transplant patients[80]; this strategy, along with a rapid decrease in steroids, can be helpful in obese patients. CNIs can also contribute to hypertension and dyslipidemia. Approaches to minimize those side effects can be helpful. mTOR inhibitors are associated with elevated triglycerides; thus, they should be avoided in patients is MAFLD. In summary, protocols with early tapering of steroids and minimal use of CI:N should be considered in post-transplant patients with already risk factors for MAFLD and to minimize the development of those.

CONCLUSION

Given MAFLD is the fastest growing indication for liver transplantation; both *de novo* and recurrent graft steatosis in the context of MetS or MAFLD are common in the post-transplant settings. The role of noninvasive tests in detecting graft steatosis and fibrosis is challenging. Given the performance of image-based techniques is promising, larger cohort studies with histologic validation are necessary. Liver biopsy remains the gold standard for detecting graft steatosis and different degree of graft fibrosis. Although *de novo* and recurrent MAFLD after transplant have common pathways, it appears that recurrent MASH is more severe than *de novo*. Recurrent graft steatosis with the progression of fibrosis is found to be more frequent in patients being transplanted for hepatic steatosis compared to those with *de novo* graft steatosis. Even

though graft steatosis has an impact on CV events and incidence of *de novo* neoplasms, the patient and graft survival seem to be not affected by either *de novo* or recurrent graft steatosis. Management is mainly focused on weight control and tailoring of immunosuppressive therapy. The clinical significances and outcomes of both *de novo* and recurrent MAFLD in post LT population is summarized in Table 2. There are many knowledge gaps in the field of post LT MAFLD and MASH. Further studies are required for long-term outcomes of post LT MAFLD and MASH population and management strategies.

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MINIREVIEWS

Liver dysfunction as a cytokine storm manifestation and prognostic factor for severe COVID-19

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Abstract

Liver damage in severe acute respiratory coronavirus 2 infection occurs in patients with or without preexisting liver disorders, posing a significant complication and mortality risk. During coronavirus disease 2019 (COVID-19), abnormal liver function is typically observed. However, liver injury may occur because of the treatment as well. Ischemia, cytokine storm, and hypoxia were identified as the three major factors contributing to liver damage during COVID-19. Indeed, raised liver enzymes during hospitalizations may be attributed to medications used, as well as sepsis and shock. As a result, the proportion of hospitalized patients afflicted with COVID-19 and pathological liver biomarkers varies from 14% to 53%. Aminotransferases and bilirubin are found most often elevated. Usually, increased gamma-glutamyltransferase, alkaline phosphatase, and decreased serum albumin levels are demonstrated. Additionally, although there is no specific treatment for COVID-19, many of the drugs used to treat the infection are hepatotoxic. In this mini-review, we focus on how liver dysfunction can be one of the features associated with the COVID-19 cytokine storm. Furthermore, data show that liver injury can be an independent predictor of severe COVID-19, the need for hospitalization, and death.

Key Words: Liver dysfunction; Liver damage; Cytokine storm; Prognostic factor; COVID-19; Severe COVID-19; SARS-CoV-2; Aspartate aminotransferase; Alanine aminotransferase; Bilirubin; Interleukin-6

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Core Tip: Looking at the liver tests in patients with severe coronavirus disease 2019 (COVID-19), C-reactive protein (CRP) showed a strong correlation with the aspartate aminotransferase (AST) levels. This was observed in both intensive care units (ICU) and non-ICU patients. However, CRP levels were higher in non-ICU patients with liver damage, whereas alanine aminotransferase (ALT) was higher in ICU COVID-19 patients. Thus, like interleukin-6 (IL-6), ferritin, and CRP correlated directly with AST and ALT levels in non-ICU patients, there is a direct correlation of IL-6 and acute phase proteins with AST in severe COVID-19 cases. These observations confirm the critical impact of systemic inflammation and specifically elevated IL-6 during severe acute respiratory coronavirus 2 cytokine storm on liver injury.

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INTRODUCTION

The newly emerged severe acute respiratory coronavirus 2 (SARS-CoV-2) and the disease that causes coronaviral disease 2019 (COVID-19) are still unclear regarding all virulence factors, immunological effects and deteriorations of human organs during infection[1]. However, it is assumed that the interaction between the SARS-CoV-2 virus and the individual's immune system substantially influences the disease's onset and progression and the pathological effects on many organs. Both humoral and cell-mediated immune mechanisms participate in the immune response to a viral infection [2].

However, in some patients, these antiviral immunological mechanisms escape the regulatory control and eventually contribute to the multiorgan failure caused by the virus, including liver failure. Furthermore, an overreaction of the host immune system triggers a systemic inflammatory state that causes significant tissue and organs damage due to high cytokine release. The latter phenomenon is known as cytokine storm, leading to extreme tissue damage[2]. Therefore, the mortality rate and the COVID-19 complications in the elderly and patients with preexisting medical comorbidities, such as diabetes, asthma and cardiovascular disease, are even higher. Furthermore, the risk of severe COVID-19 might be increased by the underlying liver disease. In addition, it can cause direct or indirect damage to the liver by creating a multisystem inflammation[3].

Liver damage in SARS-CoV-2 infection occurring during disease progression in patients with or without preexisting liver diseases is a substantial challenge for clinical practice. Abnormal liver function is expected during COVID-19 infection because of SARS-CoV-2 direct and indirect impact on the liver. Additionally, certain hepatotoxic medications, especially for COVID-19 treatment, are connected with drug-induced liver damage. However, liver injury is defined as any liver damage occurring during disease and treatment. Therefore, hospitalized patients infected with COVID-19 with abnormal liver biomarkers range from 14% to 53%; this is most often observed for aminotransferase and bilirubin[1]. In addition, increased levels of gamma-glutamyl transferase (GGT), alkaline phosphatase, and decreasing serum albumin levels are also observed[4].

As significant liver biomarkers changes are observed in patients with severe COVID-19, more frequent in adults in the intensive care unit, studies documented that elevation of liver enzymes is associated with severity of COVID-19. Additionally, male sex and CRP were demonstrated as independent risk factors of COVID-19 complicated by liver injury[5].

This mini-review discusses how liver dysfunction can be one of the manifestations of the COVID-19-associated cytokine storm. Furthermore, liver damage might be an independent prognostic factor for severe COVID-19 and hospitalization and death.

LIVER DYSFUNCTION AS A MANIFESTATION OF THE CYTOKINE STORM

Cytokine storm syndrome occurring in some of the COVID-19 infected patients involved many organs, such as lungs, kidneys, heart, and liver[2]. COVID-19 may also lead to multiorgan failure and severe consequences owing to systemic inflammatory conditions caused by a cytokine cascade with pulmonary, cardiac, and hepatic involvement, as described above[6].

Three main factors are associated with liver damage during COVID-19: ischemia, cytokine storm, and hypoxia. Other influential contributors are the direct cytopathic effect of the virus on cholangiocytes (*via* ACE2 receptors), preexisting liver disease (*i.e.*, steatosis, hepatitis, cholangitis, thrombosis, Kupfer cell proliferation, liver impairment), severe inflammatory responses/sepsis[6].

Direct or indirect effects of SARS-CoV-2 on other organs are described beyond the respiratory system. In addition, it was shown that additional receptors might facilitate the virus to enter and infect the human cells *via* spike protein, including the liver. This suggests that there might be additional receptor pathways for infection with COVID-19 that can be targeted with specific treatment.

SARS-CoV-2 caused dysfunction and inducing a systemic inflammatory response leading to severe liver injury by binding to ACE2 receptors on cholangiocytes. In detail, spike protein binds the asialoglycoprotein receptor located on human hepatocytes. It was recently published that in vitro, SARS-CoV-2 spike protein can bind the asialoglycoprotein receptor 1 Located on primary human hepatocytes and hepatocyte-like cells[7]. In line with this, the serum GGT as a diagnostic marker for cholangiocyte injury has been found at elevated levels in up to 72% of severe COVID-19 patients[8].

Hypoxic liver injury (HLI) is not rare in patients with severe COVID-19 and has a high mortality. Its leading causes are lung and cardiac failure and may be associated with the immune-mediated inflammatory response. Patients with HLI have high mortality as a result of the deterioration of multiple organ failures. Levels of total bilirubin (TBIL), C-reactive protein (CRP), procalcitonin, and interleukin-6 (IL-6) show a statistically significant elevation in HLI cases compared with that in non-HLI cases. Besides, the median survival time of patients with HLI is significantly shorter than that of those not developing HLI[9].

Massive cytokine release causes a cytokine storm (also known as cytokine release syndrome) and is characterized by elevated CRP, IL-6, lactate dehydrogenase (LDH), and ferritin concentrations[10]. Furthermore, the subsequent organ dysfunction (*i.e.*, acute respiratory distress syndrome, progressive liver damage, and liver failure). As a result, systemic pro-inflammatory cytokine release appears to be a driver of disease progression in COVID-19[11-13].

Notably, COVID-19 patients had hepatic lymphocyte infiltration, centrilobular sinusoidal dilation, and patchy necrosis following the SARS-CoV-2 directly binding to ACE2-expressing cholangiocytes. However, the cause of the liver damage is unknown and may be due to systemic inflammation, SARS-CoV-2 infection, or drug administration[14].

Effenberger *et al*[10] discovered a clear link between systemic inflammation (as measured by IL-6, CRP, and ferritin) and liver damage. IL-6 development can be attributed to immune cells, fibroblasts, endothelial cells, and hepatocytes, orchestrating an acute phase response in the liver. Though IL-6 signaling impacts hepatic regeneration, clinical trials (for example, testing the effect of IL-6 administration in cancer patients) have shown that this pathway is essential in hepatic injury and hepatotoxicity[10]. The authors also found a strong association between acute-phase proteins and IL-6 in the serum of COVID-19 patients with elevated aspartate aminotransferase (AST), which is consistent with the importance of systemic inflammation and, in particular, IL-6 on liver injury.

The main sources of IL-6, which is the chief stimulator of the production of most acute phase proteins, are macrophages and monocytes at inflammatory sites. It has been shown that macrophages and monocytes produce high amounts of IL-6 in response to SARS-CoV-2 proteins[15].

COVID-19 patients with gastrointestinal complaints (nausea, vomiting, diarrhea, *etc.*) had higher AST and alanine aminotransferase (ALT) levels. Furthermore, there was a significant increase in enzymes among COVID-19 patients, primarily in the intensive care unit (ICU) facilities[16]. A relationship between liver enzyme elevation and disease activity has been also demonstrated[17].

Furthermore, the incidence of elevated AST levels was found to be greater than that of ALT levels and significantly higher in patients with severe COVID-19 (45.5%) relative to non-severe cases (15.0%). Thus, Lei *et al*[18] established a link between liver

injury and inpatient mortality in COVID-19 patients. They also found a correlation between AST abnormality and mortality risk compared to other liver injury measures during hospitalization[18].

Liver biopsies revealed moderate microvesicular steatosis with slight lobular and portal inflammation, indicating either direct viral or drug-induced liver damage[19]. It is proposed that a direct virus-mediated cytopathic effect exists. The latter can result after triggered immunological reactions and inflammatory cytokines, leading to liver injury[20,21]. Monocyte and macrophage dysfunction contribute to the progression of liver damage. Activation of liver-resident macrophages (Kupffer cells) and damage-associated molecular patterns result in recruitment of effector cells to the injured liver. Early monocyte infiltration is a major factor in the progression of local tissue destruction. Furthermore, the local inflammation results in the secretion of more and more pro-inflammatory cytokines that drive systemic inflammatory response syndrome[22].

Additionally, predominated parenchymal liver damage according to the elevated AST (23.2%) and ALT (21.2%), rather than bile duct injury, as shown by GGT (9.7%) and ALP (4.0%) levels in COVID-19 patients[16]. Patients with mild COVID-19 also have liver damage which resolves without any specific treatment. Most of the patients with liver failure during hospitalization, associated with severe COVID-19, are due to several drugs' hepatotoxicity.

Different drugs can impair liver function. However, the hepatotoxicity of medications varies on race, sex, and age of the patients[23]. Thus, the knowledge on the potential contributors to liver failure is significant. In addition, some medications can induce asymptomatic elevations of liver enzymes, acute hepatitis.

Many of the patients required treatment with antibiotics, anti-inflammatory, and antiviral agents. Antibiotics, anti-inflammatory, and antiviral medications used to treat COVID-19 patients are among the medicines that can induce liver harm[24,25]. Some of them cause asymptomatic elevation of the liver enzymes, while others lead to acute hepatitis. In some cases (*e.g.*, acetaminophen), these effects are dose-dependent. In contrast, in other medications, liver damage occurs independently of the drug dosage [24].

Hydroxychloroquine alone or in combination with azithromycin, lopinavir / ritonavir, remdesivir, darunavir, umifenovir, interferon beta, baricitinib, imatinib exert hepatotoxicity. Their immediate availability has led to off-label use for COVID-19 treatment in many countries[26].

There is currently no specific antiviral medication for SARS-CoV-2. Still, many COVID-19 patients are given antivirals approved for different uses (*i.e.*, remdesivir, lopinavir, or ritonavir, and other medications[27], all of which have been linked to hepatotoxicity and liver impairment[26].

Incorrect liver metabolization may also result in COVID 19-induced liver impairment which increases the risk of poisoning. However, a combination of patient records and thorough laboratory tests is carried out to diagnose drug-induced liver impairment to exclude other hepatic diseases and identify the relationship between hepatic injuries and probable causative medications.

More COVID 19 individuals suffer from fever, and hepatotoxicity can be triggered by antipyretics and analgesics (*i.e.*, paracetamol). This is associated with liver injuries, resulting in a potentially deadly combination, generally in the most severe phases of COVID-19. Furthermore, some antiviral drugs - remdesivir, lopinavir, ritonavir, IL-6 inhibitors (*i.e.*, tocilizumab), antibiotics - azithromycin, may cause idiosyncratic drug-induced liver failure[26].

Mechanisms involved in liver injury during COVID-19 infection and cytokine storm are presented on Figure 1.

LIVER FAILURE AS A PROGNOSTIC FACTOR IN SEVERE COVID-19 PATIENTS

Different risk factors can be associated with severe liver injury. Specifically, preexisting liver diseases - obesity with non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, cirrhosis - all of them correlate with Child-Pugh class and model for end-stage liver disease score. Moreover, autoimmune liver diseases, chronic hepatitis B infections could be reactivated and contribute to high levels of AST/ALT [28,29].

Patients with cirrhosis have a high risk of mortality from respiratory failure following severe SARS-CoV-2 infection. This risk might occur through multiple

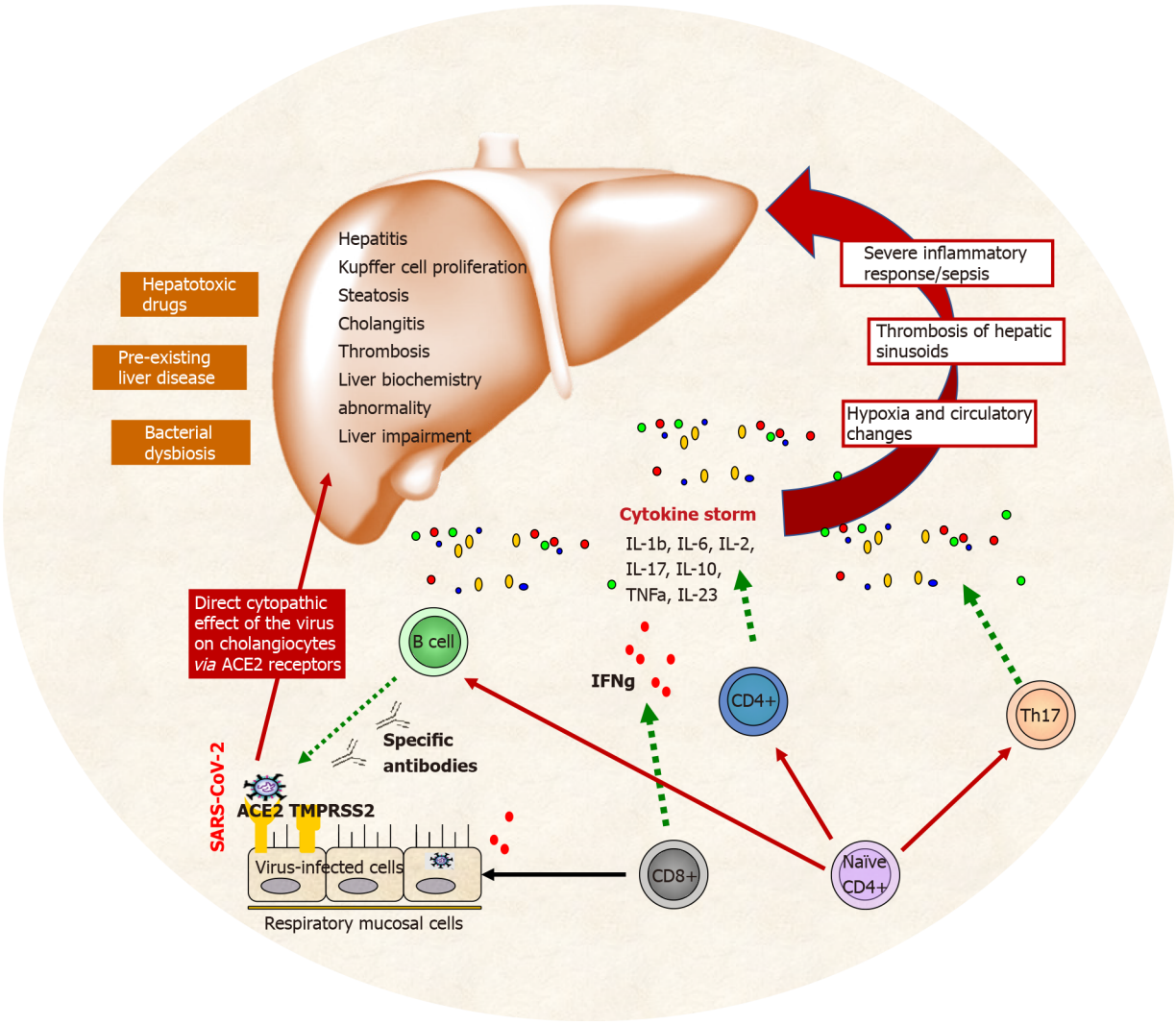

Figure 1 Liver dysfunction defined by the negative effects of cytokine storm (severe inflammation, thrombosis, hypoxia, *etc.*) during coronavirus disease 2019 infection. Other contributing factors for liver injury are also presented – preexisting liver condition, direct cytopathic action of severe acute respiratory coronavirus 2 and treatment with hepatotoxic drugs.

converging pathways, including contributions from cirrhosis-associated immune dysfunction, acute hepatic decompensation, and systemic inflammatory response. Cirrhosis-associated immune dysfunction could also lead to defective immune responses following future SARS-CoV-2 vaccination[20]. Patient with abnormal liver tests had a higher mortality rate (28.9% *vs* 9.0%, $P < 0.001$) and higher chance to develop systemic inflammatory response[30,31].

Interestingly, abnormal liver tests and liver injury can be associated with the progression of severe pneumonia[12]. The abnormalities can be hepatocellular, cholesteric, or mixed. Some clinical research studies show that patients with abnormal liver test results, especially in hepatocyte or mixed type ALT/AST and ALP/GGT at admission or during hospitalization, had significantly higher odds of progressing to severe COVID-19[28].

As we mentioned above, the pattern of liver injury is predominantly hepatocellular rather than cholestatic, although elevations in TBIL and ALT may be more common than reported in earlier studies. Since the ACE2 receptor is predominantly expressed in cholangiocytes than in hepatocytes, it is suggested that the most prevalent mechanism of liver impairment is not due to a direct cytopathic effect of the SARS-CoV-2 virus[32].

Raised liver enzymes during hospitalizations could be partly due to drugs used for treatment and might be due to sepsis and shock[28]. Looking at the liver tests, CRP showed a strong correlation with the AST levels, especially in hospitalized patients. Additionally, for both ICU and non-ICU patients, where this association was demonstrated at admission. However, CRP levels were higher in non-ICU patients with liver damage, whereas ALT was higher in ICU COVID-19 patients[33]. IL-6,

ferritin, and CRP correlated directly with AST and ALT levels in non-ICU patients.

Further analysis revealed a direct correlation of IL-6 and acute phase proteins with AST. In severe COVID-19 cases. To sum up, these observations confirm the critical impact of systemic inflammation and specifically IL-6 on liver injury. Furthermore, these observations led to the establishment of abnormal AST and direct bilirubin (DBil) at hospital admission as independent risk factors for increased COVID-19 mortality [33].

We can emphasize that the pathological examination of liver tissues from deceased patients with COVID-19 confirmed that liver involvement of COVID-19 was characterized by microvesicular steatosis, focal necrosis with lymphocytes infiltration, and micro thrombosis in the portal area[34]. Furthermore, pathological levels DBil were often found during the hospitalization of deceased COVID-19 patients. Both baseline and higher AST and DBil levels were independently associated with in-hospital death in patients with COVID-19. While liver anomalies are typical in COVID-19, these findings indicate that the liver is unlikely to be the primary organ driving COVID-19 mortality.

Since the number of people who develop severe and fatal COVID-19 is increased in elderly patients and those with liver failure and NAFLD, it is typically advised that older COVID-9 patients on hepatotoxic medication be closely followed up. Moreover, NAFLD can make the liver more sensitive to the most recommended and widespread antipyretic medication treatment for symptomatic diseases, such as acetaminophen[35, 36]. However, while the association of the COVID-19 with the liver steatosis disease is still unknown, a recent histological study of a COVID-19 patient's liver revealed microvesicular liver steatosis[19,37].

CONCLUSION

We can conclude that the pathological mechanisms of liver damage during COVID-19 confirmed that liver involvement was often observed with an increased risk for complications and death. Furthermore, the incidence of abnormal liver enzymes, significantly elevated AST and ALT levels were observed in patients with severe COVID-19 than non-severe cases. Additionally, a link between liver injury and inpatient mortality in COVID-19 patients was established. Moreover, recent studies confirmed that if liver dysfunction, preexisting or acquired during COVID-19 treatment, is a prognostic factor for severe COVID-19, development of complications and death.

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MINIREVIEWS

COVID-19 and the liver: A brief and core review

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Abstract

Coronavirus disease 2019 has a wide range of clinical spectrum from asymptomatic infection to severe infection resulting in death within a short time. Currently, it is known that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) does not only cause a respiratory tract infection but a more complicated disease that can lead to multiple system involvement including the liver. Herein, we evaluate the epidemiology, the impact of liver injury/dysfunction on disease prognosis, the pathophysiological mechanisms and management of liver injury. More than one-fourth of the patients have abnormal liver function tests, mostly a mild-to-moderate liver dysfunction. Liver injury is significantly associated with a poor clinical outcome. Direct cytotoxic effect of SARS-CoV-2, the immune response ("cytokine storm"), the complications related to the disease, and drugs used in the treatments are the pathophysiological mechanisms responsible for liver injury. However, the exact mechanism is not yet clearly explained. The binding of SARS-CoV-2 to the angiotensin-converting enzyme 2 receptors and entering the hepatocyte and cholangiocytes can cause cytotoxic effects on the liver. Excessive immune response has an important role in disease progression and causes acute respiratory distress syndrome and multi-organ failures accompanied by liver injury. Treatment drugs, particularly lopinavir/ritonavir, remdesivir and antibiotics are a frequent reason for liver injury. The possible reasons should be meticulously investigated and resolved.

Key Words: COVID-19; SARS-CoV-2; Liver injury; Liver dysfunction; Chronic liver disease; Pathophysiology

Core Tip: The study evaluated the incidence of liver injury in coronavirus disease 2019 (COVID-19) patients and its impact on clinical outcomes and pathophysiological mechanism of liver injury. More than one-fourth of COVID-19 patients had suffered

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from liver injury, mostly a mild-to-moderate liver dysfunction. Liver involvement is independently associated with adverse clinical outcomes. Direct viral cytotoxic effect, complications of the disease, and drugs used in the treatments are the pathophysiological mechanisms suggested for liver injury. However, the exact mechanism was not clearly explained. The actual cause should be carefully investigated in the presence of abnormal liver function tests, and appropriate treatments provided for possible factors.

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INTRODUCTION

The emergence of the novel coronavirus disease 2019 (COVID-19) pandemic was a breaking point that deeply affected the whole world and changed medical priorities in daily practice. From the early time of the pandemic, it has been understood that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is not only a respiratory system virus that causes severe lung disease but a systemic disease agent that can affect all systems. Numerous studies from around the world have shown that the liver is damaged in varying degrees in patients with SARS-CoV-2 infection[1]. Recent studies have shown that a considerable part of the COVID-19 patients showed abnormality in liver function tests[2-5]. Liver injury causes a poorer outcome in affected patients, however, its effect on the disease may be more profound than it appears. Herein, we aimed to evaluate the epidemiological characteristics and impact of the liver injury on the clinical outcome, the interaction between pre-existing chronic liver diseases (CLDs) and COVID-19, the pathophysiology of liver involvement and hepatic histopathological findings, and management of liver injury.

DEFINITION

The liver is a vital organ that is mainly responsible for protein synthesis, storage of glycogen and regulation of blood glucose levels, metabolism of toxic substances, and many other physiological processes[1]. A great majority of studies revealed that a mild-to-moderate liver involvement was present in a considerable part of COVID-19 patients. However, what liver damage means has not been clearly defined. Zheng *et al*[6] pointed out that there is no clarity on what liver damage means in their letter to the editors. There are no standardized diagnostic criteria to be considered as a liver injury. The cut-off value of liver function tests varies among studies. The World Health Organization defined the severity of acute COVID-19 as mild, moderate, severe, and critical illness based on respiratory and other systemic findings using technical guidelines[7]. However, the degree of liver and other organ involvement has not been defined yet. There is no standard for cut-off values of liver function tests established by the consensus of researchers. Researchers usually have used different cut-off values, as Zheng Ye *et al*[6] emphasized. Most of them defined any elevated value above the upper limit of normal (ULN) as liver injury, others preferred values 2-3 times higher than UNL[6,8]. Cai *et al*[8] defined liver test abnormalities as two groups, elevations of liver enzymes (higher than ULN) and liver injury. Aspartate transaminase (AST)/alanine transaminase (ALT) values above 3 times ULN, or alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and total bilirubin values above 2 times ULN were accepted as liver injury.

Lv *et al*[9] stated concern about the possible misinterpretation of AST data. Determining liver injury incidences based on AST may have led to overestimation. It is believed that ALT is more specific for liver disease and reflects the real hepatic injury. AST is a less specific marker for the liver due to being produced by other tissue such as kidneys, cardiac, and skeletal muscles rather than the liver. Therefore, to be sure of the source of AST, isoform analysis should be done that is not available in routine practice. In addition, antibiotics and antivirals used during the disease also contribute

frequently to the elevation of the AST value[5]. A recent study showed that the first rising enzyme is AST followed by ALT[10]. These raise the question of whether the increase in AST may have been caused by other tissues or causes. On the other hand, the studies reported the association between AST level and the disease severity regardless of its source.

In addition, previous diagnosed or undiagnosed CLDs such as chronic viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), may also result in abnormal liver tests. The use of an established set of standards for liver dysfunction/liver injury by researchers is essential in terms of comparability of study results. Therefore, there is an urgent need to define clearly what liver dysfunction/injury means.

EPIDEMIOLOGY AND PROGNOSIS

Incidence of liver dysfunction

Numerous studies have reported liver injury and varying levels of liver dysfunctions in SARS-CoV-2 infection[3,5]. Most infections manifested as mild to moderate liver disorders presented with abnormal liver function tests [AST/ALT elevations, GGT/ALP elevations, and in some cases hypoproteinemia and prolonged prothrombin time (PT)][2-4,11-15]. In their meta-analysis, Kulkarni *et al*[5] reported liver function test abnormality in 19% of 1290 non-severe COVID-19 patients from nine articles. Cai *et al*[8] reported liver injury in 24.9% of non-severe cases. Emerging data from cohort studies have pointed out that liver dysfunction is a commonly encountered entity, usually in more than usually in more than one-third of hospitalized COVID-19 patients[11,16,17]. However, as pointed out above, the incidence of liver injury varies between cohorts, sometimes due to reasons such as differences among study and patient populations, the variety of the drug treatments, and their usage rates. Herein, we mostly addressed several meta-analyses and reviews which evaluated and summarized liver involvements in SARS-CoV-2 infections. A meta-analysis reported the pooled incidence of liver dysfunction as 23.1% at early presentation and 24.1% through the disease course among 15407 patients[5]. The incidence of abnormal levels of liver function was also reported as 29% in another meta-analysis evaluating a total of 38 studies with 3062 COVID-19 patients[17].

In a review, Alqahtani *et al*[18] analyzed more than thirty published, ahead of print and preprint reports which consisted of mostly case series. They summarized the details of the study types, patients' numbers, hepatobiliary function markers, inflammatory markers, and proposed possible mechanisms of liver injury. More than 20 publications included in the review had reported abnormal levels of aminotransferase, up to 61.1% of cases. Almost all cases had a modest liver injury except one who had an AST reaching a maximum of 1263 U/L and ALT reaching 2093 U/L. Another retrospective study by Chen *et al*[19], included in the review, reported that one case had experienced severe hepatitis with an AST of 1445 and ALT of 7590 U/L. A negligible part of patients had pre-existing liver disease. COVID-19 causes usually mild-to-moderate liver injury presented with modest abnormality in liver function tests, and it occasionally resulted in severe hepatitis.

In a comprehensive review evaluating the incidence of hepatic abnormalities in SARS, the Middle East respiratory syndrome, and SARS-CoV-2, Kukla *et al*[20] analyzed 2541 patients infected with SARS CoV-2 in 11 studies reported from China and reported that liver involvement had occurred with predominantly mild to moderately high transaminases, hypoalbuminemia, and prolongation of PT. A large-scale study of 5700 patients hospitalized with COVID-19 reported elevations of ALT and AST in 39.0% and 58.4% of the patient population, respectively[21]. Cai *et al*[8] reported 76.3% abnormal liver function tests (higher than ULN) and 21.5% liver injury (defined higher than 3 × AST/ALT or 2 × ALP/GGT/total bilirubin) at admission.

A slight hyperbilirubinemia is accompanied by elevated transaminase in COVID-19. Its incidence was reported as 13.4% in Kulkarni *et al*[3]'s study. The studies also reported the increase in other liver function tests (ALP, GGT), prolonged PT and decrease in albumin level. Cai *et al*[8] reported GGT elevation in more than 15% of the patients at admission and in approximately half of the patients during hospitalization. The pooled incidence of prolonged PT was 9.7% in adults with a meta-analysis[5]. As a result, although the incidence rates are in a wide range in studies, the incidence of liver injury was present in at least one-fourth of patients or more.

Liver dysfunction and clinical outcomes

Accumulated data since the beginning of the pandemic shows that liver dysfunction is significantly associated with a poor outcome in SARS-CoV-2 infection[3,8,11,16,17,22]. Cai *et al*[8] reported that patients with liver injury had a 9-fold-greater risk of severe COVID-19. A meta-analysis involving 3722 cases in 13 studies revealed that mortality and clinical severity were associated with liver injury in COVID-19 patients[3]. Fu *et al*[16] reported a higher mortality rate in patients with abnormal liver function tests compared to those with normal liver function tests (29.6% *vs* 6.5%, $P < 0.001$), especially AST elevation and total bilirubin elevation groups. Serum AST level was higher in deceased patients and severe COVID-19 cases than in surviving patients and non-severe cases [odds ratio (OR) = 4.48, 95% confidence interval (CI): 3.24-7.21, $P < 0.001$][3]. A comprehensive meta-analysis investigating the incidence of elevated liver functions, and the association of the patients' outcomes with liver dysfunction and CLDs upon 15407 patients revealed that COVID-19 patients with elevated liver functions had an increased risk for mortality (OR = 3.46, 95%CI: 2.42-4.95, P <0.001) and severe disease (OR = 2.87, 95%CI: 2.29-3.6, P <0.001) compared to patients without elevated liver functions[5]. In another meta-analysis, a higher level of AST, ALT, and bilirubin values, prolonged PT, and a lower level of serum albumin value were found to be associated with severe COVID-19[23]. In consequence, the elevated transaminase and abnormality of other liver function tests were common in COVID-19 patients and independently associated with adverse clinical outcomes.

PATHOPHYSIOLOGY OF LIVER INJURY

Although much has been learned about SARS-CoV-2 in the elapsed time since the beginning of the pandemic, there remain many points that need to be clarified, particularly its pathogenesis. There is still a dilemma about whether SARS-CoV-2 increases transaminases directly by viral cytotoxic effect or by the consequences of the disease such as hyperinflammation, sepsis, and drugs[24]. Although not yet fully clarified, the pathogenesis of COVID-19 associated liver injury appears to be related to direct viral hepatitis, or the disease-induced complications such as severe respiratory involvement related to hypoxia [*e.g.*, acute respiratory distress syndrome (ARDS)], sepsis, cytokine storm, or drug-related liver enzyme elevations during the infection[9,20,25]. Possible mechanism of liver injury is given in Figure 1.

Direct cytopathic effect of SARS-CoV-2 on the liver

Recent studies show that SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) receptors, mainly expressed in type 2 alveolar cells of the lungs, to enter the body[26,27]. ACE2 receptors are also mainly localized in the heart, kidney, testes, and other tissues[8]. The liver is a potential target organ for the virus due to its containing high levels of ACE2 receptors[28]. The direct cytotoxic effect and/or inflammatory response of the body to SARS-CoV-2 may be responsible for liver injury. It has been suggested that the binding of SARS-CoV-2 to the ACE2 receptors and entering the hepatocyte and cholangiocytes can cause a direct viral cytotoxic effect on the liver[5], a suggestion that is supported by the findings of a previous study where SARS-CoV-2 RNA was detected in a liver sample[29]. Nardo *et al*[30] reviewed the pathological findings of COVID-19 patients and proposed that the pathological findings of COVID-19 might be caused by hepatocellular infection with direct cytopathic effect of SARS-CoV-2 and cytokine storm, hypoxic conditions due to ARDS and drug-induced liver injury (DILI) may contribute to these findings. Previous studies had extensively investigated the cell entry mechanism of SARS-CoV-2, and reported that viral entry is triggered by the binding of receptor-binding domain of ACE receptors to the target cells such as alveolar type 2 cells, hepatocytes or cholangiocytes and activated by human proteases such as TMPRSS2[31-33]. However, more data is required to assess the relevance between virus and liver damage. Interestingly, ACE2 expression in cholangiocytes is at similar levels to the lungs, and higher than in the hepatocytes[28]. This may explain the increase in ALP, GGT, and total bilirubin levels. However, COVID-19 patients do not commonly denote a cholestatic pattern of hepatic dysfunction; increased transaminase levels are more predominant. This can be explained by the possibility that hepatic dysfunction predominantly results from secondary causes such as hypoxia and cytokine storm than the direct viral cytopathic effect of the virus[28,34]. Further studies are required to explain why serum transaminases are elevated more than ALP and bilirubin, and to assess the relevance between virus and liver injury.

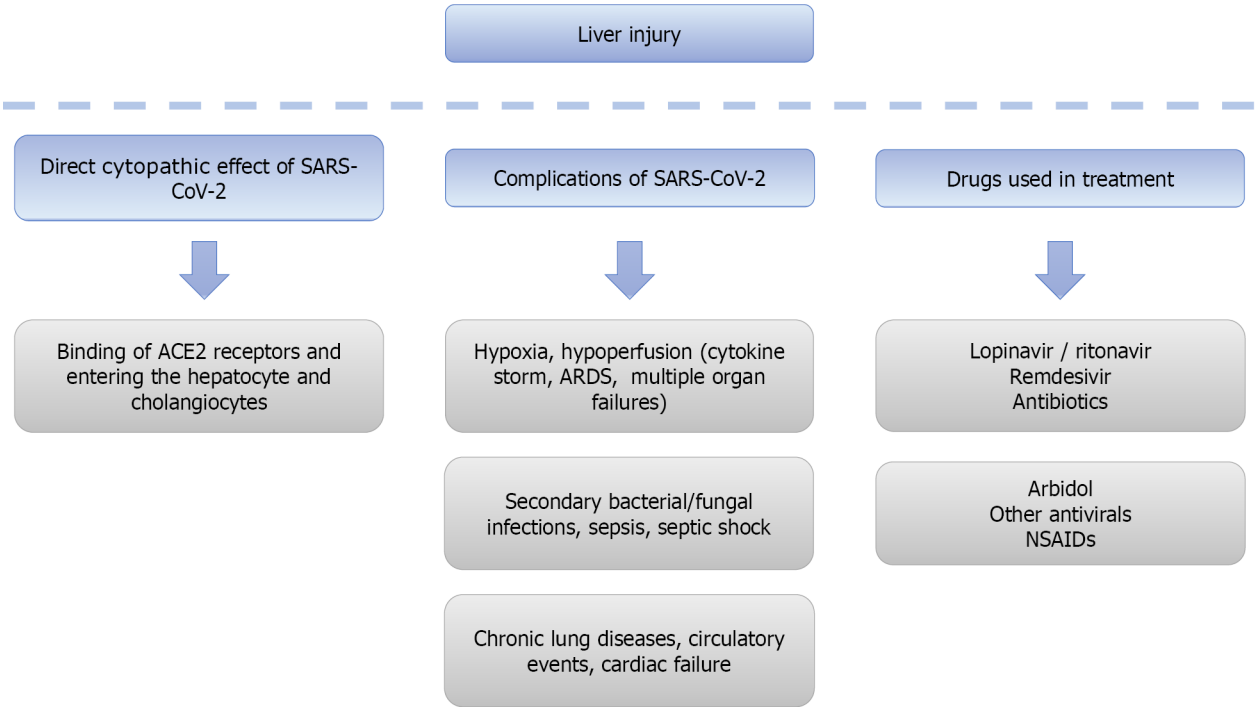

Figure 1 Possible mechanisms of liver injury in coronavirus disease 2019. ACE2: Angiotensin-converting enzyme 2; ARDS: Acute respiratory distress syndrome; NSAIDs: Non-steroidal anti-inflammatory drugs; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

Complications of SARS-CoV-2

COVID-19 has a wide range of clinical spectrum from asymptomatic infection to severe infection resulting in death within a short time. COVID-19 patients particularly with severe illness suffer from various degrees of respiratory system involvement and multiple organ failure. Its pathogenesis is complicated and mainly based on immune system dysfunction, at local and systemic levels[35]. Accumulated data on COVID-19 pathogenesis indicates that SARS-CoV-2 induces an excessive cytokine release, known as cytokine storm in some patients, and causes ARDS and multiple organ failures including heart, liver, and kidney[35-37]. Cytokine storm is the life-threatening overactivation of immune cells and dysregulated inflammatory cytokine and chemical production in relation to a triggering factor such as bacterial, fungal and viral pathogens, and is accepted as the main cause of multiple organ injury. It was confirmed that a high level of inflammatory markers such as C-reactive protein, cytokines [interleukin (IL) 1, IL-6, IL-18, tumor necrosis factor, granulocyte-colony stimulating factor], and chemokines are associated with severe infection[11,34,35,38-43]. Cytokines and chemokines stimulate both the innate and adoptive immune system resulting in apoptosis of the infected cells and immune cell hyperstimulation. Therefore, cytokine storm may play a role in the appearance of abnormal liver function tests.

Thromboembolic events are frequent in COVID-19 patients, and another possible explanation of liver involvement is endothelial injury and hyper-coagulability[44]. In a preliminary study, the signs of acute (thrombosis, luminal ectasia) and chronic (fibrous thickening of the vascular wall or phlebosclerosis, presence of abnormal portal intrahepatic system) hepatic vascular involvement was found in all specimens in varying degrees among the main pathological findings[45].

Multiple organ dysfunction induced by other COVID-19-related complications probably contribute to elevated liver function tests. COVID-19 patients, particularly with a severe and critical illness, are at risk for secondary bacterial and fungal infections[46]. Sepsis is a common condition in COVID-19 patients, especially those who are followed up in the intensive care unit and can cause multiple organ dysfunction, including the liver. Besides, the development of septic shock increases the risk of hepatotoxicity through hypoperfusion[47]. Hypoxia and cardiac failure in affected COVID-19 patients can lead to liver injury[34]. Circulatory events, underlying CLD disorders are other secondary reasons for liver injury[11,28,34].

Therapeutic drugs

Liver injury may be partially attributed to the drugs used in COVID-19 treatment[5,11]. Liver damage has been reported with the use of lopinavir/ritonavir as an antiviral in SARS-CoV-2 infection[5,8,11]. Cai *et al*[8] did not detect any significant evidence for increased risk for liver injury in patients using suspected drugs (including antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), ribavirin, herbal medication used in Chinese medicine, and interferon), except for lopinavir/ritonavir. Patients who used lopinavir/ritonavir had a higher GGT and ALP level. Similarly, Cichoż-Lach *et al*[11] reported that they did not find any association between the use of antibiotics, NSAIDs, ribavirin, and interferon, and hepatic complications. Only lopinavir/ritonavir had provoked the deterioration of liver function. In a study, the rate of lopinavir/ritonavir use had been detected higher in the patients with hepatic dysfunction than in those without hepatic dysfunction[48]. Kulkarni *et al*[5] also reported that drug-induced liver injury due to the use of lopinavir/ritonavir, remdesivir, and arbidol is common, but not resulting in life-threatening conditions. The incidence of abnormal liver function tests with lopinavir/ritonavir ranges from 22.7% to 54.6%. Remdesivir is another drug that causes frequent increases (15.2%) in liver function tests. Elevated liver function tests were reported at a rate of 18.7% with the use of arbidol.

Hydroxychloroquine, an antimalarial drug, is one of the most used and studied as immunomodulatory drugs in the treatment of COVID-19[49,50]. Although there is conflicting information about its effectiveness in COVID-19, hepatotoxicity is not a common side effect of hydroxychloroquine. Hydroxychloroquine has been used in the treatment of systemic lupus erythematosus, rheumatoid arthritis, and related diseases for over 70 years[51]. There are only a few case reports of hepatoxicity with hydroxychloroquine[34,52].

Interpreting the data on whether antibiotics, NSAIDs, and other drugs used to treat COVID-19 patients cause hepatotoxic effects is a complicated issue. As discussed above, elevated AST and ALT levels are seen in severe cases or occur during the disease course even if it is normal on admission. These cases stay longer in hospital and combat unfavorable conditions such as secondary bacterial and fungal infections, sepsis, and cytokine storm which require the administration of certain other medications. Rather than thinking that liver enzyme elevation is related solely to the drugs used, it seems more plausible to account that all factors contribute.

HISTOPATHOLOGICAL FINDINGS OF THE LIVER

Understanding histopathological findings of COVID-19 has an important role in elucidating the pathogenesis of the disease and how liver damage develops. The most common finding in histopathology is steatosis. In a review that involved 9 biopsies and 226 autopsies, histopathology findings of COVID-19 cases in the published studies were evaluated and the most important histopathological findings of lung, heart, liver, and kidney were summarized[53]. Although a limited number of samples was performed in biopsy/autopsy, the most remarkable findings have been detected as steatosis and inflammation. Similarly, Díaz *et al*[24] reported detecting hepatic steatosis and vascular thrombosis as major and prevalent histological liver findings. Portal and lobular inflammation and Kupffer cell hyperplasia or proliferation were other frequent findings. Steatosis was higher than the normal population. It should be noted that these findings may lead to a bias since patients with more severe illnesses are included in the autopsy or biopsy studies. Besides, it can also be explained by the co-existence of other common causes of steatosis (*e.g.*, diabetes, obesity, NAFLD, hypertension, and heart diseases) in severe COVID-19 patients[9,24].

PRE-EXISTING LIVER DISEASES

The prevalence of CLDs among COVID-19 patients is low. Kulkarni *et al*[5] reported the pooled prevalence of underlying CLDs as 3.6% (95%CI: 2.5-5.1) among 15407 patients in 50 articles, and as 3.9% among 1587 severely ill patients in 15 articles that reported it. However, there are higher rates of its prevalence in different studies. Oyelade *et al*[54] reported its prevalence as 3%-11% in their meta-analysis. Fu *et al*[16] reported the prevalence of CLDs as 19.9% (viral hepatitis 8.9% and NAFLD 1%) in their study population and did not find any significant associations between CLDs and elevated liver function tests. Certain studies reported that underlying CLDs are

associated with higher mortality[55-57]. Contrary to this, in the comprehensive meta-analysis by Kulkarni *et al*[5], the presence of CLDs was not associated with severe COVID-19 (OR = 0.8, 95%CI: 0.31-2.09, P = 0.67). Similar to Kulkarni, Lippi *et al*[58] could not find any association between CLDs and COVID-19 severity (OR = 0.96, 95%CI: 0.36–2.52) and its mortality (OR = 2.33, 95%CI: 0.77–7.04). Conflicting results in the literature about the relation between SARS-CoV-2 infection and pre-existing liver disease may be associated with the heterogeneity of the study populations and the type (*e.g.*, alcoholic liver disease, NAFLD, viral hepatitis) and severity of the underlying liver diseases (*e.g.*, cirrhosis, decompensated disease or hepatocellular carcinoma), and further investigation is needed to clearly understand.

An observational study found the presence of alcohol-related liver disease, decompensated cirrhosis, and hepatocellular carcinoma as independent risk factors for higher mortality in patients with CLDs[55]. In APCOLIS study (APASL COVID-19 Liver Injury Spectrum Study), patients with obesity (in cirrhotic) and diabetes mellitus (in non-cirrhotic) were vulnerable to liver injury[59]. In fact, it appears that chronic liver patients in advanced stages, rather than all chronic liver patients, have a higher risk of severe infection and mortality[56].

The individual risk to being infected with COVID-19 in patients with CLDs depends on several factors including comorbidity, etiology of chronic disease, and baseline liver disease stage[56,60]. Controlled viral hepatitis B and C was not accepted as an exact predisposing factor to SARS-CoV-2 infection[25]. Patients with cirrhosis or hepatocellular carcinoma may be more vulnerable to SARS-CoV-2 infection because of the impairment of patients' immune systems[61]. However, many more studies are needed to clarify the issue of whether chronic viral hepatitis creates a predisposition to SARS-CoV-2 infection.

MANAGEMENT OF LIVER INJURY

In mild cases of COVID-19, liver injury usually resolves spontaneously[61]. If liver injury develops during the COVID-19 clinical course, it should first be investigated whether the abnormal liver function tests are related to the drugs including antivirals, antibiotics, NSAIDs used in the treatment, and if necessary, the drug held responsible for liver damage should be discontinued[34]. However, severe liver injury may require a more meticulous evaluation and careful treatment. The actual cause of liver injury should be investigated, and appropriate treatment provided for possible factors. If present, hypoxia and hypoperfusion should be regulated. Timely control of immune-mediated systemic inflammation and cytokine storm improve the prognosis and reduce respiratory cell infiltration and hypoxia. Anti-inflammatory treatments such as dexamethasone or other corticosteroids that have been found to reduce mortality by suppression of inflammation are used. Dexamethasone 6 mg IV or orally for 10 d (or until discharge if earlier), is recommended in severe cases of COVID-19 particularly with end organ dysfunction. Alternatively, methylprednisolone 32 mg and prednisone 40 mg which are equivalent doses to dexamethasone 6 mg can also be used[62-64]. Corticosteroids are also one of the treatment options in hemophagocytic lymphohistiocytosis, a type of cytokine storm associated with deepening laboratory abnormalities including elevated liver function tests and seen in COVID-19 patients[35]. Other immunomodulatory and cytokine antagonists can be used in the treatment[35]. Adding tocilizumab to standard of care is recommended for progressive severe and critical cases of COVID-19 who have elevated markers of systemic inflammation[62]. Thus, liver damage due to hypoxia or hyperinflammation can be reduced with appropriate and on-time treatment.

To prevent the risks that may arise with COVID-19 infection, EASL recommends SARS-CoV-2 vaccination as early as possible in patients with CLDs, hepatocellular carcinoma, and candidates for liver transplantations as the potential benefits of the vaccine outweigh the risks associated with the vaccine. In transplanted patients, the optimal time of vaccination is 3-6 mo after transplantation[60].

CONCLUSION

In conclusion, we summarized the epidemiological characteristics of liver involvement in COVID-19 infection and the effects of liver dysfunction on the COVID-19 prognosis. We also evaluated the data on the pathophysiology of liver injury. Abnormal liver function tests have been detected in more than one-fourth of patients with COVID-19

and were associated with poorer outcomes. Abnormal liver function tests in COVID-19 need to be carefully investigated. The detection of real mechanisms on liver injury is a complicated and concurrent condition. Direct viral cytotoxic effect, the disease-induced complications and drugs used in COVID-19 treatment can cause singular or joined liver injury. Appropriate treatment should be provided for the possible reasons of liver injury.

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MINIREVIEWS

Newer variants of progressive familial intrahepatic cholestasis

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Abstract

Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous group of disorders characterized by defects in bile secretion and presentation with intrahepatic cholestasis in infancy or childhood. The most common types include PFIC 1 (deficiency of FIC1 protein, ATP8B1 gene mutation), PFIC 2 (bile salt export pump deficiency, ABCB11 gene mutation), and PFIC 3 (multidrug resistance protein-3 deficiency, ABCB4 gene mutation). Mutational analysis of subjects with normal gamma-glutamyl transferase cholestasis of unknown etiology has led to the identification of newer variants of PFIC, known as PFIC 4, 5, and MYO5B related (sometimes known as PFIC 6). PFIC 4 is caused by the loss of function of tight junction protein 2 (TJP2) and PFIC 5 is due to NR1H4 mutation causing Farnesoid X receptor deficiency. MYO5B gene mutation causes microvillous inclusion disease (MVID) and is also associated with isolated cholestasis. Children with TJP2 related cholestasis (PFIC-4) have a variable spectrum of presentation. Some have a self-limiting disease, while others have progressive liver disease with an increased risk of hepatocellular carcinoma. Hence, frequent surveillance for hepatocellular carcinoma is recommended from infancy. PFIC-5 patients usually have rapidly progressive liver disease with early onset coagulopathy, high alpha-fetoprotein and ultimately require a liver transplant. Subjects with MYO5 B-related disease can present with isolated cholestasis or cholestasis with intractable diarrhea (MVID). These children are at risk of worsening cholestasis post intestinal transplant (IT) for MVID, hence combined intestinal and liver transplant or IT with biliary diversion is preferred. Immunohistochemistry can differentiate most of the variants of PFIC but confirmation requires genetic analysis.

Key Words: Progressive familial intrahepatic cholestasis; Tight junction protein; Hepatocellular carcinoma; Biliary diversion; Microvillous inclusion disease

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Core Tip: Progressive familial intrahepatic cholestasis (PFIC) manifests with a varying spectrum of clinical features, with some variants progressing rapidly into end stage liver disease. Recently, newer variants of PFIC have been described including PFIC 4 due to tight junction protein 2 (TJP2) mutation, PFIC 5 due to NR1H4 mutation and MYO5B related cholestasis also sometimes known as PFIC 6. TJP2 related PFIC also has a risk of hepatocellular carcinoma. This article describes the pathogenesis and clinical features of the newer variants of PFIC.

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INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous group of intrahepatic cholestatic disorders caused by a defect in bile transport and secretion. It manifests in infancy or childhood and can progress to end-stage liver disease[1-3]. Genetically confirmed PFIC accounts for 12%-13% of cholestatic disorders in infants and children[4]. Disease variants are classified based on the specific bile transporter defects and all of them have an autosomal recessive inheritance. The three most prominent varieties are familial intrahepatic cholestasis-1, 2 and 3, which are caused by mutations in ATP8B1 gene encoding FIC1, ABCB11 gene encoding bile salt export pump, and ABCB4 gene encoding multidrug resistance protein-3 respectively (Figure 1). Nearly two-thirds of subjects with normal gamma-glutamyl transpeptidase (GGT) cholestasis (normally associated with PFIC except PFIC 3) do not have any mutations identified in ATP8B1 or ABCB11 genes[3]. Detailed mutational analysis in patients with this phenotype has led to the identification of 3 more conditions, often known as PFIC 4, 5, and 6. PFIC 4 is caused by the loss of function of tight junction protein 2 (TJP2)[5], and PFIC 5 is due to NR1H4 mutation causing farnesoid X receptor (FXR) deficiency[6,7]. MYO5B mutation, known to cause microvillous inclusion disease (MVID), is also reported to cause isolated cholestasis and is sometimes known as PFIC 6 though it is not yet recognized by the Online Mendelian Inheritance in Man [8]. The exact incidence of newer variants of PFIC is not known due to the limited number of studies, which are mostly case reports or small case series. Based on the available literature, this review attempts to sensitize physicians to the disease.

GENETICS AND PATHOGENESIS

PFIC 4

TJP2 gene, located in chromosome 9q21 was first discovered in 1991 by Gumbiner *et al* [9]. It encodes a protein called tight junction protein 2 or zona occludens-2. Though named as tight junction protein, it is not present in the tight junction. Instead, TJP2 is a cytosolic protein, involved in maintaining cell-to-cell adhesion by linking the transmembrane tight junction proteins like claudin with the actin cytoskeleton. There are two types of claudin *i.e.*, claudin-1 (CLDN1) and claudin-2 (CLDN2), both of which are localized to the bile canalicular membrane[10]. In TJP2 mutation, CLDN1 fails to localize to the bile canalicular membrane (Figure 2). This results in reduced integrity of the canalicular membrane and reflux of toxic bile acids through the paracellular spaces into hepatocytes, causing hepatocyte damage and cholestasis[11]. TJP2 has a widespread expression, including the respiratory and central nervous systems. This may explain the systemic features reported in a few cases[11]. The detergent action of the bile potentiates damage in the liver, which explains the predominant hepatic manifestations in this condition.

PFIC 5

PFIC 5 is related to a deficiency of the FXR due to loss of function mutation in the *NR1H4* gene located in chromosome 12q23. NR1H4 related PFIC 5 is a less commonly

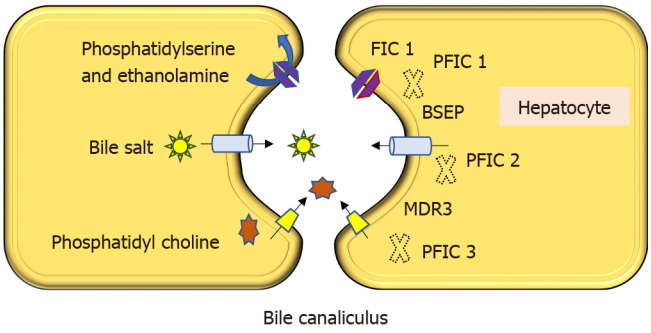

Figure 1 Pathogenesis of progressive familial intrahepatic cholestasis 1, 2 and 3. Familial intrahepatic cholestasis protein 1 is a flippase that helps in movement of phosphatidylserine and phosphatidylethanolamine from the outer to inner leaflet of the plasma membrane of hepatocyte; Bile salt exporter pump exports bile acid from hepatocytes to bile canaliculus; Multidrug resistance protein 3 is a floppase involved in transporting phosphatidylcholine into bile canaliculus. PFIC: Progressive familial intrahepatic cholestasis; FIC1: Familial intrahepatic cholestasis protein 1; BSEP: Bile salt exporter pump; MDR3: Multidrug resistance protein 3.

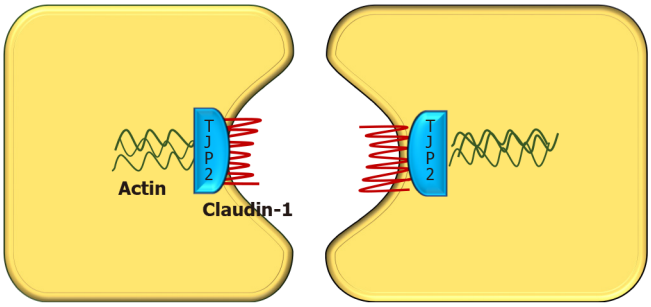

Figure 2 Diagrammatic representation of interaction between various tight junction proteins in hepatocytes. Claudin, tight junction proteins (TJP2), and actin form intercellular cytoskeletal support. Tight junctions prevent mixing of bile and blood. Absence of TJP2 causes a failure of claudin-1 localization at the canalicular membrane, leading to loss of compactness of the tight junctions and leakage of the bile through the paracellular space. TJP2: Tight junction proteins 2.

reported variant, with < 10 cases reported by 2020. FXR, a protein translated from the NR1H4 gene was first described in 1995 by Forman *et al*[12]. It belongs to a nuclear receptor group activated by farnesyl, an intermediate metabolite of the mevalonic acid synthesis pathway. FXR is the master regulator of cholesterol, bile acid, triglyceride and various sterol ring-containing compounds (Vitamin D, carotenoids, retinoids, *etc.*) [13]. In the liver, the FXR acts as a nuclear bile acid-sensing receptor involved in the expression of bile salt export protein (BSEP) and sometimes MDR3[6,14]. Apart from the liver, FXR is also expressed in the small intestine. Whenever bile acid levels are elevated in the ileal enterocytes, FXR is activated to induce the synthesis of fibroblast growth factor 19 (FGF19). FGF 19 is then transported *via* enterohepatic recirculation to the liver, where it binds to the fibroblast growth factor receptor 4/β-Klotho complex, and causes inhibition of bile acid synthesis by repressing CYP7A1. Elevated bile acid inside hepatocytes also activates FXR which induces ABCB11gene transcription, BSEP synthesis, and bile acid export from the liver. Hence, the NR1H4 mutation causes loss of BSEP expression, leading to the accumulation of toxic bile and hepatocellular damage (Figure 3). FXR is also involved in the regulation of coagulation factor synthesis by transactivating fibrinogen and kininogen genes. Thus, the FXR mutation leads to the development of vitamin K independent, early-onset coagulopathy, well before liver failure sets in[6].

Homozygous or compound heterozygous loss of function mutations (c.526C>T and c.419 420insAAA/intragenic 31.7-kb deletion, respectively) have been described[7]. In one woman with intrahepatic cholestasis of pregnancy, *NR1H4* heterozygous variant (c.-1G>T) was found to be associated with cholestasis[15].

PFIC 6

The *MYO5B* gene located in chromosome 18q21.1 encodes an actin-associated molecular motor protein called MYO5B. MYO5B and RAS-related GTP-binding protein 11A (RAB11A) is essential for the epithelial cell polarization in multiple tissues (Figure 4). In hepatocytes, it is important for the localization of ATP-dependent bile canalicular transporters like BSEP to the canalicular membrane, and in the intestine, it

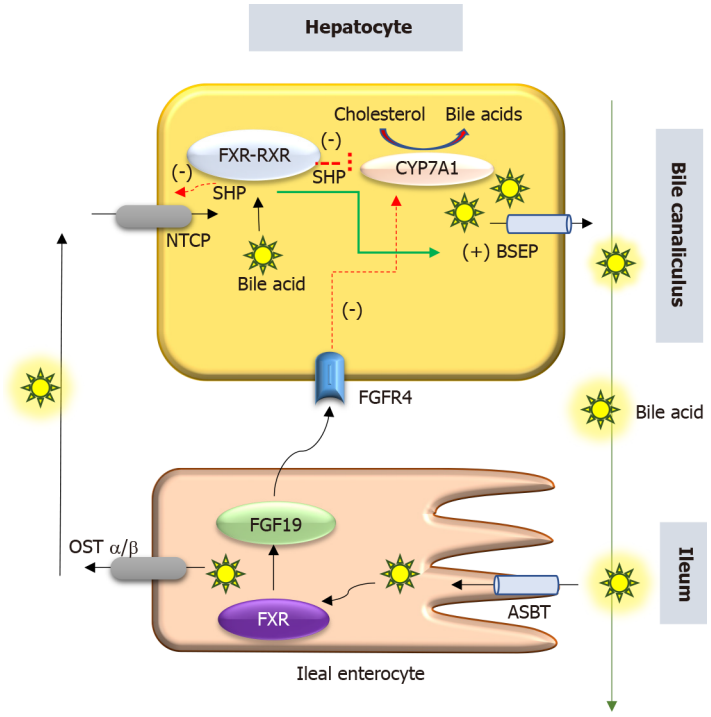

Figure 3 Schematic representation of role of farnesoid X receptor in hepatocyte. Bile acids are transported into the hepatocyte by NTCP. De novo synthesis of bile acids from cholesterol is mediated by CYP7A1. Bile acids and farnesoid X receptor (FXR) interact and enter the nucleus to promote expression of bile salt export protein and short heterodimer partner (SHP). SHP suppresses expression of NTCP and CYP7A1. FXR also induces FGF-19 in ileal enterocytes which inhibits CYP7A1 *via* FGFR4. ASBT: Apical sodium bile transporter, BSEP: Bile salt export pump; FGF-19: Fibroblast growth factor-19; FGFR-4: Fibroblast growth factor receptor-4; FXR: Farnesoid X receptor; NTCP: Na+-taurocholate co-transporting polypeptide; OST α/β: Organic solute transporter; RXR: Retinoid X receptor; SHP: Short heterodimer partner.

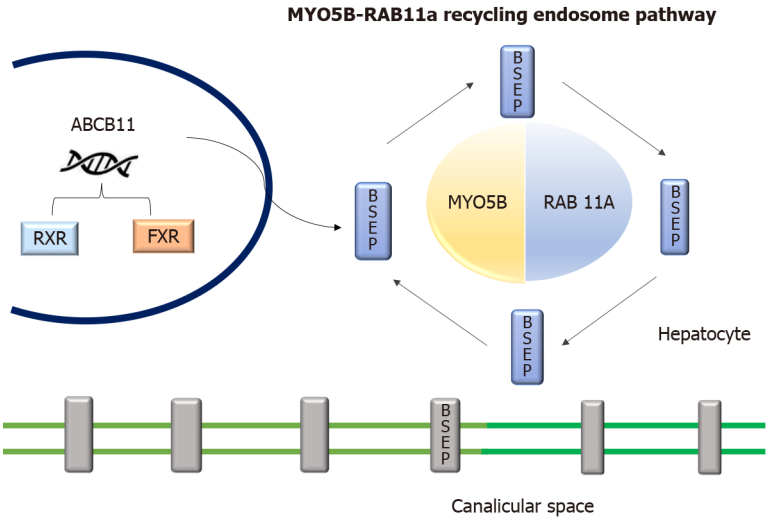

Figure 4 Diagrammatic representation of role of MYO5B and RAS-related GTP-binding protein 11A interaction and endosome recycling pathway and bile salt export pump expression. MYO5B and RAS-related GTP-binding protein 11A (RAB11A) interaction is essential for epithelial cell polarization and BSEP localization to the canalicular membrane. Diminished MYO5B/RAB11A recycling endosome pathway leads to disruption of bile salt export pump localization. ABCB11: ATP Binding Cassette Subfamily B Member 11; BSEP: Bile salt export pump; FXR: Farnesoid X receptor; RAB11A: RAS-related GTP-binding protein 11A; RXR: Retinoid X receptor.

is important for maintaining enterocyte polarity[16]. MYO5B mutations disrupt the MYO5B/RAB11A recycling endosome pathway leading to defective targeting of BSEP [17]. MYO5B gene mutations can result in cholestatic liver disease with or without associated MVID, which presents as intractable diarrhea in infancy[8,18]. Staining of BSEP and MDR3 by immunohistochemistry in these patients is sub-canalicular in the location instead of the regular localization in the canalicular membrane[8].

There is a suggestion that the type of MYO5B mutation affects the clinical presentation[18,19]. Less severe mutations have a loss of canalicular transporter function in hepatocytes without any loss of enterocytes functionality. These patients present with isolated cholestasis. In severe variants of mutations, there is a dysfunction of both bile canalicular transporter and enterocyte polarization. However, a severe loss of enterocyte function leads to a reduced bile acid absorption in the intestine and in turn decreased bile acid load to the hepatocyte, potentially preventing cholestatic manifestations[18]. Patients with MVID more often have biallelic severe mutations in MYO5B. Biallelic mutations in the MYO5B-RAB11A interaction domain are more in MVID than those with isolated cholestasis[20]. Thus, isolated cholestasis appears to reflect relatively mild MYO5B functional deficiency, whereas severe mutations in MYO5B primarily cause MVID[20].

CLINICAL PRESENTATION

Intrahepatic cholestasis is the hallmark of how these 3 genetic conditions present. Most often, patients present with variable combinations of pruritus, jaundice, pale stools, and failure to thrive. The published literature on each of these three entities (TJP2, FXR, and MYO5B) is limited and has been summarized in Tables 1-3 respectively.

PFIC 4

A varying spectrum of clinical presentation, ranging from mild anicteric illness, recurrent jaundice to severe progressive liver disease has been described[5,11]. Incomplete penetrant, homozygous, missense mutations affecting both isoforms of TJP2 have been shown to cause familial hypercholanemia in the Amish population which manifests as a mild anicteric disease with pruritus and steatorrhea. In this condition, the binding of TJP2 to claudins is impaired[21]. Milder mutations of TJP2 are also known to be associated with intrahepatic cholestasis of pregnancy[15].

In the 12 cases reported by Sambrotta *et al*[5], 9 (75%) required liver transplantation (LT) while 2 had portal hypertension. In contrast, none of the 7 cases reported by Zhang *et al*[22] required LT, and cholestasis responded to medical therapy in a majority. Zhang *et al*[22] also showed that truncating or canonical splice-site biallelic TJP2 mutations caused a more severe presentation due to a complete loss of protein expression. In their study, 3 children with severe mutations had growth failure. While the other 3 cases with missense variants had normal growth and sustained response in pruritus with ursodeoxycholic acid (UDCA) and cholestyramine.

All homozygous mutations are predicted to abolish protein translation and a complete loss of function[5]. Mutations involving missense and frame deletion lead to less severe clinical disease due to residual TJP2 protein expression[22]. This suggests the presence of a genotype-phenotype correlation based on the amount of remnant functional TJP2 activity.

There is a higher risk of developing hepatocellular carcinoma (HCC) in these cases, similar to that seen in PFIC 2 patients. Subjects can either present with a space-occupying lesion (SOL) in the liver or are detected to have HCC after LT on histology of the explanted liver[23,24]. This predisposition to HCC highlights the importance of close follow-up and regular monitoring.

PFIC 5

FXR is the master player of bile acid regulation and plays an important role in reducing bile acid-induced hepatotoxicity. Rapidly progressive liver disease and early onset vitamin K independent coagulopathy are the main features of this condition. The details of the 8 published cases are given in Table 2. A majority of patients presented early in the first 3 mo of life and progressed rapidly to liver failure. Patients have markedly increased alpha-fetoprotein and deranged international normalized ratio. Without a liver transplant, 5/8 died in infancy itself. Three cases survived post-liver transplant, of which 2 were found to have liver function abnormality with graft steatosis in the follow-up[6]. This post-transplant hepatic damage may be attributed to the altered enterohepatic circulation and FXR signalling in these cases. The absence of FXR in the intestine leads to low FGF 19 levels and this allows for continued and increased synthesis of bile acids by the liver[25]. Intrahepatic cholestasis of pregnancy has been reported and attributed to the downregulation of BSEP in this condition[26].

Table 1 Clinical characteristics and outcome in patients with *TJP2* mutation

Ref.	*n*	Age at onset of symptoms	Symptoms	Other symptoms	Treatment	Liver transplant	Outcome
Sambrotta *et al*[5]	12	1 wk-3 mo	NC-12/12	Chronic respiratory disease-1, recurrent unexplained hematoma-1	UDCA, PEBD-2	9/12 cases at the age of 1.5-10 yr	Post-transplant-9 (doing well, no disease recurrence); Stable liver disease with PHT-2; Mortality-1 at 13 mo age
Zhang *et al*[22]	7 (M = 6, F = 1)	3 d-2 mo	NC-6/7, pruritus at 7 mo-1/7	Gallstones 2/7	Response to UDCA, cholestyramine	None	Resolved cholestasis (n = 6) over 7-26 mo; Persisting icterus-1
Ge *et al*[46]	1 (F)	6mo	Jaundice, pruritus, FTT	-	Responded to medical treatment	None	Resolved cholestasis
Mirza *et al*[47]	1 (M)	4 yr	Jaundice, pruritus	-	Medical treatment	None	Cirrhosis, PHT with variceal bleed at 15 yr
Wei *et al*[24]	Index case (M) with multiple affected family members[1]	19 yr	Cirrhosis, PHT with variceal bleed, HCC at 22 yr	-	Medical treatment including EVL	23 yr	Well in post-transplant period

[1]Variable severity of liver disease: Cholestatic liver disease requiring transplant, cholestatic liver disease and intrahepatic cholestasis of pregnancy in other affected members.

EVL: Endoscopic variceal ligation; F: Female; FTT: Failure to thrive; HCC: Hepatocellular carcinoma; M: Male; NC: Neonatal cholestasis; PEBD: Partial external biliary diversion; PHT: Portal hypertension; UDCA: Ursodeoxycholic acid.

PFIC associated with MYO5B defects

Patients with MYO5B mutations can present with isolated cholestasis, isolated MVID, or both MVID and cholestasis. Typically, the child presents with jaundice, pruritus, and hepatomegaly. In patients with MVID and cholestasis, the onset of cholestasis may be pre or post-small bowel transplant. The exact explanation as to why some MVID cases develop cholestasis while others do not is unclear but it may be related to the severity of mutation (vide supra). The summary of the clinical presentation of 29 cases with MYO5B mutation, as reported in 4 papers, is shown in Table 3. Even in siblings with the same mutation and presentation with cholestasis, the disease severity may vary[20]. This suggests the possible role of modifier genes or environmental factors. Among Han Chinese children, defects in MYO5B accounted for approximately 20% of cases of idiopathic low-normal GGT intrahepatic cholestasis[20].

INVESTIGATIONS AND THE APPROACH TO DIAGNOSIS

The main steps for making a diagnosis of PFIC and determining the specific type in any given child with cholestasis are as follows: Step 1: Detailed history and physical examination including family history, consanguinity, extraintestinal symptoms, growth, nutritional deficiencies, and features of advanced liver disease; Step 2:

Table 2 Clinical characteristics and outcome in patients with *NR1H4* mutation

Ref.		Sex	Age at onset of symptoms	Age at initial evaluation	Symptoms	Lab parameters			Histology/IHC	Age at LTx	Outcome
						GGT	INR (at onset)	AFP ng/mL			
Gomez-Ospina *et al* [6], 2016	All cases had homozygous mutations										
	[1]Patient 1	F	2 wk	20 mo	J, FTT	53	2	716	Cirrhosis	22 mo	10 yr[4]
	[1]Patient 2	M	2 wk	7 wk	J, FTT	45	2	146000	Fibrosis	4.4 mo	15 mo[4]
	[2]Patient 3	F	6 wk	6 wk	J	59	1.4	13900	Fibrosis	ND	Died 8 mo
	[2]Patient 4	M	Birth	Birth	J, ascites, pleural effusion, ICB		-	-	Fibrosis	ND	Died at 4 wk
Himes *et al*[7], 2020	Patient 5 and 7 had homozygous mutations										
	Patient 5	M	16 mo	17 mo	J, ascites	81	1.9	9610	Cirrhosis	20 mo	Alive at 8 yr of age, no graft steatosis
	[3]Patient 6	M	3 wk	1 mo	J, FTT, hydrothorax	-	-	-	-	ND	Died at 8 mo, liver failure
	[3]Patient 7	F	1 wk	4 mo	J, FTT, hydrothorax	-	-	> 100000	-	ND	Died at 7 mo, liver failure
Chen *et al*[27], 2019	Patient had compound heterozygote mutation										
	Patient 8		N/A	3 mo	J, splenomegaly		3.0	> 80000	-	ND	Died at 5 mo

[1]Family 1.
[2]Family 2.
[3]Family 3.
[4]Post transplant both cases have hepatic steatosis and liver function test abnormalities.
AFP: Alpha fetoprotein; BSEP: Bile salt export pump; F: Female; FTT: Failure to thrive; FXR: Farnesoid X receptor; GGT: Gamma-glutamyltransferase; ICB: Intracranial bleed; IHC: Immunohistochemistry; INR: International normalized ratio; J: Jaundice; LTx: Liver transplantation; MDR3: Multidrug resistance protein 3; M: Male; N/A: Not applicable; ND: Not done.

Complete liver function test with GGT. Low-normal GGT is seen in ATP8B1, ABCB11, TJP2, NR1H4, and MYO5B disease. Early-onset of vitamin K unresponsive coagulopathy is a feature of NR1H4 disease; Step 3: Radiologic imaging. Ultrasonography (USG) of the abdomen is useful to exclude structural causes of neonatal cholestasis, like biliary atresia or choledochal cyst. The presence of biliary radicle dilatation may suggest sclerosing cholangitis, which needs to be confirmed by MRCP. USG is also useful to document features of advanced liver disease like ascites, splenomegaly, dilated portal vein, and collaterals. Gall stones have been reported in TJP2 disease, as also in PFIC 2 and 3. The presence of hepatic SOL raises suspicion of HCC and needs evaluation by triple-phase CT and alpha-fetoprotein. Early HCC is a feature of TJP2 disease; Step 4: Liver histology including immunohistochemistry and next-generation sequencing (NGS). Liver biopsy shows canalicular cholestasis in all three

Table 3 *MYO5B* mutation clinical characteristics and outcome

Ref.		Age at onset of symptom	Age at initial evaluation	Symptoms	Treatment	Lab parameters			Outcome
						GGT (IU/L)	AST (IU/L)	ALT (IU/L)	
Qiu *et al* [20], 2017	*n* = 10, M-8, F-2, 4 had affected siblings	2 d-19 mo	1 mo-10 yr	Jaundice and pruritus; No diarrhea	UDCA, cholestyramine	9-99	24-255	41-432	Recurrent-3, persistent-2, transient cholestasis-2, lost to follow-3, listed for LT -1 (died)
Cockar *et al* [19], 2020	*n* = 6, M-3, F-3	-	6 mo-15 yr	Pruritus with pale stools-6, Jaundice-3; FTT-3; Diarrhea-2, (intractable and settled at 3 yr and 7 yr), gallstone-1	Antipruritic medications-6; PIBD-1; PIBD followed by PEBD-1; ENBD followed by PEBD-1	10-22	-	15-177	1-LT for poor QOL and pruritus; 5-Partial response with mild pruritus while on medications
Gonzales *et al*[8], 2017	*n* = 5, M-4, F-1	-	7-15 mo	Pruritus-5; Jaundice-5; Pale stools-5 hepatomegaly-5; Language delay-1 episodes of severe diarrhea before 3 yr of age-1	UDCA and rifampicin-5; PEBD-1	7-11	31-170	57-207	Followed till 3.5-13.5 yr of age; Fluctuating cholestasis-4; Cholestasis resolved after 1 mo of PEBD, well till 7 yr of age
Girard *et al* [17], 2014	*n* = 8/28 MVID, patients with cholestasis M-5, F-3	3-60 mo		Jaundice, pruritus, hepatomegaly-8; Pre Int Tx-5, post Int Tx-3	Antipruritic medications-8; PIBD followed by PEBD-1; PIBD-1; PEBD-1; Combined liver and Int Tx-1	8-42	51-124	52-121	Follow up till 2.8-14 yr of age, remission-6, partial remission-2; Removal of small bowel graft due to acute rejection in 2 cases improved cholestasis

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ENBD: Endonasal biliary drainage; F: Female; FTT: Failure to thrive; GGT: Gamma glutamyl transferase; Int Tx: Intestinal transplant; LT: Liver transplantation; M: Male; MVID: Microvillus inclusion disease; PEBD: Partial external biliary drainage; PIBD: Partial internal biliary drainage; QOL: Quality of life; UDCA: Ursodeoxycholic acid.

types (TJP2, NR1H4, and MYO5B defects) along with a variable degree of fibrosis and giant cell transformation[6]. On electron microscopy, the tight junctions appear elongated and lack the densest part of the zona occludens in PFIC 4[6]. In subjects with MYO5B and liver disease, electron microscopy will show dilatation of the bile canalicular lumen, canalicular thickening, and disappearance of the microvilli apart from cholestasis[17]. Inclusion bodies are not seen in the hepatocytes on transmission electron microscopy, in contrast to the findings in intestines in MVID[17]. The comparative features at histology in these three types are given in Table 4.

A complete panel of immunohistochemistry including BSEP, MDR3, TJP2, FXR, MYO5B, and Claudin1 can help in identifying the subtype of PFIC as shown in Table 4. However, simultaneous NGS for multiple genes (cholestasis panel) is a rapid and affordable way of confirming the molecular diagnosis[27]. A recent study has shown that a molecular genetic diagnosis can be made in a quarter of cases with neonatal cholestasis using NGS[28]. A study with a 66-gene cholestasis panel in 2171 cholestatic children and young adults, had a diagnostic yield of 12% and turnaround time of only 21 d[29]. The simultaneous testing for multiple genes helps in not only confirming the diagnosis but also in excluding other conditions. NGS is becoming the test of choice in the primary evaluation of patients with PFIC phenotype as it is non-invasive in comparison to liver biopsy and immunohistochemistry. For cases in which

Table 4 Comparison of clinical features, laboratory profile and outcome in progressive familial intrahepatic cholestasis 4, 5 and 6

	PFIC 4	PFIC 5	PFIC 6
Gene mutation	TJP2/Zona occludens-2 located in 9q21.11	NR1H4/FXR-located in 12q23.1	MYO5B located in 18q21.1
Clinical features			
Clinical features	Cholestatic jaundice with pruritus	Rapidly progressive neonatal-onset cholestasis with uncorrectable coagulopathy	Cholestasis with pruritus, with/without transient, recurrent or progressive diarrhea (association with MVID)
Extrahepatic features	Neurological and respiratory symptoms	-	-
ICP	Yes	Yes (uncommon)	No
Laboratory parameters			
AST/ALT	Elevated	Moderate elevation	Mild to moderate elevation
GGT	Normal or mild elevation	Normal	Normal
Coagulopathy	Late-onset	Early-onset	Late-onset
Alpha fetoprotein	Normal, elevated in cases with HCC	Elevated	Normal
S. Bile acids	Elevated	Elevated	Elevated
Histopathology			
Canalicular cholestasis	Yes	Yes	Yes
Portal/lobular fibrosis	Yes	Yes	Yes
Giant-cell transformation	Yes	Diffuse	Sparse
Ductular reaction	No	Yes	Yes
Hepatocyte necrosis	Yes	-	-
Cirrhosis	Yes	Yes	Less common
Immunohistochemistry			
BSEP	Present	Absent BSEP staining on bile canaliculus	Abnormally thick, irregular and granular positivity that overflows into subcanalicular area
MDR3	Present	Present	Thickened canalicular staining granular and patchy pattern overflows into subcanalicular area
TJP2	Absent expression in canalicular membrane	Present	Present
Claudin1	Absent or reduced staining on bile canaliculi	Present	Present
FXR	Normal	Absent staining on bile canaliculus	Normal
MYO5B/RAB11	Normal	Normal	Intense, granular staining pattern in hepatocyte cytoplasm, and weak/loss of canalicular expression
Progression	Rapid	Very rapid	Slow
Complications	Hepatocellular carcinoma	Post-transplant graft steatosis similar to PFIC1	Worsening of cholestasis post intestinal transplant
Treatment			
Medical management	UDCA, Rifampicin	Minimal role	UDCA, rifampin, cholestyramine
Biliary diversion	PEBD some role	Not tried	Cholestasis subsides after BD in MVID patients with cholestasis
Liver transplant	Yes	Yes	Yes. Combined liver intestinal transplant in children with MVID and ongoing cholestasis

ALT: Alanine aminotransferase; ASBT: Apical sodium-dependent bile acid transporter; AST: Aspartate aminotransferase; BD: Biliary diversion; BSEP: Bile salt export pump; FXR: Farnesoid X receptor; GGT: Gamma-glutamyl transferase; HCC: Hepatocellular carcinoma; MDR3: Multidrug resistance class 3 glyco-protein; ICP: Intrahepatic cholestasis of pregnancy; MVID: Microvillus inclusion disease; MYO5B: Myosin-5b; NBD: Nasobiliary drainage; PEBD: Partial external biliary drainage; PFIC1Progressive familial intrahepatic cholestasis-1; RAB11: RAS-related GTP-binding protein-11; TJP2: Tight junction protein-2; UDCA: Ursodeoxycholic acid.

the panel yields a negative result and the index of suspicion is high, further testing by the whole exome (WES) or whole-genome (WGS) sequencing may be done. The presence of variables of unknown significance and monoallelic pathogenic/likely pathogenic variants in a significant proportion of cases highlights the complexity of analysis and the need for expertise for proper interpretation. Also, the ongoing discovery of new genes requires expansion of the genetic testing panel from time to time.

DIFFERENTIAL DIAGNOSIS

The main differentials to be considered in a patient with intrahepatic cholestasis with low-normal GGT (< 100 U/L) include bile acid synthetic defect (BASD), arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome, and USP53 related cholestasis, apart from the different types of PFIC (1, 2, 4, 5 and MYO5B associated). Type 3 PFIC (ABCB4) has raised GGT[1,30]. The serum bile acids are raised in PFIC and ARC syndrome, while they are low in the BASD. These entities can be differentiated by their distinct clinical presentation and liver histopathology with immunohistochemistry. However, the confirmation of diagnoses is best done by genetic analysis.

Bile acid synthetic defects

In bile acid synthetic defects (BASD) there is an accumulation of toxic bile acid intermediates in the hepatocytes due to deficiency of various enzymes involved in bile acid synthesis. Patients present with cholestatic jaundice, overt steatorrhea and florid manifestations of fat-soluble vitamin deficiencies like rickets. Pruritus is distinctly uncommon. Sometimes they may also present with neonatal liver failure, and cholestatic liver disease along with neurological manifestations like hypotonia, seizures[31]. BASD is diagnosed with fast-atom bombardment mass spectrometry of urine, which shows the accumulation of the distinct bile acid intermediaries due to a block in the bile acid synthesis pathway. Genetic analysis is confirmatory. Supplementation of cholic acid (CA) and chenodeoxycholic acid (CDCA) along with fat-soluble vitamins is the mainstay of therapy[32].

ARC syndrome

ARC syndrome (MIM 208085) is a rare multisystem disorder with autosomal recessive inheritance. It includes a triad of arthrogryposis, renal tubular acidosis, and neonatal cholestatic jaundice. Some patients may have accompanying features like ichthyosis (approximately 50%), platelet anomalies (approximately 25%), agenesis of the corpus callosum (> 20%), congenital cardiovascular anomalies (approximately 10%), and deafness. The clinical features are very useful to suspect the diagnosis, which is confirmed by a demonstration of mutations in the VPS33B or VIPAR gene. Histopathology shows bile duct paucity, giant cell transformation, bile plugs, and portal fibrosis. Caution is required before proceeding with a renal or liver biopsy due to the increased risk of a life-threatening bleed. Treatment is supportive and includes management of joint contractures, renal tubular acidosis, and cholestasis (UDCA, fat-soluble vitamins)[33].

USP53 related cholestasis

USP53 encodes an enzyme known as ubiquitin carboxyl-terminal hydrolase 53, which belongs to the de-ubiquitinating enzyme family and helps in maintaining cell integrity by interacting with TJP2 in hepatocytes. Whole-exome sequencing in 69 Han Chinese infants, with low GGT cholestasis without any pathological variants in ATP8B1, ABCB11, NR1H4, TJP2, and MYO5B genes, showed the presence of biallelic *USP53* mutations (homozygous or compound heterozygous) in 7 patients[34]. All these children had cholestatic jaundice in infancy and responded to medications (UDCA, cholestyramine). Liver biopsy showed varying levels of lobular disarray and hepatocellular and canalicular cholestasis, rosetting, portal tract fibrosis, ductular prolif-

eration, and giant-cell transformation. Ultrastructural examination in 2 cases revealed abnormality of tight junction complexes and expression of TJP2 and CLDN1 were reduced. Two children also had sensorineural hearing loss. In another report on the novel USP53 mutation, three members from the same family (2 sisters and a cousin) had low-GGT cholestasis, pruritus, elevated transaminases, very high alkaline phosphatase, and sensorineural hearing loss (n = 2). One of them required LT because of intractable pruritus[35].

Alagille syndrome

Alagille syndrome is also known as arteriohepatic dysplasia or syndromic paucity of interlobular bile ducts. This disorder is autosomal dominant with variable phenotypic penetrance. Alagille syndrome is one of the commonest causes of genetic cholestasis [36]. The defining feature is cholestasis with multisystemic involvement. Features include neonatal cholestasis in 95%, extrahepatic biliary hypoplasia, pruritus, xanthoma and associated facial dysmorphism. Structural cardiac defects such as peripheral pulmonary stenosis and septal defects are seen in 88%. Vertebral anomalies, ocular abnormalities most commonly posterior embryotoxon, renal dysplasia, vascular anomalies like Moyamoya disease, carotid and subclavian artery aneurysm are the other systemic features. Genetic analysis reveals *JAG1* mutation in the majority (approximately 90%) and *NOTCH2* mutation in minority[35].

Citrin deficiency

It is caused due to SLC25A13 (Solute Carrier family 25) gene mutation located in chromosome 7q21.3. The disease spectrum includes neonatal intrahepatic cholestasis, failure to thrive and dyslipidemia, and adult-onset type II citrullinemia. Chubby cheeks in infancy are a hallmark finding. These children also have a characteristic history of aversion to carbohydrates and a dietary preference towards a protein and lipid-rich diet[37].

Neonatal ichthyosis-sclerosing cholangitis syndrome

Neonatal Ichthyosis Sclerosing cholangitis is a rare cause of neonatal cholestasis with an autosomal recessive inheritance pattern. It is caused due to a mutation in the CLDN1 gene which encodes the CLDN1 protein located at the tight junction. This condition presents with neonatal cholestasis, cicatricial alopecia, ichthyosis and pruritus. Magnetic resonance cholangiopancreatography will show features of sclerosing cholangitis[38].

Other PFIC subtypes

Amongst the different PFIC subtypes, PFIC 1, 2, 4, 5 and 6 have low-normal GGT cholestasis. The presence of diarrhea is a feature of PFIC 1 and MYO5B disease. While neurological symptoms may be seen in ARC syndrome and sometimes in patients with MVID, a higher risk of HCC is a feature of TJP2 and BSEP deficiency. Table 4 gives the comparison of the clinical features and investigations in TJP2, FXR, and MYO5B defects. A detailed description and comparison of PFIC 1 and 2 are given elsewhere [39].

TREATMENT

Medical management

The main components include counselling of parents in detail, providing adequate nutrition, correcting vitamin deficiencies, controlling pruritus, managing complications like ascites, variceal bleeding *etc.*, growth monitoring, and vaccination[40].

Nutritional therapy: A diet that provides adequate calories (125%-140% of RDA) and protein (2-3 g/kg) with supplementation of medium-chain triglyceride and fat-soluble vitamins is recommended[41]. The doses of vitamin supplementation may need modification based on clinical signs and symptoms of vitamin deficiency and serum level monitoring (if available). Anemia, if present, needs to be corrected. Age-appropriate immunization including vaccination against hepatotropic viruses (hepatitis A and hepatitis B) is essential.

Management of pruritus: Pruritus is one of the most disabling symptoms in these children. Apart from skincare, medications such as UDCA, cholestyramine, rifampicin, naltrexone, and sertraline are used for controlling pruritus. These aspects have been

addressed in detail elsewhere[42]. There are no published reports on the use of FXR agonists like obeticholic acid, or apical sodium–bile acid transporter inhibitors like maralixibat in PFIC 4, 5 and MYO5B related diseases. Long-term follow-up includes growth monitoring, monitoring for nutritional deficiencies, and HCC surveillance, especially in TJP2 related cholestasis.

Biliary diversion

Biliary diversion (BD) takes away bile from the intestine, thereby reducing the reabsorption of bile acids through the enterohepatic circulation[43]. It has an important role in the alleviation of pruritus that is refractory to medical management in PFIC 1 and 2[44]. The role of BD is not well known in the newer variants of PFIC. BD has been tried in MVID patients who developed worsened cholestasis post intestinal transplant and was found to be helpful[17]. In MYO5B mutation, the ongoing cholestatic liver disease worsens after the intestinal transplant, leading to progressive liver fibrosis. Hence combined liver and intestinal transplantation are preferred. But in cases of isolated intestinal transplants, gallbladders should be preserved so that in case the cholestasis worsens, partial external biliary drainage can be attempted. The ileal bypass should be avoided as it removes a part of the transplanted bowel and doesn't result in long-term remission of cholestasis[16].

Liver transplant

LT is to be considered in children with decompensated chronic liver disease, growth failure (not amenable to dietary modification), refractory pruritus, or associated complications like hepato-pulmonary syndrome. In NR1H4 related PFIC, an early transplant may be required due to progressive liver disease with decompensation. Post liver transplant graft steatosis may develop in patients with NR1H4 mutation-associated cholestasis[6].

Genetic counseling

Once a child is confirmed to have PFIC, parents need to be counselled about the nature of the disease and the autosomal recessive pattern of inheritance. A geneticist should be involved in counselling about future pregnancies and testing during pregnancy[45].

CONCLUSION

TJP2, FXR, and MYO5B are recent additions to the three well-known types of PFIC (1, 2, and 3). This review has described the genetics, clinical profile, investigative findings, and treatments of these newer entities. There are gaps in our understanding of these conditions due to the limited literature at present. Advances in bioinformatics and techniques of next-generation gene-sequencing will help us study the genotype-phenotype correlation and synergistic effect of multiple mutations. Despite the recognition of these entities, not all cases with the PFIC phenotype have a confirmed genetic diagnosis, which indicates the presence of other causative genes that are waiting to be discovered.

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MINIREVIEWS

Deep learning in hepatocellular carcinoma: Current status and future perspectives

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Abstract

Hepatocellular carcinoma (HCC) is among the leading causes of cancer incidence and death. Despite decades of research and development of new treatment options, the overall outcomes of patients with HCC continue to remain poor. There are areas of unmet need in risk prediction, early diagnosis, accurate prognostication, and individualized treatments for patients with HCC. Recent years have seen an explosive growth in the application of artificial intelligence (AI) technology in medical research, with the field of HCC being no exception. Among the various AI-based machine learning algorithms, deep learning algorithms are considered state-of-the-art techniques for handling and processing complex multimodal data ranging from routine clinical variables to high-resolution medical images. This article will provide a comprehensive review of the recently published studies that have applied deep learning for risk prediction, diagnosis, prognostication, and treatment planning for patients with HCC.

Key Words: Hepatocellular carcinoma; Artificial intelligence; Deep learning

Core Tip: There are emerging roles for deep learning technology in the field of hepato-

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cellular carcinoma (HCC) including HCC risk prediction, as well as diagnosis, prognostication, and treatment planning leveraging readily available data from radiologic and histopathologic medical images. This article will provide a comprehensive review of the recently published studies that have applied deep learning for risk prediction, diagnosis, prognostication, and treatment planning for patients with HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive primary liver cancer that develops in the setting of chronic parenchymal liver diseases, and is among the top causes of cancer incidence and mortality worldwide[1,2]. While the burden of HCC has been declining with effective antiviral therapy against hepatitis B virus (HBV) and hepatitis C virus (HCV), HCC incidence related to metabolic syndrome will likely continue to rise due to the dramatic increase in the prevalence of non-alcoholic fatty liver disease (NAFLD) in the general population[3]. Decades of HCC research led to the development of a screening protocol, non-invasive diagnostic modalities based on imaging, and various treatment modalities including surgical, locoregional and systemic therapies[4,5]. However, the overall outcomes of patients with HCC continue to remain poor and there are areas of significant unmet need in risk prediction, early detection, accurate prognostication, and individualized treatments for patients with HCC.

Patients with HCC generate enormous amounts of health data. While promising for researchers, ensuring that such high volumes of data are turned into actionable knowledge can be a significant challenge. Artificial intelligence (AI) is thought to be capable of synthesizing and analyzing multimodal data with superhuman degrees of accuracy or reliability, and recent years have seen a rapid growth in the application of AI to many fields of medicine including hepatology[6]. This "AI revolution" over the past decade has been possible due to the advent of deep learning technology. Deep learning algorithms can process a broad spectrum of medical data from structured numeric data such as vital signs and laboratory values, high dimensional data from multi-omics studies, as well as digitized high-resolution images from various radiologic and histopathologic studies. This review aims to provide an overview as well as highlight examples of the many potential applications of deep learning to improve the care of patients with HCC.

AI, MACHINE LEARNING, AND DEEP LEARNING

AI-based approaches provide a variety of methods for a range of tasks and clinical application including image classification, organ and lesion segmentation, accurate extraction of key imaging features and measurements, tumor detection, stratification of high-risk subjects, prediction of disease and treatment outcome (Figure 1). Advancements in AI in recent years, particularly in the realm of medical image processing and analysis, offer an enormous range of automated tools for extracting precise measurements of biomarkers, revealing complex features, quantifying tissue characteristics and performing radiomics for deep analysis of raw imaging data.

The term "artificial intelligence" encompasses a broad range of technology that enables machines to perform tasks typically thought to require human reasoning and problem-solving skills[7]. "Machine learning" is a branch of AI in which computer algorithms train on sample data to build a mathematical model that makes predictions or decisions without being explicitly programmed to do so[8]. Machine learning algorithms can be broadly divided into supervised and unsupervised learning. Supervised learning algorithms train on sample data with labeled outcome data, and their goal is to learn the relationship between the input data and the outcomes to make

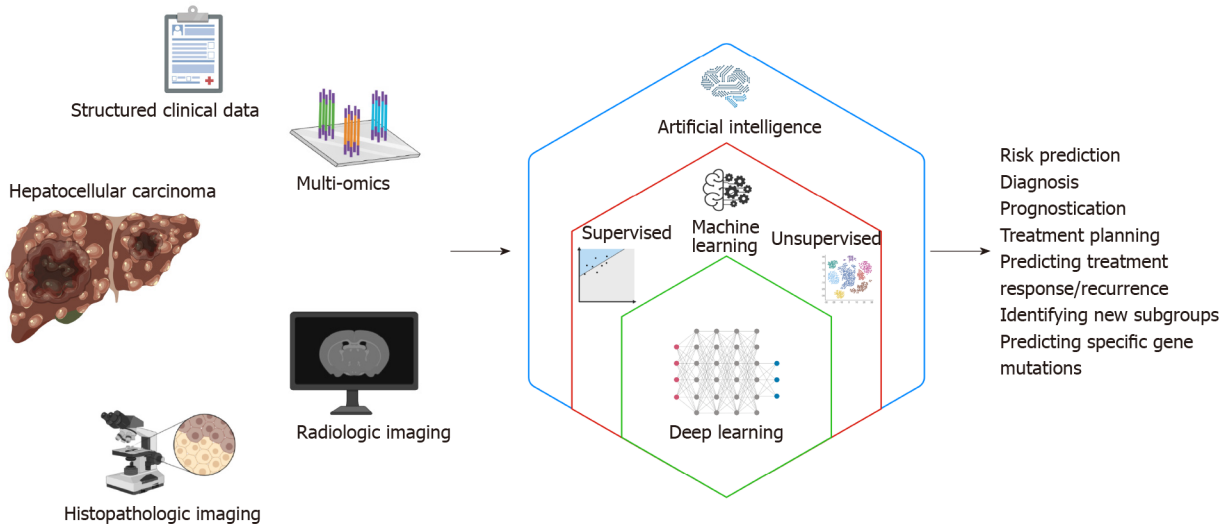

Figure 1 Schematic representation of the relationships between the terms artificial intelligence, machine learning, and deep learning, and how deep learning can utilize multimodal data to improve care for patients with hepatocellular carcinoma.

accurate predictions about the outcome when provided with a new set of input data [9]. Examples of supervised learning algorithms include traditional techniques such as linear regression and logistic regression, as well as more sophisticated techniques including support vector machines, random forest and gradient boosting. On the other hand, unsupervised learning algorithms train on unlabeled sample data and analyze the underlying structure or distribution within the data to discover new clusters or patterns[10]. Examples of unsupervised learning algorithms include K-means and principle component analysis among many others.

Among the various AI-based machine learning algorithms, artificial neural networks (ANNs) consist of layers of interconnected mathematical formulas that enable them to analyze complex non-linear relationships[11]. "Deep learning (DL)" refers to highly complex AI models utilizing multiple layers of ANNs and has recently emerged as a state-of-the-art AI technique for analyzing complex, high-dimensional healthcare data. Some of the commonly used DL techniques include convolutional neural networks (CNNs) and recurrent neural networks (RNNs)[12]. CNNs have connective patterns resembling those of an animal visual cortex and can detect inherent spatial features of high dimensional images. RNNs have connections forming a directed graph along a temporal sequence, and therefore can be highly useful in time series prediction.

It is crucial to recognize that any AI-based machine learning algorithms require external validation in an independent dataset as models could be overfitted and end up overestimating the performance. In this review article, the performance characteristics of the various DL models are from the validation cohorts, and not the original derivation cohorts used to train the algorithms.

HCC CLINICAL DATA

Despite multiple available risk prediction tools for HCC, none have been rigorously validated or endorsed by major liver societies. Currently, HCC surveillance is recommended for patients with cirrhosis and high risk patients with chronic HBV infection [13]. Accurate prediction models utilizing more specific risk factors for HCC development at individual levels would allow health systems to implement targeted screening strategies. Ioannou *et al*[14] trained a RNN to predict HCC development within 3 years using 4 baseline variables and 27 longitudinal variables from 48151 patients with HCV-related cirrhosis in the national Veterans Health Administration. The RNN model significantly outperformed logistic regression and exhibited an area under the curve (AUC) of 0.759 among all samples and an AUC of 0.806 among patients with sustained virologic response. Phan *et al*[15] surveyed 1 million random samples from Taiwan's National Health Insurance Research Database between 2002 to 2010 to predict liver cancer among patients with viral hepatitis. The disease history of

each patient was transformed into a 108 × 998 matrix and applied to a CNN, which predicted liver cancer with an AUC of 0.886 and an accuracy of 0.980. Another study by Nam *et al*[16] constructed a deep neural network to predict 3-year and 5-year incidence of HCC in 424 patients with HBV-related cirrhosis on entecavir therapy. When applied to an external validation cohort of 316 patients, the DL model achieved a Harrell's C-index of 0.782 and significantly outperformed 6 previously reported models based on traditional modeling. The same group also developed another DL model called the AI-based Model of Recurrence after Liver Transplantation (MoRAL-AI) to predict HCC recurrence after liver transplantation using variables such as tumor diameter, age, alpha-fetoprotein (AFP), and prothrombin time[17]. The MoRAL-AI showed significantly better predictive performance compared to conventional models such as the Milan, UCSF, up-to-seven, and Kyoto criteria (C-index = 0.75 *vs* 0.64, 0.62, 0.50, 0.50, respectively; $P < 0.001$).

HCC MULTI-OMICS

Serum AFP has been widely used as a predictive and prognostic biomarker for HCC [18], but AFP has limited sensitivity for detecting early-stage HCC and its levels do not reliably correlate with disease progression[19]. Recent advances in multi-omics related to HCC are expected to address this unmet need for novel biomarkers. Multi-omics refers to an approach to biological analysis which utilizes data sets from multiple "omics", such as the genome, epigenome, transcriptome, proteome, metabolome and microbiome. Multi-omics experiments generate an enormous amount of information, and various machine learning techniques including DL that can help with the computational challenges of processing and analyzing such high dimensional data. Xie *et al*[20] used gene expression profiling of peripheral blood to build an ANN model that classifies HCC patients from a control group. Using a nine-gene expression system, the ANN was able to distinguish HCC patients from controls with an AUC of 0.943, 98% sensitivity, and 85% specificity, although it should be noted that the control group was healthy individuals rather than patients with cirrhosis, which could have overestimated the performance of the model. Choi *et al*[21] proposed a novel network-based DL method to identify prognostic gene signatures *via* G2Vec, a modified Word2Vec model originally used for natural language processing (NLP). When applied to gene expression data for HCC from the Cancer Genome Atlas (TCGA), G2Vec showed superior prediction accuracy for patient outcomes compared to existing gene selection methods and was able to identify two distinct gene modules significantly associated with HCC prognosis. Chaudhary *et al*[22] used RNA se×quencing, miRNA, and methylation data of 360 HCC patients from TCGA to build an autoencoder, which is an unsupervised feed-forward neural network. Using this DL model, they were able to distinguish patients with survival differences and identify specific mutations and pathways as predictors of aggressive tumor behavior.

RADIOLOGY

HCC diagnosis and segmentation

In recent years, there have been remarkable advances in the application of AI for the interpretation of medical imaging, primarily due to the use of DL algorithms using CNN[23]. CNN algorithms trained on ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI) images have shown excellent performances in detection of lesions, classification of lesions, segmentation of organs or anatomic structures, and imaging reconstruction[24].

In 2012, Streba *et al*[25] prospectively studied contrast-enhanced ultrasound images of 112 patients to train an ANN that classified five different types of liver tumors. The ANN showed promising performances with accuracies of 94.5% in the training set and 87.1% in the testing set. In 2017, Hassan *et al*[26] reported using the stacked sparse auto-encoder, an unsupervised DL technique, to segment and classify liver lesions on ultrasound images with a classification accuracy of 97.2%. Additionally, Bharti *et al*[27] built a CNN using echotexture and roughness of liver surface on 754 segmented ultrasound images, which differentiated between normal liver, chronic liver disease, cirrhosis, and HCC with a classification accuracy of 96.6%. Schmauch *et al*[28] also created a CNN which detects and characterizes benign and malignant focal liver lesions on 2-D ultrasound images from 367 patients from various institutions. When

applied to a new dataset of 177 patients, the model achieved a weighted mean AUC of 0.891. Recently, Brehar *et al*[29] conducted a study comparing CNN's performance for HCC detection on ultrasound images against conventional machine learning algorithms including multi-layer perceptron, support vector machines, random forest and AdaBoost. The CNN achieved an AUC of 0.95% with 91.0% accuracy, 94.4% sensitivity, and 88.4% specificity and significantly outperformed the conventional machine learning algorithms. Beyond detecting the actual presence of HCC on ultrasound images, studies have also attempted to predict the risk of future HCC development based on analyzing the ultrasound images of liver parenchyma in patients without HCC. For example, Jin *et al*[30] performed a DL radiomics analysis on 2-D shear wave elastography and corresponding B-mode ultrasound images of 434 chronic HBV patients, which predicted 5-year HCC development with AUC of 0.900 in the test cohort.

In addition to ultrasound images, cross-sectional imaging from CT or MRI studies serve as an extremely abundant and promising source of data for DL. In 2018, Yasaka *et al*[31] used CT image sets of liver masses from 460 patients to train a CNN that can classify liver lesions into five categories of: (1) HCC; (2) Other malignant tumors; (3) Indeterminate masses; (4) Hemangiomas; and (5) Cysts with a median AUC of 0.92. Shi *et al*[32] showed that incorporation of a CNN enabled identification of HCC using a three-phase CT imaging protocol with a diagnostic accuracy similar to that of a four-phase protocol, which would allow patients to receive lower doses of radiation. Segmentation of HCC, liver parenchyma, and other organs on CT scan is very important for determination of tumor extent and treatment planning, but manual contouring of the images is highly time-consuming and subject to inter-observer variability. The 2017 International Conference On Medical Image Computing Computer Assisted Intervention called for a Liver Tumor Segmentation Benchmark (LITS) challenge, encouraging researchers to develop automatic segmentation algorithms to segment liver lesions using 200 CT scans (training: 130; testing: 70) provided by clinical sites around the world. Several teams participating in the challenge have developed DL algorithms with promising performances for HCC segmentation using CT images[33-37]. Beyond the LITS challenge, there are ongoing research efforts to improve segmentation using different architectures of DL networks [38-42].

Hamm *et al*[43] used MRI images from 494 patients to train a CNN which can classify hepatic lesions into six different categories. When applied to random cases in the test set, the CNN outperformed expert radiologists (90% sensitivity and 98% specificity *vs* 82.5% sensitivity and 96.5% specificity) and especially for HCC detection (90% sensitivity *vs* 60%-70% sensitivity). The same group conducted additional studies to make their CNN interpretable by generating highlighted feature maps corresponding to liver lesions[44]. Wu *et al*[45] built a CNN using multiphase MRI images and achieved an AUC of 0.95 for distinguishing Liver Imaging Reporting and Data System (LI-RADS) grade 3 from LI-RADS 4 and 5 lesions for HCC diagnosis. Zhen *et al* [46] also trained a CNN model combining unenhanced MRI images and clinical variables from 1210 patients with liver tumors, which demonstrated diagnostic performances on par with three experienced radiologists using enhanced MRI images.

HCC prognostication, treatment planning, and response to treatment

In addition to serving as accurate and efficient tools for diagnosis of HCC, DL models utilizing radiology data can also be used for prognostication, treatment planning, and assessing tumor response to therapy. Vascular invasion is a key prognostic element in patients with HCC. Recent studies developed CNN models with promising ability to detect microvascular invasion on MRI images of HCC patients undergoing surgical resection[47-49]. An *et al*[50] used an unsupervised CNN-based deformable image registration technique to assess the relationship between ablative margins and local tumor progression in 141 patients with single HCC who underwent microwave ablation, and demonstrated that patients with ablative margins < 5 mm were at significantly higher risk of local tumor progression. Liu *et al*[51] developed a DL radiomics model to predict responses to trans-arterial chemoembolization (TACE) using ultrasound images of 130 HCC patients, which accurately predicted TACE response with an AUC of 0.93. The same group also assessed their ultrasound-based DL radiomics model to predict 2-year progression-free survival among 419 HCC patients and facilitate optimized treatment selection. Peng *et al*[52] trained a residual CNN model to predict response to TACE using CT images from 562 patients with intermediate-stage HCC undergoing TACE, which showed accuracies of 85.1% and 82.8% in two external validation cohorts. Another study developed a DL score for disease-specific survival by using CT images in a cohort of 243 patients with HCC

treated with TACE, with a higher score predicting poor prognosis [hazard ratio (HR): 3.01; 95% cumulative incidence (CI): 2.02-4.50][53]. Finally, Zhang *et al*[54] built a DL-based model predicting overall survival using CT images from 201 patients with unresectable HCC treated with TACE and sorafenib, which achieved superior predictive performance compared to the clinical nomogram (C-index of 0.730 *vs* 0.679, $P = 0.023$).

HCC PATHOLOGY

Automated interpretation of histopathologic images from liver biopsy is another major area of medical imaging in patients with HCC where DL can be utilized. In addition to effectively replicating the human pathologists' jobs of diagnosing and grading HCC, DL models can help identify and analyze additional complex imaging features and patterns which are related to specific mutations and disease prognosis. Lin *et al*[55] used images from multiphoton microscopy of 113 HCC patients to train a CNN with over 90% accuracy for determining HCC differentiation. Kiani *et al*[56] developed a CNN-based "Liver Cancer Assistant" which accurately differentiated hematoxylin and eosin (H&E) images of HCC and cholangiocarcinoma and helped improve the diagnostic performance of nine pathologists. Liao *et al*[57] used TCGA dataset for training a CNN that distinguished HCC from adjacent normal tissues with perfect performance (AUC: 1.00) and predicted the presence of specific somatic mutations with AUCs over 0.70. Wang *et al*[58] trained a CNN for automated segmentation and classification of individual nuclei at single-cell levels on H&E-stained tissue sections of HCC tumors from TCGA, and performed feature extraction to identify 246 quantitative image features. Then, a clustering analysis by an unsupervised learning approach identified three distinct histologic subtypes which were independent of previously established genomic clusters and had different prognosis. Chen *et al*[59] trained a CNN for automatic grading of HCC tumors on histopathological H&E images, which showed 96% accuracy for benign and malignant classification and 89.6% accuracy for the degree of tumor differentiation, and predicted the presence of specific genetic mutations.

Lu *et al*[60] applied three pre-trained CNN models to extract imaging features from HCC histopathology and performed Cox proportional hazards analysis to predict overall survival and disease-free survival, and observed significant correlations between the imaging features and established biological pathways. Saillard *et al*[61] used two DL algorithms based on whole-side digitized histological slides from 194 patients with HCC to predict the survival of patients treated by surgical resection. When tested on an independent validation set from TCGA, both DL models had a higher discriminatory power than a score combining all baseline variables associated with survival. Shi *et al*[62] built an interpretable DL framework using pathologic images from 1445 patients with HCC and developed a "tumor risk score" which showed prognostic performances independent of and superior to clinical staging systems and stratified patients into five groups of different prognosis. A recent study by Yamashita *et al*[63] developed a histopathology-based DL based system which stratified patients with risk scores for postsurgical recurrence of HCC.

FUTURE DIRECTION

There are several key issues to address before DL-based AI models can be universally implemented in real world clinical practice settings. Due to their complexity, DL models are traditionally considered to be "black-box" models, meaning humans cannot understand how the DL models make their predictions. Interpretability of the DL models are crucial for physicians to accept and trust them in everyday clinical practice, and for troubleshooting and improving the models for rare cases. This is being addressed by recent developments in various "explainable AI" techniques but currently there is no clear consensus on the best methodology. Another potential limitation is the generalizability of the individual DL algorithms. Concerns have been raised that AI algorithms developed at highly specialized academic medical centers using their own patients' data may over-represent certain groups of patients and not accurately reflect the real-world population of patients seen at local community hospitals. Finally, AI models, like other prediction models, are often not publicly available, limiting external validation. Independent validation of the proposed model and comparison to old models are as important as deriving new models. Large-scale,

Table 1 Studies applying deep learning for hepatocellular carcinoma

Study	Cohort	Data source	Deep learning	Input	Output	Main findings
Predicting HCC risk using clinical variables						
Ioannou *et al*[14] 2020	48151 HCV cirrhosis (T: 90%, V: 10%)	VHA database	RNN	Clinical variables	Risk of HCC development	RNN predicted HCC development with AUC of 0.759, and AUC of 0.806 among those who achieved SVR
Phan *et al*[15] 2020	6052 HBV and HCV (T: 70%, V: 30%)	Taiwanese NHIRD	CNN	Disease history data	Risk of HCC development	CNN achieved an accuracy of 0.980 and AUC of 0.886 for predicting HCC development among viral hepatitis patients
Nam *et al*[16] 2020	T: 424 HBV cirrhosis; V: 316 HBV cirrhosis	2 Korean centers	ResNet	Clinical variables	Risk of HCC development	DL model achieved an accuracy of 0.763 and AUC of 0.782 in the validation cohort and outperformed previous models
Nam *et al*[17] 2020	T: 349 LT recipients; V: 214 LT recipients	3 Korean LT centers	ResNet	Clinical variables	Recurrent HCC after LT	DL model significantly outperformed conventional models in prediction of post-T HCC recurrence with AUC of 0.75
Multi-omics-based HCC diagnosis and prognostication						
Xie *et al*[20] 2018	T: 133 HCC/54 HV; V: 52 HCC/34 HV	1 center in China	ANN	Gene expression	HCC detection	ANN using nine genes had an AUC of 0.943, 98% sensitivity, and 85% specificity for classifying HCC
Choi *et al*[21] 2018	135 HCC (10-fold CV)	TCGA	G2Vec	Gene expression	HCC prognosis	G2Vec showed significantly higher prediction accuracy for patient outcomes compared to existing gene selection tools
Chaudhary *et al* [22] 2018	T: 360 HCC; V: 220, 221, 166, 40, 27 HCC	TCGA; 5 external datasets	Auto-encoder	RNA-seq, miRNA-seq, methylation	HCC prognosis	DL model distinguished groups with survival differences and identified mutations and pathways predicting aggressive tumor behavior
Radiology-based HCC diagnosis/prediction						
Streba *et al*[25] 2012	112 FLL (10-fold CV)	1 center in Romania	ANN	US images	FLL type	ANN had 87.12% testing accuracy, 93.2% sensitivity, and 89.7% specificity for classifying 5 classes of liver lesions
Hassan *et al*[26] 2017	110 FLL (10-fold CV)	1 center in Egypt	Auto-encoder	US images	FLL type	The proposed system had 97.2% accuracy, 98% sensitivity, and 95.70% specificity for classifying liver lesions
Bharti *et al*[27] 2018	24 normal, 25 CLD, 25 cirrhosis, 20 HCC	1 center in India	CNN	US images	Liver stages	CNN achieved 96.6% classification accuracy for differentiating normal liver, CLD, cirrhosis, and HCC
Schmauch *et al* [28] 2019	T: 367 FLL; V: 177 FLL	Centers in France	ResNet	US images	FLL type	DL model reached mean AUC of 0.935 for focal liver lesion detection and 0.916 for focal liver lesion characterization
Brehar *et al*[29] 2020	T: 200 HCC; V: 68 HCC	1 center in Romania	CNN	US images	HCC detection	CNN achieved AUC of 0.95, accuracy of 0.91, 94.4% sensitivity and 88.4% specificity for HCC detection
Jin *et al*[30] 2021	434 HBV (3:1:1 split)	1 center in China	DL radiomics	US images	Risk of HCC development	DL radiomics model predicted 5-yr HCC development risk with AUC of 0.900 in the test set
Yasaka *et al*[31] 2018	T: 460 liver masses; V: 100 liver masses	1 center in Japan	CNN	CT images	Liver mass type	CNN classified liver lesions into five categories with a median AUC of 0.92
Shi *et al*[32] 2020	449 FLL; (T: 80%, V: 20%)	1 center in China	CNN	CT images	FLL type	CNN applied to three-phase CT protocol images achieved AUC of 0.925 for differentiating HCC

						from other FLLs
Hamm *et al*[43] 2019	T: 434 FLL; V: 60 FLL	1 center in United States	CNN	MRI images	FLL type	CNN achieved 90% sensitivity and 98% specificity for classifying FLLs and AUC of 0.992 for HCC classification
Wang *et al*[44] 2019	T: 434 FLL; V: 60 FLL	1 center in United States	CNN	MRI images	FLL type	Interpretable DL system achieved 76.5% PPV and 82.9% sensitivity for identifying correct radiological features
Wu *et al*[45] 2020	89 liver tumors; (60: 20: 20)	1 center in United States	CNN	MRI images	LI-RADS grading	CNN achieved AUC of 0.95, 90% accuracy, 100% sensitivity and 83.5% PPV for LI-RADS grading of liver tumors
Zhen *et al*[46] 2020	T: 1210 liver tumors; V: 201 liver tumors	1 center in China	CNN	MRI images	Liver tumor type	CNN combined with clinical data showed AUC of 0.985 for classifying HCC with 91.9% agreement with pathology
Radiology-based HCC prognostication, treatment planning, and response to treatment						
Zhang *et al*[47] 2021	T: 158 HCC; V: 79 HCC	1 center in China	CNN	MRI images	MVI in HCC	CNN achieved AUC of 0.72, 55% sensitivity, and 81% specificity for preoperative MVI in HCC patients
Wang *et al*[48] 2020	T: 60 HCC; V: 40 HCC	1 center in China	CNN	MRI images	MVI in HCC	Fusion of deep features from MRI images yielded AUC of 0.79 for MVI prediction in HCC patients
Jiang *et al*[49] 2021	405 HCC; (T: 80%, V: 20%)	1 center in China	CNN	CT images	MVI in HCC	CNN achieved AUC of 0.906 for prediction of MVI. Mean survival was significantly better in the group without MVI
An *et al*[50] 2020	141 single HCC resect MWA	1 center in China	CNN	MRI images	Ablative margin	Deep learning model accurately estimated ablative margins and risk of local tumor progression
Liu *et al*[51] 2020	T: 89 HCC resect TACE; V: 41 HCC rec. TACE	1 center in China	CNN	Ultrasound images	Response to TACE	Deep learning radiomics model predicted tumor response to TACE with AUC of 0.93
Peng *et al*[52] 2020	T: 562 HCC resect TACE; V:227 HCC rec. TACE	3 centers in China	CNN	CT images	Response to TACE	Deep learning model had accuracies of 85.1% and 82.8% for predicting TACE response in 2 validation cohorts
Liu *et al*[53] 2020	243 HCC resect TACE (6:1:3 split)	1 center in China	CNN	CT images	Post-TACE survival	Higher DL score was an independent prognostic factor and predicted overall survival with AUCs of 0.85-0.90
Zhang *et al*[54] 2020	201 HCC resect TACE + sorafenib (T: 120, V: 81)	3 centers in China	CNN	CT images	OS on TACE + sorafenib	Deep learning signature achieved C-index of 0.714 for predicting OS in HCC patients receiving TACE + sorafenib
Histopathology-based HCC diagnosis, subtyping, and outcome predictions						
Lin *et al*[55]2019	113 HCC	1 center in China	CNN	Histopath images	HCC differentiation	CNN achieved an accuracy of 0.941 for determining HCC differentiation on multiphoton microscopy
Kiani *et al*[56] 2020	70 WSI (35 HCC, 35 CC)	TCGA	CNN	Histopath images	HCC *vs* CC	CNN-based "Liver Cancer Assistant" accurately differentiated HCC *vs* cholangiocarcinoma
Liao *et al*[57] 2020	T: 491 HCC; V: 455 HCC	TCGA; 1 center in China	CNN	Histopath images	HCC detection, mutations	CNN distinguished HCC from adjacent tissues with AUC of 1.00 and predicted specific mutations with AUC over 0.70
Wang *et al*[58] 2020	T: 99 HCC; V: 205 HCC	TCGA	CNN	Histopath images	Histological HCC subtype	Unsupervised clustering identified 3 histological subtypes complementing molecular pathways and

						prognostic value
Chen *et al*[59] 2020	T: 402 HCC/89 normal; V: 67 HCC/34 normal	GDC portal; 1 center in China	CNN	Histopath images	HCC grade mutations	CNN achieved 89.6% accuracy for tumor differentiation stage and predicted presence of specific gene mutations
Lu *et al*[60] 2020	421 HCC/105 normal (6-fold CV)	GDC portal	CNN	Histopath images	HCC prognosis	Pre-trained CNN predicted OS using pathology images and identified HCC subgroups with different prognosis
Saillard *et al*[61] 2020	T: 194 HCC; V: 328 HCC	1 French center TCGA	CNN	Histopath images	Survival after HCC resection	CNN models using pathology images predicted survival with C-index 0.75-0.78 and outperformed conventional models
Shi *et al*[62] 2021	T: 1125 HCC; V: 320 HCC	1 center in China; TCGA	CNN	Histopath images	HCC outcomes	Deep learning-based "tumor risk score" was superior to clinical staging and stratified 5 groups of different prognosis
Yamashita *et al* [63] 2021	T: 36 WSI; V: 30 WSI	1 center in United States; TCGA	CNN	Histopath images	Post-surgical recurrence	CNN risk scores outperformed TNM system for predicting recurrence and identified high- and low-risk subgroups

ANN: Artificial neural network; AUC: Area under the curve; CC: Cholangiocarcinoma; CNN: Convolutional neural network; CV: Cross-validation; FLL: Focal liver lesion; GDC: Genomic Data Commons; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HV: Healthy volunteers; LT: Liver transplant; MVI: Microvascular invasion; MWA: Microwave ablation; OS: Overall survival; PFS: Progression-free survival; RFA: Radio-frequency ablation; RNN: Recurrent neural network; SR: Surgical resection; STS-net: Spatial transformed similarity network; SVR: Sustained virologic response; T: Training; TCGA: The Cancer Genome Atlas; V: Validation; VHA: Veterans Health Administration; WSI: Whole slide image; CT: Computed tomography; MRI: Magnetic resonance imaging; NHIRD: National Health Insurance Research Database; TNM: Tumor, Nodes, Metastasis; TACE: Trans-arterial chemoembolization; LI-RADS: Liver Imaging Reporting and Data System.

prospective, multi-centered studies involving diverse populations with external validation will be necessary before DL algorithms can be widely accepted.

A currently under-explored, but highly promising and exciting area for the application of DL is the field of autonomous robotics. In a recent editorial, Gumbs *et al* [64] state that while the current form of robotic surgery seems like a form of minimally invasive surgery, the true power of robotic surgery exists in its potential to create autonomous actions. Recently, a DL-based surgical instrument tracking algorithm was able to closely track the instruments during robotic surgery and evaluate the surgeons' performance, demonstrating that DL algorithms can learn the correct steps of robotic surgery[65]. With the help of DL and other AI technologies, it may be possible to imagine a future where fully autonomous robots perform resection of large, complex HCC in ways that no human surgeons can mimic. However, there are significant barriers before the idea of fully autonomous robotic surgery can become a reality, including the current technical limitations of autonomous surgical robotics, as well as the hesitation of patients and providers to fully trust autonomous robots to perform invasive operations. "Explainability" of the DL algorithms will be critical here, as humans would need to be able to understand and correct every single mistake that an autonomous robot makes during surgery. Therefore, for the foreseeable future, DL will most likely remain as a helpful, adjunctive tool to assist human surgeons.

CONCLUSION

This review has provided a comprehensive overview of various ways in which DL algorithms can be employed to assist medical providers and enhance the care of patients with HCC (Table 1). DL algorithms not only can efficiently and accurately replicate the same jobs performed by human physicians, but more importantly can help discover novel biologic pathways and disease subgroups with clinical significance by processing and analyzing complex high-dimensional data in ways impossible for the human brain.

Despite some important limitations to overcome, application of state-of-the-art AI technologies such as DL for the care of patients with HCC is no longer a futuristic idea but is rapidly becoming a reality. Most of the studies covered in this review were published within the past two years, and the number of studies utilizing DL continues

to increase exponentially. We anticipate that DL algorithms will soon take a major role in the diagnosis, prognostication, and treatment of patients with HCC.

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ORIGINAL ARTICLE

Basic Study

Gut dysbiosis and systemic inflammation promote cardiomyocyte abnormalities in an experimental model of steatohepatitis

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Abstract

BACKGROUND

Cardiovascular disease is the main cause of death in metabolic-associated fatty liver disease, and gut microbiota dysbiosis is associated with both of them.

AIM

To assess the relationship between gut dysbiosis and cardiovascular risk (CVR) in an experimental model of steatohepatitis.

METHODS

Adult male Sprague-Dawley rats were randomized to a control group (n = 10) fed a standard diet and an intervention group (n = 10) fed a high-fat choline-deficient diet for 16 wk. Biochemical, molecular, hepatic, and cardiac histopathology. Gut microbiota variables were evaluated.

RESULTS

The intervention group had a significantly higher atherogenic coefficient, Castelli's risk index (CRI)-I and CRI-II, interleukin-1β, tissue inhibitor of metalloproteinase-1 (all P < 0.001), monocyte chemoattractant protein-1 (P = 0.005), and plasminogen activator inhibitor-1 (P = 0.037) than the control group. Gene expression of miR-33a increased (P = 0.001) and miR-126 (P < 0.001) decreased in the intervention group. Steatohepatitis with fibrosis was seen in the intervention group, and heart computerized histological imaging analysis showed a significant decrease in the percentage of cardiomyocytes with a normal morphometric appearance (P = 0.007), reduction in the mean area of cardiomyocytes (P = 0.037), and an increase of atrophic cardiomyocytes (P = 0.007). There were significant correlations between the cardiomyocyte morphometry markers and those of progression and severity of liver disease and CVR. The intervention group had a lower Shannon diversity index and fewer changes in the structural pattern of gut microbiota (both P < 0.001) than controls. Nine microbial families that are involved in lipid metabolism were differentially abundant in intervention group and were significantly correlated with markers of liver injury and CVR.

CONCLUSION

The study found a link between gut dysbiosis and significant cardiomyocyte abnormalities in animals with steatohepatitis.

Key Words: Animal model; Cardiovascular diseases; Gut microbiota; Metabolic-associated fatty liver disease; Predicted lipid metabolism; Risk cardiovascular; Steatohepatitis

Core Tip: Cardiovascular disease is the main cause of death in metabolic-associated fatty liver disease (MAFLD) and gut microbiota dysbiosis is associated with both. Among the risk factors, we report significant correlations between the presence of atherogenic dyslipidemia, systemic inflammation, endothelial dysfunction, liver fibrogenesis, and gut dysbiosis, all of which contributed to the progression of MAFLD and increased cardiovascular risk.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common form of liver disease and a leading cause of morbidity and mortality in both developed and developing countries[1]. The natural course the disease encompasses a pathological spectrum of liver injury ranging from simple steatosis to steatohepatitis and progressive liver fibrosis that can result in cirrhosis and other complications, including liver decompensation and hepatocellular carcinoma (HCC)[1,2]. Recently, a new nomenclature, metabolic-associated fatty liver disease (MAFLD) was suggested because the disease is not only confined to the liver only, but rather represents a major part of a multisystemic disease that includes cardiovascular manifestations[3-6]. Indeed, cardiovascular disease (CVD) is the leading cause of death in patients with MAFLD, accounting approximately 40%–45% of the total deaths[4,7,8].

The association of steatohepatitis with CVD is related to the metabolic risk factors that they have in common, such as obesity, diabetes mellitus, hypertension, and dyslipidemia. However, multiple studies have shown that steatohepatitis is also independently associated with several markers of subclinical atherosclerosis[4,7,8]. Although the putative pathophysiological mechanisms that link steatohepatitis and CVD are still not completely explained, many nontraditional and emerging risk factors, including proinflammatory cytokines and procoagulant factors (*e.g.*, fibrinogen, plasminogen, and vascular adhesion molecules) are associated with the process[7,9]. Recently, the intestinal microbiome and its highly complex and interdependent interaction with host metabolism, immunity, and disease have opened a new horizon of investigation into the link between these clinical conditions[4,9,10]. Gut microbiota, or the bacterial components and metabolites carried to the liver through the portal vein, overstimulate immune cells and may result in more severe liver damage, inflammation, and fibrosis, thus accelerating the development of steatohepatitis and inducing the systemic inflammation and endothelial dysfunction that promotes increased cardiovascular risk (CVR)[4,10]. Despite considerable progress, understanding of the molecular mechanisms governing microbiota-host interactions is far from complete. Experimental studies are needed to further explore the mechanisms whereby gut microbiota contribute to steatohepatitis-associated CVR.

The goal of this study was to assess the relationships of the gut microbiota, steatohepatitis, and CVR, by describing the crosstalk among gut dysbiosis, associated metabolic predictions, systemic inflammation, endothelial dysfunction, paracrine cell signaling, and cardiomyocyte morphology in an experimental nutritional steatohepatitis model that mimics the metabolic changes found in humans.

MATERIALS AND METHODS

Animals and experimental model

Twenty 60-day-old adult male Sprague-Dawley rats weighing 280-350 g were used. The animals were kept in groups inside two polypropylene boxes in a controlled-temperature environment (22 ± 2 °C) and a 12-h light/dark cycle. All experimental procedures were approved by the Ethics Committee for the Use of Animals (No. 17-0021 and No. 17-0531) and were conducted following the international guidelines for animal welfare. Measures were taken to minimize animal pain and discomfort.

After acclimatization to the environment, the animals were randomized to two experimental groups according to their weight, as previously described[11]. The control group (n = 10) received a standard diet (Nuvilab CR-1, Quimtia S.A., Brazil). The intervention group (n = 10) received a high-fat, choline-deficient diet consisting of 31.5% total fat and enriched with 54.0% trans fatty acids (Rhoster Ltda., Brazil) to induce steatohepatitis. Both groups received water and food ad libitum during the study. After 16 wk of treatment, the animals were fasted for 8 h, anesthetized with isoflurane, and euthanized by cardiac exsanguination. Blood samples were collected and centrifuged to obtain the serum, which was kept at −80 °C until the analyses were performed. Pieces of hepatic and cardiac tissue were fixed in 10% formaldehyde for histopathological evaluation. Feces present in the intestine were collected aseptically and kept at −80 °C for analysis of the gut microbiota.

Atherogenic ratios

Serum total cholesterol (TC), low density lipoprotein-cholesterol (LDLC), high-density lipoprotein cholesterol (HDLC) and triglycerides (TG) were assayed with a Labmax 560[11]. Atherogenic ratios were calculated from the lipid profile and used as a tool for

the prediction of CVR. The ratios included Castelli's risk index (CRI)-I = TC/HDLC, CRI-II = LDLC/HDLC and the atherogenic coefficient (AC) = (TCH − DLC)/HDLC [12].

Systemic inflammation and endothelial dysfunction

The serum markers of inflammation and endothelial dysfunction markers included in the analysis were monocyte chemoattractant protein (MCP)-1, tissue inhibitor of metalloproteinase (TIMP)-1 and plasminogen activator inhibitor (PAI)-1, and were determined by multiplex assay with the Luminex platform (Millipore, Germany). The results were expressed as ng/mL. Serum interleukin (IL)-1β was measured with an enzyme-linked immunosorbent assay kit (Thermo Scientific, United States). Absorbance was measured spectrophotometrically at a wavelength of 450 nm with a Zenyth 200rt microplate reader (Biochrom). The results were expressed in pg/mL. All procedures were performed in duplicate following the manufacturer's instructions.

Analysis of circulating microRNAs

Total RNA was extracted from serum using miRNeasy serum/plasma kits (Qiagen, United States). A cel-miR-39 (1.6×10^8 copies) spike-in control (Qiagen, United States) was added to provide an internal reference. cDNA conversion was performed with 10 ng of total RNA using TaqMan microRNA reverse transcription kits (Applied Biosystems, United States). Amplification of miR-33a, miR-126, miR-499, miR-186 and miR-146a, was performed by quantitative real-time PCR using the TaqMan assay (Applied Biosystems, United States) and expression as normalized against cell-miR-39. The sequences and codes of the assessed miRNAs are listed in Supplementary Table 1 (Private sharing link for Figshare data https://figshare.com/s/2d858620da6b13fe2fec). Values were calculated by the $2^{-(\Delta\Delta Ct)}$ method.

Hepatic histopathological analysis

Formalin-fixed liver tissue samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and picrosirius red. Histopathological lesions of the different evolutionary stages of liver disease were scored as previously described by Liang *et al*[13]. The score is highly reproduceable and applicable to experimental models in rodents. The analysis was performed by an experienced pathologist who was blinded to the experimental groups. Fibrosis was quantified by morphometric analysis after picrosirius red staining. Ten randomly selected fields were observed *per* animal to measure staining intensity using an Olympus BX51 microscope, and QCapture 64-bit (QImaging) at × 200 magnification. The evaluation was performed using ImageJ (version 1.51p, https://imagej.nih.gov/ij/).

Cardiomyocytes morphometric analysis

Cardiomyocyte morphometric analysis (CMA) was performed based on adaptations of the nuclear morphometric analysis developed by Filippi-Chiela *et al*[14]. Cardiomyocyte size and shape were measured using Image Pro Plus 6.0 (IPP6, Media Cybernetics). H&E images from hearts of animals were acquired. Five different fields were photographed in tissue from each animal using QCapture 64-bit software and an Olympus BX51 microscope. At least 50 cross-sectioned cardiomyocytes of each animal were analyzed. The outlines of single cells were marked using the magic wand tool of IPP6, followed by acquisition the cell area, aspect, area/box, radius ratio, and roundness. The last four measurements were used to define the cardiomyocyte irregularity index (CII) of each cell (CII = area + aspect – area/box + roundness). These variables were used to report the size and shape of single cardiomyocytes. In addition to the average size and regularity, the plot of area *vs* CMA also defined the percentage of normal, hypertrophic, and atrophic cells.

DNA extraction, 16S rRNA sequencing and bioinformatics analysis

A detailed description of the methods used for 16S ribosomal RNA gene sequencing and analyses is provided in the Supplementary Information (Private sharing link for Figshare data https://figshare.com/s/2d858620da6b13fe2fec). Briefly, after DNA extraction, the V4 hypervariable region of the 16S rRNA gene was amplified using 515F–806R primer pair and sequencing was performed with Ion Torrent (Thermo Fisher Scientific, United States). A custom pipeline in Mothur was used for 16S rRNA reads processing. Subsequent analysis of the sequence dataset and data visualization were performed in R using the vegan, phyloseq, ggplot2, and MicrobiomeAnalystR packages or QIIME.

Correlations between analyzed markers

For this analysis, we selected the histopathological NAFLD score, quantification of liver collagen, TIMP-1, MCP-1, and IL-1β as markers of severity and progression of steatohepatitis. For the correlation of CVD risk factors and lipid metabolism, we selected miR-33a, miR-126, PAI-1, CRI-I, CRI-II and AC. We selected the percentage of normal cardiomyocytes, percentage average area of cardiomyocytes, and percentages of atrophic cardiomyocyte morphological characteristics. The overall microbiota composition was correlated with the variables.

Statistical analysis

Data symmetry was tested using the Shapiro-Wilk test. Student-*t* and Mann-Whitney U tests were performed. Spearman's correlation coefficient was performed, with moderate ($0.3 < r < 0.6$), strong ($0.6 < r < 0.9$), or very strong ($0.9 < r < 1.0$) correlations. Quantitative variables were expressed as means ± standard deviation or medians with minimum and maximum values. $P \leq 0.05$ was considered statistically significant. Data were analyzed with SPSS 18.0 (IBM Corp., United States).

RESULTS

Atherogenic ratios, inflammation, and endothelial dysfunction to assess CVR

The results obtained for these parameters are shown in Table 1. There were significant increases in AC), CRI-I, and CRI-II (all $P < 0.001$) in the intervention group, indicating that the animals had an increased CVR. There were significant increases in the serum concentrations of IL-1β ($P = 0.001$), MCP-1 ($P = 0.005$), TIMP-1 ($P < 0.001$), and PAI-1 ($P = 0.037$) in the intervention group compared with the control group. Together, the results suggest the study intervention had increased systemic inflammation and endothelial dysfunction.

Level of circulating microRNAs related to CVR

The levels of circulating microRNAs related to CVR are shown in Figure 1. There was a significant increase in the gene expression of miR-33a ($P = 0.001$) in the intervention group compared with the control group, the opposite was reported for miR-126 ($P < 0.001$). There were no between-group differences in the expression of miR-499 ($P = 0.171$), miR-186 ($P = 0.151$), and miR-146a ($P = 0.151$).

Liver histopathological analysis

No abnormalities were seen in the livers of the control group animals, whereas animals in the intervention group had predominantly microvesicular steatosis along with macrovesicular steatosis of moderate intensity, inflammatory activity, and a mild degree of fibrosis. In the histopathological staging of lesions, seven animals in the intervention group had steatohepatitis and three had simple steatosis. Picrosirius red staining of collagen was more intense ($P < 0.001$) in animals in the intervention group than in the control group (4.10, range: 3.02-6.04 *vs* 1.35, range: 1.21-1.55) relative luminescence units, indicating a significant increase in the deposition of connective tissue fibers in the liver.

Morphometric and histopathological evaluation of cardiomyocytes

Myocardial steatosis was not observed in either the control of intervention group. The evaluation of cardiomyocyte morphometry (*i.e.* size and shape) demonstrated the percentages of normal size, large, or small cells and their shape regularity (Figure 2A). There was a significant decrease in the percentage of cardiomyocytes with a normal morphometric appearance ($P = 0.007$) in the intervention group compared with the control group (Figure 2B). Among the most clinically relevant morphometric changes, there was a significant reduction in the mean area of cardiomyocytes ($P = 0.037$, Figure 2C) and a significant increase in the percentage of atrophic cardiomyocytes in the intervention group ($P = 0.007$, Figure 2D) in relation to the control group. Finally, we separated the animals in the intervention group into two subgroups by the median percentages of normal cardiomyocytes (Figure 2E) and atrophic cardiomyocytes (Figure 2F) and the average area (Figure 2G) and then compared the data. Animals with a percentage of normal cardiomyocytes higher than the median had higher liver tissue levels of TIMP-1, IL-1β, IL-6 and myeloid differentiation primary response (Myd)-88, and lower levels of IL-1β/IL-10 (Figure 2E). Animals with a percentage of atrophic cardiomyocytes above the median had lower liver tissue levels of IL-1β

Table 1 Atherogenic ratios, inflammation and endothelial dysfunction markers in a nutritional model of steatohepatitis

Variable	Control (*n* = 10)	Intervention (*n* = 10)	*P* value
AC	0.6 (0.2–0.9)	2.5 (1.5–3.4)	< 0.001[a]
CRI-I	1.6 (± 0.4)	3.5 (± 1.1)	< 0.001[a]
CRI-II	0.3 (± 0.1)	0.8 (± 0.2)	< 0.001[a]
IL-1β (pg/mL)	367.7 (± 31.2)	465.9 (± 52.7)	0.001[a]
MCP-1 (ng/mL)	2.7 (± 0.6)	3.8 (± 0.9)	0.005[a]
TIMP-1 (ng/mL)	7.1 (± 1.4)	12.4 (± 2.3)	< 0.001[a]
PAI-1 (ng/mL)	0.11 (± 0.05)	0.17 (± 0.06)	0.037[a]

Data are means ± standard deviation or medians (25^{th}-75^{th} percentiles).

[a]$P \leq 0.05$ was considered statistically significant.

AC: Atherogenic coefficient; CRI: Castelli's risk index; IL: Interleukin; MCP: Monocyte chemoattractant protein; PAI: Plasminogen activator inhibitor; TIMP: Tissue inhibitor of metalloproteinase.

(Figure 2F). Animals with an average cardiomyocytes area greater than the median had lower liver tissue levels of tumor necrosis factor-α/IL-10 (Figure 2G).

Gut microbiota diversity and composition

The Shannon diversity index was significantly lower ($P < 0.001$) in intervention than in the control group (Figure 3A). In addition, analysis of similarities (ANOSIM) revealed that the structural pattern of the gut microbiota in intervention group was clearly distinct from that of the control group ($P < 0.001$) by principal coordinates analysis (PCoA) using the Bray-Curtis distance metric (Figure 3B). In terms of composition (*i.e.* taxonomic identification), 1266 bacterial taxa (operational taxonomic units) that belonged to 112 genera, 41 families, and eight phyla were identified. *Firmicutes* (53.1%) and *Bacteroidetes* (43.1%) were the most abundant phyla in all samples. The most abundant families were *Muribaculaceae* (21.7%), *Lachnospiraceae* (20.8%), *Ruminococcaceae* (18.5%), and *Bacteroidaceae* (15.4%, Figure 3C). The four families represented 76.4% of all observed taxa. Differential abundance analysis identified nine families that were associated with the intervention group and one family associated with control group (Linear discriminant analysis score > 2.0; Figure 3D).*Bacteroidaceae*, *Ruminococcaceae*, *Peptostreptococcaceae*, *Peptococcaceae*, *Erysipelotricaceae*, *Clostridiaceae*, *B- urkholderiaceae*, *Streptococcaeae*, and *Tannerellaceae* were differentially abundant in the intervention group. *Lachnospiraceae* was differentially abundant in control group. The distribution of the 41 families and their features are shown in Figure 3E. Most of the taxa prevalent in control group were less prevalent or absent in intervention group. The reverse was also observed.

Lipid metabolism prediction

PCoA using the Bray-Curtis distance metric indicated that the clustering of the predicted lipid metabolic pathways in the study groups was clearly distinct (ANOSIM, $P < 0.001$) As shown in Figure 4A, two samples, R01 and R11, were considered outliers and were not included in further statistical analysis (*e.g.*, LefSe analysis). The distribution of the predicted lipid metabolic pathways is shown in Figure 4B. In total, 12 metabolic pathways were identified in which the between-group difference in the relative frequency was significant ($P < 0.001$, linear discriminant analysis score > 2.0; Figure 4C). The results showed that metabolic pathways involved in sphingolipid metabolism, fatty acid biosynthesis, fatty acid metabolism, steroid hormone biosynthesis, and arachidonic acid metabolism were significantly increased in intervention group, and glycerophospholipid metabolism, glycerolipid metabolism, synthesis and degradation of ketone bodies, biosynthesis of unsaturated fatty acids, alpha-linolenic acid metabolism, linoleic acid metabolism, and ether lipid metabolism were significantly increased in control group.

Correlations between steatohepatitis, CVR, and gut microbiota

The correlations between markers of liver disease progression and severity, CVR factors, cardiomyocyte morphometry and microbiota composition are shown in Table 2. Additional correlations can be found in Supplementary Table 2 (Private

Table 2 Correlation of steatohepatitis, cardiovascular risk, and microbiota composition

Variable[1]		Severity and progression of liver injury				CVR factors and metabolism of lipids						Cardiomyocyte morphometry			Microbiota composition
		Quantification of collagen (picrosirius)	TIMP-1	MCP-1	IL-1β	miR-33a	miR-126	PAI-1	CRI-I	CRI-II	AC	% Normal CAR	Average area of CAR	% Atrophic CAR	
Severity and progression of liver injury	**NAFLD score**	0.879[2]	0.791[2]	0.673[2]	0.347	0.639[2]	-0.777[2]	0.444[3]	0.809[2]	0.820[2]	0.809[2]	-0.519[3]	-0.630[2]	0.721[2]	0.694[2]
	Quantification of collagen (picrosirius)		0.611[2]	0.456[3]	0.752[2]	0.571[3]	-0.683[2]	0.415	0.819[2]	0.821[2]	0.819[2]	-0.205	-0.312	0.238	0.378[2]
	TIMP-1			0.803[2]	0.726[2]	0.728[2]	-0.812[2]	0.535[3]	0.691[2]	0.747[2]	0.691[2]	-0.694[2]	-0.405	0.607[2]	0.539[2]
	MCP-1				0.567[3]	0.492[3]	-0.623[2]	0.336	0.549[3]	0.561[3]	0.549[3]	-0.490[3]	-0.390	0.498[3]	0.232[3]
	IL-1β					0.809[2]	-0.688[3]	0.544[3]	0.645[3]	0.688[2]	0.645[3]	-0.437[3]	-0.393	0.382	0.293[3]
CVR factors and metabolism of lipids	**miR-33a**						-0.655[2]	0.363	0.529[3]	0.603[3]	0.529[3]	-0.704[2]	0.038	0.232	0.160[3]
	miR-126							-0.634[2]	-0.712[2]	-0.730[2]	-0.712[2]	0.459[3]	0.320	-0.364	0.368[2]
	PAI-1								0.487[3]	0.671[2]	0.487[3]	-0.317	0.389	-0.289	0.103
	CRI-I									0.863[2]	1.000[2]	-0.234	-0.459[3]	0.386	0.469[2]
	CRI-II										0.863[2]	-0.399	-0.492[3]	0.551[3]	0.584[2]
	AC											-0.236	-0.457[3]	0.389	0.477[2]
Cardiomyocyte morphometry	**% Normal cardiomyocytes**												0.105	-0.058	
	% Average area of cardiomyocytes													-0.818[2]	
	% Atrophic cardiomyocytes														

[1]Variables were evaluated by Spearman's *r* correlation coefficient: moderate ($0.3 < r < 0.6$), strong ($0.6 < r < 0.9$) or very strong ($0.9 < r < 1.0$).

[2]Correlation significant at the 0.01 level.

[3]Correlation significant at the 0.05 level.

AC: Atherogenic coefficient; CAR: Cardiomyocytes; CRI: Castelli's risk index; CVR: Cardiovascular risk; IL: Interleukin; MCP: Monocyte chemoattractant protein; NAFLD: Nonalcoholic fatty liver disease; PAI: Plasminogen activator inhibitor; TIMP: Tissue inhibitor of metalloproteinase.

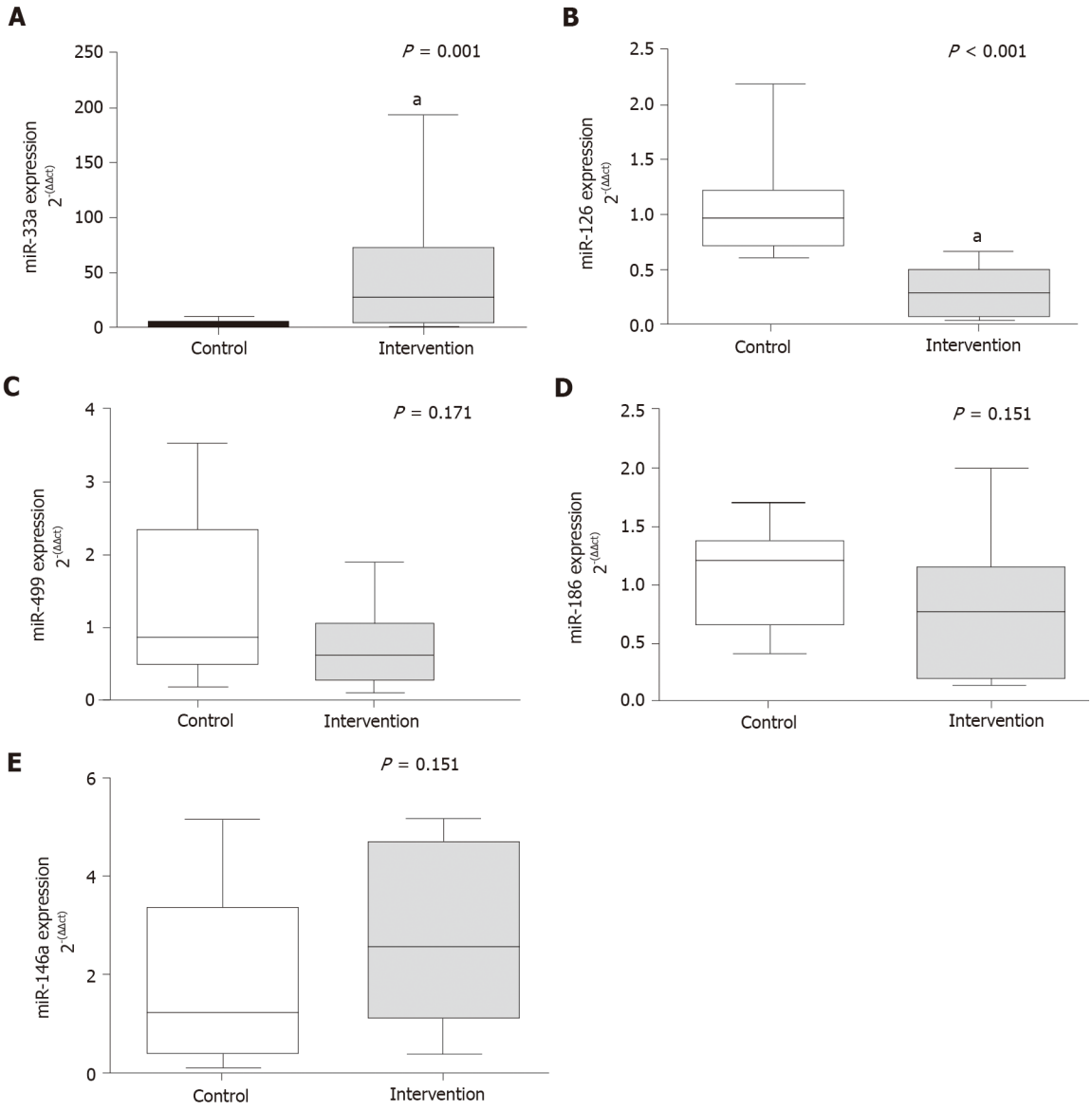


Figure 1 Gene expression of circulating microRNAs. A: miR-33a ($P = 0.001$); B: miR-126 ($P < 0.001$); C: miR-499 ($P = 0.171$); D: miR-186 ($P = 0.151$); E: miR-146a ($P = 0.151$). $^{a}P < 0.05$, Significant effect of the high-fat and choline-deficient diet. Data are medians (25th-75th percentile), Mann-Whitney U test.

sharing link for Figshare data https://figshare.com/s/2d858620da6b13fe2fec). There was a positive correlation between the markers of steatohepatitis severity and progression with CVR factors, such as miR-33a, PAI-1, and atherogenic ratios. Negative correlations were observed for miR-126. Regarding cardiomyocyte morphometry, there were negative correlations between the average area and the percentage of normal cardiomyocytes with the NAFLD score. There was a positive correlation of histopathological NAFLD score with the percentage of atrophic cardiomyocytes, a negative correlation between the percentage of normal cardiomyocytes with MCP-1 and TIMP-1 and a positive correlation of those markers with the percentage of atrophic cardiomyocytes. Furthermore, the average area of cardiomyocytes correlated negatively with atherogenic ratios, CRI-I, CRI-II and AC. miR-33a correlated negatively and miR-126 and positively with the percentage of normal cardiomyocytes.

The composition of the microbiota was positively correlated with markers of liver injury and CVR. The correlation of each family of microorganisms with markers of liver disease progression and severity and CVR factors are shown in Table 3. Significant moderate and strong correlations were observed between nearly all families of bacteria and the hepatic histopathology score, collagen fiber deposition in hepatic tissue, TIMP-1, microRNAs, and atherogenic ratios. Families of interest in the underlying disease including *Bacteroidaceae*, *Clostridiaceae*, *Firmicutes* and *Lactobacillaceae* were correlated with the evaluated markers. No correlation was observed

Table 3 Correlation of gut microbiota at family level, steatohepatitis, and cardiovascular risk factors

Variable[1] (Family)	Severity and progression of liver injury				CVR factors and metabolism of lipids					
	NAFLD score	Quantification of collagen (picrosirius)	TIMP-1	MCP-1	miR-33a	miR-126	PAI-1	CRI-I	CRI-II	AC
Actinomycetaceae					0.584[2]					
Aerococcaceae										
Anaeroplasmataceae		-0.553[2]						-0.614[2]		-0.614[2]
Atopobiaceae	0.627[2]	0.610[2]						0.592[2]	0.663[2]	0.592[2]
Bacillales_unclassified						0.549[2]		-0.548[2]	-0.533[2]	-0.548[2]
Bacteroidaceae	0.836[2]	0.746[2]	0.784[2]		0.689[2]	-0.754[2]		0.662[2]	0.732[2]	0.662[2]
Bacteroidales_unclassified		-0.560[2]							-0.589[2]	-0.492[2]
Burkholderiaceae									0.564[2]	
Clostridiaceae	0.807[2]	0.723[2]	0.645[2]		0.593[2]	-0.669[2]		0.676[2]	0.638[2]	0.676[2]
Clostridiales_unclassified	-0.628[2]	-0.529[2]	-0.535[2]		-0.576[2]				-0.586[2]	-0.525[2]
Clostridiales_vadinBB60	-0.602[2]	-0.671[2]	-0.527[2]		-0.558[2]	0.524[2]		-0.626[2]	-0.502[2]	-0.626[2]
Corynebacteriaceae	-0.669[2]	-0.545[2]	-0.680[2]		-0.782[2]	0.611[2]		-0.571[2]	-0.622[2]	-0.571[2]
Desulfovibrionaceae	-0.806[2]	-0.603[2]	-0.872[2]	-0.776[2]	-0.631[2]	0.755[2]		-0.729[2]	-0.746[2]	-0.729[2]
Eggerthellaceae									0.490[2]	
Firmicutes_unclassified	-0.797[2]	-0.637[2]	-0.687[2]		-0.655[2]	0.594[2]		-0.629[2]	-0.699[2]	-0.629[2]
Gastranaerophilales	-0.822[2]	-0.656[2]	-0.644[2]		-0.643[2]	0.657[2]		-0.698[2]	-0.586[2]	-0.698[2]
Lachnospiraceae	-0.850[2]	-0.653[2]	-0.789[2]	-0.788[2]	-0.613[2]	0.766[2]		-0.643[2]	-0.629[2]	-0.643[2]
Lactobacillaceae	-0.616[2]	-0.633[2]				0.795[2]			-0.529[2]	
Lactobacillales_unclassified										
Micrococcaceae	0.669[2]		0.534[2]			-0.528[2]			0.493[2]	
Mollicutes_RF39_fa	-0.650[2]	-0.618[2]	-0.590[2]		-0.609[2]	0.713[2]		-0.857[2]	-0.768[2]	-0.857[2]
Moraxellaceae	-0.669[2]	-0.536[2]	-0.557[2]		-0.543[2]			-0.599[2]	-0.473[2]	-0.599[2]
Muribaculaceae	-0.816[2]	-0.794[2]			-0.576[2]	0.693[2]	-0.684[2]	-0.827[2]	-0.846[2]	-0.827[2]
Pasteurellaceae										
Prevotellaceae		-0.705[2]				0.603[2]			-0.522[2]	-0.486[2]

Rikenellaceae				-0.679[2]				
Saccharimonadaceae	-0.737[2]	-0.559[2]	-0.619[2]	-0.674[2]	0.656[2]	-0.776[2]	-0.759[2]	-0.776[2]
Staphylococcaceae	-0.734[2]	-0.647[2]	-0.808[2]	-0.838[2]	0.716[2]	-0.616[2]	-0.679[2]	-0.616[2]
Streptococcaceae	0.790[2]	0.726[2]	0.637[2]	0.595[2]	-0.622[2]		0.724[2]	0.515[2]

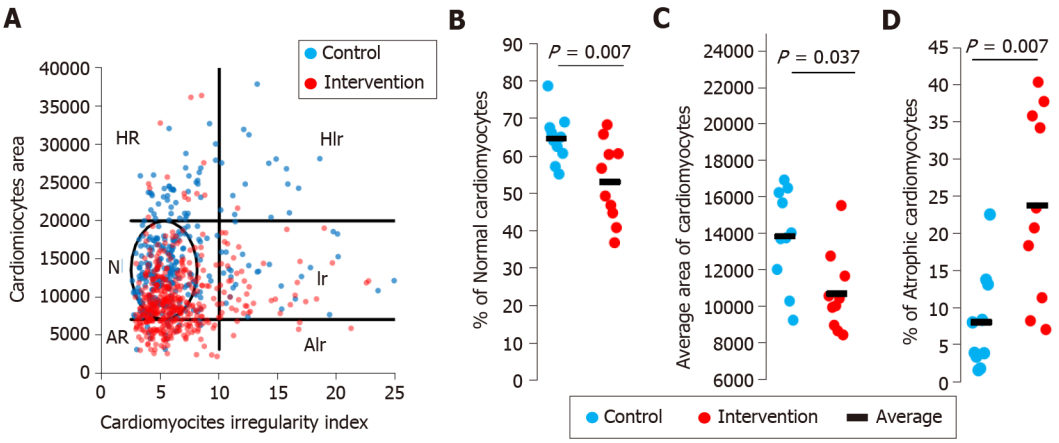
[1]Variables were evaluated by Spearman's *r* correlation coefficient, moderate ($0.3 < r < 0.6$) or strong ($0.6 < r < 0.9$).
[2]Correlation significant at the 0.05 level.
AC: Atherogenic coefficient; CRI: Castelli's risk index; CVR: Cardiovascular risk; MCP: Monocyte chemoattractant protein; NAFLD: Nonalcoholic fatty liver disease; PAI: Plasminogen activator inhibitor; TIMP: Tissue inhibitor of metalloproteinase.

between families of gut microbiota and measurements of cardiomyocyte morphometry.

DISCUSSION

Steatohepatitis and CVD are both associated with metabolic risk factors, including glucose abnormalities, dyslipidemia, chronic inflammation, endothelial dysfunction, and gut dysbiosis. The relationship is recognized in the clinical setting, but the links among steatohepatitis, CVD, and gut dysbiosis needs to be better understood. This study provided evidence of the role of MAFLD as an adjuvant risk factor for the development of CVD. We found that dysbiotic bacteria and their metabolites were translocated to the liver through the ruptured intestinal barrier, causing impaired hepatic triglyceride metabolism, inflammatory responses, and fibrogenesis, which are necessary for the development and progression of MAFLD[11]. We also found significant correlations between the activation of pathophysiological pathways that link MAFLD and increased risk of developing cardiovascular events, such as atherogenic dyslipidemia, systemic inflammation, endothelial dysfunction, gut dysbiosis, and changes in cardiomyocyte morphometry. In this study, the significant associations between steatohepatitis and CVR, justify the screening of MAFLD and its associated risk factors in high-risk patients, in order to intervene effectively, with a focus on new approaches aimed at directing the composition of the intestinal microbiota as a potential therapeutic target.

In a recent publication, we reported that the experimental nutritional model developed in this study is capable of causing marked deposition of body and liver fat, changes in biochemical parameters, activation of microRNAs, receptors, mediators, and inflammatory cytokines, an increase in intestinal permeability, and hepatic histopathological changes, similar to steatohepatitis in humans[11]. This robust experimental model of steatohepatitis of metabolic origin allows evaluating pathophysiological mechanisms related to the development of CVD in MAFLD. We



E

% Normal cardiomyocytes	TIMP-1	IL-1β	Myd88	IL-6 (liver)	IL-1β/IL-10 (liver)
< Median	13.5	3.9	7.3	52914	0.52
> Median	11.2	1.1	2.9	45436	0.64
T Test	0.05	0.04	0.04	0.02	0.01

F

% Atrophic cardiomyocytes	IL-1β (liver)
< Median	2560
> Median	3309
T Test	0.003

G

Average area	TNF-α/IL-10 (liver)
< Median	2.3
> Median	3.4
T Test	0.028

Figure 2 Cardiomyocytes morphometric analysis. The area and cross-sectional shape of cardiomyocytes were determined from images of hematoxylin and eosin-stained tissue. A: Dot plot of cardiomyocyte area *vs* cardiomyocyte irregularity index in control (blue) and intervention (red) groups. Each dot represents a population of cardiomyocytes with different morphometry. N–normal area and shape, Ir–normal area and irregular shape, HR–hypertrophic and regular cardiomyocytes, HIr–hypertrophic and irregular cardiomyocytes, AR–atrophic and regular cardiomyocytes, AIr–atrophic and irregular cardiomyocyte; B: Average area of cardiomyocytes; C: Percentage of normal cardiomyocytes; D: Percentage of atrophic cardiomyocytes; E-G: We segregated the animals in the intervention group into two subgroups and the data were compared. IL: Interleukin; TNF: Tumor necrosis factor.

demonstrated that abnormalities of lipid metabolism and atherogenic ratios were related to greater propensity to develop CVD associated with steatohepatitis. The results are consistent with other experimental and clinical studies[7,15-18]. In addition, we report a significant increase of systemic markers of inflammation and endothelial dysfunction in animals with steatohepatitis. The worsening of the inflammatory state in MAFLD is associated with worse cardiometabolic outcomes. PAI-1 is a marker of endothelial dysfunction, being released in response to low-grade inflammation, free fatty acids, and atherogenic lipoproteins[19,20]. A previous study reporting that an increase in PAI-1 was correlated with the histological severity of MAFLD and alterations in the lipid profile, promoting a more atherogenic phenotype[21]. PAI-1 also plays a vital role in liver fibrosis, promoting increased deposition of extracellular matrix in liver tissue, in which TIMP-1 performs a similar function[22]. In that sense, liver fibrosis can lead to severe hepatic dysfunction and even life-threatening conditions such as liver cirrhosis and HCC. The mechanism of liver fibrosis is multifaceted and, in this study, animals with steatohepatitis had an increase in TIMP-1 concentration and deposition of collagen fibers in liver tissue, markers that significantly correlated with increased CVR.

Assessment of microRNAs has been used for the early detection and monitoring of the progression of MAFLD, and to assess clinical and subclinical CVD. miR-33a inhibits genes involved in high-density lipoprotein synthesis and the reverse transport of cholesterol[23,24]. In this study, animals with steatohepatitis had a significant increase in miR-33a expression that was positively correlated with atherogenic ratios and markers of severity and progression of liver injury. miR-126 expression, which is high in endothelial cells and regulates the migration of inflammatory cells, formation of capillary networks, and cell survival[25], was decreased in animals with steatohepatitis. In fact, there was an inverse correlation between miR-126 expression and


A
Shannon index
4.0
3.5
3.0
B
Control
Intervention
Axis.2 [12.6%]
0.8
0.4
0
-0.4
-0.8
-0.50
-0.25
0.00
0.25
0.50
Axis.1 [45.7%]
C
Control
Intervention
Relative abundance
1.00
0.75
0.50
0.25
0.00
R01 R02 R03 R04 R05 R06 R07 R08 R09 R10
R11 R12 R13 R14 R15 R16 R17 R18 R19 R20
Family
Bacteroidaceae
Muribaculaceae
Lachnospiraceae
Ruminococcaceae
Peptococcaceae
Others
Peptostreptococcaceae
Rikenellaceae
Erysipelotrichaceae
Clostridiaceae_1
D
Control
Bacteroidaceae
Ruminococcaceae
Peptostreptococcaceae
Peptococcaceae
Erysipelotrichaceae
Clostridiaceae_1
Burkholderiaceae
Streptococcaceae
Tannerellaceae
Christensenellaceae
Desulfovibrionaceae
Gastranaerophilales_fa
Lachnospiraceae
Intervention
-5
-4
-3
-2
-1
0
1
2
3
4
5
LDA score

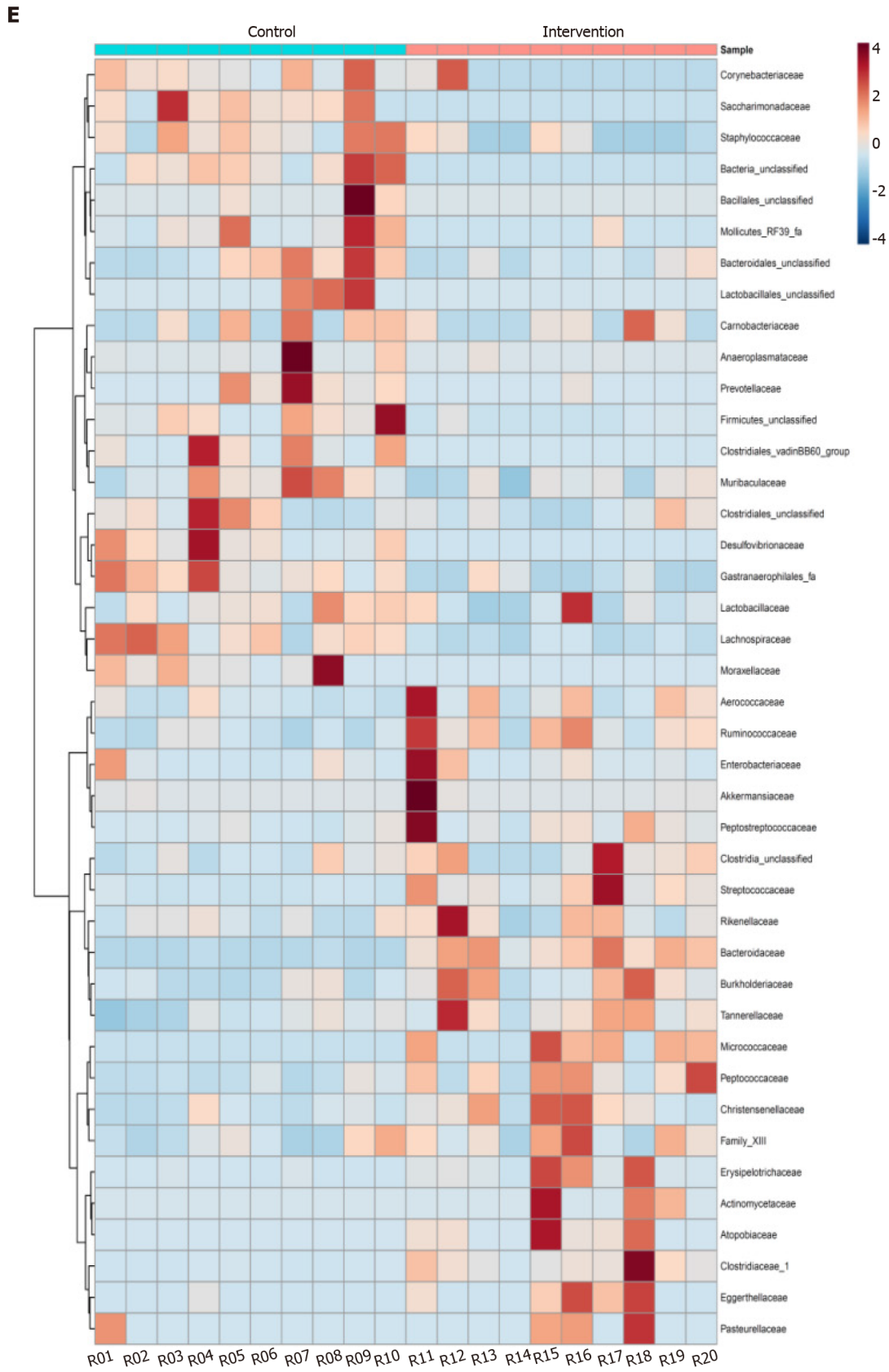


Figure 3 Gut microbiota changes in intervention and control groups. A: Shannon diversity index; B: Principal coordinate analysis based on Bray-Curtis distance metric; C: Relative abundance of gut microbiota at the family level; D: Differential abundance by linear discriminant analysis; E: Heatmap distribution of the 41 families among the samples. LDA: Linear discriminant analysis.

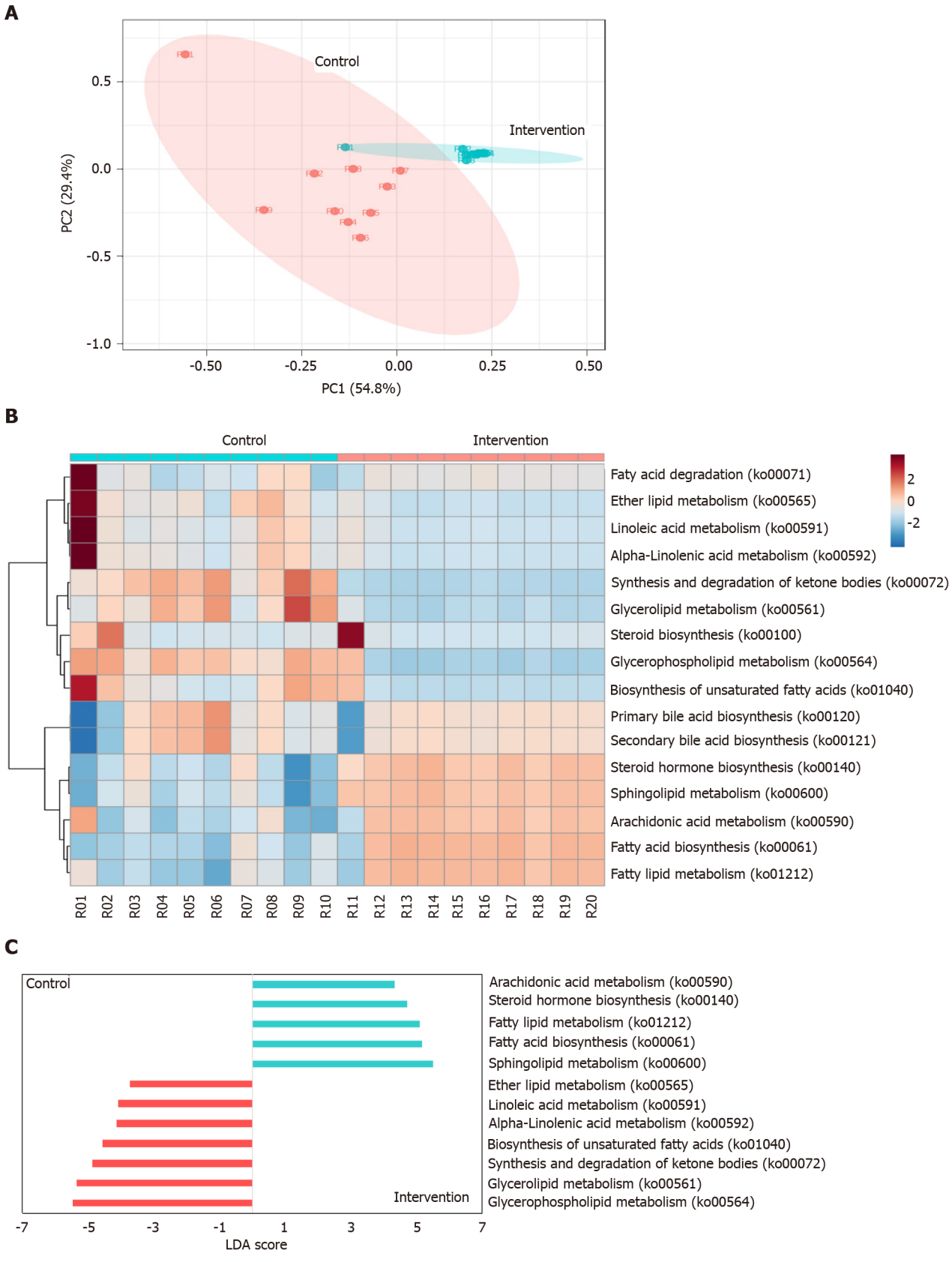


Figure 4 Sixteen predicted functional Kyoto Encyclopedia of Genes and Genomes lipid metabolism pathways in intervention and control group. A: Principal coordinate analysis; B: Heatmap distribution; C: Linear discriminant analysis (LDA) of the 16 differentially abundant KEGG lipid metabolism pathways.

atherogenic ratios, endothelial dysfunction, inflammation, fibrogenesis, and severity of liver injury. As established in the literature, microRNAs act in the epigenetic regulation of intricate processes[24,25]. In this study, we clearly demonstrated that the

expression of miR-33a and miR-126 was involved in the regulation of cholesterol, lipid metabolism, and endothelial dysfunction, and contributed to the development of metabolic disorders and CVD related to steatohepatitis.

The morphometric evaluation of cardiomyocytes was an interesting and innovative analysis in this study, and it found that animals with steatohepatitis had a significant decreases in the percentage of cardiomyocytes with a normal appearance and the mean area of cardiomyocytes relative to the control group. In addition, animals with steatohepatitis had a significant increase in the percentage of atrophic cardiomyocytes. To the best of our knowledge, morphometric analysis of cardiomyocytes in MAFLD has not been previously reported, which makes it difficult to discuss the data obtained. Several cellular processes can be inferred through morphometric analysis, and the method can be used in the diagnosis and prognosis of some clinical conditions[14,26,27]. In this study, we reported that the percentage of normal cardiomyocytes was negatively correlated with the histological severity of liver damage, fibrogenesis, and inflammation. Furthermore, the percentage of atrophic cardiomyocytes correlated positively with the liver injury markers. Clinical manifestations of MAFLD, such as steatosis and inflammation, are additional risk factors for the development of CVD[3,9]. However, the exact mechanisms for this complex relationship are unclear[3,9]. It is likely that several highly interrelated factors contribute to the increase of CVR in steatohepatitis and changes in the morphometry of cardiomyocytes. However, more studies are needed to evaluate the morphometry of cardiomyocytes in more advanced stages of MAFLD.

The "multiple parallel hits" hypothesis highlights the importance of the gut microbiota and seems to provide a more accurate explanation of the pathogenesis of steatohepatitis and its contribution to the increase in CVR[3,10]. The liver is closely related to the intestine both anatomically and functionally, and recent evidence demonstrates that the type and quantity of intestinal microorganisms determine important characteristics related to the pathogenesis and progression of these clinical conditions[28-30]. Our data corroborate with experimental and clinical studies reporting that the development and progression of MAFLD is associated with a significant decrease in the diversity and structure of the bacterial communities of the gut microbiota[29,31,32]. In this study, we report an increase in the abundance of family *Bacteroidaceae* and a decrease in the abundance of *Prevotellaceae* in animals with steatohepatitis. It is known that the diet directly influences the composition of the gut microbiota. Western diets abundant in fat, animal protein, and sugar have been associated with steatohepatitis and increased risk of CVD. That diet favors the abundance of family *Bacteroidaceae*; while diets high in fiber, starch, and plant polysaccharides promote the abundance of family *Prevotellaceae*[30,33,34]. In this study, we report an increase in the abundance of family *Bacteroidaceae* and a decrease in the abundance of *Prevotellaceae* in animals with steatohepatitis, which is consistent with another study[30]. Regarding the increase in the relative abundance of family *Ruminococcacea* observed in the animals of the intervention group, a previous report that demonstrated the *Ruminococcus increased* in more severe disease, especially if advanced hepatic fibrosis was diagnosed. The decrease in its abundance has also been reported in lean steatohepatitis patients[30,35]. There are reports that associate the abundance of *Ruminococcaceae* with the development of CVD[36,37]. However, we found no correlations between the presence of *Ruminococcaceae* and the CVR markers that were assessed in this study. Genus *Ruminococcus* is quite heterogeneous, including both beneficial and deleterious bacteria, making data discussion difficult. Family *Ruminococcaceae* is associated with aerobic fermentation that leads to the production of short chain fatty acids and alcohol, and this can have detrimental effects on intestinal permeability and hepatic inflammation[30,35].

Some of the metabolites produced by gut flora are already biologically active, whereas others are further metabolized by the host, generating secondary mediators that influence the microbiota-host interaction. In this study, we predicted the lipid metabolic pathways that were expressed as a result of the gut dysbiosis observed in steatohepatitis. Animals with steatohepatitis had a significant increase in sphingolipid metabolism. The sphingolipids are membrane lipids that participate in cell division, differentiation, gene expression, and apoptosis. The study data corroborate emerging evidence that support the role of sphingolipids in hepatocellular death, which contributes to the progression of MAFLD[38]. Additionally, there are reports that dysregulation of circulating sphingolipids was independently associated with CVD and subclinical atherosclerosis[39,40]. In this study, arachidonic acid metabolism was significantly increased in animals with steatohepatitis. In addition, a significant decrease in linoleic acid metabolism was reported in this experimental group. Arachidonic acid is synthesized from polyunsaturated fatty acids, and can be derived

from linoleic acid, which is an essential fatty acid[41]. The products resulting from arachidonic acid metabolism are linked to the inflammation and vasodilation of MAFLD and CVD, mainly by the action of the enzyme cyclooxygenase[41,42]. Therefore, as reported in this study, an increase in arachidonic acid metabolism in steatohepatitis and CVD is expected. We report an increase in glycerophospholipid metabolism in animals in the control group. As described by Schnabl and Brenner[43], a high-fat diet causes the gut microbiota to convert choline in the diet to methylamines, consequently reducing the plasma levels of phosphatidylcholine, which is a glycerophospholipid. Phosphatidylcholine is an important constituent of the cell membrane of very low density lipoproteins. Without its presence triglycerides cannot attach to the lipoprotein and start to accumulate in the liver tissue, causing MAFLD [43]. In parallel, there were increases in plasma trimethylamine, and its hepatic metabolism to trimethylamine-N-oxide has been associated with the appearance of CVD. This compound is considered harmful, as it changes the way cholesterol and steroids are metabolized and inhibits the reverse transport of cholesterol, causing the accumulation of fat on the internal walls of arteries[44,45]. Therefore, in this study, the predicted lipid metabolism in animals with steatohepatitis did not include expression of glycerophospholipid metabolism, probably because of the action of the gut microbiota in the metabolic pathway.

CONCLUSION

In summary, it is known that steatohepatitis and CVD have many risk factors in common. Among those, we report significant correlations between the presence of atherogenic dyslipidemia, systemic inflammation, endothelial dysfunction, liver fibrogenesis, and gut dysbiosis, all of which contribute to the progression of MAFLD and increased CVR. In addition, we infer, through the composition of the gut microbiota, which lipid metabolism pathways are activated in animals with steatohepatitis and their relationship with CVR. Subsequent metabolomic studies may aid in elucidating the influence of gut microbial function with the development of cardiometabolic disorders related to steatohepatitis. The gut microbiota may be a potential therapeutic target for both clinical conditions.

ARTICLE HIGHLIGHTS

Research background

Metabolic-associated fatty liver disease (MAFLD), in addition to being a progressive liver disease, is an independent and significant risk factor for the development of cardiovascular disease, and dysbiosis of the intestinal microbiota is associated with both.

Research motivation

The motivation was to explore the mechanisms whereby gut microbiota contribute to steatohepatitis-associated increased cardiovascular risk.

Research objectives

The objective was to assess the relationship between gut dysbiosis and cardiovascular risk in an experimental model of steatohepatitis.

Research methods

Adult male Sprague-Dawley rats were randomized to a control group given a standard diet or an intervention of a high-fat and choline-deficient diet for 16 wk of ten animals each. Biochemical, molecular, hepatic, and cardiac histopathology and gut microbiota variables were evaluated.

Research results

We reported significant correlations between the presence of atherogenic dyslipidemia, systemic inflammation, endothelial dysfunction, liver fibrogenesis and gut dysbiosis, all of which contributed to the progression of MAFLD and increased CVR.

Research conclusions

This study shows that there is a link between gut dysbiosis and significant cardiomyocyte abnormalities in animals with steatohepatitis.

Research perspectives

Metabolomic studies may aid in elucidating the association of gut microbial function with the development of cardiometabolic disorders related to steatohepatitis. The gut microbiota may be a potential therapeutic target for both clinical conditions.

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ORIGINAL ARTICLE

Case Control Study

Leukocyte cell-derived chemotaxin-2 and fibroblast growth factor 21 in alcohol-induced liver cirrhosis

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Author contributions: Sak JJ and Prystupa A were involved in the conception of the study, data collection and analysis, drafting and revision of the manuscript; Kiciński P and Luchowska-Kocot D were involved in the data collection and analysis; Kurys-Denis E was involved in the drafting and revision of the manuscript; Bis-Wencel H contributed to the data collection and revision of the manuscript.

Institutional review board statement: The study protocol was approved by the Bioethics Committee at Medical University of Lublin, Poland.

Informed consent statement: All patients gave their written informed consent for participation in the study.

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Abstract

BACKGROUND

The importance of early diagnosis of alcoholic liver disease underscores the need to seek better and especially non-invasive diagnostic procedures. Leukocyte cell-derived chemotaxin-2 (LECT2) has been widely studied to determine its usefulness in monitoring the course of non-alcoholic fatty liver disease but not for alcoholic liver cirrhosis (ALC).

AIM

To determine the concentration of LECT2 in the blood serum of patients in relation to progressive stages of ALC, its relation to fibroblast growth factor 1 (FGF-1) and FGF-21, and to examine the possible wider use of LECT2 in diagnosing ALC.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at jaroslaw.sak@umlub.pl. Participants gave informed consent for data sharing.

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METHODS
A retrospective case-control study was conducted with 69 ALC cases and 17 controls with no ALC. Subjects were recruited from the region of Lublin (eastern Poland). Liver cirrhosis was diagnosed based on clinical features, history of heavy alcohol consumption, laboratory tests, and abdominal ultrasonography. The degree of ALC was evaluated according to Pugh-Child criteria (the Pugh-Child score). Blood was drawn and, after centrifugation, serum was collected for analysis. LECT2, FGF-1, and FGF-21 were determined using enzyme-linked immunosorbent assay kits.

RESULTS
The LECT2 Levels in the control group were 18.99 ± 5.36 ng/mL. In the study groups, they declined with the progression of cirrhosis to 11.06 ± 6.47 ng/mL in one group and to 8.06 ± 5.74 ng/mL in the other ($P < 0.0001$). Multiple comparison tests confirmed the statistically significant differences in LECT2 Levels between the control group and both test groups ($P = 0.006$ and $P < 0.0001$). FGF-21 Levels were 44.27 ± 64.19 pg/mL in the first test group, 45.4 ± 51.69 pg/mL in the second ($P = 0.008$), and 13.52 ± 7.51 pg/mL in the control group. The difference between the control group and the second test group was statistically significant ($P = 0.007$).

CONCLUSION
We suggest that LECT2 may be a non-invasive diagnostic factor for alcohol-induced liver cirrhosis. The usefulness of LECT2 for non-invasive monitoring of alcohol-induced liver cirrhosis was indirectly confirmed by the multiple regression model developed on the basis of our statistical analysis.

Key Words: Leukocyte cell-derived chemotaxin-2; Fibroblast growth factor 21; Fibroblast growth factor 1; Alcoholic liver cirrhosis; Pugh-Child score

Core Tip: Leukocyte cell-derived chemotaxin-2 (LECT2) was first described in 1996 as a novel chemotactic factor for neutrophils. It has been widely studied to determine its usefulness for monitoring the course of non-alcoholic fatty liver disease but not for alcoholic liver cirrhosis (ALC). We suggest that LECT2 may be used for the non-invasive diagnosis of ALC.

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INTRODUCTION

Alcoholic liver disease (ALD) occurs in three stages: fatty liver, alcoholic hepatitis, and liver cirrhosis. In the present study, the role of leukocyte cell-derived chemotaxin-2 (LECT2) in the development of alcohol-induced liver cirrhosis was investigated.

In recent decades, there have been significant developments in research on the biochemical possibilities for the early diagnosis and monitoring of non-alcoholic fatty liver disease (NAFLD)[1]. Hepatokines were found to be extremely useful for NAFLD monitoring[2]. Moreover, relationships between the stages of NAFLD and fetuin-A[3,4], selenoprotein-P[5,6], and fibroblast growth factor 21 (FGF-21)[7] have been demonstrated. Fibroblast growth factor mimicking has been developed as a novel therapeutic option[8]. The analogues of hepatokines, such as a pegylated FGF-21 analogue[9], have been used in NAFLD therapies. However, finding similar diagnostic options for ALD remains valid[10]. ALD is among the most prevalent diseases in Western countries. It has recently been recognized as an increasingly serious epidemi-

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ological and therapeutic problem in developing countries[11,12].

Therefore, finding new possibilities for the early diagnosis of ALD, especially novel and precise non-invasive diagnostic procedures, is a real challenge for modern hepatological practice.

LECT2 has been widely studied to determine its usefulness in monitoring the course of NAFLD. According to the available study findings, serum LECT2 concentrations increase with the advancement of NAFLD[13,14]. LECT2 was first described by Yamagoe *et al*[15] in 1996 as a novel chemotactic factor for neutrophils. Subsequent studies identified its expression in human hepatocytes and classified it as a hepatokine [16-18]. Clinical observations have demonstrated that LECT2-associated amyloidosis is a frequent cause of hepatic amyloidosis in the United States[19]. Studies in animal models have reported that LECT2 overexpression increases fibrosis, promotes sinusoid capillarization, and inhibits portal angiogenesis. LECT2 is a functional ligand of Tie1. Xu *et al*[20] suggested that serum LECT2 Levels may be a potential biomarker for the diagnosis or screening of liver fibrosis, and LECT2/Tie1 signaling may be used for the development of new drugs.

It seems that LECT2 could be of great importance in the diagnosis of fatty liver. In a cross-sectional study, Okumura *et al*[13] showed statistically significant higher levels of LECT2 in fatty liver and obesity. However, the possibility of diagnosing and monitoring the course of alcohol-induced liver cirrhosis using LECT2 has not yet been assessed.

The aim of our study was to determine the concentration of LECT2 in the blood serum of patients at progressive stages of alcoholic liver cirrhosis to determine the relation to FGF-1 and FGF-21, and to discuss the possible wider use of LECT2 in the diagnosis of ALC.

MATERIALS AND METHODS

The study protocol was approved by the Bioethics Committee. All patients gave their written informed consent prior to participating in the study.

Patients

The study was conducted at the Department of Internal Medicine, Medical University of Lublin, Poland, and included 69 patients from the region of Lublin (eastern Poland) with alcoholic cirrhosis. Liver cirrhosis was diagnosed based on clinical features, history of heavy alcohol consumption, laboratory tests, and abdominal ultrasonography. Heavy alcohol consumption was defined according to the guidelines of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as consuming more than four drinks on any day or more than 14 drinks per week for men and three drinks on any day or more than seven drinks per week for women[21]. Patients with alcoholic hepatitis, hepatocellular carcinoma, or viral and autoimmune diseases were excluded from the study. Other exclusion criteria were type 2 diabetes, obesity, acute infections (*e.g.*, pneumonia, spontaneous bacterial peritonitis), acute and chronic heart failure (> NYHA I—*i.e.* slight or marked limitation of physical activity, ordinary physical activity results in fatigue, palpitation, dyspnea), acute and chronic respiratory disorders resulting in respiratory insufficiency, acute kidney injury, and chronic kidney disease (> stage G2—*i.e.* an estimated glomerular filtration rate < 60 mL/min). Both clinical assessments and laboratory tests were used to exclude underlying liver diseases in the control group. The degree of liver cirrhosis was evaluated according to Pugh-Child criteria (the Pugh-Child score), and on that basis, patients were assigned to one of three groups: Pugh-Child (P-Ch) A (n = 21) with stage A, P-Ch B (n = 23) with stage B, and P-Ch C (n = 28) with stage C liver cirrhosis (Table 1). The control group consisted of 17 healthy individuals without liver disease who did not abuse alcohol. Detailed demographic, clinical, and biochemical characteristics of the patients are presented in Tables 1 and 2.

Biochemical measurements

Blood was drawn, and after centrifugation, serum was collected for analysis. Human LECT2, FGF-1, and FGF-21 were determined using enzyme-linked immunosorbent assay (ELISA) kits. All absorbance readings were conducted using an Epoch Microplate Spectrophotometer (BioTek Instrumentals, Inc., Winooski, VT, United States). LECT2 concentrations were determined using a BioVendor Human LECT2 ELISA kit (BioVendor, Laboratorni medicina a.s., Brno, Czech Republic). FGF-1 and FGF-21 concentrations were quantified using sandwich enzyme immunoassay kits

Table 1 Patients' demographics and clinical characteristics

	Control group (*n* = 17)	Liver cirrhosis		*P* value
		Pugh-Child A + B (*n* = 37)	Pugh-Child C (*n* = 32)	
Age (yr)	43.7 ± 14.6	55.7 ± 12.1	55.9 ± 10.2	0.021
Percentage of males (%)	64.3%	73%	72.7%	0.52
Body weight (kg)	67.6 ± 8.9	73 ± 11.4	75.5 ± 12.8	0.17
Height (cm)	173 ± 5.9	174 ± 8	173 ± 7.6	0.64
Duration of alcohol abuse (yr)	-	15.7 ± 8.2	18.7 ± 8.3	0.98
Oesophageal varices (%)	-	32.4%	81.8%	< 0.0001
Encephalopathy (%)	-	32.4%	83.9%	< 0.0001
Ascites (%)	-	40.5%	90.9%	< 0.0001
Total bilirubin (mg/dL)	0.6 ± 0.3	4.6 ± 6.9	10.5 ± 9.2	< 0.0001
INR	-	1.36 ± 0.35	1.95 ± 0.56	< 0.0001
Albumin (g/dL)	-	3.1±0.8	2.4±0.4	0.0002
Total protein (g/dL)	6.3 ± 0.3	6.4 ± 1	5.9 ± 0.9	0.16
Alanine aminotransferase (U/L)	17.9 ± 6	65.3 ± 139.9	50.6 ± 87.3	0.018
Aspartate aminotransferase (U/l)	18.3 ± 7	128.1 ± 173.5	120 ± 164.7	< 0.0001
Platelets (G/L)	231.4 ± 29.8	173 ± 105.4	127.8 ± 72.3	0.0004
Mean corpuscular volume (fL)	84.8 ± 3.5	91.2 ± 9.1	95.5 ± 9	0.0002
Urea (mg/dL)	-	27.5 ± 16.1	58.2 ± 43.7	0.065
Sodium (mmol/l)	140 ± 3.3	133.8 ± 5	131.9 ± 6.7	< 0.0001
Potassium (mmol/L)	4.4 ± 0.4	3.8 ± 0.7	3.9 ± 0.8	0.019
C-reactive protein (mg/L)	2.5 ± 2.3	19.8 ± 21	32.7 ± 27.8	< 0.0001
Angiotensinogen (ng/mL)	1006.91 ± 610.49	1117.04 ± 873.69	1468.7 ± 817.33	0.22

INR: International normalized ratio.

Table 2 Levels of selected biochemical parameters according to the stage of liver cirrhosis

	Control group	Liver cirrhosis		*P* value
		Pugh-Child A + B	Pugh-Child C	
LECT2 (ng/mL)	18.99 ± 5.36	11.06 ± 6.47	8.06 ± 5.74	< 0.0001
FGF-1 (pg/mL)	37.94 ± 40.4	144.77 ± 14.42	164.52 ± 169.46	0.01
FGF-21 (pg/mL)	13.52 ± 7.51	44.27 ± 64.19	45.4 ± 51.69	0.008

LECT2: Leukocyte cell-derived chemotaxin-2; FGF-1: Fibroblast growth factor 1; FGF-21: Fibroblast growth factor 21.

produced by Cloud-Clone Corp. (Katy, TX, United States). Serum samples had been suitably diluted (20-fold dilution for LECT2) or used without dilution (FGF-1 and FGF 21) prior to testing, in accordance with the manufacturers' recommendations. Testing was carried out in accordance with the typical standard applicable for enzyme-linked immunoassays: samples, standards, and blanks were applied to a plate pre-coated with a factor-specific antibody. Subsequently, horseradish peroxidase conjugated avidin was added to each well, and the plate was incubated for one hour at room temperature (LECT2) or at 37°C (FGF-1 and FGF-21). Next, TMB substrate was added; the wells containing biotin-conjugated antibody and enzyme-conjugated avidin exhibited a change in color. The enzyme-substrate reaction was terminated by adding acidic solution, and the absorbance of the complex formed was measured at a

wavelength of 450 nm. The concentrations of the study parameters were determined using a standard curve. Results were multiplied by the dilution factor, when necessary.

Statistical analysis

Statistica 13.3 (TIBCO Software, Inc.) was used for data analysis. Continuous variables were expressed as mean ± SD. Before calculations, variables were checked for normality using the Shapiro-Wilk test. To compare the results between more than two groups, one-way ANOVA and the Kruskal-Wallis test were used, depending on distribution. Correlations among variables were tested using Pearson's and Spearman's correlation tests, depending on distribution. Qualitative variables were shown as indicators of structure (percentage). For intergroup comparisons, the χ^2 test was used. For all tests, $P < 0.05$ was considered statistically significant.

RESULTS

The study group consisted of 69 patients (50 men), including 37 with P-Ch A or P-Ch B cirrhosis and 32 with P-Ch C. The control group included 17 gender-matched individuals ($P = 0.52$). The age of patients in the control group was lower than that of patients with cirrhosis ($P = 0.021$). The duration of alcohol abuse in the study group was, on average, 15.7 ± 8.2 years in the P-Ch A + B subgroup and 18.7 ± 8.3 years in the P-Ch C subgroup.

As expected, patients with liver cirrhosis were characterized by significantly lower albumin levels and higher total bilirubin (TB), alanine aminotransferase, aspartate aminotransferase (AST), international normalized ratio, and C-reactive protein levels (Table 1).

Angiotensinogen levels increased with the progression of cirrhosis, reaching the highest in the P-Ch C group of 1468.7 ± 817.33 ng/mL. However, the differences observed were not statistically significant ($P = 0.22$).

The LECT2 Levels in the control group were 18.99 ± 5.36 ng/mL. With the progression of cirrhosis in the P-Ch A + B group, this value dropped to 11.06 ± 6.47 ng/mL and to 8.06 ± 5.74 ng/mL in the P-Ch C group ($P < 0.0001$) (Table 2). Multiple comparisons confirmed the statistically significant differences in LECT2 Levels between the control group and the P-Ch A + B ($P = 0.006$) and between the control group and P-Ch C ($P < 0.0001$) (Figure 1).

Otherwise, the lowest FGF-1 Level was found in the control group—37.94 ± 40.4 pg/mL—and was higher in patients with cirrhosis, increasing to 144.77 ± 1 in the P-Ch A + B group and to 164.52 ± 169.46 pg/mL in the P-Ch C group ($P < 0.01$). The difference between the control group and P-Ch C was statistically significant ($P = 0.002$) (Table 2).

A similar trend was observed for FGF-21. Its concentration in the control group was 13.52 ± 7.51 pg/mL, 44.27 ± 64.19 pg/mL in the P-Ch A + B group, and 45.4 ± 51.69 pg/mL in the P-Ch C group ($P = 0.008$). The difference between the control group and the P-Ch C group was statistically significant ($P = 0.007$) (Table 2).

The strongest correlations were observed between LECT2 and TB ($r = –0.59$; $P < 0.0001$) and angiotensinogen ($r = –0.51$; $P < 0.0001$) (Table 3).

In the multiple regression model, angiotensinogen, AST, TB, and age were observed to be independent LECT2-related variables (Table 4). This model was statistically significant ($P < 0.0001$) and explained less than two-thirds of variability (adjusted $R^2 = 0.59$).

DISCUSSION

ALD is a serious health consequence of excessive alcohol consumption. The spectrum of clinical-histologic ALD changes includes fatty liver, alcoholic hepatitis, and cirrhosis [22]. It is estimated that over 90% of all heavy drinkers have fatty liver; about 25% of them have alcoholic hepatitis, and 15% have cirrhosis. According to a meta-analysis conducted by Askgaard *et al*[23], the probability of alcoholic liver cirrhosis reaches 16% after 8–12 years of alcoholization; 45% of patients with cirrhosis had been consuming more than 110 g of alcohol daily. The above results correspond to our observations based on a relatively small sample. Alcohol-induced liver cirrhosis accounts for half of all cirrhosis cases in the United States. In recent years, the importance of finding new non-invasive methods to diagnose more severe forms of

Table 3 Correlations between leukocyte cell-derived chemotaxin-2 and other clinical and laboratory parameters (only those statistically significant were included)

Pair of variables		Correlation coefficient	
		R	*P* value
LECT2	Age	-0.29	0.048
	Total bilirubin	-0.59	< 0.0001
	Platelets	0.34	0.02
	Alanine transaminase	-0.43	0.003
	C-reactive protein	-0.4	0.008
	Angiotensinogen	-0.51	< 0.0001
	FGF-1	-0.38	0.004
	FGF-21	-0.39	0.004

LECT2: Leukocyte cell-derived chemotaxin-2; FGF-1: Fibroblast growth factor 1; FGF-21: Fibroblast growth factor 21.

Table 4 Independent factors associated with leukocyte cell-derived chemotaxin-2 concentration (multiple regression)

Effect	B*	SE with B*	B	SE with B	*P* value
Constant			30.64	3.68	< 0.0001
Angiotensinogen	-0.423	0.114	-0.004	0.001	0.001
Alanine aminotransferase	-0.341	0.115	-0.02	0.005	0.005
Total bilirubin	-0.279	0.108	-0.25	0.099	0.014
Age	-0.275	0.109	-0.16	0.064	0.016

B*: Standardized coefficient (Beta). Model: $R = 0.79$; $R^2 = 0.64$, adjusted $R^2 = 0.59$; $P < 0.0001$.

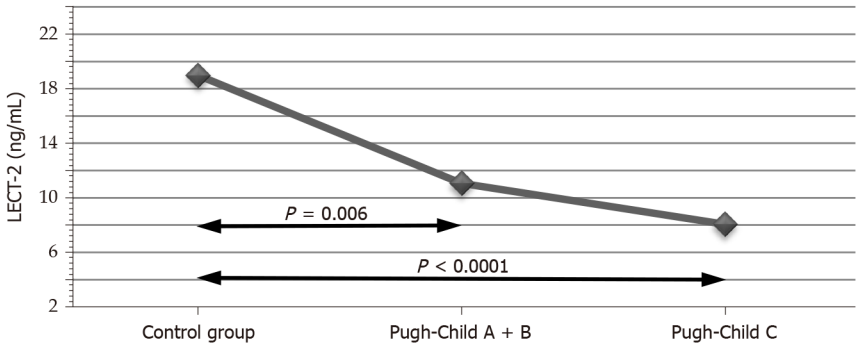

Figure 1 Concentration of leukocyte cell-derived chemotaxin-2 according to the stage of alcoholic liver cirrhosis. LECT-2: Leukocyte cell-derived chemotaxin-2.

ALD and predict prognosis has been strongly emphasized[24,25].

In our study, the serum levels of FGF-1 and FGF-21 in the study groups and control group were determined to obtain biochemical reference points for levels of LECT2. FGF-1 is an angiogenic factor that modifies the migration and proliferation of endothelial cells and regulates the metabolism of lipids and carbohydrates. FGF-1 is involved in response to injury and fibrosis. The highest expression of FGF has been observed in the late stages of hepatic morphogenesis in animal models, as well as during hepatic differentiation in the adult liver. FGF-1 is present in perisinusoidal hepatic stellate cells (HSCs) during liver regeneration. The chronic activation of nonparenchymal HSCs (also called Ito cells and fat-storing cells) is the major contributor to liver fibrogenesis resulting from chronic toxic insult primarily through

its production of extracellular matrix components.

FGF-1 reduces hepatic lipid accumulation independently of insulin and is important in the pathogenesis of NAFLD. Moreover, it has therapeutic potential for the treatment of ischemic disease[26]. Previous studies have demonstrated an inverse relationship between this factor and portal pressure in patients after liver transplantation[27]. In animal model studies, the protective effect of FGF-1 on liver cells was confirmed, as it prevented acute inflammation and apoptosis induced by acetaminophen[28]. The main source of FGF-1 in the human body is liver cells. However, this protein is also expressed in the pancreas, testes, duodenum, and adipose tissue. For this reason, its use as an indicator of liver function is clearly limited, and in recent years this problem has not been studied. Among fibroblast growth factors, FGF-21 has been tested as a marker of liver function[29,30]. According to a Chinese prospective study, this protein is an independent predictor of NAFLD[31]. The possible use of FGF-21 as an NAFLD marker has also been described in an American study conducted in children[32]. However, the above study demonstrated significant relationships between the level of this marker and the prevalence of obesity, with or without insulin resistance. In a study on ALD, Yang *et al*[33] suggested that FGF-21 may indicate a progression from heavy drinking to alcoholic cirrhosis. In their latest study, Willis *et al*[34] indicated that acute high-fat overfeeding augments circulating concentrations of FGF-21, LECT2, and fetuin-A in healthy men. Perhaps a slightly opposite effect than in this subgroup occurs in patients with cirrhosis with regard to correlation of LECT2 and FGF-21. The results of our study showed that LECT2 Levels correlated inversely with FGF-1 and FGF-21 in ALD. However, based on our results, it is not possible to state whether this is specific to ALD. Previous studies have shown that LECT2 could be of great importance in the diagnosis of NAFLD[13,14]. We suggest the need for further, more extensive, including prospective, studies.

Our study is the first attempt to assess the usefulness of LECT2 in the non-invasive diagnosis of alcohol-induced liver cirrhosis. Therefore, the points of reference are scarce. However, considering the above-mentioned studies on the marker function of FGF-21, it is worth noting that our results are compatible with those reported by Yang *et al*[33] In our study, the concentration of FGF-21 in the control group, that is, patients without cirrhosis, was significantly lower compared to both subgroups of the study group. However, the differences in FGF-21 concentrations between the two subgroups (P-Ch A + B and P-Ch C) were not statistically significant. FGF-21 may play an important role in supporting non-invasive diagnostics of alcohol-induced liver cirrhosis and in monitoring the course of NAFLD. We did not find it useful in non-invasive monitoring of alcohol-induced liver cirrhosis, contrary to the level of serum taurine/glycine-conjugated bile acids as a non-invasive marker to predict the severity of alcohol-induced liver cirrhosis, as tested by Yang *et al*[33]. Our results suggest that LECT2 might be used as a diagnostic and monitoring marker to determine the severity of alcohol-induced liver cirrhosis. Its highest statistically significant concentration was observed in the control group. In the study groups, as cirrhosis progressed, the plasma levels of LECT2 dropped. The lowest values of LECT2 were observed in P-Ch C stage patients, that is, in the most advanced stage of the disease.

LECT2 Levels correlated inversely with TB, AST, and angiotensinogen (AGT). Although strong correlations were identified between LECT2 and cirrhosis progression, and between AGT and LECT2, we did not observe an analogous relationship between AGT and cirrhosis progression. We suggest that this may be caused by low sample size and decreased power. The liver's renin-angiotensin system plays an important role in the development of liver cirrhosis. The levels of total bilirubin, AST, and AGT increase as alcohol-induced liver cirrhosis progresses. Higher serum concentration of AGT indicates unfavorable histological remodeling of the liver parenchyma closely related to liver dysfunction. Previous studies on animal models have indicated that AGT plays an important role in NAFLD[35-37]. AGT is an important precursor of hepatic fibrogenesis, which has been confirmed in animal studies[38]. According to the reported data, AGT inhibition could be an effective anti-liver fibrosis strategy.

CONCLUSION

Our research suggests that LECT2 may be used for the non-invasive diagnosis of alcohol-induced liver cirrhosis. The usefulness of LECT2 for non-invasive monitoring of alcohol-induced liver cirrhosis was indirectly confirmed by the multiple regression model developed on the basis of our statistical analysis.

ARTICLE HIGHLIGHTS

Research background

Leukocyte cell-derived chemotaxin-2 (LECT2) has been widely studied to determine its usefulness for monitoring the course of non-alcoholic fatty liver disease but not for alcoholic liver cirrhosis (ALC).

Research motivation

The aim of our study was to assess and discuss LECT2's possible wider use in the diagnosis of ALC.

Research objectives

The purpose of this study was to determine the concentration of LECT2 in the blood serum of patients in accordance with progressive stages of ALC and its relation to fibroblast growth factor 1 (FGF-1) and FGF-21.

Research methods

A study was conducted with an ALC group and a control group with no ALC. The extent of ALC was evaluated according to Pugh-Child criteria (the Pugh-Child score). LECT2, FGF-1, and FGF-21 were determined using enzyme-linked immunosorbent assay kits.

Research results

Our study showed strong correlations between LECT2 and cirrhosis progression. LECT2 levels correlated inversely with FGF-1 and FGF-21.

Research conclusions

LECT2 may be used for the non-invasive diagnosis of alcohol-induced liver cirrhosis.

Research perspectives

Further prospective studies should be conducted to explore whether the inverse correlation of LECT2 and FGF-21 is specific to ALD.

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ORIGINAL ARTICLE

Retrospective Study

Biliary complications in recipients of living donor liver transplantation: A single-centre study

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Abstract

BACKGROUND

Biliary complications (BCs) after liver transplantation (LT) remain a considerable cause of morbidity, mortality, increased cost, and graft loss.

AIM

To investigate the impact of BCs on chronic graft rejection, graft failure and mortality.

METHODS

From 2011 to 2016, 215 adult recipients underwent right-lobe living-donor liver transplantation (RT-LDLT) at our centre. We excluded 46 recipients who met the exclusion criteria, and 169 recipients were included in the final analysis. Donors' and recipients' demographic data, clinical data, operative details and postoperative course information were collected. We also reviewed the management and outcomes of BCs. Recipients were followed for at least 12 mo post-LT until December 2017 or graft or patient loss.

RESULTS

The overall incidence rate of BCs including biliary leakage, biliary infection and biliary stricture was 57.4%. Twenty-seven (16%) patients experienced chronic

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graft rejection. Graft failure developed in 20 (11.8%) patients. A total of 28 (16.6%) deaths occurred during follow-up. BCs were a risk factor for the occurrence of chronic graft rejection and failure; however, mortality was determined by recurrent hepatitis C virus infection.

CONCLUSION
Biliary complications after RT-LDLT represent an independent risk factor for chronic graft rejection and graft failure; nonetheless, effective management of these complications can improve patient and graft survival.

Key Words: Biliary complications; Living donor liver transplantation; Retrospective analysis; Bile leak; Biliary stricture; Risk factors; Mortality; Graft rejection

Core Tip: We included 169 right lobe living-donor liver transplantation recipients in this retrospective study. The overall incidence rate of biliary complications including biliary leakage, biliary infection and biliary stricture was 57.4%. Twenty-seven (16%) patients experienced chronic graft rejection. Graft failure developed in 20 (11.8%) patients. A total of 28 (16.6%) deaths occurred during follow-up. Biliary complications were an independent risk factor for the occurrence of chronic graft rejection and failure; however, mortality was determined by unresolved recurrent hepatitis C virus infection. In conclusion, biliary complications represent an independent risk factor for chronic graft rejection and graft failure; nonetheless, effective management of these complications can improve patient and graft survival.

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INTRODUCTION

Liver transplantation (LT) is a life-saving therapeutic modality for patients with end-stage hepatic disease[1]. Despite considerable progress in LT surgical performance and peri-operative management, post-LT biliary complications (BCs) remain a considerable cause of morbidity, mortality, increased cost, and graft loss[2,3].

Living-donor liver transplantation (LDLT) is a well-established substitute to deceased-donor LT (DDLT)[4,5]. LDLT has potential advantages over DDLT, such as lower cost, superior graft vitality, shorter cold ischemia time, and lower prevalence of steroid-resistant graft rejection[6]. However, it has been reported that LDLT is related to higher post-LT morbidity, hospitalization rates and duration of stay. This is mainly referred to the higher incidence rate of BCs in LDLT ranging from 10% to 67% compared to DDLT[7-9], which could be attributed to the technically challenging biliary reconstruction during LDLT[9]. Technical skilfulness is mandatory to reduce the incidence of BCs[10], and the most critical key step is to maintain the blood supply to the biliary ducts in donor surgery[11].

Post-LT BCs include biliary strictures (BSs), biliary leaks (BLs), and biliary infection. There are two types of BLs post-LDLT: Anastomotic and cut surface BLs[12,13]. BLs occur commonly at the T-tube insertion site and less frequently at the anastomosis site [14]. Most BLs occur within the first post-transplant month and are mostly related to inadequate surgical skills or biliary duct ischemia[15].

BSs are the most common BC, accounting for 40% of BCs following LT. Like BLs, BSs are more prevalent post-LDLT when compared to DDLT, mostly due to the more technically challenging biliary anastomosis in LDLT due to the small-sized ducts requiring multiple biliary anastomoses[7,16]. BSs typically present after one month post-LT; in addition, they can be anastomotic or non-anastomotic[12]. Anastomotic

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strictures account for approximately 80% of post-LT BSs and commonly occur in LDLT and at the anastomotic site[7,17]. Non-anastomotic strictures account for approximately 10%-25% of post-LT BSs[18]. BSs are mainly linked to surgical skills, patients with small-sized ducts, donor-recipient bile duct size mismatch, longer operative time, total ischemia time, local ischemia, chronic rejection, older donor age, donor and recipient gender matching and initial disease recurrence like primary sclerosing cholangitis (PSC)[2,3,19,20].

Duct-to-duct anastomosis (DDA) has developed into the preferred biliary reconstruction method due to its benefits of a shorter total operative time, less incidence of post-operative infections, more physiological enteric functions and the enablement of access to the biliary tree in case of complications. Roux-en-Y hepaticojejunostomy (RYHJ) is performed in the case of re-transplantation or short or diseased bile ducts[21]. However, diversity in the results regarding the superiority of both of the two biliary reconstruction and suturing techniques is still present[3,8,15,22].

Similarly, the use of biliary drainage remains controversial[10]. The post-LT stent represents a method for biliary tract decompression, as well as the facilitation of postoperative cholangiography[22]. However, this technique is predisposed to BL at the entry site and thus has become less commonly used[14]. Also, temporary internal biliary stents may be applied to cross the anastomosis site[19]; however, it has been reported that the incidence of BCs may increase with this technique[23].

There is considerable overlap in the diagnostic and therapeutic modalities in patients with post-LT BCs. Frequently used diagnostic modalities include abdominal ultrasonography, computed tomography scan, magnetic retrograde cholangiopancreatography (MRCP), magnetic resonance imaging, percutaneous transhepatic cholangiography (PTC) and endoscopic retrograde cholangiopancreatography (ERCP). Currently, the preferred imaging method for the biliary tract is MRCP; it provides a guide for further interventional approaches[14].

In the case of isolated deranged liver functions post-LT, it is crucial to make an accurate diagnosis of other parenchymal hepatic diseases such as acute or chronic rejection, drug-induced hepatotoxicity, recurrence of primary cholestatic disease or viral hepatitis to further apply the appropriate management plan. Liver biopsy is a conclusive diagnostic procedure for these patients[4,7].

The management of BCs depends on a multidisciplinary approach including endoscopic, percutaneous and surgical interventions. Currently, ERCP is the preferable first-line therapeutic modality, especially in cases of DDA[4,17]. The success rate of this technique is variable, ranging from 51% to 100%[24]. If ERCP fails, PTC can be tried; also, it is the preferred therapeutic modality in cases of RYHJ. Surgical intervention is a last option for BCs management[2,20]. However, the optimal strategy for managing post-LT BCs remains undefined.

Based on the published literature, BC causes significant morbidity following LDLT. If not managed properly, it leads to cholestasis, progressive bridging fibrosis, secondary biliary cirrhosis and eventually graft failure. Hence, we aimed to investigate its impact on chronic graft rejection, graft failure and mortality.

MATERIALS AND METHODS

Study design

This retrospective cohort study was conducted at Ain Shams Centre for Organ Transplantation, Ain Shams Specialized Hospital, Cairo, Egypt, from January 2011 to December 2016. This study was performed according to the ethical guidelines of the Declaration of Helsinki and was approved by the ethical review board of the Faculty of Medicine, Ain Shams University (No. FMASU MD 187/2016), which waived the requisite of informed consent owing to the retrospective nature of the study.

During the study period, 215 adult recipients underwent right lobe-LDLT (RL-LDLT) at our centre. We excluded 46 patients who met the exclusion criteria, and 169 recipients were enrolled in the final analysis. We included cirrhotic patients who met the transplantation criteria of our institution [a Child-Pugh score of ≥ 7 and model for end-stage liver disease (MELD) score of ≥ 15]. Patients with hepatocellular carcinoma (HCC) were enrolled if they met the Milan criteria, defined as a single lesion ≤ 5 cm or up to three lesions of ≤ 3 cm each with the absence of vascular invasion and extrahepatic metastases[25]. We excluded patients with cholestatic hepatic diseases [primary biliary cirrhosis (PBC) or PSC] and early postoperative mortality and patients lost on follow-up (Figure 1).

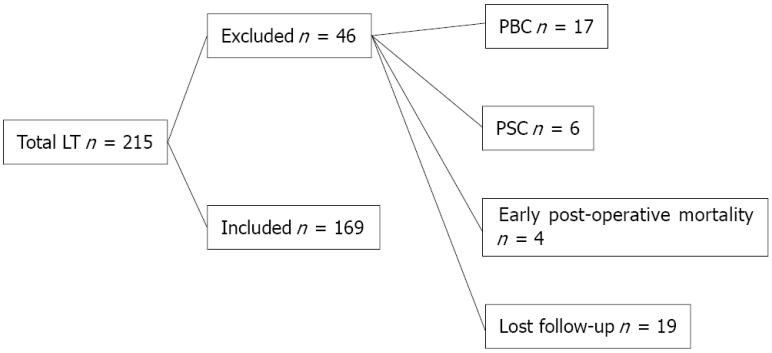

Figure 1 Flow chart of study cohort. LT: Liver transplantation; PBC: Primary biliary cholangitis; PSC: Primary sclerosing cholangitis.

Donors' and recipients' demographic data, clinical data, operative details and postoperative course information were collected. We also reviewed the management and outcomes of BCs. Recipients were followed for at least 12 mo post-LT until December 2017 or graft or patient loss.

Study definitions

The following BCs and their management were recorded from data files:

BL: Clinically suspected due to the existence of bile in the surgical drains or the presence of an intra-abdominal biloma and confirmed by imaging studies.

Biliary infection: Clinically suspected due to fever, abdominal pain, rigours, biochemical cultures and elevated inflammatory markers, including levels of C-reactive protein.

BS: Clinically suspected due to jaundice, pruritus, and elevated levels of serum bilirubin and/or alkaline phosphatase and confirmed by imaging studies as a narrowing at any site of the biliary tree whether at an anastomotic or non-anastomotic site with proximal dilatation.

Diagnosis of other clinical outcomes

Graft failure: Confirmed by histological evidence as graft cirrhosis, the need for re-transplantation because of graft failure and/or allograft-associated mortality.

Chronic ductopenic graft rejection: Proven by liver biopsy.

Recurrent hepatitis C virus (HCV) infection: Proven by high viral load, elevated transaminases and liver biopsy.

Institutional surgical technique for right lobe living donor liver transplantation

A right-lobe graft was used without the middle hepatic vein by the piggyback technique. Biliary anastomosis was done by DDA with an end-to-end interrupted style using absorbable polydioxanone (PDS-II; Ethicon) 6-0 sutures[26]. A ductoplasty was conducted if one duct was approximately twice the size of the other. A routine external biliary stent was inserted for three months post-operation. Three drains were placed postoperatively: In the right subphrenic space, the right Morrison's pouch and at the cut surface of the graft. Internal biliary stents were used selectively if indicated. Arterial reconstruction was described previously[27]. The ratio of graft weight to recipient body weight was used to assess the relation of the graft size for recipients [27]. The accepted ratio was 1.2 ± 0.2%. All recipients had the same ABO blood group as the donors.

Statistical methods

Data were analysed using IBM© SPSS© Statistics version 23 (IBM© Corp., Armonk, NY) and MedCalc© version 18.2.1 (MedCalc© Software bv, Ostend, Belgium). Non-parametric numerical variables were presented as medians and interquartile ranges, whereas between-group differences were analysed using the Mann-Whitney test and, in the case of paired data, the Wilcoxon signed-rank test. Parametric numerical data were shown as mean ± standard deviation, and between-group differences were analysed using a *t*-test and, in the case of paired data, a paired *t*-test. Nominal

variables were shown as number and percentage, and differences were analysed using Pearson's chi-squared test or Fisher's exact test. Ordinal data were analysed using the chi-squared test for trend. Multivariable binary logistic regression analysis was used to define the independent risk factors. Univariable time-to-event analysis was done using the Kaplan-Meier method. Cox proportional hazard regression analysis was used for multivariable time-to-event analysis. Two-sided *P* values of < 0.05 were considered statistically significant.

RESULTS

This study included 169 adult RL-LDLT recipients. At the time of operation, the mean age of the recipient was 50 ± 8 years, and 150 (88.8%) were male. The indications for LT were HCC [60 (35.5%)] and liver cirrhosis because of HCV [148 (87.6%)], hepatitis B virus (HBV) [5 (3%)], HCV and HBV coinfection [4 (2.4%)], and other aetiologies including vascular, autoimmune, and cryptogenic cirrhosis [12 (7.1%); Tables 1 and 2].

Prior to LT, 33 (19.52%) patients were HCV RNA negative, and 136 (80.46%) were HCV RNA positive. Thirty-one (18.3%) patients received antiviral treatment prior to LT. Forty-one (24.3%) patients experienced recurrent HCV infection, which was resolved in 37 (90.2%) patients (Table 1). Before the direct-acting antivirals (DAA) era, a Peg-interferon alfa-2a/Ribavirin (Peg-IFN/RBV) regimen was used for eligible patients, whereas after the availability of DAA therapy, sofosbuvir/daclatasvir ± RBV, sofosbuvir/simeprevir and ledipasvir/sofosbuvir regimens were used.

The majority of grafts had one or two ducts [both *n* = 78 (46.2%)], and the majority of patients needed one anastomosis [109 (64.5%)]. One to two stents were used in the majority of grafts [71 (42%) and 79 (46.7%), respectively; Table 1].

Fourteen (8.3%) patients experienced arterial complications; 12 patients had hepatic artery thrombosis (HAT), and two patients had hepatic artery stenosis (HAS; Table 1). In case HAT was detected not beyond two weeks post-LT, re-exploration was done, and after implementing inflow from the hepatic artery as well as backflow from the graft artery by embolectomy, re-anastomosis was conducted. In case of late presented HAT, interventional radiology and anticoagulation were done. In the case of HAS, a stent was inserted.

Development and management of BCs

Among the 169 RT-LDLT recipients included in this study, minor BLs occurred in 55 patients (32.5%) and stopped spontaneously without further management. Only in nine (16.4%) patients were pigtail insertion and further interventional management needed. Ninety-seven (57.4%) patients suffered from biliary infection; it mostly occurred early [91 (93.81%)], and 13 (7.7%) patients had three or more episodes (Table 1).

Sixty (35.5%) patients developed BS, most of which were anastomotic [59 (98.33%)], presented late [45 (75%)] and in one to two episodes [43 (25.4%)]. Most patients [45/60 (75%)] were HCV PCR positive during the occurrence of BS. Twenty-seven (45%) patients were not eligible for HCV antiviral treatment, while 14 (23.3%), 13 (21.7%) and 6 (10%) patients were treated before, during, and after the occurrence of BS, respectively (Table 1). Risk factors for BS were BL, biliary infection (especially if early or frequent), chronic graft rejection and longer graft arterialization time (Tables 3, 4 and Figure 2). In the multivariate analysis, graft arterialisation time > 130 min and biliary infection were the two determinants of BS (Table 5).

With respect to the management of BCs, ERCP with stenting ± dilatation was done for 60 (35.5%) patients, with 18 (10.7%) patients needing ≥ 3 ERCP sessions. PTC was attempted only in 8 (4.7%) patients, with one patient needing another session. These methods only failed in one patient who needed surgical reconstruction of BSs (Table 1).

Chronic graft rejection

Twenty-seven (16%) patients experienced chronic graft rejection. It was determined by biliary infection (especially if early or frequent), BS (especially if early or frequent), the need of ERCP (especially if multiple sessions), the number of stents used for BS treatment, hospital admission (especially if frequent) and recurrent HCV infection (Tables 1, 6 and Figure 3). The impact of these parameters on graft rejection was further demonstrated by multivariate analysis and Kaplan-Meier analysis (Table 7, Figure 4, and Supplementary material).

Table 1 Descriptive categorical data for the whole study population

Variable		*n* (%)
Etiology of cirrhosis	HCV	148 (87.6)
	HBV	5 (3)
	Combined HCV & HBV	4 (2.4)
	Others	12 (7.1)
Hepatocellular carcinoma	-	109 (64.5)
	+	60 (35.5)
Donors' gender	Male	141 (83.4)
	Female	28 (16.6)
Recipients' gender	Male	150 (88.8)
	Female	19 (11.2)
HCV PCR viremia prior to transplantation	Negative	33 (19.52)
	Below 200 000 IU	59 (34.91)
	200000 to 2 million	69 (40.82)
	More than 2 million	8 (4.73)
Antiviral treatment for HCV prior to transplantation	-	138 (81.7)
	+	31 (18.3)
Arterial complications	-	155 (91.7)
	+	14 (8.3)
Number of anastomosis	1 Anastomosis	109 (64.5)
	2 Anastomosis	57 (33.7)
	3 Anastomosis	3 (1.8)
Number of ducts	1 Duct	78 (46.2)
	2 Ducts	78 (46.2)
	3 Ducts	12 (7.1)
	4 Ducts	1 (0.6)
Number of stents introduced at surgery	Nil	7 (4.1)
	1 Stent	71 (42)
	2 Stents	79 (46.7)
	3 Stents	11 (6.5)
	4 Stents	1 (0.6)
Immunosuppressant	Tacrolimus	118 (69.8)
	Cyclosporine	51 (30.2)
Biliary leakage	-	114 (67.5)
	+	55 (32.5)
Need of pigtail catheter for biloma	-	46 (83.6)
	+	9 (16.4)
Biliary infection	-	72 (42.6)
	+	97 (57.4)
Frequency of biliary infection	1-2 Episodes	84 (49.7)
	≥ 3 Episodes	13 (7.7)
Biliary stricture	-	109 (64.5)

	+	60 (35.5)
Frequency of biliary stricture	1-2 Episodes	43 (25.4)
	≥ 3 Episodes	17 (10.1)
Need for ERCP	-	109 (64.5)
	+	60 (35.5)
Frequency of ERCP	1-2 ERCP	42 (24.9)
	≥ 3 ERCP	18 (10.7)
Need for PTC	-	161 (95.3)
	+	8 (4.7)
Frequency of PTC	1 PTC	7 (4.1)
	2 PTC	1 (0.6)
Surgical intervention for stricture	-	168 (99.4)
	+	1 (0.6)
HCV PCR during occurrence of stricture	Negative	15 (25)
	Below 200 000 IU	15 (25)
	200000 to 2 million	19 (31.7)
	More than 2 million	11 (18.3)
HCV antiviral treatment in relation to stricture diagnosis	No treatment	27 (45)
	Before stricture	14 (23.3)
	During occurrence of stricture	13 (21.7)
	After stricture	6 (10)
Admission related to BC	-	95 (56.2)
	+	74 (43.8)
Mortality	-	141 (83.4)
	+	28 (16.6)
Cause of mortality (total number: 28)	Biliary sepsis	5 (17.9)
	Graft rejection	4 (14.3)
	Recurrent HCV	3 (10.7)
	Other causes	16 (57.1)
Chronic rejection	-	142 (84)
	+	27 (16)
Recurrent HCV infection	-	128 (75.7)
	+	41 (24.3)
Resolution of recurrent HCV	-	4 (9.8)
	+	37 (90.2)
Graft failure	-	149 (88.2)
	+	20 (11.8)
Causes of graft failure (total number: 20)	Biliary sepsis	5 (25)
	Graft rejection	6 (30)
	Recurrent HCV	3 (15)
	Other causes	6 (30)
Early biliary infection (total = 97)	-	6 (6.18)
	+	91 (93.81)

Early biliary stricture (total = 60)	-	45 (75)
	+	15 (25)

Data presented in number (*n*) and percentage (%). HCV: Hepatitis C virus; HBV: Hepatitis B virus; PCR: Polymerase chain reaction; ERCP: Endoscopic retrograde cholangiopancreatography; PTC: Percutaneous transhepatic cholangiography; BC: Biliary complication.

Table 2 Descriptive numerical data for the whole study population

Variable	Data
MELD score	16 ± 4
Child score	10 ± 2
Donors' age (yr)	27 ± 6
Donors' BMI (kg/m^2)	24 ± 3
Recipient's age (yr)	50 ± 8
Recipient's BMI (kg/m^2)	28 ± 4
Total bilirubin (mg/dL)	2.6 (1.9-3.8)
Direct bilirubin (mg/dL)	1.3 (0.7-2.1)
Alkaline phosphatase (IU/L)	104 ± 48
Gamma-glutamyl transferase (IU/L)	36 (19-61)
Platelets (10^9/L)	79 ± 35
Cold ischemia time (min)	49 ± 24
Warm ischemia time (min)	48 ± 20
Graft arterialization time (min)	141 ± 51
Time to biliary infection (d)	16 (11-30)
Time to biliary stricture (d)	150 (120-218)
Time to mortality (d)	285 (55-808)
Time to chronic graft rejection (d)	490 (230-920)
Time to recurrent HCV (d)	391 (180-714)
Time to graft failure (d)	556 (135-1267)

Data are presented as mean ± SD or median and range. MELD: Model for end-stage liver disease; BMI: Body mass index; HCV: Hepatitis C virus.

Graft failure

Graft failure developed in 20 (11.8%) patients; the causes were chronic graft rejection [6 (30%)], biliary infection [5 (25%)], recurrent HCV infection [3 (15%)], and other causes [6 (30%); Table 1]. BL, the need for pigtail catheter insertion, biliary infection (especially if frequent), recurrent HCV infection and non-response to HCV therapy were the risk factors of graft failure (Tables 8, 9 and Figure 5). Kaplan-Meier survival analysis further proved the impact of major BL and biliary infection on graft survival (Figure 6).

Mortality

A total of 28 (16.6%) deaths occurred during follow-up. The aetiologies of mortality were biliary infection [5 (17.9%)], chronic graft rejection [4 (14.3%)], recurrent HCV infection [3 (10.7%)], and other causes [16 (57.1%); Table 1]. Unresolved recurrent HCV infection was the only risk factor for mortality (Table 10 and Figure 7). This was further proved by Kaplan-Meier survival analysis (Figure 8).

Table 3 Risk factors for biliary strictures: Categorical factors

Variable		Biliary strictures		OR	CI		*P* value[1]
		No stricture (*n* = 109)	Stricture (*n* = 60)		95% LCL	95% UCL	
		n, Row %	*n*, Row %				
Etiology of cirrhosis	HCV	95 (64.2)	53 (35.8)				0.142[2]
	Isolated HBV	5 (100)	0 (0)				
	Combined HCV & HBV	1 (25)	3 (75)				
	Causes other than viral hepatitis	8 (66.7)	4 (33.3)				
Donors' gender	Male	90 (63.8)	51 (36.2)	0.8	0.4	2.0	0.684
	Female	19 (67.9)	9 (32.1)				
Recipients' gender	Male	96 (64)	54 (36)	0.8	0.3	2.3	0.704
	Female	13 (68.4)	6 (31.6)				
HCV PCR viremia prior to transplantation	Negative	20 (60.6)	13 (39.4)				0.768[3]
	Below 200000 IU	41 (69.5)	18 (30.5)				
	200000 to 2 million	44 (63.8)	25 (36.2)				
	More than 2 million	4 (50)	4 (50)				
Antiviral treatment prior to transplantation	-	92 (66.7)	46 (33.3)	1.6	0.7	3.6	0.214
	+	17 (54.8)	14 (45.2)				
Hepatocellular carcinoma	-	71 (65.1)	38 (34.9)	1.1	0.6	2.1	0.815
	+	38 (63.3)	22 (36.7)				
Arterial complications	-	102 (65.8)	53 (34.2)	1.9	0.6	5.8	0.255[2]
	+	7 (50)	7 (50)				
Number of anastomoses	One	70 (64.2)	39 (35.8)				0.910[3]
	Two	37 (64.9)	20 (35.1)				
	Three	2 (66.7)	1 (33.3)				
Number of ducts	1 Duct	50 (64.1)	28 (35.9)				0.857[3]
	2 Ducts	52 (66.7)	26 (33.3)				
	3 Ducts	6 (50)	6 (50)				
	4 Ducts	1 (100)	0 (0)				
Number of stents	Nil	5 (71.4)	2 (28.6)				0.578[3]
	1 Stent	43 (60.6)	28 (39.4)				
	2 Stents	53 (67.1)	26 (32.9)				
	3 Stents	7 (63.6)	4 (36.4)				
	4 Stents	1 (100)	0 (0)				
Immunosuppressant	Tacrolimus	81 (68.6)	37 (31.4)	1.8	0.9	3.5	0.087
	Cyclosporine	28 (54.9)	23 (45.1)				
Biliary leakage	-	80 (70.2)	34 (29.8)	2.1	1.1	4.1	**0.026**
	+	29 (52.7)	26 (47.3)				
Biliary infection	-	62 (86.1)	10 (13.9)	6.6	3.0	14.4	**< 0.001**
	+	47 (48.5)	50 (51.5)				
Frequency of biliary infection	Nil	62 (86.1)	10 (13.9)				**< 0.0013**

	1-2 Episodes	45 (53.6)	39 (46.4)				
	≥ 3 Episodes	2 (15.4)	11 (84.6)				
Early biliary infection	-	64 (82.1)	14 (17.9)	4.7	2.3	9.5	**< 0.001**
	+	45 (49.5)	46 (50.5)				
Chronic graft rejection	-	99 (69.7)	43 (30.3)	3.9	1.7	9.2	**0.001**
	+	10 (37)	17 (63)				
Recurrent HCV	-	87 (68)	41 (32)	1.8	0.9	3.8	0.096
	+	22 (53.7)	19 (46.3)				

[1]Pearson chi-squared test unless otherwise indicated.
[2]Fisher's exact test.
[3]Chi-squared test for trend.
Data are presented as number (*n*) and percentage (%). OR: Odds ratio; LCL: Lower confidence limit; UCL: Lower confidence limit. HCV: Hepatitis C virus; HBV: Hepatitis B virus; PCR: Polymerase chain reaction.

Table 4 Risk factors for biliary stricture: Numerical factors

Variable	No biliary stricture (*n* = 109)	Biliary stricture (*n* = 60)	*P* value[1]
MELD score	15 (13-18)	15 (13-19)	0.588
CHILD score	10 (9-11)	9 (8-11)	0.198
Donors' age (yr)	27 (23-30)	25 (24-30)	0.727
Donors' BMI (kg/m^2)	25 (23-26)	24 (22-26)	0.155
Recipient's age (yr)	51 (46-56)	52 (48-55)	0.961
Recipient's BMI (kg/m^2)	27 (25-30)	27 (26-30)	0.219
Total bilirubin (mg/dL)	2.6 (1.9-3.7)	2.5 (1.9-4.1)	0.911
Direct bilirubin (mg/dL)	1.3 (0.8-2.1)	1.3 (0.7-1.9)	0.405
Alkaline phosphatase (IU/L)	99 (70-118)	84 (68-143)	0.982
GGT (IU/L)	36 (19-63)	34 (22-60)	0.992
Platelets (10^9/L)	70 (51-104)	68 (51-102)	0.830
Cold ischemia time (min)	45 (30-60)	45 (30-60)	0.929
Warm ischemia time (min)	45 (35-60)	45 (35-60)	0.860
Graft arterialization time (min)	120 (90-150)	155 (120-205)	**< 0.001**

[1]Mann-Whitney U test.
Data are presented as median and interquartile range (IQR). MELD: Model for end-stage liver disease; BMI: Body mass index.

Table 5 Multivariable binary logistic regression model for prediction of biliary stricture

Variable	*P* value	Odds ratio	95%CI
Graft arterializations time > 130 min	**0.001**	3.705	1.669-8.224
Biliary leakage	0.649	1.208	0.536-2.726
> 1 Episode of biliary infection	**< 0.0001**	9.892	4.086-23.952
Chronic graft rejection	0.173	2.088	0.725-6.014

CI: Confidence interval.

DISCUSSION

LT is considered the only curative therapeutic option for patients with end-stage hepatic disease. Several complications, especially BC, still endanger its short and long-term outcomes[21,28,29]. Many studies have focused on BC to improve care for transplanted recipients; however, data on long-term outcomes remains scarce[28].

The BC incidence rate is extremely diverse between centres. The overall incidence of BC, including BL, biliary infection and BS, in our study was 57.4%. This rate is comparable to previous reports[17,30-33]; however, it is higher than other published data[8,15,21,34,35]. This difference can be attributed to the heterogeneous structure between the different studies regarding the type of graft, surgical techniques and the inconsistent inclusion of biliary infection and bile stones as a part of BC.

In addition to surgical techniques, several risk factors for BC have been defined in the published literature[3,7,14,21,36], such as older recipients and donors, female recipients and recipients of female donors, ABO mismatch, a prolonged anhepatic phase and prolonged ischemia times. However, the current study and other investigators[15,22,34] were unable to establish any of these conditions as risk factors for BC. This may be attributed to the inclusion of only ABO-matched living grafts, the younger age of our donors and recipients and the male predominance in our cohort.

Additionally, cholestatic liver diseases and the use of RYHJ technique were independent risk factors for BS in previous reports[15,37]. However, this is not the case in our study because DDA was used in all the grafts; besides, we excluded patients with PBC and PSC from the final analysis to avoid the bias of primary disease recurrence as a confounding factor during analysis of BC.

In accordance with published data[15,17], no association between BC and MELD score was observed. This result differs from studies recognizing a higher MELD score as a risk factor for BC[3,28,34]. This can be explained by the lower MELD scores in our patients. Also, these conflicting results may reflect the well-established limits of the MELD score in predicting post-LT outcomes[38].

The ideal material and style of sutures in biliary reconstruction has been argued since the early development of LT. Kaldas *et al*[17] reported that the use of non-absorbable sutures for biliary reconstruction was an independent risk factor for BC. However, this was not the case in the present study due to the different suture material.

In accordance with previous results[22], we observed that the occurrence of BS was not related to the number of bile ducts or stent insertion. In contrast, Miyagi *et al*[8] and Ogiso *et al*[34] identified the number of bile ducts as a risk factor for BC. Furthermore, Senter-Zapata *et al*[15] reported that internal biliary stents and T-tube insertion were risk factors for BC post-LT. However, in our centre, we prefer external drainage for easy accessibility of biliary ducts for postoperative cholangiography to manage any strictures[22]; on the contrary, other centres do not prefer this due to the higher incidence of postoperative BL and biliary infections[14].

BCs are mostly identified in the first three to 12 mo post-LT[8,17]. Similarly, in consistence with other reports[7,15,17,30,31,33], we detected BL early in 55/169 (32.5%) patients, and BS in 60/169 (35.5%) patients. The majority of BSs were anastomotic and presented late.

In a similar management plan as other centres[22,24,29,30,34], minor BLs were treated conservatively; nonetheless, major BL required percutaneous drainage and/or stenting. ERCP was the treatment of choice for all patients. PTC was the treatment option if ERCP failed, and surgical intervention was performed as a last option.

In consistence with our results, other investigators[7,8,21,39] observed that BL and cholangitis were risk factors for the development of BS. This can be explained by the inflammatory process with the resultant progression of fibrosis and stricture formation [40].

In agreement with Rammohan *et al*[39], we identified longer arterialization time as a risk factor for BS. This finding is predictable because biliary tract vascularization is supplied exclusively by the hepatic artery[41-43], and a longer arterialization time of the graft may cause biliary ischemia and subsequently BS[28].

In contrast to the present and Ogiso *et al*[34] studies, other investigators[15,17,28,29,41] reported that hepatic artery complications were linked to the incidence of BC. This conflicting result can be attributed to the low incidence of arterial complications in our cohort as well as the early effective intervention for such complications.

It was previously reported that graft rejection and BC are interrelated conditions[15,35]; however, there are limited data concerning the impact of BC on chronic graft rejection. The incidence rate of chronic ductopenic rejection in our study was 27 (16%) patients; 23 (85.18%),17 (63%) and 13 (48.1%) of them had biliary infection, BS and BL,

Table 6 Relation between biliary complications and chronic graft rejection

Variable		No Chronic graft rejection (*n* = 142), *n* (%)	Chronic graft rejection (*n* = 27), *n* (%)	OR	CI		*P* value[1]
					95% LCL	95% UCL	
Biliary leakage	-	100 (87.7)	14 (12.3)	2.2	1.0	5.1	0.059
	+	42 (76.4)	13 (23.6)				
Insertion of pigtail catheter for biliary leakage	-	135 (84.4)	25 (15.6)	1.5	0.3	7.9	0.637[2]
	+	7 (77.8)	2 (22.2)				
Biliary infection	-	68 (94.4)	4 (5.6)	5.3	1.7	16.1	**0.001**
	+	74 (76.3)	23 (23.7)				
Frequency of biliary infection	Nil	68 (94.4)	4 (5.6)				**< 0.0013**
	1-2 Episodes	68 (81)	16 (19)				
	≥ 3 Episodes	6 (46.2)	7 (53.8)				
Early biliary infection	-	73 (93.6)	5 (6.4)	4.7	1.7	13.0	**0.002**
	+	69 (75.8)	22 (24.2)				
Biliary stricture	-	99 (90.8)	10 (9.2)	3.9	1.7	9.2	**0.001**
	+	43 (71.7)	17 (28.3)				
Frequency of biliary strictures	Nil	99 (90.8)	10 (9.2)				**0.0013**
	1-2 Episodes	32 (74.4)	11 (25.6)				
	≥ 3 Episodes	11 (64.7)	6 (35.3)				
Early biliary stricture	-	134 (87)	20 (13)	5.9	1.9	17.9	**0.0032**
	+	8 (53.3)	7 (46.7)				
Need for ERCP	-	99 (90.8)	10 (9.2)	3.9	1.7	9.2	**0.001**
	+	43 (71.7)	17 (28.3)				
Frequency of ERCP	Nil	99 (90.8)	10 (9.2)				**0.0013**
	1-2 ERCP	31 (73.8)	11 (26.2)				
	≥ 3 ERCP	12 (66.7)	6 (33.3)				
Number of stents introduced for stricture	Nil	102 (91.1)	10 (8.9)				**0.0023**
	1-2 stents	25 (73.5)	9 (26.5)				
	≥ 3 stents	15 (65.2)	8 (34.8)				
Need for PTC	-	136 (84.5)	25 (15.5)	1.8	0.3	9.5	0.615[2]
	+	6 (75)	2 (25)				
Frequency of PTC	Nil	136 (84.5)	25 (15.5)				0.190[3]
	1 PTC	6 (85.7)	1 (14.3)				
	2 PTC	0 (0)	1 (100)				
Surgical intervention for stricture	-	141 (83.9)	27 (16.1)	0.8	0.8	0.9	1.000[2]
	+	1 (100)	0 (0)				
HCV PCR at occurrence of stricture	Negative	10 (66.7)	5 (33.3)				0.660[3]
	Below 200000 IU	12 (80)	3 (20)				
	200000 to 2 million	15 (78.9)	4 (21.1)				
	More than 2 million	6 (54.5)	5 (45.5)				
Antiviral treatment in relation to stricture	Not given	21 (77.8)	6 (22.2)				0.536[2]
	Before stricture	9 (64.3)	5 (35.7)				

	After stricture	4 (66.7)	2 (33.3)				
	During occurrence of stricture	9 (69.2)	4 (30.8)				
Admission related to BC	-	85 (89.5)	10 (10.5)	2.5	1.1	5.9	**0.028**
	+	57 (77)	17 (23)				
Frequency of admissions related to biliary complications	Nil	85 (89.5)	10 (10.5)				**0.0023**
	1-2	35 (87.5)	5 (12.5)				
	≥ 3	22 (64.7)	12 (35.3)				
Recurrent HCV	-	116 (90.6)	12 (9.4)	5.6	2.3	13.3	**< 0.001**
	+	26 (63.4)	15 (36.6)				
Resolution of recurrent HCV	-	1 (25)	3 (75)	0.2	0.0	1.7	0.130[2]
	+	25 (67.6)	12 (32.4)				

[1]Pearson chi-squared test unless otherwise indicated.
[2]Fisher's exact test.
[3]Chi-squared test for trend.
Data are presented as number (*n*) and percentage (%). OR: Odds ratio; LCL: Lower confidence limit; UCL: Lower confidence limit; ERCP: Endoscopic retrograde cholangiopancreatography; PTC: Percutaneous transhepatic cholangiography; PCR: Polymerase chain reaction; HCV: Hepatitis C virus; BC: Biliary complication.

Table 7 Multivariable binary logistic regression model for prediction of chronic graft rejection

Variable	*P* value	Odds ratio	95%CI
Biliary infection	**0.001**	4.301	1.97-8.224
Early biliary infection	0.061	1.105	0.89-1.20
Frequency of biliary infection	**0.025**	1.208	0.536-2.726
Biliary stricture	**< 0.0001**	3.882	4.056-9.952
Need for ERCP	**0.02**	2.91	1.85-7.97
Frequency of ERCP	0.074	1.098	0.99-1.114
Number of stents	0.62	1.22	0.57-2.42
Admission related to BCs	0.082	1.102	0.99-1.40
Frequency of admission	0.51	1.73	0.56-7.5
Recurrent HCV	**0.032**	3.11	1.97-8.07

CI: Confidence interval; ERCP: Endoscopic retrograde cholangiopancreatography; HCV: Hepatitis C virus; BC: Biliary complication.

respectively. Additionally, chronic graft rejection was a risk factor for BS. Similar findings were reported by other investigators[44]. This is consistent with the histopathological findings of chronic ductopenic rejection where ductal inflammation and proliferation are seen in early stages and biliary duct fibrosis with progressive ductopenia is seen in late stages, which is manifested as intrahepatic BS by MRCP[45].

Biliary infection was a risk factor for chronic graft rejection and graft failure, which is explained by interrupted immunosuppressive therapy during times of sepsis[46,47].

In agreement with previous results[15,17,34,48], we found that the main reasons for graft failure were chronic ductopenic rejection, biliary infection, BL, and recurrent HCV infection, while Egeli *et al*[49] reported that HCC recurrence was the main cause of graft failure. This is justified by the inclusion of many patients beyond Milan criteria in their study.

In contrast to Mathur *et al*[50] and in consistence with other investigators[8,17,34,41], there was no association between BS and graft failure. This proves that early detection and efficient management of BS can prevent graft loss.

Table 8 Relation between biliary complications and graft failure

Variable		No graft failure (*n* = 149), *n* (%)	Graft failure (*n* = 20), *n* (%)	OR	CI		*P* value[1]
					95% LCL	95% UCL	
Biliary leakage	-	105 (92.1)	9 (7.9)	2.9	1.1	7.5	**0.022**
	+	44 (80)	11 (20)				
Insertion of pigtail catheter	-	144 (90)	16 (10)	7.2	1.8	29.6	**0.012**[2]
	+	5 (55.6)	4 (44.4)				
Biliary infection	-	68 (94.4)	4 (5.6)	3.4	1.1	10.5	**0.029**
	+	81 (83.5)	16 (16.5)				
Frequency of biliary infection	Nil	68 (94.4)	4 (5.6)				**0.021**[3]
	1-2 Episodes	71 (84.5)	13 (15.5)				
	≥ 3 Episodes	10 (76.9)	3 (23.1)				
Early biliary infection	-	73 (93.6)	5 (6.4)	2.9	1.0	8.3	**0.043**
	+	76 (83.5)	15 (16.5)				
Biliary stricture	-	98 (89.9)	11 (10.1)	1.6	0.6	4.0	0.345
	+	51 (85)	9 (15)				
Frequency of biliary stricture	Nil	98 (89.9)	11 (10.1)				0.168[3]
	1-2 Episodes	38 (88.4)	5 (11.6)				
	≥ 3 Episodes	13 (76.5)	4 (23.5)				
Early biliary stricture	-	137 (89)	17 (11)	2.0	0.5	7.9	0.392[2]
	+	12 (80)	3 (20.0)				
Need for ERCP	-	98 (89.9)	11 (10.1)	1.6	0.6	4.0	0.345
	+	51 (85)	9 (15.0)				
Frequency of ERCP	Nil	98 (89.9)	11 (10.1)				0.188[3]
	1-2 ERCP	37 (88.1)	5 (11.9)				
	≥ 3 ERCP	14 (77.8)	4 (22.2)				
Number of stents introduced for stricture	Nil	101 (90.2)	11 (9.8)				0.136[3]
	1-2 Stents	30 (88.2)	4 (11.8)				
	≥ 3 Stents	18 (78.3)	5 (21.7)				
Need for PTC	-	142 (88.2)	19 (11.8)	1.1	0.1	9.2	1.000[2]
	+	7 (87.5)	1 (12.5)				
Frequency of PTC	Nil	142 (88.2)	19 (11.8)				0.374[3]
	1 PTC	7 (100)	0 (0)				
	2 PTC	0 (0)	1 (100)				
Surgical intervention for stricture	-	148 (88.1)	20 (11.9)	0.9	0.8	0.9	1.000[2]
	+	1 (100)	0 (0)				
HCV PCR at occurrence of stricture	Negative	13 (86.7)	2 (13.3)				0.292[3]
	Below 200000 IU	13 (86.7)	2 (13.3)				
	200000 to 2 million	18 (94.7)	1 (5.3)				
	More than 2 million	7 (63.6)	4 (36.4)				
Antiviral treatment in relation to stricture	Not given	24 (88.9)	3 (11.1)				0.836[2]
	Before stricture	11 (78.6)	3 (21.4)				
	After stricture	5 (83.3)	1 (16.7)				

	During occurrence of stricture	11 (84.6)	2 (15.4)				
Admission related to BC	-	85 (89.5)	10 (10.5)	1.3	0.5	3.4	0.551
	+	64 (86.5)	10 (13.5)				
Frequency of admissions related to BC	Nil	85 (89.5)	10 (10.5)				0.119
	1-2 ERCP	38 (95)	2 (5)				
	≥3 ERCP	26 (76.5)	8 (23.5)				
Recurrent HCV infection	-	118 (92.2)	10 (7.8)	3.8	1.5	10.0	**0.010**[2]
	+	31 (75.6)	10 (24.4)				
Resolution of recurrent HCV	-	0 (0)	4 (100)	6.2	3.0	12.8	**0.002**[2]
	+	31 (83.8)	6 (16.2)				

[1]Pearson chi-squared test unless otherwise indicated.
[2]Fisher's exact test.
[3]Chi-squared test for trend.
Data are presented as number (*n*) and percentage (%). OR: Odds ratio; LCL: Lower confidence limit; UCL: Lower confidence limit; ERCP: Endoscopic retrograde cholangiopancreatography; PTC: Percutaneous transhepatic cholangiography; PCR: Polymerase chain reaction; HCV: Hepatitis C virus; BC: Biliary complication.

Table 9 Multivariable binary logistic regression model for prediction of graft failure

Variable	*P* value	Odds ratio	95%CI
Biliary leakage	**0.021**	1.82	1.34-5.57
Insertion of pigtail catheter	**0.010**	3.76	1.45-11.83
Biliary infection	**0.032**	3.11	1.03-9.06
Early biliary infection	0.05	1.34	0.65-2.86
Frequency of biliary infection	**0.001**	2.52	1.28-4.91
Nonresponse to HCV anti-viral therapy	**0.001**	3.6	1.8-9.34
Recurrent HCV	**0.001**	3.56	1.86-10.71

CI: Confidence interval; HCV: Hepatitis C virus.

In the current study, recurrent HCV infection was a risk factor for chronic graft rejection, graft failure and mortality. This is predictable due to the aggressive course of HCV recurrence in LT recipients through direct cytotoxic effects on the graft, resulting in graft failure[48,49,51-53]. It is noteworthy that DAA were not FDA approved during the first three years of the study duration; thus, many patients were ineligible for the Peg-IFN/RBV regimen at that time.

Similar to Takagi *et al*[54] study, the overall mortality rate for recipients was 28 (16.56%). Unresolved HCV recurrence was the only significant risk factor for mortality, while BC had no impact on recipients' survival in the present study. This is similar to previous results[17,21,39,41,49]. In contrast, other investigators[15,33] observed a worse survival rate in recipients with BC. This indicates that early detection and effective management of BC can improve recipients' survival[2,17].

This study has the strength of being large volume with a long duration of follow-up, as well as the exclusion of LDLT recipients because of cholestatic hepatic diseases; however, it is limited by being a single-centre retrospective study. Multi-centre large-scale studies are required to comprehensively investigate the risk factors for the occurrence and impacts of BC.

CONCLUSION

In conclusion, biliary complications after RT-LDLT represent an independent risk

Table 10 Relation between biliary complications and mortality

Variable		Survivors (*n* = 141), *n* (%)	Non-survivors (*n* = 28), *n* (%)	OR	CI		*P* value[1]
					95% LCL	95% UCL	
Biliary leakage	-	97 (85.1)	17 (14.9)	1.4	0.6	3.3	0.405
	+	44 (80)	11 (20)				
Biliary infection	-	60 (83.3)	12 (16.7)	1.0	0.4	2.2	1.000[2]
	+	81 (83.5)	16 (16.5)				
Frequency of biliary infection	Nil	60 (83.3)	12 (16.7)				0.940[3]
	1-2 Episodes	70 (83.3)	14 (16.7)				
	≥ 3 Episodes	11 (84.6)	2 (15.4)				
Early biliary infection	-	64 (82.1)	14 (17.9)	0.8	0.4	1.9	0.655[2]
	+	77 (84.6)	14 (15.4)				
Biliary stricture	-	89 (81.7)	20 (18.3)	0.7	0.3	1.7	0.401[2]
	+	52 (86.7)	8 (13.3)				
Frequency of biliary strictures	Nil	89 (81.7)	20 (18.3)				0.396[3]
	1-2 Episodes	37 (86)	6 (14)				
	≥ 3 Episodes	15 (88.2)	2 (11.8)				
Early biliary stricture	-	128 (83.1)	26 (16.9)	0.8	0.2	3.6	1.000[2]
	+	13 (86.7)	2 (13.3)				
Need for ERCP	-	89 (81.7)	20 (18.3)	0.7	0.3	1.7	0.401[2]
	+	52 (86.7)	8 (13.3)				
Frequency of ERCP	Nil	89 (81.7)	20 (18.3)				0.375[3]
	1-2 ERCP	36 (85.7)	6 (14.3)				
	≥ 3 ERCP	16 (88.9)	2 (11.1)				
Number of stents introduced for stricture	Nil	92 (82.1)	20 (17.9)				0.520[3]
	1-2 Stents	29 (85.3)	5 (14.7)				
	≥ 3 Stents	20 (87)	3 (13)				
Need for PTC	-	134 (83.2)	27 (16.8)	0.7	0.1	6.0	1.000[2]
	+	7 (87.5)	1 (12.5)				
Frequency of PTC	Nil	134 (83.2)	27 (16.8)				0.674[3]
	1 PTC	7 (100)	0 (0)				
	2 PTC	0 (0)	1 (100)				
Surgical intervention for stricture	-	140 (83.3)	28 (16.7)				1.000[2]
	+	1 (100)	0 (0)				
HCV PCR at occurrence of stricture	Negative	12 (80)	3 (20)				0.849[3]
	Below 200 000 IU	14 (93.3)	1 (6.7)				
	200000 to 2 million	18 (94.7)	1 (5.3)				
	More than 2 million	8 (72.7)	3 (27.3)				
Antiviral treatment in relation to stricture	Not given	23 (85.2)	4 (14.8)				1.000[2]
	Before stricture	12 (85.7)	2 (14.3)				
	After stricture	5 (83.3)	1 (16.7)				
	During occurrence of stricture	12 (92.3)	1 (7.7)				

Admission related to BC	-	75 (78.9)	20 (21.1)	0.5	0.2	1.1	0.076[2]
	+	66 (89.2)	8 (10.8)				
Recurrent HCV	-	108 (84.4)	20 (15.6)	1.3	0.5	3.2	0.560[2]
	+	33 (80.5)	8 (19.5)				
Resolution of recurrent HCV (n = 41)	-	0 (0)	4 (9.7)	9.3	3.7	23.3	**0.001**[2]
	+	33 (80.4)	4 (9.7)				

[1]Pearson chi-squared test unless otherwise indicated.
[2]Fisher's exact test.
[3]Chi-squared test for trend.
Data are presented as number (n) and percentage (%). OR: Odds ratio; LCL: Lower confidence limit; UCL: Lower confidence limit; ERCP: Endoscopic retrograde cholangiopancreatography; PTC: Percutaneous transhepatic cholangiography; PCR: Polymerase chain reaction; HCV: Hepatitis C virus; BC: Biliary complication.

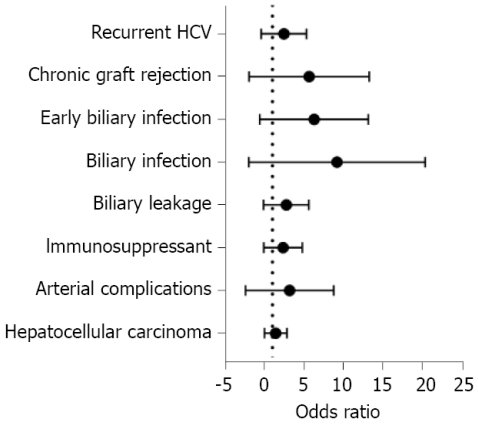

Figure 2 Forest plot for risk factors for biliary strictures. HCV: Hepatitis C virus.

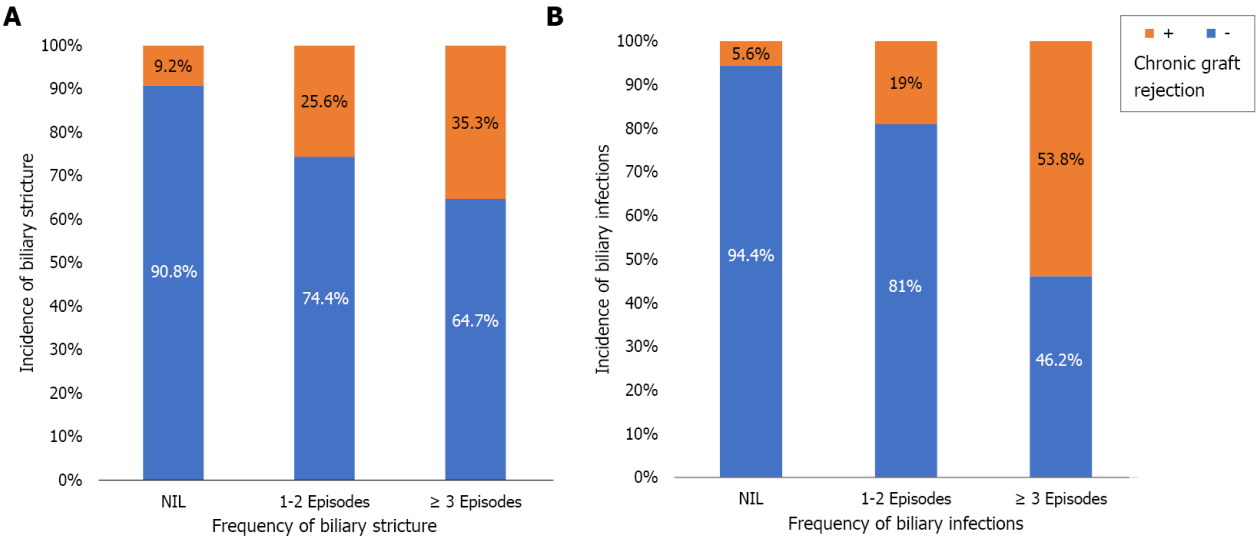

Figure 3 Incidence of chronic graft rejection according to the occurrence of biliary strictures (A) and biliary infections (B).

factor for chronic graft rejection and graft failure; nonetheless, effective management of these complications can improve patient and graft survival.

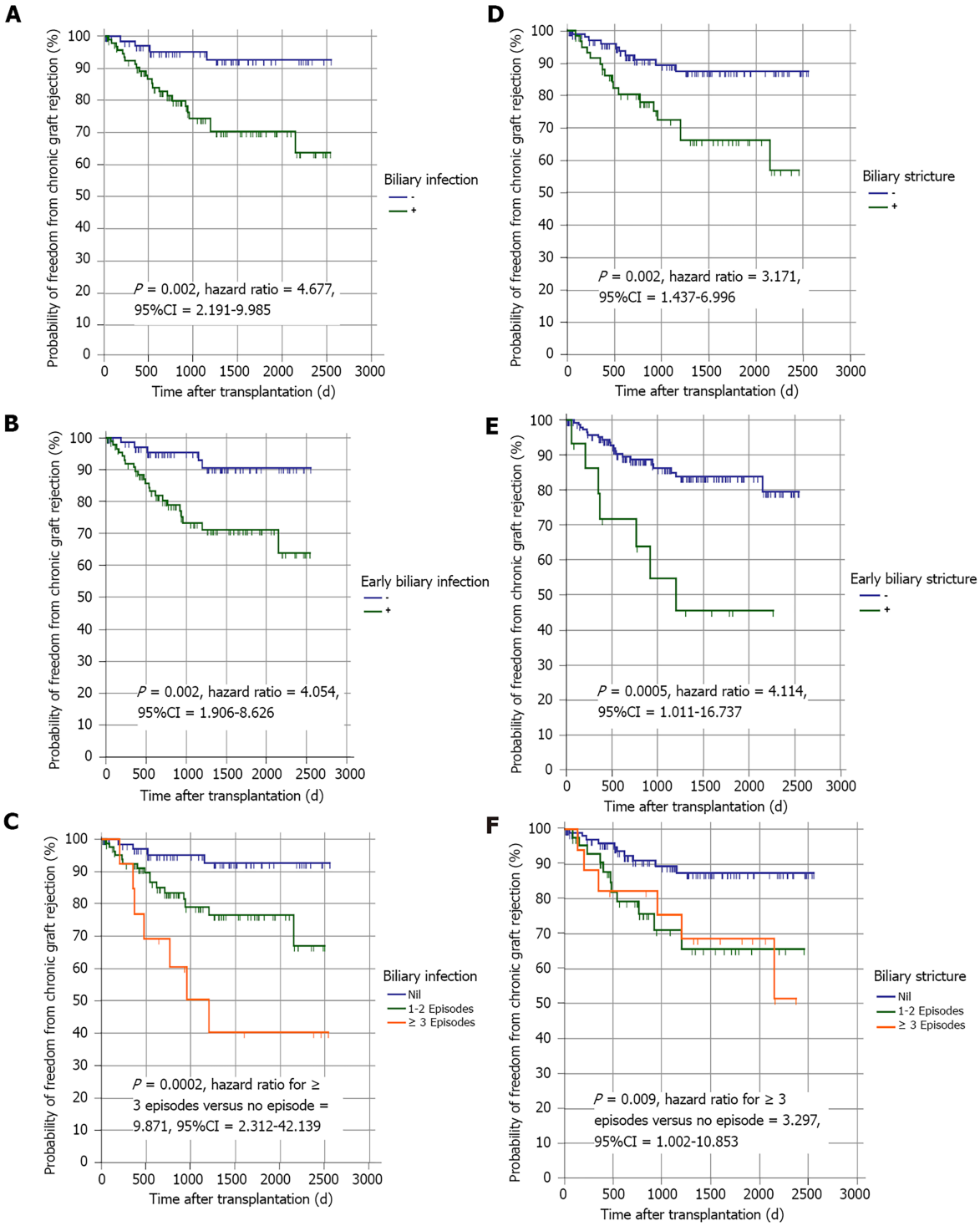

Figure 4 Kaplan-Meier curves. A-C: The curves showing the probability of chronic graft rejection in patients regarding the occurrence (A), timing (B), and frequency (C) of biliary infection; D-F: The curves showing the probability of chronic graft rejection in patients regarding the occurrence (D), timing (E), and frequency (F) of biliary strictures.

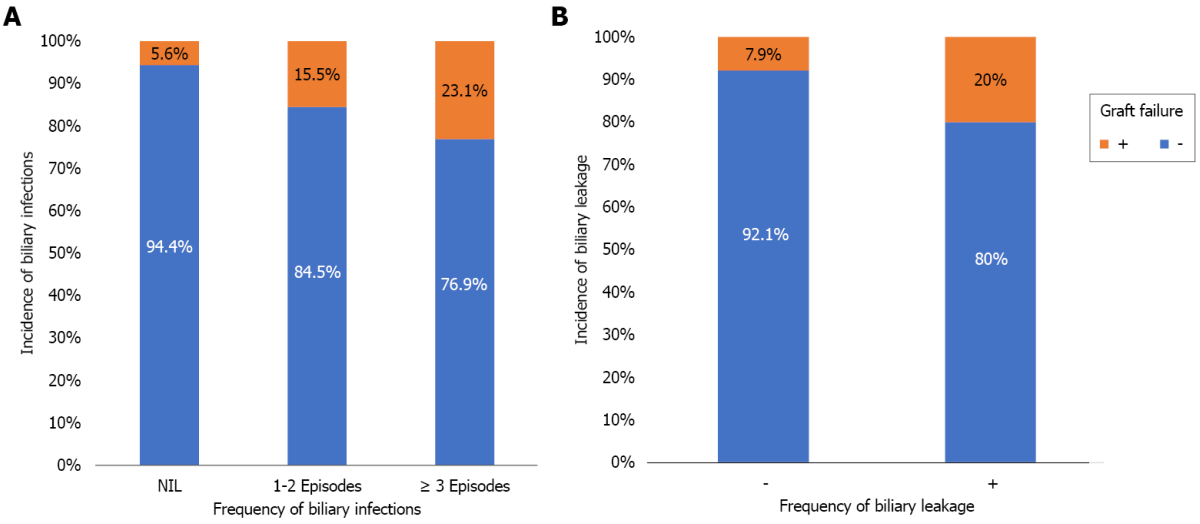

Figure 5 Incidence of graft failure according to the occurrence of biliary infections (A) and biliary leakage (B).

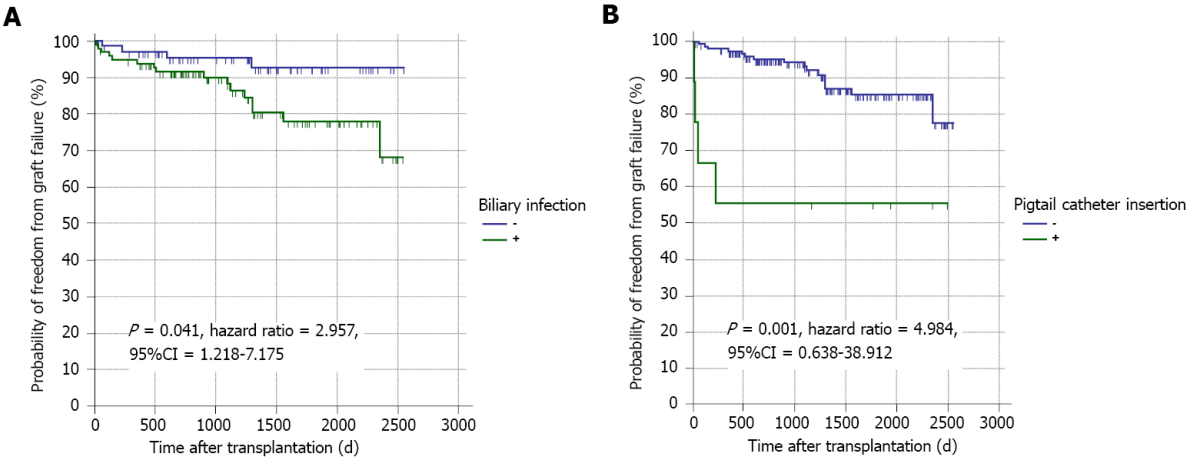

Figure 6 Kaplan-Meier curves. The curves showing the probability of graft failure in patients regarding the occurrence of biliary infection (A) and large bile leaks as indicated by pigtail insertion (B).

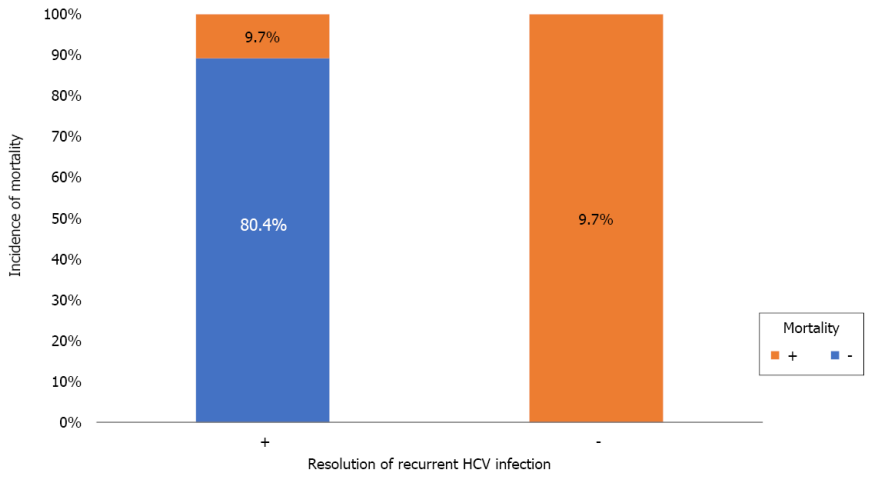

Figure 7 Mortality rate in patients with or without resolution of recurrent hepatitis C virus in patient with biliary stricture. HCV: Hepatitis C virus.

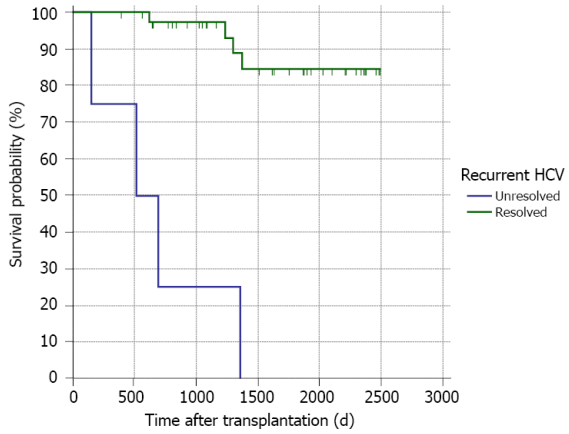

Figure 8 Kaplan-Meier curves showing the survival probability of patients with resolved or unresolved hepatitis C virus. HCV: Hepatitis C virus.

ARTICLE HIGHLIGHTS

Research background

Despite considerable progress in liver transplantation (LT) surgical performance and peri-operative management, post-LT biliary complications (BCs) remain a considerable cause of morbidity, mortality, increased cost, and graft loss.

Research motivation

Many studies have focused on biliary complications to improve care for transplanted recipients; however, data on long-term outcomes remain scarce.

Research objectives

We aimed to investigate the impact of BCs after right lobe-LDLT (RL-LDLT) on chronic graft rejection, graft failure and mortality.

Research methods

From 2011 to 2016, 215 adult recipients underwent RL-LDLT at our centre. We excluded 46 recipients who met the exclusion criteria, and 169 recipients were included in the final analysis. Donors' and recipients' demographic data, clinical data, operative details and postoperative course information were collected. We also reviewed the management and outcomes of BCs. Recipients were followed for at least 12 mo post-LT until December 2017 or graft or patient loss.

Research results

The overall incidence rate of BCs including biliary leakage, biliary infection and biliary stricture was 57.4%. Twenty-seven (16%) patients experienced chronic graft rejection. Graft failure developed in 20 (11.8%) patients. A total of 28 (16.6%) deaths occurred during follow-up. BCs were a risk factor for the occurrence of chronic graft rejection and failure; however, mortality was determined by recurrent hepatitis C virus infection.

Research conclusions

Biliary complications after RT-LDLT represent an independent risk factor for chronic graft rejection and graft failure; nonetheless, effective management of these complications can improve patient and graft survival.

Research perspectives

Multi-centre large-scale studies are required to comprehensively investigate the risk factors for the occurrence and impacts of BC.

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ORIGINAL ARTICLE

Retrospective Study

Liver function tests and metabolic-associated fatty liver disease: Changes in upper normal limits, does it really matter?

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Abstract

BACKGROUND

Metabolic-associated fatty liver disease (MAFLD) is the commonest cause of abnormal liver function tests (LFTs). Current upper normal of limit (UNL) of LFTs was derived from a "healthy" population, where undiagnosed MAFLD and viral hepatitis might be suspected.

AIM

To evaluated potential implications of changes in UNL of alanine aminotransferase (ALT) in MAFLD.

METHODS

We retrospectively assessed consecutive first referrals with a diagnosis of MAFLD from 2010 to 2017. The conventional UNL of ALT was 45 IU/L for men and 34 IU/L for women, while a low UNL of ALT was 30 IU/L for men and 19 IU/L for women. The UNL of aspartate aminotransferase (AST) was 40 IU/L.

RESULTS

Total 436 patients were enrolled; of these, 288 underwent liver biopsy. Setting a lower UNL reduced the percentage of those with significant disease despite normal ALT; specifically, patients with advanced fibrosis (F ≥ F3) or definite "metabolic-associated steato-hepatitis (MASH)" (NAS ≥ 5) within normal ALT decreased from 10% to 1% and from 28% to 4% respectively. However, the proportion of those with elevated ALT and no evidence of advanced fibrosis or "definite MASH" increased from 39% to 47% and from 3% to 19%. Overall, LFTs performed poorly in distinguishing "definite MASH" from simple steatosis (receiver operating characteristic areas under the curves 0.59 for ALT and 0.55 for

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AST).

CONCLUSION
Liver function tests might both under- and overestimate MASH-related liver disease. Reducing the UNL might not be beneficial and imply an increase in healthcare burden. Risk stratification in MAFLD should rely on a combination of risk factors, not on LFTs alone.

Key Words: Metabolic-associated fatty liver disease; Liver function tests; Alanine aminotransferase; Fibrosis; Stiffness

Core Tip: In the United Kingdom, the hepatologists receive increasing demand for secondary care services to investigate liver function tests (LFTs), especially with the suspicion of metabolic-associated fatty liver disease (MAFLD). With current upper normal limit (UNL), patients without liver diseases but elevated LFTs is high (27%), as well as those with significant fibrosis or metabolic-associated steato-hepatitis and normal LFTs (10%). Here, we aimed to evaluate the potential implications of changes in UNL of LFTs. Our data show that reducing the UNL would lead to an increase in overall healthcare burden. In MAFLD, the risk-stratification should rely on a combination of risk factors, rather than on LFTs alone.

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INTRODUCTION

Metabolic-associated fatty liver disease (MAFLD) is emerging as the most prevalent chronic liver disease worldwide secondary to the epidemic of obesity and metabolic syndrome. MAFLD also represents the commonest cause of abnormal liver function tests (LFTs) in Western countries[1]. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are enzymes which transfer amino groups to different substrates, with ALT being more liver-specific[2]. Notably, the patient's metabolic status (such as the presence of obesity and/or insulin resistance) may directly influence LFTs values[3,4]. Moreover, current upper normal limits (UNL) were derived in a population with highly-prevalent MAFLD but unrecognised as a disease entity at the time. As such, several studies have questioned whether current UNL of ALT should be revised although giving contrasting results[5,6].

LFTs are often the first-line investigation for any suspected liver disease with or without imaging[2]. However, the role of LFTs in diagnosing metabolic-associated steato-hepatitis (MASH)-related liver disease, such as the presence of advance fibrosis and/or steatohepatitis, is currently limited. In particular, the full spectrum of MAFLD has been reported in patients with normal LFTs[7,8]. Although histology represents the "gold standard" for diagnosing and staging MASH, the costs and invasive nature of the procedure limit its widespread applicability. Therefore, non-invasive markers are an established part of the investigation of MAFLD. In particular, transient elastography has been validated as marker of fibrosis and represents the typical second-line investigation for MAFLD[2,9].

The aim of this study was to evaluate potential implications of lowering the UNL of ALT in patients with a clinical or histological diagnosis of MAFLD.

MATERIALS AND METHODS

Study population

We retrospectively assessed all consecutive referrals with a clinical or histological diagnosis of MAFLD followed-up at the Liver Unit of St. Mary's Hospital, Imperial College Healthcare NHS Trust, from January 2010 to May 2017.

At the time of liver biopsy or Liver Stiffness Measurement, clinical parameters were recorded, including demographic, anthropometric and biochemical data. The use of steatogenic drugs, chronic alcohol consumption, as well as other liver disease were considered as exclusion criteria[9]. Fibrosis-4 index and non-alcoholic fatty liver disease (NAFLD) fibrosis score were calculated based on published formulas[10,11].

The conventional upper normal limit (UNL) of ALT from the Imperial College NHS Trust laboratory was 45 IU/L for men and 34 IU/L for women. The effect of the application of a lower value of ALT was then investigated. This UNL was set at 30 IU/L for men and 19 IU/L for women, in keeping with previous studies aiming to increase the sensitivity in diagnosing active chronic hepatitis C in the general population[5]. Similarly, this lower ALT UNL helped with differentiating active from inactive chronic hepatitis B carriers[12].

The whole study population was then stratified into three subgroups: the group with ALT higher than the conventional UNL (ALT ≥ 45 IU/L for men and ≥ 34 IU/L for women), the group with ALT within the conventional and the low UNL (ALT 31-45 IU/L for men and 20-34 UI/L for women), and the group with ALT lower than the low UNL (ALT ≤ 30 IU/L for men and ≤ 19 IU/L for women). The UNL for AST was set as 40 IU/L, as per laboratory range.

Liver stiffness measurement

Liver stiffness measurement (LSM) was obtained using FibroScan™. Scans were performed after 4 h fasting. LSM was interpreted according to interquartile range/median ratio: "poorly reliable" LSM values were not considered[13]. Advanced fibrosis was defined as LSM ≥ 8.1 kPa[14].

Liver histology

Liver biopsies were performed using a 16-Gauge Trucut needle (Argon, Athens Tx, USA). Specimens were formalin-fixed and paraffin-embedded; thick sections were stained with Hematoxylin and Eosin and Sirius Red. All biopsies were scored using the NASH CRN scoring system. Advanced fibrosis was defined as fibrosis stage ≥ F3. "Definite MASH", "possible MASH" and "non-MASH" were defined as per NAFLD activity score (NAS)[15].

Statistical analysis

The distribution of variables was explored using the Shapiro-Wilk test. Since the variables were normally distributed, continuous variables were expressed as medians and SD, and categorical variables were expressed as relative frequencies. Differences between the groups were tested using one-way ANOVA for categorical and Mann-Whitney or Kruskal Wallis for categorical variables. Correlation was measured using Pearson's Rho coefficient. Receiver operating characteristic (ROC) areas under the curves (AUROC) were used to assess the diagnostic performance of ALT and AST. Statistical analysis was performed using SPSS© (version 24.0; SPSS Inc. Chicago, IL).

Ethics

This study was considered a service evaluation project, using routinely collected patient data, therefore no ethical approval was required under the United Kingdom (UK) policy framework for health and social care.

RESULTS

Alanine aminotransferase and liver stiffness measurement

Four hundred thirty-six patients underwent LSM. Overall, 330 (76%) patients had ALT higher than the conventional UNL, 73 (17%) had ALT within the conventional and the low UNL and 33 (7%) had ALT lower than the low UNL. AST and γ-GT levels only were significantly different between the three groups ($P < 0.0001$ and $P = 0.008$ respectively). There was no difference in terms of use of statin therapy between the groups (Table 1).

Table 1 Anthropometric and clinical characteristics of the whole population, stratified into three groups according to alanine aminotransferase levels

Variable	ALT lower than the low cut-off (*n* = 33)	ALT within the conventional and the low cut-off (*n* = 73)	ALT higher than the conventional cut-off (*n* = 330)	*P* value
Age (yr)	52 ± 13.3	52.1 ± 12.1	52.5 ± 13.1	0.52
BMI (kg/m^2)	29.9 ± 4.2	30 ± 5.5	29.3 ± 4.5	0.23
T-Cholesterol (mmol/L)	4.2 ± 1.4	4.4 ± 1	4.7 ± 2	0.3
HDL (mmol/L)	1 ± 0.3	1.1 ± 0.3	1 ± 0.8	0.81
LDL (mmol/L)	2.4 ± 1.1	2.5 ± 0.9	2.6 ± 1	0.27
Triglycerides (mmol/L)	1.9 ± 1	1.6 ± 0.9	1.7 ± 1.4	0.28
HbA1c (mmol/L)	41 ± 21	42 ± 16	45 ± 15.8	0.75
AST (IU/L)	25 ± 8	31 ± 7.7	51 ± 37	< 0.0001[1]
γGT (IU/L)	32 ± 41	38 ± 62	81 ± 76	0.008[1]
Platelet (10^9 /L)	208 ± 70	225 ± 72	229 ± 72	0.39
Albumin (g/L)	40 ± 6.1	41 ± 3.4	41 ± 3.2	0.62
Ferritin (μg/L)	58 ± 145	104 ± 150	163 ± 120	0.13
Male gender	21 (65)	52 (62)	207 (63)	0.13
Diabetes Mellitus	19 (58)	46 (55)	161 (49)	0.12
Ethnicity				
Caucasian	17 (5)	35 (48)	163 (49)	0.79
Arab	8 (24)	11 (15)	66 (20)	0.31
Hispanic and Latinos	2 (6)	5 (6)	20 (7)	0.99
South Asian	4 (12)	11 (15)	41 (12)	0.95
East Asian	1 (3)	6 (6)	25 (7)	0.26
African/Afrocaribbean	1 (3)	5 (6)	15 (4)	0.73
Hypertension	15 (45)	33 (39)	112 (34)	0.2
Dyslipidemia	13 (39)	37 (44)	141 (43)	0.93
Statin treatment	14 (42)	34 (46)	152 (46)	0.54

[1]Significantly different. Data present as mean ± SD or *n* (%). ALT: Alanine aminotransferase; BMI: Body mass index; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; HbA1c: Hemoglobin A1C; AST: Aspartate aminotransferase.

Using the conventional UNL as reference, 10% of the patients had evidence of advanced fibrosis (LSM ≥ 8.1 kPa) despite normal ALT. When the low UNL for ALT was applied, this percentage reduced to 3%. However, applying the low UNL determined also the increase in the proportion of those with elevated ALT but not showing evidence of advanced fibrosis (LSM ≥ 8.1 kPa) from 42% to 52% (Supplementary Figure 1).

In the whole population, there was no linear association between ALT and age, as Pearson's correlation was not significant (Rho = -0.86, *P* = 0.07). Moreover, the distribution of ALT across age groups was similar when patients were further stratified per gender (Kruskal Wallis).

Alanine aminotransferase and liver histology

A subgroup of 288 patients underwent a liver biopsy. Overall, 220 (78%) patients had ALT higher than the conventional UNL, 50 (17%) had ALT within the conventional and the low UNL and 18 (5%) had ALT lower than the low UNL.

Using the conventional UNL as reference, 10% of patients had advanced fibrosis (F ≥ F3) on histology despite normal ALT. When the low UNL for ALT was applied, this percentage reduced to 1%. However, applying the low UNL determined also the increase in the proportion of those with elevated ALT but not showing advanced

fibrosis from 39% to 47% (Figure 1). Similarly, lowering the UNL of ALT, the percentage of those with "definite MASH" (NAS ≥ 5) despite normal ALT decreased from 28% to 4%, whilst the percentage of patients without "definite MASH" but showing elevated ALT increased from 3% to 19% (Figure 2).

Overall, FIB-4 and NAFLD fibrosis scored performed better than ALT in diagnosing F > F3. Specifically, the AUROC of ALT for diagnosing F ≥ F3 was 0.45 (95%CI: 0.38-0.53, P = 0.05) compared to 0.71 (95%CI: 0.63-0.79, P = 0.0001) for FIB-4 and 0.65 (95%CI: 0.59-0.72, P = 0.0001) for NAFLD fibrosis score. However, ALT, FIB-4 and NAFLD fibrosis score performed similarly in diagnosing "definite MASH". In particular, the AUROC of ALT was 0.55 (95%CI: 0.47-0.62, P = 0.049), compared to 0.47 (95%CI: 0.39-0.54, P = 0.01) for FIB-4 and 0.5 (95%CI: 0.42-0.58, P = 0.05) for NAFLD fibrosis score (Figure 3A and B).

Aspartate aminotransferase and liver stiffness measurement

Overall, 235 (54%) patients had elevated AST and 201 (46%) had normal AST. ALT, γ-GT and ferritin only were significantly different between the groups (P < 0.0001, P < 0.0001 and P = 0.008 respectively). There was no difference in terms of statin therapy (Supplementary Table 1).

Advanced fibrosis (LSM ≥ 8.1 kPa) was diagnosed despite normal AST in 16% of the cases, while the proportion of those with elevated AST but LSM <8.1 kPa was 27%.

In the whole population, there was no linear association between AST and age, as Pearson's correlation was not significant (Rho = 0.01, P = 0.99). Moreover, the distribution of AST across age groups was similar when patients were further stratified per gender (Kruskal Wallis).

Aspartate aminotransferase and liver histology

In the subgroup of patients who underwent a liver biopsy, 155 (54%) patients had elevated AST and 133 (46%) had normal AST. Advanced fibrosis (F ≥ F3) was diagnosed despite normal AST in 21% of the cases, while the proportion of those with elevated AST and no advanced fibrosis (F ≥ F3) was 26%. "Definite MASH" was diagnosed in presence of normal AST in 37% cases.

Overall, FIB-4 and NAFLD fibrosis scored performed better than AST in diagnosing F > F3, while the three performed similarly in diagnosing "definite MASH". Specifically, the AUROC of AST for diagnosing F ≥ F3 was 0.56 (95%CI: 0.49-0.64, P = 0.05) and 0.59 (95%CI: 0.52-0.67, P = 0.049) for diagnosing "definite MASH" (Figure 3A and B).

DISCUSSION

Metabolic-associated Fatty Liver Disease is a major cause of chronic liver disease and the commonest cause of elevated liver enzymes[16,17]. In the UK, referrals for abnormal LFTs are increasing (> 300 referrals/year), and this often represents the first step in diagnosing MAFLD[18].

Several factors may influence ALT, such as age, gender, BMI, insulin resistance and triglycerides[3,4,19]. Overall, ALT is more commonly elevated than AST in chronic liver disease, with the notable exception of alcohol-induced liver injury[20]. Since transaminases are released following hepatocellular injury, AST and ALT are markers of cytolysis and not necessarily associated with inflammation or steatosis[21]. Nevertheless, LFTs are often used as a surrogate markers to assess the anti-inflammatory effect in clinical trials in MAFLD[22].

While the diagnosis and management of MAFLD has been streamlined in secondary and tertiary care centres, there is still a high variability in how the disease is assessed within the community. In particular, general practitioners (GPs) in primary care rely heavily on LFTs measurement, consistent with pragmatic guidelines which have been developed only recently in the UK[2]. It is also evident from a recent survey study that diagnosing MAFLD is perceived as challenging even to experienced GPs, with the overall perception of overlooking the disease especially in high-risk groups[23].

In this retrospective cohort of patients diagnosed with MAFLD, LFTs were frequently normal despite the presence of advanced liver disease. Moreover, transaminases could not distinguish simple steatosis from "definite MASH" (AUROC 0.59 for ALT and 0.55 for AST) at first referral, giving false reassurance in 10%-15% of patients. Conversely, decision-making based on LFTs alone might have implied unnecessary second-line investigations in approximately 27%-42% of cases. Our results confirm that non-invasive markers based on blood tests (*i.e.*, FIB-4 and NAFLD fibrosis score)

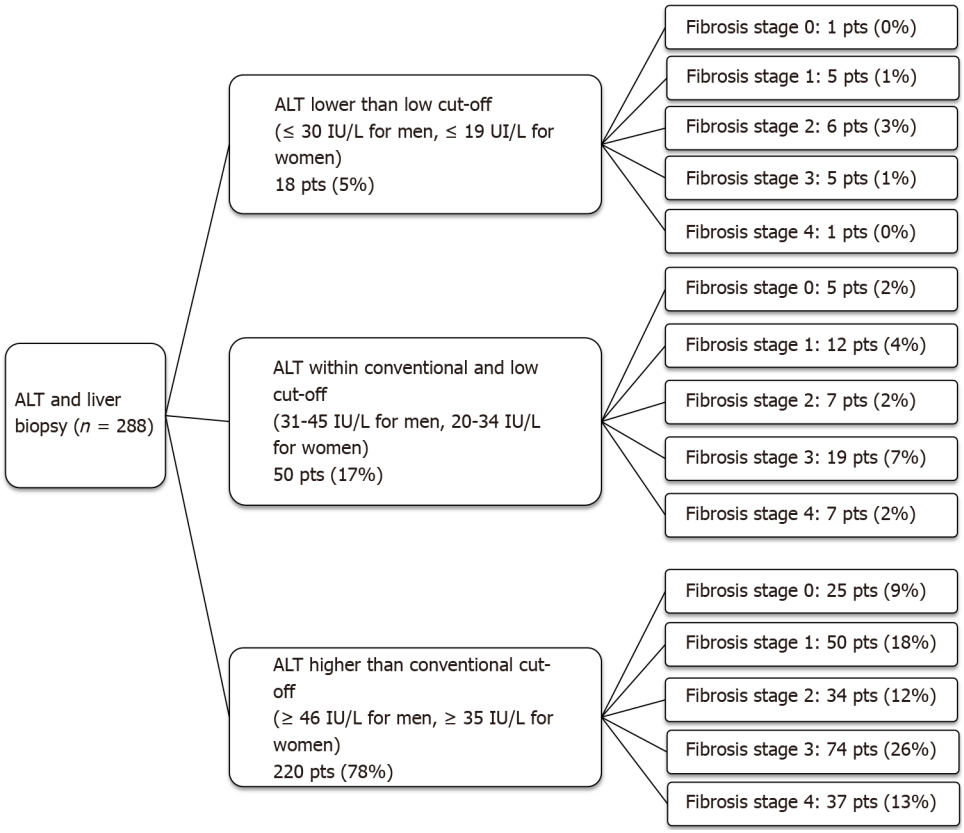

Figure 1 Fibrosis stage in three subgroups of patients stratified for alanine aminotransferase levels. ALT: Alanine aminotransferase; pts: Patients.

perform better than LFTs alone in assessing the severity of liver disease from NAFLD.

The actual normal ALT value is an area of ongoing controversy. Differences in the UNL used between studies are consistent, resulting from laboratory setting and populations tested[24]. Interestingly, the ALT normal range has been derived from "healthy" subjects in the general population[1], where MAFLD and obesity were highly prevalent[24]. Moreover, the UNL was first described in the 1980s, when LFTs were used to rule out 'non-A and non-B hepatitis' positivity amongst blood donors, in a time when anti-HCV antibodies were not available[25]. As such, both undiagnosed cases of MAFLD and chronic viral hepatitis may have contributed to the current definition of the UNL.

In this cohort, when a lower UNL was applied, the proportion of patients with advanced fibrosis or definite MASH on biopsy and normal biochemistry fell substantially, providing a rationale for revising current UNL. However, reducing the ALT normal range might lead to an increase in unnecessary second-line investigations (from 27% to 33% in based on histology this population) for a disease which is already highly prevalent in the general population. As a result, health costs would overwhelm the healthcare system with no clear clinical benefit[5].

CONCLUSION

Liver function tests might both underestimate and overestimate MASH-associated liver disease. Changing the UNL of ALT is not beneficial, as it might increase healthcare burden. Referral/management pathways and risk-stratification strategies are most needed for primary and they should rely on a combination of risk factors and non-invasive markers, not on LFTs alone.

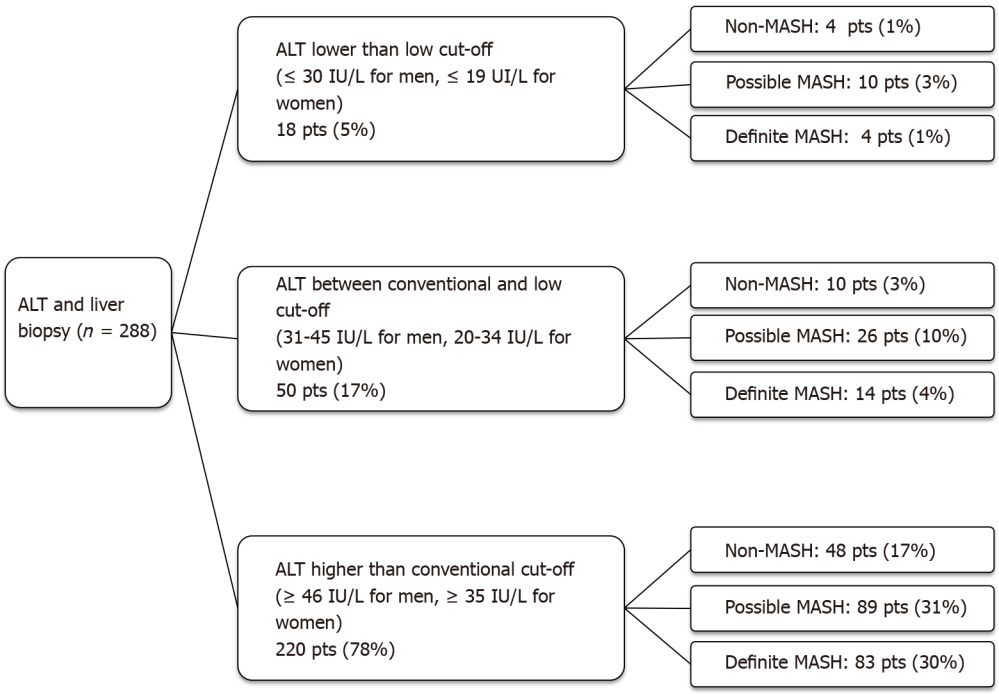

Figure 2 Diagnosis of metabolic-associated steato-hepatitis in three subgroups of patients stratified for alanine aminotransferase levels. ALT: Alanine aminotransferase; pts: Patients; MASH: Metabolic-associated steato-hepatitis.

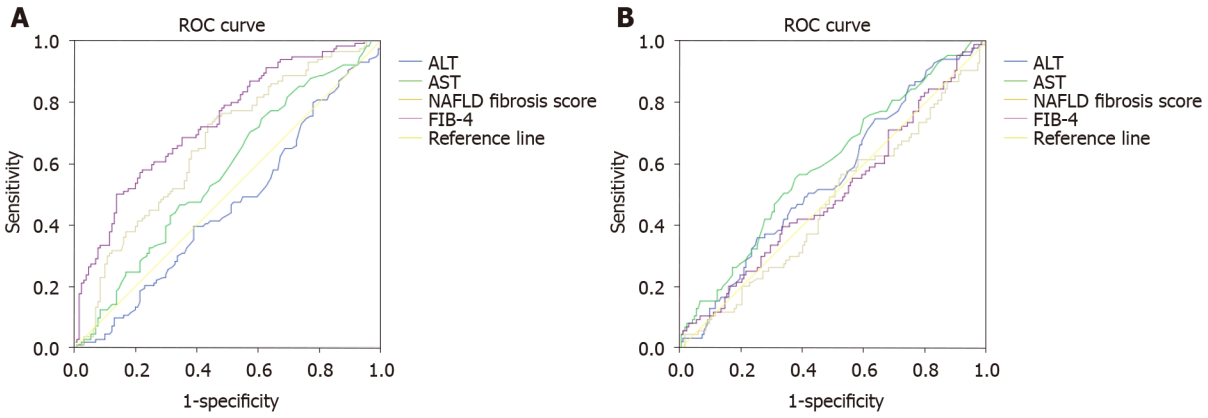

Figure 3 Receiver operating characteristic areas under the curves for liver function tests and non-invasive markers of fibrosis for diagnosis advanced fibrosis (F ≥ F3) and definite metabolic-associated steato-hepatitis (Non-alcoholic fatty liver disease activity score ≥ 5). A: Liver function tests and non-invasive markers of fibrosis for diagnosis advanced fibrosis (F ≥ F3); B: Liver function tests and non-invasive markers of fibrosis for diagnosis definite metabolic-associated steato-hepatitis (Non-alcoholic fatty liver disease activity score ≥ 5). ROC: Receiver operating characteristic; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NAFLD: Non-alcoholic fatty liver disease; FIB: Fibrosis.

ARTICLE HIGHLIGHTS

Research background

Elevated liver function tests (LFTs) often represent the main reason for referring patients with metabolic-associated fatty liver disease (MAFLD) to secondary and tertiary care.

Research motivation

In MAFLD, liver function tests may both under and over-estimate liver disease. Moreover, difference in upper normal limit (UNL) of LFTs is consistent across the literature.

Research objectives
As such, we investigated the potential use of different UNLs of LFTs in MAFLD.

Research methods
We evaluated the use of a lower UNL of ALT *vs* histology and liver stiffness measurement in a cohort of 436 patients with non-alcoholic fatty liver disease in a tertiary care centre.

Research results
Modifying the upper normal limit of LFTs does not improve the diagnostic performance of the test in MAFLD.

Research conclusions
In MAFLD, the risk-stratification should rely on a combination of risk factors and non-invasive markers, rather than on LFTs alone.

Research perspectives
Future research should focus on identifying biomarkers for diagnosing metabolic-associated steato-hepatitis and advanced fibrosis.

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ORIGINAL ARTICLE

Retrospective Study

Use of oral vancomycin in children with autoimmune liver disease: A single centre experience

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Abstract

BACKGROUND

Previous reports showed some beneficial effect of oral vancomycin treatment (OVT) in children with primary sclerosing cholangitis; conversely, the experience in patients with other autoimmune liver diseases (AILD), including autoimmune hepatitis (AIH) and autoimmune sclerosing cholangitis (ASC), is scant.

AIM

To assess the response to immunosuppressive treatment (IS) and to OVT in children diagnosed with AILD.

METHODS

Retrospective study of children diagnosed with AIH (normal biliary tree at cholangiography) and ASC (abnormal biliary tree at cholangiography) in the last 10 years. All underwent standard immunosuppressive therapy (IS), but non-responders received also OVT. Biochemical remission [normal aspartate aminotransferase (AST)] and immunological remission (normal IgG and negative autoantibodies) rates and Sclerosing Cholangitis Outcomes in Pediatrics (SCOPE) index were assessed and compared during the follow up.

RESULTS

75 children were included [69% female, median age 10.5 years (5.6-13.4 years), AIH = 54, ASC= 21]. Sixty-three patients (84%, AIH = 52, ASC = 11) were treated with standard IS and 61 achieved biochemical remission, whereas 12 not responding to IS [16%, F = 75%, median age 13.5 years, (12.2-15.7), 10 with ASC] required OVT and 8 achieved biochemical remission. Overall OVT increased the biochemical remission rate of the whole group of AILD patients from 81% (61/75) to 92% (69/75). Median values of AST, alanine aminotransferase (ALT) and

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gamma-glutamyl transferase (GGT) decreased significantly after OVT start ($P < 0.05$). Complete normalization of livers enzymes (AST, ALT and GGT) was observed in 6/12 patients (50%). Decrease in SCOPE index score was reported in 5/12 patients (42%). At last follow up (median of 4.4 years, range 0.6-13.8 years) all 75 patients are alive, 6 (8%, 1 with ASC) successfully discontinued medications, 1 (with ASC) required liver transplantation.

CONCLUSION
Children with AIH and ASC respond well to IS treatment. OVT may represent a valuable treatment option to achieve biochemical remission in patients not responding to standard IS. These promising preliminary results suggest that a prospective study is indicated to define the efficacy of OVT in AILD.

Key Words: Autoimmune hepatitis; Autoimmune sclerosing cholangitis; Autoimmune liver disease; Vancomycin; Children; Liver transplantation

Core Tip: Experience with oral vancomycin in children with autoimmune liver disease (AILD) is limited. We enrolled 75 children [median age 10.5 years (5.6-13.4)], 54 with autoimmune hepatitis and 21 with autoimmune sclerosing cholangitis; 63/75 achieved remission by standard immunosuppressive therapy (IS), whereas 12/75 (16%) required oral vancomycin treatment (OVT). In 6/12 patients (50%) the response was complete, whereas it was partial in 2/12 (17%), and absent in 4/12 (33%). Overall OVT increased the remission rate of the whole group of AILD patients from 81% to 92%. OVT may represent a valuable treatment option in children with AILD who do not respond to standard IS.

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INTRODUCTION

Pediatric autoimmune liver disease (AILD) is a progressive inflammatory condition including autoimmune hepatitis (AIH), (diagnosed with the standard criteria) and autoimmune sclerosing cholangitis (ASC), (defined as patients fulfilling the criteria for AIH but with an abnormal biliary tree at cholangiography)[1-3].

Children with AILD respond well to immunosuppressive (IS) treatment, although some patients progress to cirrhosis despite normal liver enzymes; a low proportion (30%-40%) achieve immunological remission (normal IgG and negative autoantibodies), and only a small percentage (10%-20%) can stop medications successfully, maintaining remission off treatment[3,4]. Furthermore, children with ASC have a higher need for liver transplantation (LT) compared to AIH, suggesting that bile duct damage may progress despite IS treatment[1-4].

Empirical use of candidate therapies for AILD has significantly increased in the last decades, in the attempt of finding effective medications to normalize liver enzymes and improve outcomes; oral vancomycin is one of the most common drugs empirically used in patients with SC[5-7]. Oral vancomycin is supposed to have an immunomodulatory effect by inducing an increase of T-regs lymphocytes and TGF-β (both with anti-inflammatory activity) without alterations in Th1 or Th2 cytokine production patterns [6-9]. Cox *et al*[12] reported benefits from oral vancomycin treatment (OVT) in children with primary SC (PSC) and inflammatory bowel disease (IBD). Interestingly, OVT seems to be able to modify the gut microbiota and bile acid metabolism, that may have a protective effect on PSC recurrence after LT[10-12].

Previous studies have offered information on the use of OVT in adults and children with PSC; conversely the experience with OVT in children with AIH or ASC not responding to standard IS is very limited[5-7].

In our center we empirically used oral vancomycin in a small series of children with AIH and ASC not responding to standard IS to gather insights that could guide us to the design of a prospective clinical trial.

In this study, we aimed to review our cohort of pediatric patients with AILD to assess: (1) The response to standard IS treatment; and (2) The efficacy of OVT to achieve biochemical and immunological remission in patients not responding to standard IS.

MATERIALS AND METHODS

Data collection

We reviewed retrospectively the medical records of children diagnosed with AILD (AIH or ASC) at Hospital Papa Giovanni XXIII, Bergamo, Italy, between 2010 and 2021. During this period of time all patients were diagnosed by the standard diagnostic criteria including magnetic resonance cholangiopancreatography (MRCP) performed at diagnosis; OVT was regularly adopted in patients not responding to standard treatment. Biochemical and clinical features, histology, and data on outcomes were collected in all patients and compared between the two groups divided according to the diagnosis (AIH *vs* ASC) and OVT.

Diagnosis of autoimmune liver disease

The diagnosis of AILD was based on elevated transaminases and IgG levels, positive autoantibodies, compatible liver histology, and exclusion of other liver diseases[13]. A lower threshold for autoantibody positivity was applied to children compared to adults, *i.e.*, titre ≥ 1:20 for antinuclear antibodies (ANA) and smooth muscle antibodies (SMA) and ≥ 1:10 for anti-liver kidney microsomal type 1 (anti-LKM-1) were used, as indicated by the International Autoimmune Hepatitis Group (IAIHG) consensus statement on liver autoimmune serology[14] and more recently by the European Society of Paediatric Gastroenterology, Hepatology and Nutrition[3]. Patients without cholangiopathy on MRCP were diagnosed as AIH type 1 (AIH-1, positivity for SMA and/or ANA) or type 2 (AIH-2, positivity for LKM-1 and/or LC1)[1,3]. Patients with cholangiopathy were diagnosed as ASC[1,3]. Patients with histological diagnosis of ASC but normal cholangiogram were classified as small duct ASC[3].

Clinical presentation was classified as: (1) Acute (malaise, nausea/vomiting, abdominal pain, jaundice, dark urine, pale stools); (2) Insidious (fatigue, headache, amenorrhoea, joint pain); and (3) Asymptomatic (incidental finding of abnormal liver function tests during investigation of non-hepatic conditions, including IBD). Protocol and description of autoantibodies detection and histological features suggestive for biliopathy are reported in our previous studies[4].

Treatment protocol

IS treatment consisted of first line use of oral prednisone at a dose of 2 mg/kg/d (up to a maximum of 60 mg/d) for 10-14 d followed by 4-6 wk tapering schedule to reach a total maintenance dose of 5 or 2.5 mg/d (depending on age). Blood tests during induction of remission were checked weekly to monitor the response to treatment and side effects. If the response was not satisfactory, azathioprine was added [starting dose 0.5 mg/kg/d, increased weekly to 1.5 mg/kg/d (maximum dose 2-2.5 mg/kg/d) in the absence of side effects or leukopenia] until normal transaminase levels were achieved. Mycophenolate mofetil (MMF, as second line treatment) and calcineurin inhibitors (cyclosporine or tacrolimus, as third line treatment) were used when standard treatment failed or azathioprine was contraindicated. Patients with ASC were also administered ursodehoxycholic acid (UDCA) at the dose of 15-20 mg/kg/d [3,15].

Patients not responding to standard immunosuppression underwent liver biopsy to assess the degree of inflammation and the stage of biliopathy as per criteria defined in our previous study[16].

OVT was given to patients who did not respond to first/second line treatment and who had on histology features of biliopathy without (or mild) portal-periportal inflammation. OVT was started at the dose of 50 mg/kg/d (divided in 3 doses, maximum dose 1500 mg/d), for 6 mo. In patients who did not respond, OVT was discontinued after 6 mo, whereas it was continued in responders.

Conversely, in children having on histology moderate/severe inflammatory infiltrate, a temporary increase of oral prednisone and conversion from azathioprine to MMF or from MMF to tacrolimus were prescribed[3], and OVT was not commenced.

We considered OVT-related side-effects the following symptoms: Fever, chills, rash, fatigue, gastroenterological symptoms (abdominal pain, persistent diarrhea), nephrotoxicity, neutropenia, ototoxicity, thrombocytopenia, antibiotic-resistant infections and neurological symptoms[5].

Response to treatment

Biochemical remission was defined as normal transaminase levels; immunological remission was normal transaminase and IgG levels with negative/Low titer (ANA/SMA < 1:20) of autoantibodies; histological remission was the absence of inflammation on liver histology. Relapse was defined as transaminase levels ≥ 2-fold the upper limit of normal (ULN)[3].

In patients receiving OVT, the values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), serum IgG and autoantibodies were reported before and after treatment.

Biochemical response to OVT was classified as follows:

Complete response: AST, ALT and GGT returning within normal values (NV);

Partial response: AST, ALT or GGT levels decreasing to < 1.5 × ULN, but not reaching NV;

No response: No significant changes in liver enzymes.

Discontinuation of IS treatment was attempted in patients with normal transaminases and IgG, negative or low positive titer of autoantibodies at least 3 years after starting IS treatment, and no inflammation on follow up histology[3].

SCOPE index

The Sclerosing Cholangitis Outcomes in Pediatrics (SCOPE) index includes 5 parameters which correlate with long-term outcomes in children with SC. The model stratifies patients as low, medium, or high risk based on progression to transplant or death (rates of < 1%, 3%, or 9% annually) and to hepatobiliary complications, including portal hypertension or biliary strictures (rates of 2%, 6%, and 13% annually)[17]. In this study, we assessed whether the SCOPE index score was improved, stable or worsened after OVT.

Statistical analysis

Data are reported as medians and interquartile range unless specified differently. Baseline measures and data on outcome were compared between AIH and ASC to see whether they differed. Paired *t* test/Mann-Whitney *U* test were used for continuous variables and chi-square/Fisher exact test for categorical variables. A *P* value of 0.05 or less was assigned significance. The analysis was performed with IBM-SPSS 13.0 for Windows. The statistical methods of this study were reviewed by one of the authors (EN) who is an expert statistician.

RESULTS

Seventy-five patients were diagnosed with AILD [AIH = 54 (type 1 *n* = 42, type 2 *n* = 12), ASC = 21] during the study period. Median age at diagnosis was 10.5 years (5.6-13.4) without differences between the two groups ($P > 0.05$). Female predominance was 69% (AIH = 72 %, ASC = 62%). The most common type of presentation was acute (35%, 43% in AIH *vs* 14% in ASC, $P = 0.011$), followed by asymptomatic (33%) and insidious (32%), the latter more common in ASC group (57% in ASC *vs* 22% in AIH, $P = 0.005$). IBD was reported in 18 patients [24%, ulcerative colitis (UC) in 12, Crohn's disease (CD) in 2 and IBD-unclassified (IBD-U) in 4 patients], mainly in ASC group (57% *vs* 11% in AIH group, $P < 0.001$). Associated autoimmune disorders were reported in 13/75 patients (17%, AIH = 17% and ASC = 14%) including coeliac disease in 4 (with AIH), autoimmune thyroiditis in 3 (1 with AIH), diabetes mellitus type 1 in 2 (both with AIH), psoriasis in 2 (both with AIH), idiopathic arthritis in 1 (with ASC), nephrotic syndrome in 1 (with ASC).

Baseline features

At diagnosis, all but one patient (F, with ASC, already on treatment for IBD) had raised transaminases; GGT was increased in 63 patients (84%) and normal in 12 (16%, all with AIH). Median values of AST, ALT, GGT, total bilirubin, ALT/AST ratio,

Table 1 Laboratory and histological features at diagnosis of 75 children with autoimmune liver disease

	All patients, *n* = 75	AIH, *n* = 54	ASC, *n* = 21	*P* value
Biochemical profile				
AST U/L (NV ≤ 45)	438 (129-982)	678 (204-1200)	150 (94-333)	< 0.001
GGT U/L (NV ≤ 50)	116 (60-296)	107 (54-196)	360 (68-607)	< 0.001
Total bilirubin (NV ≤ 1 mg/dL)	1.7 (0.6-4.5)	2.7 (0.6-5.3)	1.2 (0.7-2.5)	0.05
ALP (NV ≤ 350 U/L)	296 (204-469)	283 (199-462)	301 (242-494)	0.328
ALP/AST ratio	0.7 (0.3-2.2)	0.4 (0.2-1.6)	2.3 (0.7-3.5)	0.002
Albumin (NV: 30-50 g/dL)	42 (38-44)	42 (37-44)	42 (40-46)	0.082
INR (NV: 0.9-1.2)	1.2 (1.1-1.5)	1.2 (1.1-1.6)	1.1 (1.0-1.2)	< 0.05
Platelet (10^9/L)	252 (180-350)	234 (167-314)	319 (251-393)	< 0.05
Autoimmune profile				
ANA (≥ 1:20): *n* (%)	55 (73)	38 (70)	17 (81)	0.777
SMA (≥ 1:20): *n* (%)	53 (71)	38 (70)	15 (71)	1
Anti-LKM-1 (≥ 1:10): *n* (%)	12 (16)	12 (22)	0 (0)	< 0.05
Anti-LC1: *n* (%)	9 (12)	9 (17)	0 (0)	< 0.05
ANCA: *n* (%)	37 (49)	22 (41)	15 (71)	< 0.05
IgG g/dL (NV: 0.5-1.8 g/dL)	2.0 (1.4-3.2)	2.3 (1.4-3.3)	1.7 (1.5-2.2)	0.325
IgG > ULN: *n* (%)	51 (68)	37 (69)	14 (67)	1
Histology, *n* (%)				
Interface hepatitis	51 (68)	42 (78)	12 (57)	0.09
Fibrosis	61 (81)	42 (78)	19 (90)	0.324
Cirrhosis	17 (23)	15 (28)	2 (10)	0.127
Features of biliopathy[1]	62 (83)	37 (68)	17 (81)	0.764

[1]It includes at least one of the following: inflammatory injury of the bile duct, ductular reaction, periductular fibrosis, biliary metaplasia, granulomatous cholangitis[16]. Values are expressed as median and interquartile ranges.

AIH: Autoimmune hepatitis; ASC: Autoimmune sclerosing cholangitis; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; INR: International normalized ratio; ANA: Antinuclear antibody; SMA: Smooth muscle antibody; LKM-1: Liver-kidney microsome antibody type 1; LC1: Liver cytosol antibody type 1; SLA: Liver soluble antigen; ANCA: Anti-neutrophil cytoplasmic LT antibodies; ULN: Upper limit of normal; NV: Normal value.

international normalized ratio and platelets were significantly different between AIH and ASC (Table 1). Autoantibodies were positive in all (100%). No patient with ASC was positive for anti-LKM-1 and/or LC-1. Positivity for anti-neutrophil cytoplasmatic antibodies was more common in ASC patients (71% *vs* 41% in AIH, $P < 0.05$). Raised IgG was reported in 68% of patients (51/75) without differences between the two groups ($P > 0.05$). Liver biopsy was performed in all patients with similar prevalence of interface hepatitis, cirrhosis and biliary features in the two groups ($P > 0.05$) (Table 1).

Response to treatment in the whole group

Medications used in our cohort of patients are reported in Table 2. The association between prednisone/azathioprine was more common in AIH patients (52% *vs* 10% in ASC, $P < 0.001$); conversely the association between prednisone/MMF/OVT was commonly used in ASC patients (23% *vs* 2% in AIH, $P = 0.005$) (Table 2).

Sixty-nine patients (92%, AIH = 96% *vs* ASC = 81%, $P = 0.048$) normalized transaminase levels and achieved biochemical remission at a median of 0.1 years (0.1-0.5) after starting standard medical treatment; 74 patients (98%, AIH = 100% and ASC = 95%) reduced AST levels to < 2 × ULN (AST NV 45 IU/L).

Sixty-eight patients (91%) normalized GGT levels at a median of 0.3 years (0.2-0.9) after starting standard medical treatment. Median time to GGT normalization tended to be significantly higher in ASC patients ($P = 0.06$); 71 patients (95%, AIH 98% and

Table 2 Response to medical treatment and outcome of 75 patients with autoimmune liver diseases

Variables	All patients, *n* = 75	AIH, *n* = 54	ASC, *n* = 21	*P* value
Treatment, *n*				
Prednisone alone	26 (35%)	19 (35%)	7 (33%)	1
Prednisone + Azathioprine	30 (40%)	28 (52%)	2 (10%)	< 0.001
Prednisone + MMF	5 (7%)	3 (5%)	2 (10%)	0.615
Prednisone + Vancomycin	4 (5%)	1 (2%)	3 (14%)	0.064
Prednisone + Azathioprine + Vancomycin	2 (3%)	0 (0%)	2 (10%)	0.075
Prednisone + MMF + Vancomycin	6 (8%)	1 (2%)	5 (23%)	0.005
Prednisone + Tacrolimus	1 (1%)	1 (2%)	0	NA
Cyclosporine	1 (1%)	1 (2%)	0	NA
Response to treatment				
Normal AST (NV ≤ 45 U/L): *n*	69 (92%)	52 (96%)	17 (81%)	0.048
Time to normalize AST (yr)	0.1 (0.1-0.5)	0.2 (0.1-0.6)	0.1 (0.1-0.2)	0.19
GGT (< 50 UI/L), *n*	68 (91%)	51 (94%)	17 (81%)	0.811
Time to normalize GGT (yr)	0.3 (0.2-0.6)	0.3 (0.2-0.5)	0.3 (0.2-1.1)	0.062
Immunological remission[1]: *n*	25 (33%)	22 (40%)	3 (14%)	0.032
Time to immunological remission	3.1 (2.2-4.2)	3.8 (2.9-4.3)	3.4 (3.2-3.7)	0.86
Relapse of AILD during treatment, *n*				
At least one relapse	36 (48%)	22 (41%)	14 (67%)	< 0.070
1 relapse alone	26 (35%)	17 (31%)	9 (43%)	0.421
≥ 2 relapses	10 (13%)	5 (9%)	5 (24%)	0.131
Outcome at last follow up				
Median follow up, yr (range)	4.4 (0.6-13.8)	4.1 (1.2-11.7)	4.5 (0.6-13.8)	0.079
Alive	75 (100%)	54 (100%)	21 (100%)	NA
OFF-IS therapy	6 (8%)	5 (9%)	1 (5%)	0.666
Medical treatment	68 (91%)	49 (91%)	19 (90%)	1
Liver transplant	1 (1%)	0 (0%)	1 (5%)	0.28

[1]Normal aspartate aminotransferase, normal IgG, and negative or low titer autoantibodies.

AIH: Autoimmune hepatitis; ASC: Autoimmune sclerosing cholangitis; AST: Aspartate aminotransferase; MMF: Mycophenolate mofetil; GGT: Gamma-glutamyl transferase; IS: Immunosuppressive; NA: Not applicable; ALID: Autoimmune liver disease; NV: Normal value.

ASC = 85%) reduced GGT levels to < 2 × ULN (GGT < 100 U/L).

One patient with ASC (F, age at diagnosis 13.1 years, with CD) did not respond to first and second line treatment and required LT (details below). Immunological remission was achieved in 25 patients (33%, AIH 40% and ASC = 14%) at a median of 3.1 years (2.2-4.2) after starting standard IS treatment.

Thirty-six patients experienced at least 1 episode of relapse (1 episode *n* = 16 patients; ≥ 2 episodes *n* = 10) managed with a temporary increase of prednisolone dose in 10 patients, with the addition of azathioprine in 15, and conversion from azathioprine to MMF in 11. Suboptimal adherence to treatment was detected in 8% (*n* = 3, AIH = 2, ASC = 1) of those who relapsed.

Treatment with OVT in non-responders

Of 75 patients, 12 [16%, F = 75%, median age 13.5 years, (12.2-15.7)] required OVT after a median time from the diagnosis of 2.2 years (0.8-4.3) (Table 3). Ten patients were diagnosed with ASC and 2 with AIH; 10/12 had IBD (83%) (Table 3). Liver biopsy performed before starting OVT showed absent (or mild) inflammatory infiltrate in all, and biliary features including inflammatory injury of the bile duct in 8 (67%) patients,

Table 3 Demographic, biochemical and histological features of 12 patients with autoimmune liver disease treated with oral vancomycin

Patients/ diagnosis	Gender	Age at diagnosis (yr)	Type of presentation	IBD	Splenomegaly[1]	IgG > ULN	Auto-antibodies	Histology				Medications
								Interface hepatitis	Fibrosis	Cirrhosis	Biliopathy[3]	
AIH	F	4.2	Asymptomatic	IC	Not	Yes	SMA 1:40; p-ANCA	No	Yes	No	Yes	Pred/MMF/UDCA/Mesa
AIH	F	10.9	Asymptomatic	UC	Not	Yes	ANA 1:320; SMA 1:160; p-ANCA positive	Yes	Yes	No	Yes	Pred/UDCA/Mesa
ASC	F	16	Insidious	None	Yes	Not	ANA 1:160; p-ANCA positive	Yes	Yes	Yes	No	Pred/MMF/UDCA
ASC	M	4.3	Asymptomatic	CD	Not	Yes	ANA 1:160; SMA 1:160; p-ANCA +++	Yes	Yes	No	Yes	Pred/UDCA/Mesa
ASC	F	8.6	Insidious	UC	Not	Not	SMA 1:40; p-ANCA positive	No	No	No	Yes	Pred/AZA/UDCA/Mesa
ASC	F	12.1	Insidious	UC[2]	Not	Not	SMA 1:40; p-ANCA positive	No	No	No	Yes	Pred/AZA/UDCA
ASC	M	14.1	Insidious	None	Not	Not	SMA 1:40; p-ANCA positive	Yes	Yes	No	Yes	Pred/AZA/UDCA
ASC	M	14.3	Acute	UC	Not	Yes	ANA 1:320; SMA 1:320	Yes	No	No	Yes	Pred/UDCA/Mesa
ASC	F	13.8	Asymptomatic	UC	Yes	Yes	ANA 1:640; p-ANCA positive	No	Yes	Yes	Yes	Pred/MMF/UDCA/Mesa
ASC	F	5.1	Acute	IC	Not	Not	ANA 1:160; p-ANCA positive	No	Yes	No	Yes	Pred/MMF/UDCA/Mesa
ASC	F	13.1	Acute	CD	Yes	Yes	ANA 1:80; p-ANCA positive	No	Yes	No	Yes	Pred/MMF/UDCA/Mesa
ASC	F	3.6	Asymptomatic	UC	Yes	Not	ANA 1:160; SMA 1:80; p-ANCA positive	Yes	Yes	No	Yes	Pred/MMF/UDCA/Mesa

[1]Spleen size detected on liver scan o magnetic resonance cholangiopancreatography.
[2]Patient underwent colectomy at age of 14 years.
[3]It includes at least one of the following: Inflammatory injury of the bile duct, ductular reaction, periductular fibrosis, biliary metaplasia, granulomatous cholangitis[16].
AIH: Autoimmune hepatitis; ASC: Autoimmune sclerosing cholangitis; IBD: Inflammatory bowel disease; UC: Ulcerative colitis, CD: Crohn disease; IC: Indeterminate colitis; ULN: Upper limit of normal; F: Female; M: Male; ANA: Anti-nuclear antibody; SMA: Smooth muscle antibody; ANCA: Anti-neutrophil cytoplasmic antibodies; Pred: Prednisone; UDCA: Ursodeoxycholic acid; MMF: Mycophenolate mofetil; Mesa: Mesalazyne.

ductular reaction in 11 (92%), biliary metaplasia in 7 (58%), and periductular fibrosis in 6 (50%). Need for OVT was significantly higher in ASC group compared to AIH [10/12 (83%) in ASC *vs* 2/54 (4%) in AIH, $P < 0.001$]. Immunological profile, histology and medications are reported in Table 3.

Median values of AST, ALT and GGT significantly decreased during OVT [AST levels from 107 UI/L (83-158) to 38 UI/L (31-65), $P = 0.010$; ALT from 160 UI/L (140-335) to 40 UI/L (37-87), $P = 0.008$; GGT from 279 (150-498) to 63 (32-143), $P = 0.005$] (Figure 1).

AST levels decreased in 10/12 patients (83%, within normal range in 8 patients and < 1.5 × ULN in 2), ALT levels in 9/12 patients (75%, within normal range in 7 patients and < 1.5 × ULN in 2), and GGT levels in 8/12 patients (67%, within normal range in 6 patients and < 1.5 × ULN in 2) (Table 2). Median time to normalization of AST, ALT and GGT levels were 2 mo (1.7-3.2), 5 mo (2.7-6.2), and 5 mo (3.2-6.0) respectively.

A complete response to OVT (normalization of AST, ALT and GGT) was observed in 6/12 patients (50%, cases *n*. 1, 2, 4, 5, 8, 10), a partial response in 2/12 (17%, cases *n*. 3 and 9) (Table 4).

After OVT, the percentage of patients who achieved biochemical remission increased overall from 81% (61/75 patients) to 92% (69/75), [from 93% (50/54) to 96% (52/54) in AIH, and from 52% (11/21) to 81% (17/21) in ASC] (Figure 2). Similarly, the percentage of patients who normalized GGT levels increased after OVT, mainly in ASC patients (from 62% to 81%) (Figure 2). No significant changes were observed in the other biochemical parameters including total bilirubin, serum albumin, and platelet count, nor in the prevalence of high IgG and positive autoantibodies ($P > 0.05$).

Based on SCOPE index score, all 6 patients who showed a complete response to OVT were classified as low risk (cases 1, 2) or medium risk (cases 4, 5, 8, 10); the other 6 patients (cases 3, 6, 7, 9, 11, 12) were classified as high risk. Decrease in SCOPE index score was reported in 5/12 patients (42%), from high to medium risk in 2 patients (cases 7, 9) and from medium to low risk in 3 (cases 4, 5, 8) (Table 4). After a median time of 24 mo (range 1-99), none of 12 patients complained of side effects related to OVT.

Four of 12 patients (33%, cases 6, 7, 11, 12, all with ASC) did not respond to OVT. One patient (*n*. 6) underwent colectomy at the age of 14 years due to a severe form of IBD. She never normalized her liver enzymes. A course of OVT was commenced at the age of 15.2 years, was not successful and was therefore discontinued 6 mo later. At the age of 16 years she was diagnosed also with juvenile arthritis, and was treated with adalimumab. Another patient (*n*. 7) achieved histological remission 3 years after the diagnosis, and IS treatment was gradually discontinued. Six months later he developed a relapse of ASC not responding to prednisone and azathioprine. A follow up liver biopsy showed fibro-obliterative lesions around the bile ducts and OVT was commenced, though without success. One patient (*n*. 11) developed progressive cholestasis and complications of portal hypertension requiring LT at age 17 years. One year later she developed ASC disease recurrence requiring re-transplantation at age 21 years. A second ASC recurrence occurred 10 mo later leading to multiple episodes of cholangitis. A new course of OVT was commenced unsuccessfully. The patient was re-listed for the third LT.

The last patient (*n*. 12) did not respond to first and second line treatment nor to OVT and developed features of portal hypertension (splenomegaly and hypersplenism) and incomplete cirrhosis on histology.

Outcome

At last follow up (median of 4.4 years, range 0.6-13.8 years) all patients are alive. Only 1 patient (F, with ASC) underwent LT at the age of 17 years and re-LT at the age of 21 years, due to recurrence of ASC (details above). Of 74 patients not requiring LT, 68 (92%) at last follow-up were still on medical treatment. In one patient (*n*. 5) who responded to OVT, we tried to reduce the dose of vancomycin from 1500 mg/d (divided in 3 doses) to 1000 mg/d (in 2 doses). However, few weeks later, AST and GGT increased 3 × ULN and normalized again when OVT went back to full dose (1500 mg/times for day).

Based on histological remission, IS withdrawal was attempted in 8 patients [7 females, median age 10.4 years (8.1-15.1), 7 AIH-1, 1 ASC] after a median of 4.0 years (3.9-5.3) from the diagnosis; 2/8 (*n*. 1,2) received OVT at the age of 5.4 and 11.8 years respectively. Two of these 8 patients (F, both with AIH-1) relapsed 1 and 4 mo after stopping treatment and responded successfully to IS treatment re-introduction. The other 6 (8%), including 1 patient with ASC, remained off treatment. One patient (*n*.1), discontinued prednisone and MMF 7.6 years after the diagnosis remaining on OVT alone, and her AST and GGT levels remained normal. Sixteen months later (at age of

Table 4 Biochemical response to oral vancomycin and Sclerosing Cholangitis Outcomes in Pediatrics index score of 12 patients with autoimmune liver disease treated with oral vancomycin

Patients/ diagnosis	Age at OVT (yr)	AST (NV ≤ 45 U/L)				ALT (NV ≤ 45 U/L)				GGT (NV ≤ 50 U/L)				Response to OVT [1]	SCOPE index score[2]		Time on OVT (mo)	OVT side-effect	Overall FU[3] (mo)
		Before OVT	After OVT	TTN	Result	Before OVT	After OVT	TTN	Result	Before OVT	After OVT	TTN	Result		Before OVT	After OVT			
AIH	5.4	212	39	4 mo	NV	147	17	6 mo	NV	73	22	6 mo	NV	Complete	3 low risk	0 low risk	99	None	113
AIH	11.8	251	31	2 mo	NV	359	39	9 mo	NV	26	34	8 mo	NV	Complete	3 low risk	0 low risk	72	None	73
ASC	16.8	98	47	3 mo	< 1.5 NV	140	70	4	< 1.5 NV	39	83	4	< 1.5 NV	Partial	8 high risk	8 high risk	16	None	26
ASC	4.8	86	28	7 d	NV	156	38	14 d	NV	84	44	14 d	NV	Complete	4 medium risk	1 low risk	37	None	39
ASC	13.1	60	14	1 mo	NV	365	38	3 mo	NV	68	27	4 mo	NV	Complete	5 medium risk	2 low risk	31	None	84
ASC	15.2	71	40	14 mo	NV	140	56	14 mo	< 1.5 NV	52	164	12 mo	-	None	6 high risk	6 high risk	6	None	68
ASC	17.4	113	65	1 mo	< 1.5 NV	205	141	1 mo	-	49	226	1 mo	-	None	6 high risk	4 medium risk	3	None	52
ASC	15	407	30	6 mo	NV	856	35	6 mo	NV	61	28	1 mo	NV	Complete	5 medium risk	2 low risk	40	None	49
ASC	17.3	102	37	2 mo	NV	111	36	2 mo	NV	135	82	5 mo	< 1.5 NV	Partial	6 high risk	4 medium risk	18	None	61
ASC	12.5	76	31	2 mo	NV	124	40	7 mo	NV	86; TX	42	6 mo	NV	Complete	5 medium risk	4 medium risk	47	None	135
ASC	13.9	123	155	-	-	165	154	-	-	165	1800	-	-	None	8 high risk	8 high risk	6	None	86; TX
ASC	13.2	141	135	-	-	156	180	-	-	71 mo (range 26-165)	136	-	-	None	7 high risk	7 high risk	4	None	165
Response to OVT					10/12 (83%)				9/12 (75%)				8/12 (67%)				Median: 34 (range 1-99)		71 (range 26-165)

[1]Complete response is defined as "normalization of all three liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT)]", partial response as "AST, ALT or GGT levels

decreasing to < 1.5 × ULN without reaching normal value", and no response as "no significant changes in liver enzymes".
[2]Sclerosing Cholangitis Outcomes in Pediatrics: Points 0-3: Low risk; Points 4-5: Medium risk; Points 6-11: High risk.
[3]Time from diagnosis to last follow up visit.
AIH: Autoimmune hepatitis; ASC: Autoimmune sclerosing cholangitis; OVT: Oral vancomycin treatment; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; TTN: Time to normalize or to achieve the lowest value; NV: Normal value; FU: Follow up; SCOPE: Sclerosing Cholangitis Outcomes in Pediatrics.

13.8 years) on routine blood tests she had an increase of AST and GGT > 3 × ULN. The patient confessed a low adherence to treatment; once she re-started OVT regularly, AST and GGT returned normal.

DISCUSSION

In pediatrics, there are few published studies focusing on the differences between AIH and ASC. Furthermore, experience on empirical use of oral vancomycin in children with AILD not responding to standard immunosuppression is limited.

In this study, MRCP performed at diagnosis allowed us to differentiate children with AIH from those with ASC, and see whether they differ in terms of characteristics at presentation, response to medical treatment and outcome.

Our results show that characteristics at presentation were different between AIH and ASC, similarly to other studies[4,18]. All patients with ASC were positive for ANA and/or SMA, none for anti-LKM-1 confirming the rare association between LKM-1 positivity and ASC[18-20]. IBD was more common in ASC patients compared those with AIH, UC being more common[4,18-21]. On histology, cirrhosis was reported in 23% of patients, similar to previous studies (from 11% to 68%), suggesting a late diagnosis in a proportion of cases[4,18,19]. Features of biliopathy were equally reported in AIH and ASC confirming that both conditions are not easily distinguishable on histological ground making the cholangiogram the only effective tool to differentiate patients with AIH from those with ASC[16,18].

Pediatric patients with AILD respond well to IS treatment although the efficacy of second and third line treatment remains to be demonstrated, particularly in patients ASC[3].

The first study reporting benefits from OVT in children with ASC and IBD (n = 3 patients) was reported by Cox *et al*[12] in 1998. In that study OVT was administered to 3 patients (1 aged 15 years and 2 aged 14 years) diagnosed with PSC and IBD who showed improvements in gastrointestinal symptoms and liver enzymes after OVT[12].

However, this is the first study that aims to assess consistently the efficacy of OVT in a cohort of children and adolescents with AIH and ASC who did not respond to standard treatment and were treated according to a single protocol.

At our center OVT was given to children with AILD who failed to respond to first/second line IS treatment and had, on histology, features of biliopathy without (or

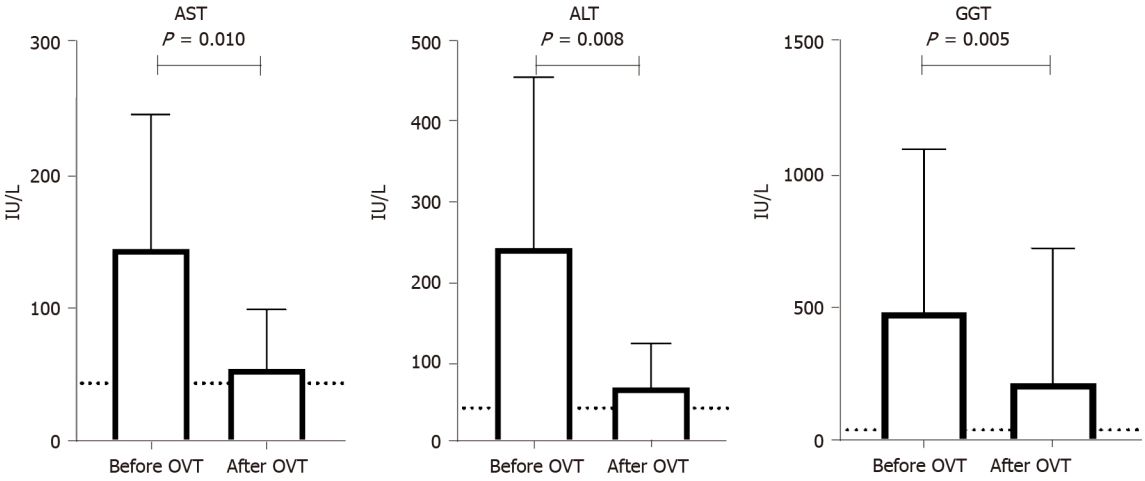

Figure 1 Aspartate aminotransferase, alanine aminotransferase and gamma-glutamyl transferase levels before and after oral vancomycin treatment (*n* = 12 patients). AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; OVT: Oral vancomycin treatment.

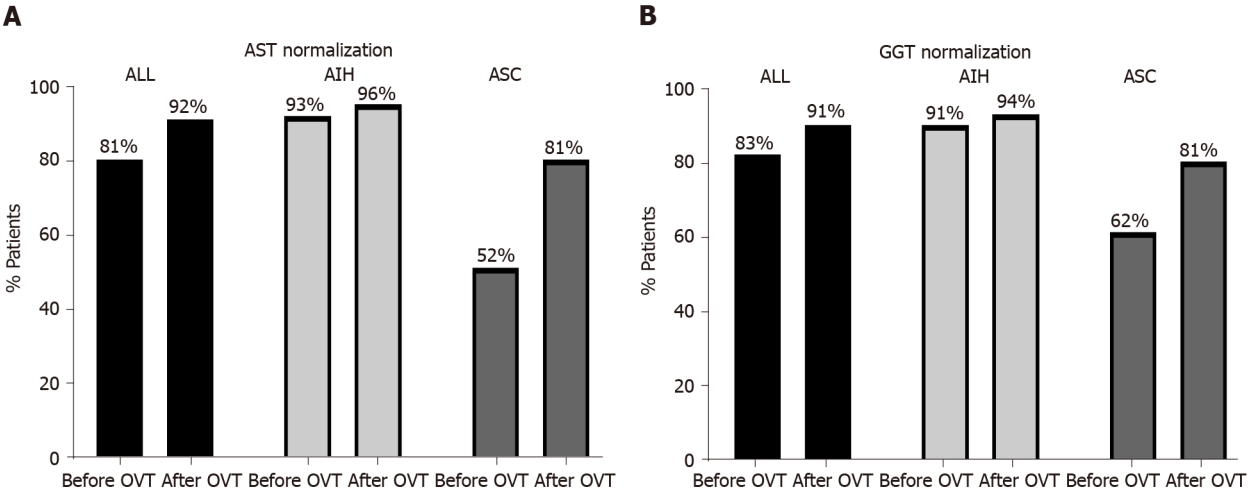

Figure 2 Percentage of patients (*n* = 75) who normalized aspartate aminotransferase and gamma-glutamyl transferase levels before and after oral vancomycin treatment. AST: Aspartate aminotrasferase; GGT: Gamma-glutamyl trasferase; OVT: Oral vancomycin treatment; AIH: Autoimmune hepatitis; ASC: Autoimmune sclerosing cholangitis.

mild) inflammation. To our opinion, in these patients an escalation of IS therapy (third line treatment) was not indicated due to the absence of significant lymphoplasmacytic infiltrate.

In this cohort a high proportion of patients normalized transaminases and GGT levels on standard IS; the majority of patients (40%) required an association between prednisone plus azathioprine, mainly in AIH group. Of interest, 10/12 patients who required OVT had ASC and 2/12 with AIH; on histology all had strong features of biliopathy, with mild or no inflammation.

Similarly to our study, improvements in liver enzymes after OVT were reported in Davies *et al*[7]'s study (*n* = 14 children with PSC and IBD), and in two randomized clinical trials on a total of 64 adult patients with PSC[5,6]. In Abarbanel *et al*[8]'s study the authors showed that all children with PSC and IBD experienced a reduction in GGT and ALT levels and improvement of biliary imaging, biopsies of the liver and intestine, and IBD symptoms while on OVT. In our study, median time to normalize liver enzymes ranged from 2 to 5 mo suggesting that a course of OVT should last at least 6 mo before assessing a biochemical response to treatment. Of note, no improvements were observed in the other biochemical parameters similar to Davies *et al*[7].

In a recent prospective study including pediatric patients (42% with small and 48% with large duct PSC), 49% (22/45), 20% (9/40), and 62.2% (28/45) of children experienced normalization of GGT, ALP, and ALT, respectively. Of note, the biochemical response to OVT was more favorable in the pediatric compared to the adult group. Besides, a significant proportion of patients showed improvements on histologic features and cholangiopathy[22]. Conversely, in a recent retrospective study on a large cohort of children with PSC the authors did not show improvement in outcomes of children treated with OVT or UDCA compared to those with "no treatment"[23], although several limitations were recorded in the study design[24]. The median OVT dose in Deneau *et al*[23]'s study was 21 mg/kg/d, which was substantially lower than the 50 mg/kg/d typically used in our and others' studies[5,6].

In Tabibian *et al*[6]'s work (n = 35 adult patients with PSC) the authors experienced a significant improvement in pruritus only in the high-dose vancomycin group. In our study we observed a temporary increase in AST and GGT levels after OVT dose reduction. In Cox *et al*[12]'s study, 3 children with SC and IBD had a normalization of liver tests while on OVT and return to abnormal values upon OVT discontinuation. These results confirm the efficacy of OVT and the importance of maintaining full doses regularly during the treatment.

The mechanisms by which OVT leads to biochemical improvement are still undefined. Previous studies suggested that OVT may have an immunomodulatory effect on regulatory T cells (Treg)[5,6-8]. Some authors suggest that the response to OVT is likely due to its antimicrobial effects on unknown pathogens or normal flora that cause abnormal immunological reactions following migration to the liver[7]. Several lines of experimental evidence from animal models demonstrate that enteric dysbiosis and/or administration of bacterial antigens can lead to hepatobiliary inflammation with various features of PSC[6]. In this study we found that the prevalence of IBD was similar in patients responding to OVT compared to those not responding, suggesting no role of IBD in the pathogenic mechanism of OVT.

Overall, the need for OVT emerged mainly in ASC group, and the percentage of patients who achieved the biochemical remission increased mainly in ASC group (from 52% to 81%) rather than in AIH (from 93% to 96%) (Figure 2)[4,25].

Of 75 patients, only 33% achieved immunological remission and no significant changes in IgG levels and autoantibody positivity were observed after OVT. This may imply an ongoing disease activity despite normal transaminase levels, possibly explaining the low proportion of children able to stop treatment successfully (8% in this study).

Interestingly, all 6 patients who showed a complete response to OVT were classified as low or medium SCOPE index strata, none as high risk, suggesting that probably the patients achieving a biochemical response to OVT are those with a milder disease activity. Similar results were reported in Deneau *et al*[17]'s study showing that a low SCOPE index at treatment initiation was an independent predictor of response. Moreover, the authors showed that the rate of response to OVT was similar in the group that started it as primary treatment and another that had it as second line[17]. Remarkably, in this study, OVT was associated with prednisone alone in 3 cases (100% responded to treatment) and with a second IS drug in the other 9 (55% responded to treatment, $P > 0.05$).

The decrease in the SCOPE index score (42% in this study) may suggest a potential benefit of OVT on long-term outcomes. Similar results were reported in a triple blinded, randomized, placebo-controlled clinical trial on adult patients with PSC where the analysis showed a significant decrease in the Mayo PSC score in the vancomycin group at the third month comparing to the baseline evaluation[5].

Similarly to previous studies, we did not observe side effects or infectious complications from long-term OVT during the study period[6,7,22]. However, whether the use of this antibiotic may lead to vancomycin-resistant organisms is still an open issue. All 4 patients not responding to OVT (all with ASC) showed a progression of liver disease. One patient developed recurrence of ASC after the LT (twice) and did not respond to OVT confirming the high recurrence rate post-LT[3]. Differently from our experience, OVT has been reported to be effective in the treatment of a pediatric patient with recurrent PSC after LT, suggesting a disease mechanism with some causes external to the liver—potentially from the gut bacteria[26].

Overall, the outcome in our cohort was excellent, with 100% of patients alive at last follow up and 8% off IS treatment. Only 1 patient required LT, although the median follow up of our cohort of patients is relatively short.

CONCLUSION

This is the first study reporting data on the consistent use of OVT in children with AILD not responding to standard treatment. Our results show that AIH and ASC have different characteristics at presentation although both respond well to medical therapy. For children not responding to standard IS, OVT may represent a valuable option to achieve biochemical remission, particularly in ASC patients. This study adds timely insights into the highly engaged discussion about the use of OVT for children with AILD, confirming the need of further structured studies assessing the efficacy and safety of OVT as well as its potential benefits on long-term outcomes.

ARTICLE HIGHLIGHTS

Research background

Pediatric autoimmune liver disease (AILD) includes autoimmune hepatitis (AIH) and autoimmune sclerosing cholangitis (ASC). Children with AILD not responding to standard immunosuppression (IS) may progress to end-stage liver disease and require liver transplantation.

Research motivation

Despite the absence of strong evidences the empirical use of candidate therapies has significantly increased in the last decades. Oral vancomycin has an immunomodulatory effect and it has been used in patients with primary sclerosing cholangitis. In pediatrics, the experience with oral vancomycin treatment (OVT) in patients with AIH or ASC is very limited.

Research objectives

In this study we evaluated: (1) The response to standard IS in a large cohort of pediatric patients with AILD; and (2) The efficacy of OVT to normalize transaminases (biochemical remission) and to achieve immunological remission in patients not responding to standard IS.

Research methods

Retrospective study of children diagnosed with AILD (AIH or ASC) at Hospital Papa Giovanni XXIII, Bergamo, Italy, in the last decade. Response to IS treatment and need for OVT was reported in all patients and compared between the two groups (AIH *vs* ASC).

Research results

Seventy-five patients diagnosed with AILD were included in this study (median age 10.5 years, range 5.6-13.4; F = 69%); 12 patients (16%, 10 with ASC) required OVT. Response to OVT was observed in 75% of patients and the percentage of those who achieved biochemical remission increased overall from 81% to 92%. Decrease in Sclerosing Cholangitis Outcomes in Pediatrics (SCOPE) index was reported in 42% of patients.

Research conclusions

This study shows that OVT may be considered as a valuable treatment option to achieve biochemical remission in children with AILD not responding to standard IS. Decrease in SCOPE index after OVT may suggest improvements in the long-term outcome.

Research perspectives

These promising preliminary results suggest that further prospective studies are needed to better define the efficacy of OVT in AILD.

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ORIGINAL ARTICLE

Retrospective Study

Trends of alcoholic liver cirrhosis readmissions from 2010 to 2018: Rates and healthcare burden associated with readmissions

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Institutional review board statement: As the National Readmission Database lacks patient and hospital-specific identifiers, this study was exempt from the Institutional Review Board (IRB) approval as per guidelines put forth by our

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Abstract

BACKGROUND

Alcoholic liver cirrhosis (ALC) is a chronic liver disease with varying disease severity. Readmissions of ALC are associated with poor outcomes.

AIM

To identify and assess trends of readmissions for ALC over an eight-year period.

METHODS

This retrospective interrupted trend study analysed 30-d readmissions of ALC in the United States from 2010 to 2018 using the National Readmissions Database. Hospitalization for ALC was the reason for index admission obtained using the International Classification of Diseases codes (571.2 and K70.3X). Biodemographic

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characteristics and hospitalization trends were highlighted over time. A multivariate regression analysis model was used to calculate the trend for risk-adjusted odds of 30-d all-cause ALC readmissions, ALC specific readmission rate, ALC readmission proportion, inpatient mortality, mean length of stay (LOS) and mean total hospital cost (THC) following adjustments for age, gender, grouped Charlson Comorbidity Index, insurance, mean household income, and hospital characteristics.

RESULTS

There was a trend towards increasing total 30-d readmissions of ALC from 7660 in 2010 to 15085 in 2018 ($P < 0.001$). Patients readmitted for ALC were noted to have an increasing comorbidity burden over time. We noted a rise in the risk-adjusted 30-d all-cause readmission of ALC from 24.9% in 2010 to 29.9% in 2018 ($P < 0.001$). ALC-specific readmission rate increased from 6.3% in 2010 to 8.4% in 2018 ($P < 0.001$) while ALC readmission proportion increased from 31.4% in 2010 to 36.3% in 2018 ($P < 0.001$). Inpatient mortality for 30-d readmissions of ALC declined from 10.5% in 2010 to 8.2% in 2018 ($P = 0.0079$). However, there was a trend towards increasing LOS from 5.6 d in 2010 to 6.3 d in 2018 ($P < 0.001$) and increasing THC from 13790 dollars in 2010 to 17150 dollars in 2018 ($P < 0.001$). The total days of hospital stay attributable to 30-d readmissions of ALC increased by 119.2% while the total attributable hospital costs increased by 149% by the end of 2018.

CONCLUSION

There was an increase in the 30-d readmission rate and comorbidity burden for ALC; however, inpatient mortality declined. Additionally, there was a trend towards increasing LOS and THC for these readmissions.

Key Words: Alcoholic liver cirrhosis; Readmissions; Epidemiology; Trends; Mortality

Core Tip: This retrospective interrupted trend study analysed 30-d readmissions of alcoholic liver cirrhosis (ALC) in the United States from 2010-2018. There was a trend towards increasing 30-d all-cause readmission rate and ALC-specific readmission rate for the study period. However, inpatient mortality was noted to have a declining trend from 10.5% in 2010 to 8.2% in 2018 ($P = 0.0079$). The total days of hospital stay attributable to ALC readmissions increased by 119.2% and total attributable hospital costs increased by 149% during the study period.

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INTRODUCTION

Alcohol use disorders are known to affect millions worldwide, and alcohol consumption is directly associated with liver disease mortality. Alcoholic liver disease varies in severity and prognosis based on several factors, including the pattern of alcohol consumption, duration of alcohol consumption, amount of alcohol consumption, the presence or absence of liver inflammation, nutritional status, genetic predisposition, and diet[1]. Alcoholic liver cirrhosis (ALC) is closely associated not only with the duration of alcohol consumption, but also the amount of undiluted alcohol consumed[1]. Although many patients with significant alcohol consumption develop fatty liver disease, not all patients with alcoholic liver disease progress to liver cirrhosis. It has also been postulated that genetic and environment factors may also

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play a key role in the development of ALC. Liver cirrhosis is reported to have significant mortality, morbidity, and reduced life expectancy. In fact, the median survival of patients with advanced ALC is reported to be around 1-2 years. Additionally, patients with decompensated cirrhosis who abstain from alcohol use have a reported 5-year survival rate of 60%, compared to the 30% survival rate in patients who continue with alcohol consumption[1]. It has previously been reported that a high proportion of patients with liver cirrhosis are readmitted within 30 d or 90 d, underscoring the risk of readmission in these patients[2].

While data on the morbidity and mortality of ALC has been reported in literature, there is paucity of information on the trends of readmissions after an index hospitalization for ALC. The purpose of this study was to identify the trends of readmissions, total hospital charges, and length of stay (LOS) over an eight-year study period while also examining changes in the demographic of ALC readmissions over time. Furthermore, as National Readmission Database (NRD) stores data on inpatient admissions in the form of International Classification of Diseases (ICD) codes, we used the codes 571.2 and K70.3X to include all patients with ALC in our study[3].

MATERIALS AND METHODS

Design and data source

This was a retrospective interrupted trends study involving adult hospitalizations for ALC in in the United States from 2010-2018. Data was extracted from the NRD which is the largest, publicly available, all-payer, inpatient healthcare readmission database in the United States, drawn from the Agency for Healthcare Research and Quality Healthcare Cost and Utilization Project (HCUP) State Inpatient Databases[3]. The NRD is an annual file constructed using one calendar year of discharge data. Discharges included in the NRD were treated at community hospitals (excluding rehabilitation or long-term acute care hospitals) and a majority of these discharges had patient linkage numbers that were verified and not questionable. Discharge weights were calculated using post-stratification for hospital characteristics (census region, urban-rural location, teaching status, bed size, and hospital control) and patient characteristics [sex and five age groups (0, 1-17, 18-44, 45-64, and 65 and older)]. The NRD contains discharge data from 28 geographically dispersed states accounting for 59.7% of the total United States population and 58.7% of all United States hospitalizations. It comprises both patient and hospital-level information. Up to 40 discharge diagnoses and 25 procedures are collected for each patient using the ICD-9 and ICD-10 codes. Diagnose were classified as principal diagnosis which was the reason for hospitalization, and secondary diagnosis which was any other discharge diagnosis. Hospitals were stratified according to ownership control, the number of beds, teaching status, urban/rural location, and geographic region. Furthermore, the NRD allows for weighted analysis to obtain 100% of the United States hospitalizations within a given year[3].

Study population

The study involved hospitalizations from NRD for 2010, 2012, 2014, 2016 and 2018 with ALC as the reason for index admission using ICD codes (571.2 and K70.3X). Individuals less than 18 years of age, December and elective hospitalizations were excluded from the study. Using unique hospitalization identifiers, index hospitalizations were identified and one subsequent hospitalization within 30 d was tagged as a readmission. Furthermore, traumatic admissions were excluded from the readmission data. December admissions were excluded when searching for index admissions as these hospitalizations would lack data for at least 30 d following discharge to determine if there was a readmission according to the study design. The comorbidity burden was assessed using Sundararajan's adaptation of the modified Deyo's Charlson Comorbidity Index[4].

Outcome measures

The biodemographic and hospitalization trends of the studied populations were highlighted over time. The 30-d all-cause ALC readmission rate, the ALC specific readmission rate, ALC readmission proportion, trends in inpatient mortality rate, mean LOS and mean THC were calculated. Total hospital cost was obtained using the HCUP Cost-to-Charge Ratio files and adjusted for inflation using the Medical Expenditure Panel Survey index for hospital care with 2018 as the reference point[5,6].

Statistical analysis

Data analysis was performed using Stata® Version 16 software (StataCorp, Texas, United States). All analyses were conducted using the weighted samples for national estimates in adjunct with HCUP regulations for using the NRD database. A multivariate regression analysis was used to calculate the trends of risk-adjusted odds of 30-d all-cause ALC readmission rate, the ALC specific readmission rate, ALC readmission proportion, trends in inpatient mortality rate, mean LOS and mean THC following adjustment for age, sex, grouped Charlson Comorbidity Index, insurance type, mean household income, and hospital characteristics. All *P* values were 2 sided with 0.05 set as the threshold for statistical significance.

Ethical considerations

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. As the NRD does not include patient-specific and hospital-specific identifiers, this study was exempt from the Institutional Review Boards as per guidelines put forth by the IRB for research on database studies.

Data availability statement

The NRD is a large publicly available all-payer inpatient care database in the United States, containing data on more than 18 million hospital stays per year[3]. Its large sample size provides sufficient data for analysis across hospital types and the study of readmissions for relatively uncommon disorders and procedures.

RESULTS

Biodemographic and hospital characteristics of ALC readmissions

Details of characteristics of readmissions for ALC within the included years for the study are shown in Table 1. There has been a yearly increase in the total number of 30-d readmissions for ALC from 7660 in 2010 to 15085 in 2018 ($P < 0.001$). Most readmissions were noted for men but there was a decreasing trend in the proportion of male readmissions ($P < 0.001$). Patients readmitted for ALC had an increasing comorbidity burden with time. We also noted a rising trend of readmissions for small bed-sized and metropolitan teaching hospitals.

Trends in ALC readmission outcomes

There was a steady rise in the rate of risk-adjusted 30-d all-cause ALC readmissions from 24.9% in 2010 to 29.9% in 2018 ($P < 0.001$). We also noted increasing risk-adjusted 30-d ALC specific readmission rate from 6.3% in 2010 to 8.4% in 2018 ($P < 0.001$) and increasing ALC readmission proportion from 31.4% in 2010 to 36.3% in 2018 ($P < 0.001$) (Table 1 and Figure 1). In-patient mortality for 30-d readmissions of ALC showed a decreasing trend from 10.5% in 2010 to 8.2% in 2018 ($P = 0.0079$). However, there was a trend towards increasing LOS from 5.6 d in 2010 to 6.3 d in 2018 ($P < 0.001$) and increasing THC from 13790 dollars in 2010 to 17150 dollars in 2018 ($P < 0.001$) (Table 2).

Cost burden of ALC readmissions

The total days of hospital stay attributable to 30-d readmissions of ALC increased by 119.2% from 43244 d in 2010 to 94789 d in 2018, while the total attributable hospital costs increased by 149% from 104 million dollars in 2010 to over 259 million dollars by the end of 2018.

DISCUSSION

Total number of readmissions and demographics of readmissions

There has been a yearly increase in the total number of 30-d readmissions of ALC in the United States. This may be due to rising alcohol use, high-risk drinking habits and DSM-IV alcohol use disorders[7]. Prior research has established a strong positive co-relation between rising alcohol use disorders and alcoholic liver disease such as ALC. In our study, most 30-d ALC readmissions were for males, but a decreasing trend was noted in the proportion of male readmissions. A study examining privately insured

Table 1 Demographic characteristics and hospitalization trends for 30-d readmissions of alcoholic liver cirrhosis in the United States from 2010–2018

Variable	Year				
	2010	2012	2014	2016	2018
Number of readmissions	7660	8211	8753	13278	15085
Mean age (yr)	53.5 ± 0.5	53.6 ± 0.4	53.6 ± 0.4	54.0 ± 0.3	54.2 ± 0.3
Male (%)	72.5	73.1	72.2	68.3	67.4
Charlson comorbidity					
Index (CCI) Score (%)					
0	2.8	2.4	2.2	0.6	0.6
1	15.7	15.1	13.0	1.4	13.2
2	7.5	6.5	6.9	7.3	6.3
≥ 3	74.0	76.0	78.0	78.4	79.8
Insurance type					
Medicare	27.6	28.2	29.3	30.0	30.5
Medicaid	40.5	42.0	42.1	41.6	40.6
Private	21.4	20.0	20.4	22.5	21.8
Uninsured	10.5	9.7	8.3	6.0	7.2
Household income					
Quartile (%)					
1	34.6	36.2	34.0	34.2	33.2
2	23.8	25.6	28.3	27.4	29.2
3	23.4	22.4	22.0	23.5	22.6
4	18.2	15.8	15.6	14.8	15.0
Hospital characteristics					
Hospital bed size (%)					
Small	9.3	9.0	12.4	11.6	14.3
Medium	22.3	22.7	26.4	25.9	25.9
Large	68.4	68.2	61.2	62.5	59.8
Teaching status (%)					
Metropolitan non-teaching	40.4	39.1	28.0	26.1	20.5
Metropolitan teaching	52.6	53.7	66.8	69.5	75.4
Non-metropolitan	7.0	7.3	5.2	4.3	4.1
Hospital Volume (Quintiles)					
Q1	2.4	2.3	2.2	1.9	1.5
Q2	6.6	5.8	6.0	5.2	5.5
Q3	12.6	12.5	12.0	10.6	11.3
Q4	21.8	22.0	20.1	20.1	20.7
Q5	56.6	57.4	59.7	62.2	61.1

individuals with alcoholic cirrhosis noted that 32% of patients with alcoholic cirrhosis were women[8]. Our findings may reflect a rise in alcohol use, alcohol use disorders, and high-risk drinking behaviours in women, which is consistent with findings in current the literature[7].

Table 2 Trends of rates and outcomes for 30-d readmissions of alcoholic liver cirrhosis in the United States from 2010–2018

Outcomes	Year					*P* value[1]
	2010	2012	2014	2016	2018	
All-cause readmission rate (%)	24.9	25.1	25.1	29.8	29.9	< 0.001[1]
ALC specific readmission rate (%)	6.3	6.2	6.2	7.7	8.4	< 0.001[1]
ALC readmission proportion (%)	31.4	30.9	30.7	33.5	36.3	< 0.001[1]
Inpatient mortality (%)	10.5	9.7	9.2	8.3	8.2	0.008[1]
Mean length of stay (d)	5.6	5.6	5.6	6.4	6.3	0.001[1]
Mean total hospital cost (USD)	13790	14206	13612	17602	17150	< 0.001[1]

[1]Statistically significant. ALC: Alcoholic liver cirrhosis. ALC: Alcoholic liver cirrhosis.

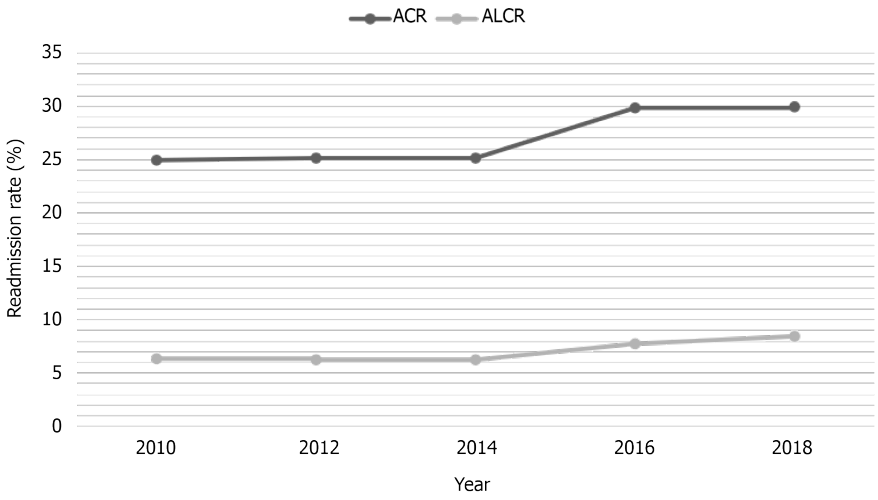

Figure 1 Trends for 30-d readmissions of alcoholic liver cirrhosis (ALC) in the United States from 2010–2018. ACR: All-cause readmissions, ALCR: Alcoholic liver cirrhosis-specific readmissions.

Recent reports have also indicated that women with alcohol use disorder may experience more barriers to treatment than men. Additionally, women are less likely to access treatment for alcohol use disorders than men. The reasons for these differences in treatment across genders are numerous and include low perception for the need of treatment, feelings of shame and guilt, concurrent disorders, economic disparities, insurance disparities, and employment status[9]. The rise of alcohol use disorders and rising consumption of alcohol by women along with differences in treatment between genders may, in part, explain the down trend noted in males over the eight-year study period. Targeted treatments plans or treatment plan elements that aim to address gaps in the treatment for alcohol use disorders may help prevent ALC and help in the management of ALC patients with alcohol use disorders. Research also suggests that treatment outcomes for women are best when given in women-only programs that have women-specific content focus[9]. Thus, creating targeted treatment programs for women may be an effective way of reducing ALC readmissions and promoting abstinence from alcohol use, a key component of ALC treatment strategies[10].

Patients readmitted for ALC had increasing comorbidity burden with time. Comorbidities are known to increase mortality and affect the overall prognosis in patients with liver cirrhosis, but it is important to recognize complications and distinguish them from comorbidities in cirrhotics[11]. Previous reports have indicated that increased alcohol consumption, high-risk consumption behaviours and increased alcohol use disorders in the United States not only constitute a public health crisis, but also increase the risk of numerous comorbidities associated with alcohol use. Alcohol use disorders and increased alcohol consumption are well known risk factors for morbidity and mortality in patients with hypertension, cardiovascular disease, stroke,

cirrhosis, certain cancers, type 2 diabetes mellitus, infections, and injuries. Moreover, alcohol use disorders and high-risk alcohol consumption are both associated with numerous psychiatric disorders[7]. As previous studies have indicated, understanding the impact of comorbidities on cirrhosis can help generate tailored treatment regimens for patients with ALC[11]. The rising comorbidity burden with time may also reflect the need for increased interventions specifically based on comorbid conditions.

Trends for ALC readmission outcomes and cost

There was a steady rise in the risk-adjusted 30-d all-cause ALC readmission rate. We also noted increasing risk-adjusted 30-d ALC specific readmission rate and ALC readmission proportion. A study investigating patients with ALC found that these patients were more likely to be disproportionately sicker at presentation and were readmitted more often than their non-ALC counterparts[8]. Additionally, hospital readmissions have been reported to occur more frequently in patients with cirrhosis. In general, research noted that early readmission reflects poor quality of care, and previous studies have reported a pooled rate of 26% for 30-d readmissions for cirrhosis. These readmissions negative impact inpatient mortality. The rising rate of readmissions in patients with ALC suggests that there may be room for improvement in caring for patients with ALC with the hope of reducing readmissions as has been suggested in previous cirrhosis-related readmissions studies[12]. Previous studies have also found that initial ALC admissions have most often resulted in readmissions secondary to acute complications from cirrhosis and substance abuse, while in patients without ALC, readmissions were most commonly due to acute cirrhosis complications and complications from cancer[2]. The rise in ALC-related readmissions found in our study may reflect increased alcohol use, closely related to the amount of undiluted alcohol consumed and the duration of consumption[1]. This reflects the need for enrolment of patients with ALC into alcohol rehabilitation programs on index admission, extensive patient education, regular outpatient follow-ups and early effective alcohol use disorder treatments in the outpatient setting to prevent development and readmissions in ALC patients.

Inpatient mortality showed a decreasing trend in our study; however, there was a trend towards increasing LOS and THC. The total days of hospital stay attributable to ALC readmissions increased by 119.2%, and total attributable hospital costs increased by 149% from 104 dollars million in 2010 to over 259 million dollars by 2018. Inpatient charges for patients with liver cirrhosis are substantial and have been consistently increasing[13]. Cirrhosis has resulted in considerable resource utilization and expenditure, despite acceptable hospital survival. Critically ill patients with liver cirrhosis have historically been perceived as not only having a poor prognosis, but also substantial costs of care, which is elucidated by our findings[14]. Alcohol liver diseases such as ALC are reportedly account for more than half of the charges associated with liver cirrhosis. This significant cost associated with ALC is driven by the volume of both admissions and readmissions of the same patients. Previous reports have suggested that effective alcohol use disorder interventions can help reduce costs related to inpatient cirrhosis management[13]. Treatments that have been proven to be cost-effective and in some cases cost-saving for ALC include one-on-one physician counselling and medication-assisted therapies[15]. These have been shown to improve outcomes in patients with compensated ALC[15].

Strengths and limitations

This study has several strengths that can be appreciated. The population used for this study is drawn from the NRD, which is believed to be a large, multi-ethnic hospital-based registry in the United States. This study also examines eight-year data and numerous demographic characteristics of ALC hospitalizations, offering a comprehensive, thorough, and contemporary overview of ALC readmissions in the United States. However, as with any study, there are limitations that should be noted. Data from the NRD is subject to all biases associated with retrospective studies. Additionally, the NRD does not contain data on the hospital course and treatment aspects of the disease. Moreover, the NRD reports information on hospitalizations rather than from individual patients. Thus, patients with numerous readmissions would be included more than once in the data set. The database also uses ICD codes to store information and therefore may have coding errors. Finally, the NRD does not include information about the severity of ALC at the time of admission.

Despite these limitations, the large sample size, outcomes of the study, and analysis techniques make for a study that provides a current perspective on a major healthcare burden while aiming to encourage further discourse and future controlled prospective studies on ALC hospitalizations and readmissions.

CONCLUSION

ALC is a chronic liver disease with a known healthcare and economic burden, morbidity, and mortality with the potential to result in readmissions. This retrospective, interrupted trends study examined adult hospitalizations for ALC in in the United States. We found a yearly increase in the total number of 30-d readmissions for ALC and an increasing comorbidity burden with time which may reflect the rise in alcohol use disorders and comorbid conditions in patients with ALC. There was a steady rise in the risk-adjusted 30-d all-cause ALC readmission rate, risk-adjusted 30-d ALC-specific readmission rate and 30-d ALC readmission proportion. This may reflect the need for better management of ALC in an outpatient setting. Medication-assisted therapies and counselling may be highly cost-effective ways to reduce ALC readmissions. Inpatient mortality notably showed a decreasing trend for the study period. However, there was a trend towards increasing LOS and THC. Ultimately, improved management of ALC and associated conditions like alcohol use disorder and high-risk alcohol consumption may help reduce readmissions and resultant healthcare burdens associated with readmissions.

ARTICLE HIGHLIGHTS

Research background

Readmissions of alcoholic liver cirrhosis (ALC) are associated with poor outcomes.

Research motivation

There is paucity of data on the trends of 30-d readmissions of ALC in the United States despite it being a significant healthcare burden.

Research objectives

The primary objective of this study was to identify and assess trends of 30-d readmissions of ALC in the United States over an eight-year period.

Research methods

This retrospective interrupted trend study used the National Readmissions Database to identify all 30-d readmissions of ALC. Multivariate regression analysis was used to calculate the trend for risk-adjusted odds of 30-d all-cause ALC readmissions, ALC specific readmission rate, ALC readmission proportion, mortality, mean length of stay (LOS) and mean total hospital cost (THC).

Research results

There was a trend towards increasing total 30-d readmissions of ALC, risk-adjusted 30-d all-cause ALC readmission, ALC specific readmission rate, and ALC readmission proportion. However, inpatient mortality declined from 10.5% in 2010 to 8.2% in 2018. The total days of hospital stay attributable to 30-d readmissions of ALC increased by 119.2% while the total attributable hospital costs increased by 149% by the end of 2018.

Research conclusions

The total number of 30-d readmissions of ALC increased; however, inpatient mortality declined. There was a trend towards increasing LOS and THC for these readmissions.

Research perspectives

Future studies are needed to investigate the treatment aspects of ALC in an inpatient setting. Additionally, the impact of early enrollment of patients into alcohol rehabilitation programs, patient education, regular outpatient follow-up and early effective alcohol use disorder treatments in the outpatient setting to prevent readmissions of ALC in is yet to be determined.

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ORIGINAL ARTICLE

Observational Study

New stem cell autophagy surrogate diagnostic biomarkers in early-stage hepatocellular carcinoma in Egypt: A pilot study

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Author contributions: Yosef T supervised the conduction of the study; Ibrahim WA and El-Nakeep S followed the clinical collection of data and the availability of patients; El-Nakeep S and Matboli M formulated the research question and its applicability and wrote the final draft; Matboli M conducted the laboratory analysis; Swilam AA collected the data from the patients; all authors revised and accepted the final submitted manuscript.

Institutional review board statement: The Internal Medicine Department, Faculty of Medicine, Ain Shams University, approved this study's protocol in 2016 for ethics of conducting the study and in accordance with the ethical standards of the Declaration of Helsinki. Informed consent was obtained from each participant. Both the patients and controls were randomly selected.

Informed consent statement: Informed consent was obtained from each participant. Both the

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Abstract

BACKGROUND

Stem cell autophagy disruption is responsible for the development of hepatocellular carcinoma (HCC). Many non-coding RNAs are linked to the activation and inhibition of certain genes. The *SQSTM1* gene controls stem cell autophagy as shown in previous studies. The upregulation of *SQSTM1* is associated with the inhibition of autophagy in cancerous stem cells in patients with HCC.

AIM

To determine whether serum microRNA, hsa-miR-519d, is linked to *SQSTM1* gene and whether they could be used as diagnostic biomarkers for early-stage HCC.

METHODS

In silico analysis was performed to determine the most correlated genes of autophagy with microRNAs. *SQSTM1* and hsa-miR-519d were validated through this pilot clinical study. This study included 50 Egyptian participants, who were classified into three subgroups: Group 1 included 34 patients with early-stage HCC, Group 2 included 11 patients with chronic liver disease, and Group 3 (control) included 5 healthy subjects. All patients were subjected to full laboratory investigations, including viral markers and alpha-fetoprotein (AFP), abdominal ultrasound, and clinical assessment with the Child–Pugh score calculation. Besides, the patients with HCC underwent triphasic computed tomography with contrast to diagnose and determine the tumor site, size, and number. Quantitative

patients and controls were randomly selected.

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real-time polymerase chain reaction was used to assess hsa-miR-519d-3p and *SQSTM1* in the serum of all the study participants.

RESULTS

Hsa-miR-519d-3p was significantly upregulated in patients with HCC compared with those with chronic liver disease and healthy subjects with an area under the curve (AUC) of 0.939, with cutoff value 8.34, sensitivity of 91.2%, and specificity of 81.8%. *SQSTM1* was upregulated with an AUC of 0.995, with cutoff value 7.89, sensitivity of 97.1%, and specificity of 100%. AFP significantly increased in patients with HCC with an AUC of 0.794, with cutoff value 7.30 ng/mL, sensitivity of 76.5%, and specificity of 72.7%.

CONCLUSION

This study is the first to show a direct relation between *SQSTM1* and hsa-miR-519d-3p; they are both upregulated in HCC. Thus, they could be used as surrogate diagnostic markers for stem cell autophagy disturbance in early-stage HCC.

Key Words: Autophagy; Hepatocellular carcinoma; miRNA; miR-519d; Stem cell; *SQSTM1*

Core Tip: Hepatocellular carcinoma (HCC) is the most common primary liver cancer. HCC is associated with poor prognosis due to difficult discovery at an early stage. The molecular pathophysiology behind HCC is not yet fully understood. Autophagy is one of the important affected pathways in HCC pathogenesis. In this study we used *in silico* analysis to determine a new molecular pathway and find the underlying background controlling genetic and epigenetic pathways. We found that autophagy-controlling gene *SQSTM1* is related to hsa-miR-519d-3p. Also, we found that their use as early detecting biomarkers for HCC diagnosis are more efficient than the currently used alpha-fetoprotein.

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INTRODUCTION

The scientists who discovered the mechanism of autophagy were awarded a Nobel Prize, and autophagy subsequently became a topic of great scientific interest for researchers. Autophagy is defined as cellular "self-eating," where lysosomal degradation of cellular elements occurs[1-3]. This process has three types: Chaperone-mediated autophagy, microautophagy, and macroautophagy. Autophagy is considered a "dynamic cellular recycling"[4] and provides cancerous cell preservation through the production of amino acids from degraded proteins[5]. The activation of autophagy increases resistance to cisplatin and sorafenib in patients with hepatocellular carcinoma (HCC); this could be reversed upon deactivation[6].

The discovery of "epigenetic–genetic" links is an important area of research. Studies on the regulation of targeted genes by microRNAs (miRNAs) must answer two questions: The mechanism of regulation and the effect of dysfunction on specific cancerous molecular pathways[7].

MiR-519d dysregulation is not only linked to the initiation and progression of many cancers as breast[8], skin[9], and gastrointestinal cancers[10,11] but also associated with obesity[12].

SQSTM1, also known as p62 protein, is a multifunctional protein responsible for various stress-induced cellular functions, including apoptosis and autophagy; its coding gene is the *SQSTM1* gene located on chromosome 5[13]. The impairment of

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autophagy causes the accumulation of p62 proteins in the hepatoma cells of mice[14]. Meanwhile, its upregulation significantly contributes to the resistance of hepatoma cells to sorafenib[15]. *SQSTM1* was initially believed to only control several cellular metabolic pathways, including the mechanistic target of rapamycin, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and mitogen-activated protein kinase signaling pathways, but later was also linked to the control of selective autophagy[16].

Here, in this study, we used *in silico* analysis to search for a new link among epigenetic–genetic biomarkers to identify and detect their relationship with early-stage HCC. We found significant *in silico* data relation between hsa-miR-519d-3p and *SQSTM1* and their link to HCC pathophysiology. We clinically validated the data by examining serum samples to assess their ability to be used in the diagnosis of HCC.

MATERIALS AND METHODS

This was a cross-sectional study conducted on randomly selected 50 Egyptian participants from outpatient clinics and inpatients attending the Gastroenterology and Hepatology Unit of the Internal Medicine Department at Ainshams University Hospitals, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

The participants were divided into three groups

Group 1: Consisted of 34 patients with HCC that were diagnosed according to the American Association for the Study of Liver Diseases practice guidelines and staged according to the Barcelona Clinic Liver Cancer as stages A to D[17].

Group 2: Consisted of 11 patients with chronic liver disease.

Group 3: Consisted of 5 healthy subjects (control), who were enlisted during routine checkups and as volunteers.

Inclusion criteria for the study

Age more than 18 years.

The ability to provide informed consent.

Proven diagnosis of HCC according to the American Association for the Study of Liver Diseases practice guidelines for group 1[17].

Exclusion criteria for the study

Patients actively undergoing chemotherapy or radiation therapy for HCC.

Patients with other malignancies or treated within the last 5 years.

The Internal Medicine Department, Faculty of Medicine, Ain Shams University, approved this study's protocol in 2016 for ethics of conducting the study and in accordance with the ethical standards of the Declaration of Helsinki. Informed consent was obtained from each participant. Both the patients and controls were randomly selected. This study was not funded.

Data of samples

The following parameters were documented for the participants: Full personal history and thorough clinical examination.

Laboratory investigations included the following: (1) Liver function: Serum albumin, prothrombin time and international normalized ratio, and total and direct bilirubin; (2) Liver enzymes: Serum aspartate transaminase, alanine transaminase, alpha-fetoprotein (AFP), hepatitis C virus antibody, and hepatitis B virus surface antigen (HBsAg); and (3) Abdominal ultrasound: Tumor size, the number of nodules, local spread, lymph node metastasis, cirrhosis, and the presence of ascites.

Triphasic spiral contrast-enhanced computed tomography in the HCC group.

Biomarker identification and bioinformatics analysis

Bioinformatics analysis was performed to retrieve biomarkers relevant to HCC based on previous microarray studies. This step included the following.

Biomarker retrieval and verification: In this concern, we used the public databases, including miRDB, miRTargetLink Human, GeneCards, and Human Protein Atlas, to choose a set of miRNAs and its targeted messenger RNA (mRNA) that is related to HCC. According to the data retrieved, we chose the microRNA-519d, hsa-miR-519d-

3p, and the targeted mRNA, *SQSTM1*, as a point to be studied in relation to HCC. *In silico* analysis is shown in detail in Figure 1.

Sample collection: Blood was collected from all participants in a plain test tube. These blood samples were left at room temperature for a minimum of 30 min to allow complete blood clotting.

The clotted blood samples were centrifuged for 20 min.

The serum was carefully separated and transferred to 1.5 mL aliquots and stored at 80 °C until assayed.

An identifier was used to label each serum sample to protect the confidentiality of the participants.

Laboratory work

Extraction of total RNA: An miRNEasy RNA isolation kit (Qiagen, Hilden, Germany) was used to extract total RNA from the serum samples according to the manufacturer's instructions. The RNA concentration and integrity were assessed using an Ultraspec 1000 UV/visible spectrophotometer (Amersham Pharmacia Biotech, Cambridge, United Kingdom). Then, 72 µL diethyl pyrocarbonate–water was added to 3 µL RNA solution (dilution 1:25). The sample was read at 260 nm for RNA detection and 280 nm for protein detection using a spectrophotometer. Next, 40 µg RNA/mL is equivalent to 1 absorbance, so the concentration of RNA in a sample (µg/mL) = sample absorbance at 260 nm × 40/1 × dilution factor (25). The samples were considered to have high RNA quality if the RNA–protein ratio (260:280 ratio) was more than 1.8–2.

Reverse transcription-polymerase chain reaction: The extracted total RNA underwent reverse transcription into cDNA using a miScript II RT Kit (Qiagen) according to the manufacturer's protocol using a Hybaid thermal cycler (Thermo Electron, Waltham, MA, United States).

Quantitative reverse transcription-polymerase chain reaction: RNA levels were examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to ensure sensitive and specific RNA detection and quantification with high amplification efficacy. All PCR primers were obtained from Qiagen. All steps followed the manufacturer's suggested protocol.

Quantitative PCR for the detection of *SQSTM1* mRNA: The relative expression of *SQSTM1* mRNA was assessed using a QuantiTect SYBR Green PCR Kit (Qiagen) on a Rotor-Gene real-time PCR detection system (Qiagen) with specific primers provided by Qiagen. Beta-actin (ACTB) was used as a housekeeping gene.

The QuantiTect SYBR Green PCR Kit provides accurate real-time quantification of cDNA targets in an easy-to-handle format. The kit can be used in real-time two-step RT-PCR of RNA targets following reverse transcription with the fluorescent dye SYBR Green I in the master mix, which enables the analysis of many targets without having to synthesize target-specific labeled probes. It uses the SYBR Green I dye to detect PCR products by binding to double-stranded DNA formed during the PCR. It binds to each new copy of double-stranded DNA generated during each PCR cycle. The result is an increase in fluorescence intensity proportional to the number of double-stranded PCR products produced.

High specificity and sensitivity in PCR are achieved using the hot-start enzyme HotStarTaq DNA Polymerase together with a specialized PCR buffer. In addition, the buffer contains ROX dye, allowing fluorescence normalization on certain cyclers. The kit has been optimized for use with any real-time cycler, including Rotor-Gene® cyclers. A melting point analysis was performed to monitor the homogeneity and specificity of the quantitative PCR (qPCR) products.

qPCR for the detection of hsa-miR-519d-3p: A relative miRNA expression level for hsa-miR-519d-3p was analyzed by mixing the total cDNA with the reagent provided in the miScript SYBR Green PCR Kit (Qiagen) according to the manufacturer's suggested protocol, in addition to the manufacturer-provided miScript universal primer. RNU-6 was used as a housekeeping gene.

For detecting mature miRNA, cDNA prepared in a reverse transcription reaction using miScript HiSpec Buffer or miScript HiFlex Buffer serves as the template for real-time PCR analysis using a miRNA-specific miScript Primer Assay (forward primer) and the miScript SYBR Green PCR Kit, which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix.

Figure 1 Bioinformatic search and validation of the newly diagnostic biomarkers. A: miR-519d-3p and *SQSTM1* as a targeted mRNA according to miRDB (http://mirdb.org/cgi-bin/search.cgi?searchType=miRNA&full=mirbase&searchBox=MIMAT0002853); B: A network of 923 genes targeted by hsa-miR-519d-3p, along with focusing on *SQSTM1* in the network (miRTargetLink Human) (https://ccb-web.cs.uni-saarland.de/mirtargetlink/network.php?type=miRNA&qval=hsa-miR-519d-3p); C: The expression of miR-519d in liver tissue and other tissues (https://www.genecards.org/); D: The tissue expression of *SQSTM1* is low in hepatocytes of healthy liver tissue (www.proteinatlas.org); E: The expression of *SQSTM1* in cancers and liver cancer specifically (www.proteinatlas.org).

PCR result analysis: The PCR program for the SYBR Green-based qPCR was as follows: Denaturation at 95 °C for 15 min; 40 cycles of denaturation for 10 s at 94 °C; annealing for 30 s at 55 °C; and extension for 34 s at 70 °C. Each reaction was performed in duplicate. A Rotor-Gene manual was used to calculate the threshold cycle (Ct) value of each sample. Any Ct value greater than 36 was considered negative. We used the melting curve analysis software of Applied Biosystems to analyze our results. The melting curves were analyzed to affirm the specificities of the amplicons for the SYBR Green-based PCR amplification. The ($2^{-\Delta\Delta Ct}$) relative quantification RQ technique was used to measure the expression of the RNA-based biomarker panel.

The housekeeping genes, ACTB and RNU-6, were used as an invariant internal control to normalize the raw data of the samples and compare these results with a reference sample.

Statistical analysis

Statistical analyses of the obtained data were performed using SPSS, version 23 (IBM Corp., Armonk, NY, United States).

To describe the studied sample, quantitative data are presented as minimum, maximum, mean, and standard deviation for parametric data and median and interquartile range (IQR) for nonparametric data. Qualitative data are presented as

count and percentage.

Student's t-test was used to compare quantitative data between two independent groups, and the Mann–Whitney U-test was used for nonparametric data.

One-way analysis of variance was performed to compare quantitative data when more than two groups were to be compared; then, a post-hoc test was used to detect the difference between individual groups for parametric data, and the Kruskal–Wallis test was used for nonparametric data.

The chi-square test and Fisher's exact test were used to compare qualitative data between different groups.

The receiver operating characteristic (ROC) curve was used to measure diagnostic validity and determine the best cutoff value for some variables.

P values less than 0.05 denote statistical significance. In addition, concerning the level of significance: *P* values represent the level of significance, *P* values more than 0.05 are non-significant, *P* values less than 0.05 are significant, and *P* values less than 0.01 are highly significant.

RESULTS

We conducted this study on 50 Egyptian participants divided into three groups: 34 patients in the HCC group, 11 patients in the chronic liver infection group, and 5 healthy participants as the control group.

The age of all participants was more than 18 years with a mean of 58.88 ± 8.08 years, 56.18 ± 16.26 years, and 55.40 ± 22.09 years in the HCC, chronic liver infection, and control groups, respectively, with a non-statistically significant *P* value (0.72). In addition, a non-significant difference was observed between the malignant and non-malignant groups (*i.e.* patients in the chronic liver infection group added to the control group) with a *P* value of 0.53.

Gender differences were observed among the study groups—HCC group: Male = 67.6% and female = 32.4%; chronic liver infection group: Male = 81.8% and female = 18.2%); and healthy group: Male = 60% and female = 40%. The difference between the three study groups was statistically non-significant with a *P* value of 0.63 (Table 1). Liver function and laboratory data are shown in Table 2.

Hepatitis C antibody was prevalent in 88.2% of the patients with HCC, whereas all patients with chronic liver disease were positive, and no subjects in the control group were positive for hepatitis C virus antibody. HBsAg was prevalent in 5.9% of the patients with HCC, whereas none of the subjects in the chronic liver disease and control groups were positive for HBsAg. These data are shown in Table 3.

Our results concerning hsa-miR-519d-3p showed data from the qRT-PCR. These data were reported in delta–delta Ct [DDCT or -(ΔΔCT)] and RQ calculated as follows: $RQ = 2^{-ddCT} = 2^{-\Delta\Delta CT}$ (Table 4 and Figure 2A).

The results of serum miRNA (miR-519d) in the three study groups, reported in RQ, showed that in the HCC group, serum miRNA was 41.94 (IQR: 18.25–139.10); in the chronic liver infection group, serum miRNA was 5.98 (IQR: 3.14–8.28), and in the control group, serum miRNA was 1.17 (IQR: 1.16–1.21), with a highly significant *P* value (< 0.001) (Table 4). These data suggest that hsa-miR-519d-3p is significantly upregulated in the HCC group compared with the chronic liver infection and control groups. The ROC curve to assess the validity of the results of qRT-PCR of hsa-miR-519d in the serum in differentiating the HCC and chronic liver infection groups with the best cutoff value of ≥ 8.34, sensitivity of 91.2%, and specificity of 81.8% is shown in Figure 3A.

The second part of this study focused on the serum level of *SQSTM1* in HCC and whether it can be used as a significant biomarker. The data we obtained from qRT-PCR using the RQ of the serum *SQSTM1* gene in comparing the three study groups from Table 4 and Figure 2B showed that *SQSTM1* was 33.91 (IQR: 14.83–132.51) in the HCC group, 3.68 (IQR: 2.28–5.50) in the chronic liver infection group, and 0.84 (IQR: 0.76–0.99) in the control group with a highly significant *P* value (< 0.001). The ROC curve to assess the validity of the results of qRT-PCR of *SQSTM1* in the serum to differentiate the HCC and chronic liver infection groups with the best cutoff value of ≥ 7.89, sensitivity of 97.1%, and specificity of 100% is shown in Figure 3B.

When we divided the groups into the malignant and non-malignant groups, we found similar results (Figure 4).

The ROC curve to assess the validity of the RQ results of qRT-PCR of hsa-miR-519d in the serum among the malignant and non-malignant groups with the best cutoff value of ≥ 8.34, sensitivity of 91.2% and specificity of 87.5% is shown in Figure 4A**.** The

Table 1 The ages in the different groups of the study (mean ± SD)

Age	HCC (*n* = 34)	Chronic liver infection (*n* = 11)	Control (*n* = 5)	F[1]	*P* value
	58.88 ± 8.08	56.18 ± 16.26	55.40 ± 22.09	0.34	0.72 NS

[1]One-way analysis of variance. HCC: Hepatocellular carcinoma; NS: Non-significant; SD: Standard deviation.

Table 2 Significance of the differences in laboratory data between the three study groups (mean ± SD)

Variable	HCC (*n* = 34)	Chronic liver infection (*n* = 11)	Control (*n* = 5)	F[1]	*P* value
INR	1.37[a] ± 0.20	1.35[a] ± 0.30	1.07[b] ± 0.08	3.93	0.03 S
Serum albumin (g/dL)	2.94 ± 0.42	3.03 ± 0.73	3.40 ± 0.25	1.95	0.15 NS
AST[2] (IU/L)	50.00[a] ± 38.00–102.00	23.00[b] ± 15.00–39.00	15.00[b] ± 14.00–18.00	16.21	< 0.001 HS
ALT[2] (IU/L)	40.50[a] ± 28.00–73.30	22.00 ± 15.00–38.00	10.00[b] ± 8.00–15.00	12.69	0.002 HS
Total bilirubin[2] (mg/dL)	1.60[a] ± 1.10–2.20	1.10 ± 0.50–1.60	0.40[b] ± 0.30–0.50	14.91	0.001 HS
Direct bilirubin[2] (mg/dL)	0.70[a] ± 0.50–1.10	0.30[b] ± 0.10–0.60	0.10[b] ± 0.10–0.20	15.84	< 0.001 HS

[1]One-way analysis of variance (a, b Post-hoc test).
[2]Kruskal–Wallis test (median and interquartile range).
[a]$P < 0.05$.
[b]$P < 0.01$.
ALT: Alanine transaminase; AST: Aspartate transaminase; HCC: Hepatocellular carcinoma; HS: Highly significant; INR: International normalized ratio; NS: Non-significant; SD: Standard deviation.

Table 3 Hepatitis virus B and C infections in the three study groups

Variable		HCC (*n* = 34), (%)	Chronic liver infection (*n* = 11), (%)	Control (*n* = 5), (%)	(X^2)[1]	*P* value
HCVAb	Positive	30 (88.2)	11 (100.0)	0 (0.0)	18.32 FE	< 0.001 HS
	Negative	4 (11.8)	0 (0.0)	5 (100.0)		
HBsAg	Positive	2 (5.9)	0 (0.0)	0 (0.0)	0.78 FE	1.00 NS
	Negative	32 (94.1)	11 (100.0)	5 (100.0)		

[1]The chi-square test (FE: Fisher's exact test). HBsAg: Hepatitis B virus surface antigen; HCC: Hepatocellular carcinoma; HCVAb: Hepatitis C virus antibody; HS: Highly significant; NS: Non-significant.

ROC curve to assess the validity of the RQ results of qRT-PCR of *SQSTM1* in the serum among the malignant and non-malignant groups with the best cutoff value of ≥ 7.89, sensitivity of 97.1%, and specificity of 100% is shown in Figure 4B.

Furthermore, in this study, AFP was 62.60 (IQR: 8.20–600.80) in the HCC group, 3.50 (IQR: 2.50–20.00) in the chronic liver infection group, and 0.70 (IQR: 0.50–1.00) in the control group (Table 5). These results show that AFP is elevated with high statistical significance in patients with HCC as compared to other groups, with a *P* value of < 0.001. The constructed ROC curve to compare AFP results between the HCC and chronic liver infection groups showed an area under the curve (AUC) of 0.794, with the best cutoff value of > 7.30 ng/mL, sensitivity of 76.5%, and specificity of 72.7% (Figure 5A). Meanwhile, the ROC curve assessing the validity of AFP for differentiating between the malignant and non-malignant groups showed an AUC of 0.854, with the best cutoff value of > 7.30, sensitivity of 76.5%, and specificity of 81.2% (Figure 5B).

Most patients had early-stage HCC, except for three patients. The full details of the radiological findings are presented in Table 6.

Table 4 Expression level of hsa-miR-519d-3p, *ACTB*, *RNU6*, and *SQSTM1* between the three study groups (mean ± SD)

Variable	HCC (*n* = 34)	Chronic liver infection (*n* = 11)	Control (*n* = 5)	F[1]	*P* value
Ct (ACTB)	30.65[a] ± 4.21	25.82[b] ± 2.31	27.34[b] ± 2.00	7.69	0.001 HS
Ct (RNU6)	36.03 ± 2.92	36.36 ± 2.82	38.55 ± 0.96	1.78	0.18 NS
Ct (miR-519d)	30.08[a] ± 3.00	33.61[b] ± 2.78	38.05[c] ± 1.08	20.48	< 0.001 HS
mRNA-*SQSTM1*	36.14 ± 3.17	34.89 ± 2.30	38.38 ± 1.86	2.48	0.10 NS
DCT (miR-519d)	−5.95[a] ± 1.98	−2.75[b] ± 0.89	−0.50[c] ± 0.40	31.17	< 0.001 HS
DDCT (miR-519d)	−5.59[a] ± 1.98	−2.39[b] ± 0.89	−0.14[c] ± 0.40	31.17	< 0.001 HS
RQ (miR-519d)[2]	41.94[a] ± 18.25–139.10	5.98[b] ± 3.14–8.28	1.17[c] ± 1.16–1.21	28.46	< 0.001 HS
DCT (*SQSTM1*)	5.49[a] ± 1.83	9.07[b] ± 0.70	11.04[c] ± 0.58	41.08	< 0.001 HS
DDCT (*SQSTM1*)	−5.51[a] ± 1.83	−1.93[b] ± 0.70	0.04[c] ± 0.58	41.08	< 0.001 HS
RQ (*SQSTM1*)[2]	33.91[a] ± 14.83–132.51	3.68[b] ± 2.28–5.50	0.84[c] ± 0.76–0.99	32.54	< 0.001 HS

[1]One-way analysis of variance (a, b post-hoc test).
[2]Kruskal–Wallis test (median and interquartile range).
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
Ct: Threshold cycle; HS: Highly significant; NS: Non-significant.

Table 5 Alpha-fetoprotein laboratory results in the three subgroups

Variable	HCC (*n* = 34), median (IQR)	Chronic liver disease (*n* = 11), median (IQR)	Control (*n* = 5), median (IQR)		*P* value
AFP[1] (ng/mL) by ELISA	62.60[a] (8.20–600.80)	3.50[b] (2.50–20.00)	0.70[c] (0.50–1.00)	19.17	< 0.001 HS

[1]Kruskal–Wallis test (median and interquartile range).
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
AFP: Alpha-fetoprotein; ELISA: Enzyme-linked immunosorbent assay; HCC: Hepatocellular carcinoma; HS: Highly significant; IQR: Interquartile range.

DISCUSSION

Our results suggest that hsa-miR-519d-3p is upregulated in the serum of the HCC group compared with the chronic liver disease and healthy control groups, with high sensitivity and specificity as a diagnostic marker. Similar results were observed by Fornari *et al*[18], where the miRNA was upregulated and considered an HCC oncogene. The study linked our target miRNA to DNA hypomethylation and p53, both of which are responsible for cell death and apoptosis. However, a recent study by Zhang *et al*[19] has linked miR-519d to the adenosine monophosphate-activated protein kinase pathway in HCC cells, regulating cellular energy metabolism by controlling the Ras-related protein (Rab10)[19]. A recent study has raised the hope of inducing autophagy in hepatoma cells by the administration of metformin through the activation of the mechanistic target of rapamycin pathway[20]. Alternatively, patients with colorectal cancer had improved survival and lower metastasis with upregulated miR-519d-3p by regulating trophinin-associated protein[11].

This study on serum mRNA of *SQSTM1* revealed significant upregulation of its serum level in the HCC group as compared to the levels in the chronic liver disease and healthy control groups. Thus, our results mean that mRNA of *SQSTM1* is upregulated in the serum of patients with HCC. This is compared to the findings of Xiang *et al*[21] who have found higher expression levels of the encoded protein p62 in hepatoma cells of patients with hepatitis B infection or those exposed to aflatoxin B1 [21], whereas our study population was mostly infected with hepatitis C virus.

Table 6 Clinical and radiological characteristics of hepatocellular carcinoma lesions.

Variable		mean ± SD
Child–Pugh score		6.76 ± 1.44
		n **(%)**
Cirrhosis	Cirrhosis	27 (79.4)
	No cirrhosis	7 (20.6)
BCLC stage	Stage A (early)	31 (91.2)
	Stage D (late)	3 (8.8)
Child–Pugh classification	A	17 (50.0)
	B	14 (41.2)
	C	3 (8.8)
Average tumor size	> 3 cm	3 (8.8)
	< 3 cm	31 (91.2)

BCLC: Barcelona Clinic Liver Cancer; SD: Standard deviation.

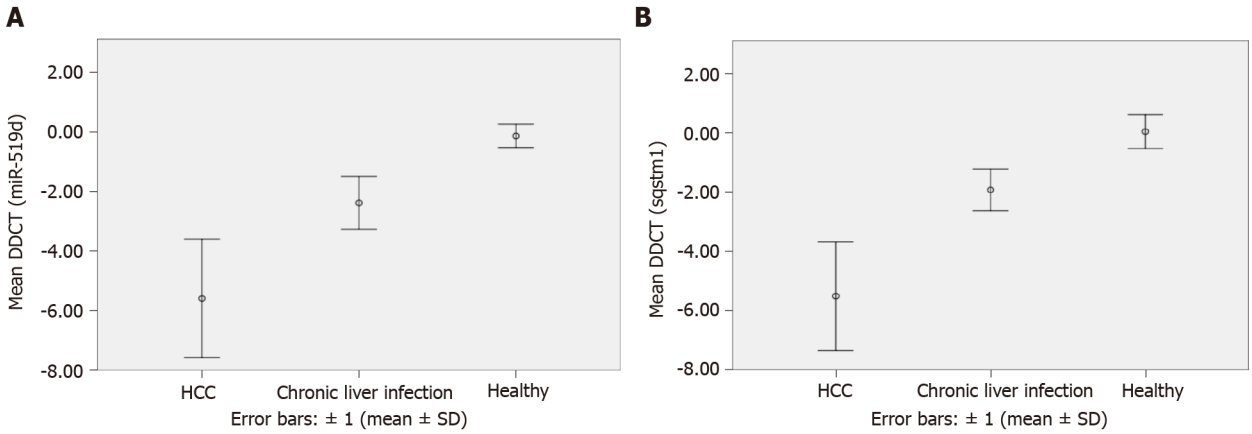

Figure 2 Box-plot figures showing the mean delta–delta threshold cycle in the new diagnostic biomarkers in different groups. A: Illustration of the mean delta–delta threshold cycle (DDCT) of the quantitative real-time polymerase chain reaction (qRT-PCR) results for hsa-miR-519d in the serum of the hepatocellular carcinoma (HCC), chronic liver infection, and control groups using error bars: ± 1 [mean ± standard deviation (SD)]; B: Illustration of the mean DDCT of the qRT-PCR results for mRNA of *SQSTM1* in the serum of the HCC, chronic liver infection, and control groups using error bars: ± 1 (mean ± SD). DDCT: Delta–delta threshold cycle; HCC: Hepatocellular carcinoma.

The *SQSTM1* gene is responsible for coding p62. This protein plays an important role as a receptor in selective autophagy, where specific cell organelles or proteins are degraded selectively by autophagosomes[22,23]. This ubiquitin-binding receptor protein is upregulated in early-stage HCC, as it is responsible for the maintenance of cancerous cells and their survival during stress[24].

In addition, our results show that hsa-miR-519d-3p is upregulated, synchronously with the upregulation of the mRNA of *SQSTM1*; this made us deduce that miRNA 519d stimulates the *SQSTM1* gene and increases the expression of its transcribed mRNA, not just increasing its translated protein level (p62) as previous studies have detected. In this study, we could not define the mechanism by which miR-519d-3p upregulates *SQSTM1*, but we have identified that the gene is upregulated at the transcriptional level, ***not*** at the post-transcriptional level. Besides, we can conclude that miR-519d-3p can affect autophagy and induce the progression of HCC through the targeted upregulation of *SQSTM1*.

In addition to these results, the sensitivity and specificity of hsa-miR-519d-3p, the mRNA of *SQSTM1*, and AFP were 91.2%–81.8%, 97.1%–100%, and 76.5%–72.7%, respectively. Also, the best cutoff values of the three parameters were ≥ 8.34 for miR-519d, ≥ 7.89 for the mRNA of *SQSTM1*, and ≥ 7.30 for AFP. Our results showed that miR-519d and the mRNA of *SQSTM1* showed higher sensitivity and specificity than

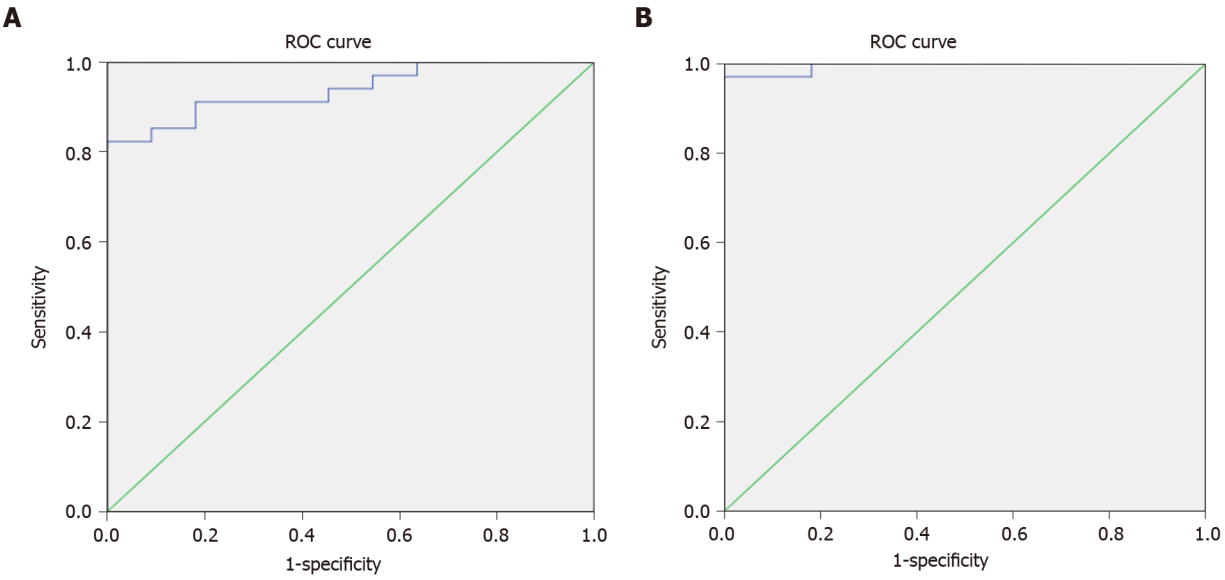

Figure 3 Receiver operating characteristic curves of the new diagnostic biomarkers studied to differentiate between hepatocellular carcinoma and chronic liver infection groups. A: Receiver operating characteristic (ROC) curve for assessing the validity of the RQ results of quantitative real-time polymerase chain reaction (qRT-PCR) for hsa-miR-519d in the serum to differentiate the hepatocellular carcinoma and chronic liver infection groups; B: ROC curve assessing the validity of the RQ results of qRT-PCR for mRNA of *SQSTM1* in the serum between hepatocellular carcinoma and chronic liver infection groups. ROC: Receiver operating characteristic.

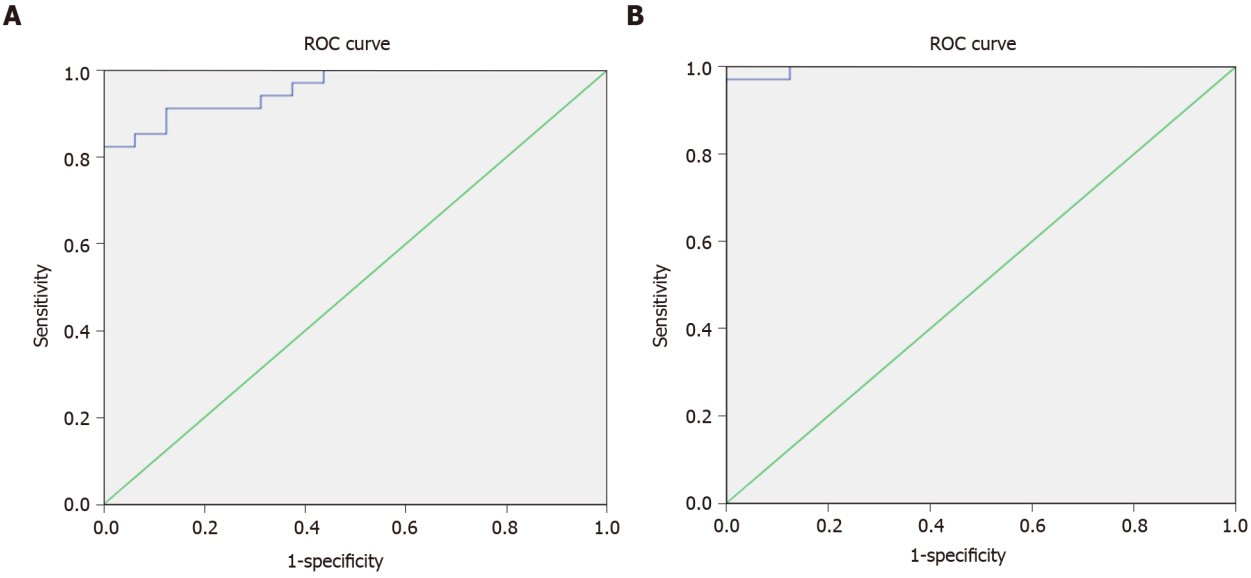

Figure 4 Receiver operating characteristic curves of the new diagnostic biomarkers studied to differentiate between the malignant and non-malignant groups. A: Receiver operating characteristic (ROC) curve assessing the validity of the RQ results of quantitative real-time polymerase chain reaction (qRT-PCR) for hsa-miR-519d in the serum among the malignant and non-malignant groups; B: ROC curve assessing the validity of the RQ results of qRT-PCR for mRNA of *SQSTM1* in the serum among the malignant and non-malignant groups. ROC: Receiver operating characteristic.

AFP, with better detection of early-stage HCC cases; thus can be used as diagnostic biomarkers for early HCC, improving the HCC outcome and prognosis. Moreover, when we compared the HCC group with the chronic liver disease group only or with the combined group of both patients with chronic liver disease and healthy subjects (malignant and non-malignant groups), we found similar results in both categories regarding hsa-miR-519d-3p, the mRNA of *SQSTM1*, and AFP.

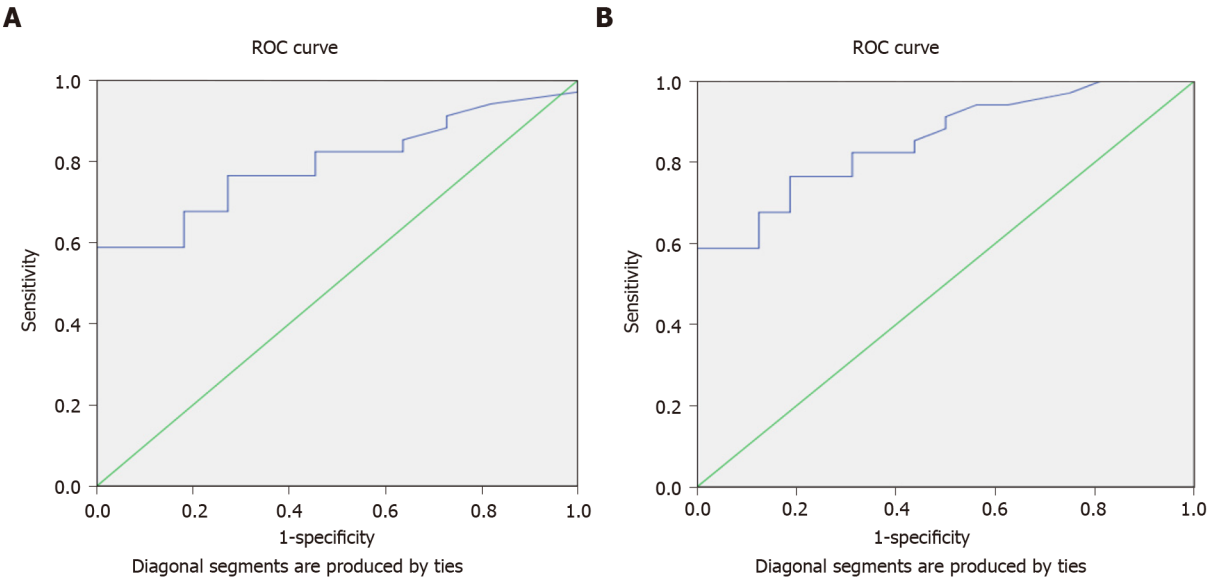

Figure 5 Receiver operating characteristic curves of the alpha-fetoprotein studied to differentiate between the hepatocellular carcinoma and chronic liver disease groups/malignant and non-malignant groups. A: Receiver operating characteristic (ROC) curve to assess the validity of alpha-fetoprotein (AFP) for the differentiation between the hepatocellular carcinoma and chronic liver disease groups; B: ROC curve assessing the validity of AFP for differentiating between the malignant and non-malignant groups. ROC: Receiver operating characteristic.

CONCLUSION

We are the first to establish a link between hsa-miR-519d-3p and the mRNA of *SQSTM1* in HCC. Hsa-miR-519d-3p and the mRNA of *SQSTM1* could be used in the diagnosis of HCC in its early stages. Further studies are needed to detect levels of miR-519d-3p and the mRNA of *SQSTM1* before and after various modalities of treatment to assess their ability to monitor treatment responses and detect recurrences. Multicentric studies with more variability to validate the use of miR-519d-3P and the mRNA of *SQSTM1* as diagnostic biomarkers of HCC on a wide scale are needed.

ARTICLE HIGHLIGHTS

Research background

Autophagy is one of the pathways affected in hepatocellular carcinoma (HCC). Genetic regulation of this pathway through the *SQSTM1* gene was established. Autophagy is responsible for the destruction of cellular components through lysosomal degradation. This process is responsible for cellular recycling and preservation. It protects from cancerous transformation, thus any imbalance in this mechanism will increase the risk of cancer.

Research motivation

We aimed to establish the genetic-epigenetic-phenotypic pathway related to the autophagic process in the pathogenesis of HCC and whether these studied biomarkers could be used as surrogate diagnostic markers for autophagy pathway in HCC.

Research objectives

We examined hsa-miR-519d microRNA effect on HCC and its association with the *SQSTM1* genetic marker. We also examined the sensitivity and specificity of those biomarkers in the diagnosis of early-stage HCC cases.

Research methods

This is an observational study. We evaluated the candidate biomarkers through bioinformatics, and after establishing a computational statistical relation, we proceeded with their clinical association through laboratory validation. We measured the genetic and epigenetic biomarkers in the serum samples taken from HCC patients, chronic liver disease patients, and healthy participants. We used reverse transcription-

polymerase chain reaction and quantitative reverse transcription-polymerase chain reaction.

Research results

We determined the sensitivity and specificity of each biomarker separately and combined as compared to the established alpha-fetoprotein (AFP) biomarker. We found that all the studied biomarkers in our study have better sensitivity and specificity than AFP, when used separately or combined, at the diagnosis of early-stage HCC.

Research conclusions

We could use the autophagy pathway biomarkers in the early-stage HCC diagnosis.

Research perspectives

More autophagy biomarkers could be examined using first *in silico* analysis then clinical laboratory confirmation. Combining computational and clinical validations in clinical studies could benefit the research process immensely.

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ORIGINAL ARTICLE

Observational Study

Determination of "indeterminate score" measurements in lean nonalcoholic fatty liver disease patients from western Saudi Arabia

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Author contributions: Khayyat YM conceived of and designed the study, collected the data, and wrote the article, providing final approval of the manuscript to be published.

Institutional review board statement: The Institutional Review Board of International Medical Centre, Jeddah, Saudi Arabia provided approval for this study (IRB No. 2019-11-215).

Informed consent statement: The requirement for consent was waived considering that there was no more than minimal risk to the subjects related to performance of FibroScan and blood tests measurements. The waiver was ensured to not adversely affect the rights and welfare of the subjects, in which tests performed were already completed, regardless of the research.

Conflict-of-interest statement: The author declares having no conflicts of interest related to this study and its publication.

Data sharing statement: The data that support the findings of this study are available from the corresponding author upon

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Abstract

BACKGROUND

Noninvasive measures to estimate liver fibrosis in lieu of biopsy in nonalcoholic liver disease (NAFLD) can broadly differentiate high *vs* low degrees of condition extent. However, an "indeterminate score" necessitates further clinical investigation and biopsy becomes essential, highlighting the need for identification of other noninvasive factors with accuracy for this midlevel extent and its prognosis. Lean NAFLD cases are of particular interest regarding this issue, as they present as otherwise healthy, and will benefit greatly from the less invasive assessment.

AIM

To estimate the agreement of two noninvasive assessment tools in lean NAFLD patients, and assess factors related to indeterminate scores.

METHODS

Ultrasound-diagnosed NAFLD patients, without sign of other chronic liver disease (n = 1262), were enrolled from a tertiary private medical centre between 2016-2019. After grouping by body mass index (obese, overweight, and lean), each participant underwent FibroScan. NAFLD fibrosis score (NFS) was used for subclassification (lower, higher, and indeterminate). No patient underwent liver biopsy. The kappa statistic was used to assess inter-rater agreement between the three groups on liver fibrosis degree assessed *via* FibroScan and NFS. Indeterminate score among the three groups was assessed to identify factors that predict its determination.

RESULTS

The NAFLD study cohort was composed of lean (159/1262, 12.6%), overweight (365/1262, 29%) and obese (737/1262, 58.4%) individuals. The lean patients were

reasonable request.

Country/Territory of origin: Saudi Arabia

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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S-Editor: Zhang H
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significantly younger (49.95 ± 15.3 years, $P < 0.05$), with higher serum high density lipoprotein (52.56 ± 16.27 mg/dL, $P < 0.001$) and lower prevalences of type 2 diabetes mellitus, hypertension and hyperlipidaemia. All groups showed a predominance of lower fibrosis degree. The lean NAFLD patients showed a significantly lower NFS ($P < 0.001$). Degree of agreement between FibroScan and NFS was fair between the lean and obese NAFLD categories, and moderate in the overweight category. NFS was predictive of indeterminate score. Age was a factor among all the body mass index (BMI) categories; other associated factors, but with less strength, were serum alanine aminotransferase in the overweight category and BMI in the obese category.

CONCLUSION
Lean NAFLD patients showed lower degree and prevalence of liver fibrosis by NFS; however, follow-up biopsy is still needed.

Key Words: Nonalcoholic fatty liver disease; Liver fibrosis; Liver biopsy; Obesity; Overweight; Lean

Core Tip: Nonalcoholic fatty liver disease (NAFLD) has emerged as a leading cause of chronic liver disease and its complications. Evaluation of fibrosis in NAFLD is of the utmost importance to early application of targeted intervention. The utilization of liver biopsy has diminished, due to patient unacceptance, sampling error, and availability of noninvasive measures of fibrosis. In this study of NAFLD cases, lean patients showed a relatively healthy metabolic profile, lower fibrosis degree and less frequent "indeterminate score" than overweight and obese patients, among which increased age and serum alanine aminotransferase level were predictive factors for determination.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a growing cause of liver-related mortality which, in recent decades, has surpassed other known causes of chronic liver diseases. It is now considered in the differential diagnoses of both overweight and lean individuals, in association with a well-established panel of metabolic abnormalities. Traditionally, the NAFLD diagnosis has been made by transabdominal ultrasound and its extent determined by the invasive assessment method of percutaneous liver biopsy. This method, despite its accuracy in staging of fibrosis, is still limited by sampling error and a hazardous risk profile of procedure-related complications, regardless of whether the approach is targeted or non-targeted[1].

Visceral obesity was long considered the sole reason for suspicion of underlying NAFLD; however, it is now recognized that lean individuals develop NAFLD. Several inflammatory cytokines have been linked to the potent effect of visceral obesity and its effects on liver fibrosis, such as the NACHT, LPR and PYD-domain containing proteins (NALPs)[2] and on hypoadiponectemia (as well as its role in liver fibrosis)[3]. The reported incidence of NAFLD among the general population is 12.1%, and within that population, lean individuals account for 40.8% and their cases do not represent healthy or benign forms of the condition[4,5]. The lean NAFLD cases add a remarkable burden to the overall landscape of NAFLD. As such, the increased clinical awareness and research focus has led to generation of novel noninvasive tests based upon mathematical modelling, serum biomarkers and liver stiffness transient elastography, providing safe alternative assessment tools by which to evaluate liver fibrosis in lieu of biopsy[6]. Such tests can be applied by specialists and non-specialists alike, partic-

ularly for the primary staging of NAFLD[7]. They have been demonstrated to have good performance, with high negative predictive values compared to liver biopsy. They are also particularly informative for NAFLD patients with high risk of advanced fibrosis, through repeated assessment by transient elastography that provides good accuracy of prediction of liver and non-liver related mortality[8].

These less invasive methods of assessment, however, are limited by uncertainty regarding the evaluation of a category of cases that falls between the low and high grades of fibrosis; such cases are scored as "indeterminate" and that label prompts further evaluation by liver biopsy (simultaneously highlighting the limited utility of the noninvasive methods early in the disease process)[9]. Complicating this situation is the fact that the increasing emergence of lean NAFLD cases has in turn increased the demand for noninvasive testing. The study described herein was, thus, designed to first determine the prevalence of indeterminate scored cases among a representative group of lean NAFLD patients, then to comparatively assess findings from bedside transient elastography or FibroScan, and ultimately to identify factors that may predispose lean NAFLD patients to obtaining an indeterminate score by noninvasive liver fibrosis tools.

MATERIALS AND METHODS

Subjects

This study was conducted at a tertiary hospital, between 2016 and 2019. Patients at least 15 years of age who received diagnosis of NAFLD (based on findings from imaging studies in accordance with ultrasonography criteria of fatty liver[10]) and those presenting components of metabolic syndrome (*i.e.* type 2 diabetes mellitus, hypertension, hyperlipidaemia, central obesity) were recruited. Patients were denied study enrolment if they were under 15-years-old, showed evidence of concurrent active medical disease that is known to impair liver function or of other secondary causes of chronic liver disease, had incomplete data, died during the study recruitment period, or refused participation in the study. Patient data collected upon enrolment included general medical history, liver disease-related history [covering other causes of chronic liver disease, such as risk factors for acquiring viral hepatitis (hepatitis B and hepatitis C virus)], medications (including over-the-counter and herbal remedies), active alcohol use or abuse, and recreational drug use. All enrolled patients were directly assessed for other causes of chronic liver disease, including hemochromatosis, Wilson's disease, and alpha 1 antitrypsin clinical manifestations, as well as autoimmune liver diseases, including autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, and hepatic vascular disease. All enrolled patients underwent complete physical examination, yielding anthropometric data on height and weight [by standard measurement protocols, used to assess body mass index (BMI)] as well as data on stigmata of chronic liver disease.

FibroScan and NAFLD fibrosis score

Each enrolled patient was fasted for 3 h and then subjected to FibroScan assessment using FibroScan 502 Touch instrument (Echosens©, Paris, France). A medium probe was applied when the skin capsule distance was ≤ 2.5 cm and an XL probe for ≥ 2.5 cm. For each patient, a median score was calculated from the values obtained from 10 successful scans performed at a single localized area.

For each enrolled patient, NAFLD fibrosis score (NFS)[11] was calculated by the following formula: -1.675 + 0.037 × age (in years) + 0.094 × BMI (as kg/m^2) + 1.13 × IFG/diabetes (with yes = 1, no = 0) + 0.99 × aspartate aminotransferase/alanine aminotransferase ratio - 0.013 × platelet count (as × 10^9/L) - 0.66 × albumin (as g/dL).

BMI categorization

After exclusion of other causes of chronic liver disease, the enrolled patients were divided into the following three groups according to their BMI: obese (BMI ≥ 30); overweight (BMI: 25-30); and lean (BMI ≤ 25). The noninvasive parameters of liver fibrosis were used to classify the BMI cohorts into low and high degree of liver fibrosis categories[12-14], with the former assigned to patients with FibroScan values < 7.9 kPa and NFS < –1.455 and the latter assigned to patients with FibroScan values > 9.5 kPa and NFS > 0.675; "indeterminate" was assigned for liver fibrosis when the measurement values fell between the low and high categorizations.

Laboratory parameters

All enrolled patients received testing for liver chemistry panel (after 4-6 h of fasting), serum glycosylated haemoglobin, and serum fasting lipid profile. Adherence to diabetic, hypertension and lipid lowering medications were verified through interviews with the patient interviews and/or immediate family relatives, as well as hospital dispensing records.

Statistical analysis

All statistical analyses were performed with SPSS software (version 26.0; IBM Corp., Armonk, NY, United States). Descriptive statistics and frequencies were calculated. Group differences were examined using one-way analysis of variance or its nonparametric equivalent, the Kruskal-Wallis test. In terms of post-hoc tests, Bonferroni correction was applied. Relationships between categorical variables were analysed with the chi-square test of independence. The kappa statistic was used to assess inter-rater agreement between the three groups on liver fibrosis degree assessed *via* FibroScan and NFS. Lastly, prediction of indeterminate NFS was determined by binary logistic regression modelling, with a P-value of < 0.005 indicating statistical significance. The statistical methods used and data interpretation were verified by an external biostatistician.

Ethical statements

The study was conducted in accordance with the Declaration of Helsinki, and all procedures were approved by the Ethics Committee of International Medical Centre (Approval No. 2019-11-115).

RESULTS

Study groups and categories

A total of 1753 patients were recruited during the study period, with 1262 meeting the criteria for enrolment and inclusion in the final analysis. A total of 491 patients had been excluded for the following reasons: incomplete data (n = 103); chronic hepatitis B (n = 185); chronic hepatitis C (n = 71); underwent weight management surgery (n = 66); active neoplastic disorders (n = 11); coexisting medical conditions known to cause liver function test alterations (n = 33); use of hepatotoxic medications(n = 8); and death during the study recruitment period (n = 13).

The entire study cohort was comprised of 159 lean NAFLD patients (12.6%), 365 overweight NAFLD patients (29.0%), and 737 obese NAFLD patients (58.4%). Tables 1 and 2 summarize the metabolic parameters and diseases among the three groups. The lean NAFLD group was of significantly younger age than the overweight and obese groups (P = 0.012).

Metabolic diseases

As shown in Table 1, the lean NAFLD group showed lower serum glycated haemoglobin (*i.e.* HbA1c) and higher serum high density lipoprotein (*i.e.* HDL) than either the overweight or obese NAFLD groups. The prevalence of various metabolic diseases differed significantly between the three BMI groups. Hyperlipidaemia was more prevalent in the overweight group (n = 205) and the obese group (n = 457) than in the lean group (n = 76, $P < 0.001$). Hypertension was also more prevalent in the overweight group (n = 144) and the obese group (n = 333) than in the lean group (n = 50, P = 0.002). Type 2 diabetes mellitus was more prevalent and to a much greater extent in the obese group (n = 405) compared to the overweight group (n = 171, $P < 0.001$) and lean group (n = 50, $P < 0.001$).

Noninvasive assessments

Transient elastography by FibroScan showed the three BMI groups to have a predominance of lower fibrosis measurements (F0-F2, *vs* higher fibrosis measurements of F3-F4) (Figure 1). In contrast, the NFS showed a significant difference between the three groups, with the lean group showing lower scores for patients in both the lower and higher fibrosis categories compared to that seen in the overweight group (P = 0.041) and the obese group ($P < 0.001$). Additionally, when the overweight group was compared with the obese group, the NFS was found to be significantly lower for the former ($P < 0.001$) (Table 2).

Table 1 Metabolic parameters in the groups classified by body mass index

Variable	Lean	Overweight	Obese	P[1]
	mean ± SD	mean ± SD	mean ± SD	
Age in yr	49.95 ± 15.34	51.34 ± 14.33	53.34 ±13.43	0.012[2]
BMI	23.14 ± 1.95	27.70 ± 1.71	35.38 ± 4.62	0.174
HbA1c, %	6.07 ± 1.41	6.51 ± 1.61	6.46 ± 1.39	0.290
ALT in U/L	37.14 ± 66.48	32.52 ± 32.16	30.73 ± 30.72	0.924
AST in U/L	28.30 ± 23.81	26.44 ± 26.96	25.04 ± 20.91	0.093
GGT in U/L	60.40 ± 81.59	56.61 ± 81.28	57.58 ± 95.50	0.141
ALKP in U/L	89.56 ± 52.69	79.77 ± 43.69	82.73 ± 38.86	0.132
Total bilirubin in mg/dL	0.74 ± 1.43	0.81 ± 1.61	0.63 ± 1.08	0.227
Direct bilirubin in mg/dL	0.35 ± 0.60	0.40 ± 1.06	0.29 ± 0.65	0.679
Total cholesterol in mg/dL	182.07 ± 48.19	172.69 ± 49.50	175.03 ± 47.37	0.222
LDL in mg/dL	118.84 ± 42.12	114.81 ± 42.00	115.38 ± 41.05	0.022
TG in mg/dL	118.69 ± 79.73	135.74 ± 88.66	132.65 ± 88.56	0.140
HDL in mg/dL	52.56 ± 16.27	47.30 ± 16.96	48.49 ± 16.50	< 0.001
FibroScan, kPa	7.43 ± 7.87	7.01 ± 8.39	8.12 ± 9.49	0.174
NFS	-2.74 ± 3.13	-2.11 ± 2.25	-1.14 ± 2.13	0.290

[1]Pairwise comparison using Bonferroni correction, with *P*-value of < 0.05 indicating statistical significance.
[2]Comparison using Kruskal-Wallis test, with *P*-value of < 0.05 indicating statistical significance.
ALKP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; GGT: Gamma-glutamyl transferase; HbA1c: Glycated haemoglobin; HDL: High density lipoprotein; LDL: Low density lipoprotein; NFS: Nonalcoholic fatty liver disease fibrosis score.

Upon evaluation of agreement between the noninvasive measures studied (FibroScan and NFS), the lean and obese groups showed fair agreement and the overweight group showed moderate agreement (Table 3).

Factors predicting "indeterminate scores"

In order to predict the possible factors that may predict an indeterminate score when NFS is used in patients with NAFLD and to compare them between the different BMI groups, single-predictor binary regression analysis was carried out with age, BMI, sex, HbA1c, AST, ALT, gamma-glutamyl transferase, alkaline phosphatase, total bilirubin, direct bilirubin, total cholesterol, low density lipoprotein, HDL, hyperlipidaemia, diabetes mellitus, and hypertension considered as independent variables (Table 4). Increasing age was found to be a statistically significant predictive factor for obtaining an indeterminate score when the NFS measurement of liver fibrosis was used. Similarly, elevated serum ALT and BMI values were found to be predictive of obtaining an indeterminate score when the NFS was used for overweight and obese groups, respectively.

DISCUSSION

The findings from this study reflect real-life data for NAFLD cases of various BMI classes and help to distinguish the distinctive metabolic phenotypes of each, providing particular insight into the lean NAFLD cases that represent a growing cohort worldwide. The lean NAFLD cases in this study were relatively young compared to other BMI groups and their phenotypic profile was closer to that of healthy individuals (in terms of having lower serum HbA1c, higher serum HDL, and less prevalence of type 2 diabetes mellitus, hypertension and hyperlipidaemia). Also, the lean group showed an overall lower fibrosis stage as measured by both FibroScan and NFS. The prevalence of cases yielding an indeterminate score was highest among the obese group (32%), followed by the overweight group (24.4%) and lean group (18.9%).

Table 2 Frequency of demographic features, metabolic diseases and noninvasive fibrosis assessment findings in the study cohort

Variable	Lean	Overweight	Obese	P[1]
Sex				0.002
Female	61 (38.4%)	142 (38.9%)	359 (48.7%)	
Male	98 (61.6%)	223 (61.1%)	378 (51.3%)	
Hyperlipidaemia				< 0.001
Absent	76 (47.8%)	130 (35.6%)	235 (31.9%)	
Present	76 (47.8%)	205 (56.2%)	457 (62.0%)	
DM				< 0.001
Non-diabetic	103 (64.8%)	171 (46.8%)	294 (39.9%)	
Diabetic	50 (31.4%)	171 (46.8%)	405 (55.0%)	
HTN				0.002
Normotensive	103 (64.8%)	198 (54.2%)	366 (49.7%)	
Hypertensive	50 (31.4%)	144 (39.5%)	333 (45.2%)	
NFS reference				< 0.001
F0-F2	85 (53.5%)	173 (47.4%)	256 (34.7%)	
F3-F4	5 (3.1%)	16 (4.4%)	84 (11.4%)	
Indeterminate score	30 (18.9%)	89 (24.4%)	237 (32.2%)	

[1]Comparison was done using chi-square test of significance, with *P*-value of < 0.05 indicating statistical significance. DM: Diabetes mellitus; HTN: Hypertension; NFS: Nonalcoholic fatty liver disease fibrosis score.

Table 3 Agreement between FibroScan and nonalcoholic fatty liver disease fibrosis score among body mass index categories

BMI class	Category	NFS < -1.455	NFS > 0.676	Agreement, κappa
Lean	Low fibrosis	72	1	0.37[c]
	High fibrosis	10	4	
Overweight	Low fibrosis	151	8	0.43[c]
	High fibrosis	9	8	
Obese	Low fibrosis	212	40	0.38[c]
	High fibrosis	30	38	

κappa: Kappa statistic used with [c]*P* < 0.001. BMI: Body mass index; NFS: Nonalcoholic fatty liver disease fibrosis score.

Upon assessment of agreement between these two modalities, the degree of agreement ranged between fair to moderate.

With the increased recognition of the importance of precision medicine in general and increased popular use of treatment algorithms in NAFLD, a proper noninvasive assessment method for liver fibrosis is needed. Indeed, advanced diagnostic methods are emerging. Transient elastography is a bedside test, easily applicable, and cost effective, with the added benefit of patient acceptance. It has been adopted clinically by non-specialist health care providers for initial assessment of liver fibrosis[15,16]. However, the drawbacks and imprecision of this technique include attenuation of the elastic shear waves by visceral obesity and subcutaneous tissues, leading to a failure rate of 3%-16%[17]. Technological enhancement of transient elastography has been made by the use of an XL probe to measure shear waves at a lower degree of fibrosis, yielding negative predictive value of 89% and specificity of 78%; nevertheless, increased BMI still carries the potential for discordance (odds ratio: 9)[14]. Since that advancement, a plethora of other noninvasive tests have been developed to overcome a variety of other obstacles using a combination of blood parameters entered into

Table 4 Logistic regression analysis for predictors of indeterminate score according to body mass index class within nonalcoholic fatty liver disease cohort

Variable	Lean			Overweight			Obese		
	OR	95%CI	*P*	OR	95%CI	*P*	OR	95%CI	*P*
Age	1.07	1.02, 1.13	0.009[b]	1.04	1.01, 1.08	0.016	1.03	1.02, 1.05	< 0.001[b]
HbA1c	1.28	0.84, 1.95	0.257	1.08	0.85, 1.36	0.541			
BMI						1.04	1.00, 1.08	.030	1.04
ALT				0.98	0.96, 0.99	0.011	1.00	0.99, 1.00	0.169
Hyperlipidaemia				0.75	0.31, 1.84	0.536	1.01	0.64, 1.57	0.981
LDL				0.99	0.98, 1.00	0.161			
DM	0.63	0.17, 2.30	0.484	0.55	0.21, 1.39	0.204	0.99	0.65, 1.50	0.946
HTN	0.61	0.19, 1.96	0.406	1.34	0.61, 2.91	0.464	0.77	0.51, 1.18	0.232

[b]*P* < 0.01. ALT: Alanine aminotransferase; BMI: Body mass index; DM: Diabetes mellitus; HbA1c: Glycated haemoglobin; HTN: Hypertension; LDL: Low density lipoprotein; OR: Odds ratio.

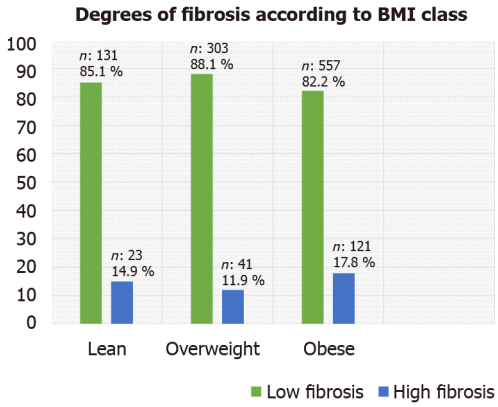

Figure 1 Grades of liver fibrosis among body mass index classified groups based on FibroScan measurements. BMI: Body mass index.

mathematical models, including direct biological and indirect markers of liver function and fibrosis[6].

Waist circumference and assessment of visceral obesity has been considered as another option to assess the degree of liver fibrosis. It is applied by means of a bedside clinical measurement of the visceral adiposity index (commonly known as the VAI); albeit, that its measurement is reportedly more robust with more advanced stages of fibrosis[18-21]. Using radiological modalities, abdominal ultrasound with assessment of the abdominal wall fat index (commonly known as the AFI)[22], and computed tomography scan with assessments of visceral fat[23], visceral adipose tissue[24] or visceral-to-subcutaneous abdominal fat ratio[25] are able to predict advanced steatohepatitis and liver fibrosis. Moreover, bioelectrical impedance estimated visceral fat (commonly known as BIA)[26] is able to predict histologically advance steatohepatitis and fibrosis.

This study found a combination of transient elastography (FibroScan) and NFS measurements in different BMI classes among individuals with predominantly lower fibrosis degree (accounting for > 80% of each BMI class). The lean NAFLD group of patients, in particular, showed fair agreement of the two tools within a lower category of fibrosis, compared to the moderate agreement shown among the overweight and obese groups. The literature includes reports of different strategies to increase the chance of proper assessment and accuracy. For example, repeat transient elastography is especially useful for when a higher degree of fibrosis is being measured (> 7.9 kPa); as shown by Chow *et al*[27], this strategy increased accuracy and subsequent normalization of the measurements in up to one-third of the patients examined. Combining FibroScan with other measures has also been shown to further increase accuracy. A

novel two-step approach to determine fibrosis in patients with high and indeterminate scores obtained with use of NFS followed by transient elastography measurement as found to minimize the need for liver biopsy compared to the use of either test alone [12]. In a Latin study by Perez-Gutiérrez *et al*[28] that correlated NFS to biopsy-based grading of liver fibrosis using Brunt criteria, up to 46% of the patients with indeterminate score showed no liver fibrosis; hence, this group would benefit from careful follow-up and possibly repeat liver biopsy.

Factors that affect interpretation of noninvasive assessment data were investigated in this study as well. A German multicentre study (known as the FLAG study) on ultrasound-based diagnosis of NAFLD in conjunction with several noninvasive assessment measures determined differences between the various noninvasive assessments of fibrosis; when groups of no-fibrosis, indeterminate score and advanced fibrosis were compared, the predictive factors were identified as increased age, waist circumference, serum AST, serum gamma-glutamyl transferase, serum ferritin, and type 2 diabetes mellitus[29]. Another study found type 2 diabetes mellitus to adversely affect the accuracy of the noninvasive parameters investigated [*i.e.* HEPASCORE, AST to platelet ratio index (the APRI) and FIB-4 tests] by down-staging their fibrosis assessment measures[30]. Similar studies have been carried out with real-life situation design. An example of such is a multi-European study that reported indeterminate scores for FIB-4 tests, ranging between 25%-30% among different NAFLD groups at primary care centres[9]. Considering the literature collectively, mitigation of liver fibrosis assessment without resorting to liver biopsy may be achieved by a combination of FibroScan measurement, NFS[12,31], serum M30 (a caspase that is cleaved to form K18 fragments that are released from apoptotic hepatocytes into the blood, where they can be detected by the M30 enzyme linked-immunosorbent assay), and APRI score[32]. Indeed, the increased accuracy achieved with this combination of tests ultimately minimized the need for liver biopsy.

In the study presented herein, patient-related characteristics, serum test results and metabolic diseases were assessed to identify potential predictive factors that may anticipate obtainment of an "indeterminate score" from NFS. Increased age and elevated serum ALT were found to increase the likelihood of need for liver biopsy. Cichoz-Lach *et al*[33] from Poland reported a similar statistically significant diagnosis of liver fibrosis in patients with indeterminate scores (constituting 30.9% of their cohort) upon analysis of NFS and BARD scores with the predictive factors of increased age, BMI > 30, and high ALT/AST ratio. In the present study, the relatively large study population provided new information of the burden of NAFLD in the region (Saudi Arabia) and the small contribution of lean NAFLD.

Importantly, lean NAFLD has long been considered as more prevalent in Asian countries. In this study, however, upon classifying NAFLD patients by BMI, we see a population prevalence of obesity similar to that in western populations; this also suggests greater generalizability of the region-specific data. Despite the fact that there was a predominantly lower degree of fibrosis in our study population, agreement was found between transient elastography and NFS. It is arguable that lean individuals may have less technical limitation for acquiring transient elastography measurement in their lean body configuration, however they still may score indeterminate score of fibrosis which subsequently impairs a precise estimation and leaves the need for liver biopsy. This limitation related to the low extent of liver fibrosis (and thus availability for the technology to detect) is an issue the merits further study. Additionally, long-term follow-up of patients with indeterminate score by NFS is needed in order to elucidate the prognosis of this measurement.

CONCLUSION

For lean NAFLD patients, noninvasive tools are valid for assessing liver fibrosis, subject to the same limitations as with obese NAFLD patients. Indeterminate score obtained by NFS is still an issue, with possible need for a subsequent histological-based assessment of liver fibrosis through invasive procedure (*i.e.* biopsy). Future studies can build upon this knowledge through efforts to determine the best follow-up strategy for such cases.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) is progressively surpassing other aetiologies of chronic liver disease, with its prevalence increasing worldwide. Earlier intervention was advocated to manage cases of less extensive fibrosis before they progress, and this process will involve the conventional invasive detection method of liver biopsy. Due to the increasing emergence of non-obese NAFLD, which is also called lean NAFLD, the need for further study of its phenotype has been recognized and related findings are expected to open new avenues for more accurate detection of fibrosis.

Research motivation

Since lean NAFLD patients are phenotypically healthy, their metabolic syndrome profile is normal. The expected degree of liver fibrosis among these cases is unknown. However, it is well recognized that use of the available noninvasive assessment tools for fibrosis in NAFLD yields a proportion of cases with "indeterminate score" who may require further assessment by liver biopsy.

Research objectives

To identify lean NAFLD characteristics distinguishing from obese NAFLD in terms of the degree of liver fibrosis using noninvasive assessment tools. Additionally, to study predictive factors that may predispose to obtainment of an indeterminate score, which may then be taken into consideration for decision-making on further affirmative evaluation by liver biopsy.

Research methods

NAFLD patients were categorized based on body mass index into lean, overweight and obese groups. Each group underwent assessment by the noninvasive tools of FibroScan and NAFLD fibrosis score (NFS). Group data based upon the subsequent subcategorizations of fibrosis degree (*i.e.* low, high and indeterminate) was applied to regression analysis to identify factors predictive of obtaining the indeterminate score.

Research results

A total of 1753 patients were recruited and 1262 of these were included in the final analysis. According to body mass index, the patients were grouped as lean (159, 12.6%), overweight (365, 29%) or obese (737, 58.4%). Lower fibrosis score was predominant within all three weight groups. Kappa statistical analysis of the FibroScan and NFS data indicated that lean and obese NAFLD cases had fair agreement between the two tools, while overweight NAFLD cases had moderate agreement. Logistic binary regression analysis performed for predictive factors of the indeterminate score obtained by NFS indicated age as a predictive factor in all three weight groups, and serum alanine aminotransferase and body mass index value as predictive in the overweight and obese groups, respectively.

Research conclusions

The lean NAFLD group showed a metabolic profile similar to healthy individuals but having a lower degree of fibrosis than their overweight and obese counterparts. The limitation of indeterminate score by NFS among obese NAFLD patients is similar to that with the lean NAFLD group; unfortunately, this is not explained by the fact that lean body mass index patients receive a more precise measurement of fibrosis than their obese counterparts. Factors that play a role in lean NAFLD patients obtaining an indeterminate score may be applied to overweight and obese counterparts; these being age and serum alanine aminotransferase of the patients.

Research perspectives

Considering lean individuals as a latent undiagnosed group among NAFLD cases, efforts to understand and properly evaluate their underlying liver fibrosis still requires systematic consideration. From the perspective of aiming to apply less invasive tools for clinical assessment of liver fibrosis, further data are needed to ascertain the benefits and limitations of the available noninvasive tools, in order to design an approach for accurate assessment of fibrosis in this newly recognized NAFLD group.

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ORIGINAL ARTICLE

Observational Study

Managing liver transplantation during the COVID-19 pandemic: A survey among transplant centers in the Southeast United States

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Abstract

BACKGROUND

The coronavirus disease-2019 (COVID-19) pandemic has had a profound worldwide impact. Indeed, it has led to a vast decrease in organ transplantation, including liver transplants (LT). There is little data regarding adjustments made by LT centers as a response to the COVID-19 pandemic.

AIM

To assess the experience of LT centers in the United States during the pandemic.

METHODS

We performed an observational survey study from May 11, 2020 to June 5, 2020. We sent out a 13 question survey to 15 LT centers across the southeastern United States.

RESULTS

Eleven LT centers responded to the survey. We found that (11/11) 100% of transplant centers made adjustments because of the COVID-19 pandemic. At least 50% of transplant centers had at least one transplant recipient infected with COVID-19. To adjust, greater than 50% of centers performed fewer LT, 100% of patients were tested for COVID-19, and most centers implemented a virtual

and revised according to the STROBE statement checklist of items.

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platform.

CONCLUSION

The COVID-19 pandemic greatly affected liver transplantation in the southeastern United States. It was evident that a concerted effort was made by LT centers to protect their patients and employees from COVID-19 but also to continue the life-saving procedure of LT in this sick patient population. Further studies are needed to assess how LT centers around the world managed the pandemic in order to learn strategies to continue life-saving procedures in this patient population.

Key Words: COVID-19; Liver transplantation; Survey; Telemedicine; Immunosuppression; Solid organ transplantation

Core Tip: The coronavirus disease-2019 (COVID-19) pandemic tremendously affected solid organ transplantation around the world, but little information has been published regarding adaptation from transplant centers. We performed a survey study of 11 Liver transplant (LT) centers in the southeastern United States. 100% of transplant centers made adjustments. COVID-19 testing of transplant candidates, virtual clinic visits, and use of remote allocation of staff were among the most commonly utilized strategies. These strategies can be advantageously used in LT centers in the future. We recommend contingency plans be in place in case of future unprecedented states of emergency.

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INTRODUCTION

The coronavirus disease-2019 (COVID-19) pandemic brought forth new challenges for transplant centers in countries all around the world. Concern for the safety of transplant donors, recipients and hospital staff, in addition to a scarcity of hospital resources allocated to organ transplantation, led to a steep decline in the number of transplanted organs worldwide[1].

In the early stages of the pandemic, limited guidance was offered to liver transplant (LT) centers in regards to the appropriate policies and practices of proceeding with transplantation. To date, there is little data regarding adjustments made by LT centers in response to the COVID-19 pandemic. In this study, we assess the impact of COVID-19 on LT centers early in the pandemic and the adjustments that these centers made in the setting of an unprecedented crisis.

MATERIALS AND METHODS

We performed an observational, survey-based study using a 13-question survey (Figure 1). The questionnaire (Table 1) was created and distributed using an emailed link to Qualtrics (Provo, UT). The questionnaire included both automatic and fill in responses. The technical functionality and ease of use of the electronic questionnaire had been tested before sending out the questionnaire. We identified transplant hepatologists from 15 LT centers in the Southeast United States. Contact information of transplant hepatologists was obtained from a database maintained by the Southeastern division of the American Liver Foundation. Participants were not compensated. Survey participants were informed of the survey details *via* electronic mail. On May 11, 2020, the questionnaire was sent *via* electronic mail. The deadline to respond to the questionnaire was June 5, 2020. Only questionnaires that were entirely completed were

Table 1 Questionnaire

Questionnaire
1 What percentage of your office staff is working remotely?
2 What percentage of your visits is now virtual?
3 How many transplants have been performed in the last 2 mo?
4 What percentage of your donors is screened for COVID-19?
5 What percentage of your candidates is screened for COVID-19?
6 Do you have a dedicated COVID-free ICU space?
7 Is there a current MELD cut-off for new evaluations to occur?
8 Are you currently rotating providers in teams to minimize exposure?
9 Are you flying out for donors?
10 Is there direct communication with UNOS regarding operations of your program?
11 What is the comparison of liver transplants in the past 2 mo to the same time frame in 2019?
12 How many of your transplanted patients contracted the COVID-19 virus?
13 What were the outcomes of those infected?

COVID-19: Coronavirus disease-2019; ICU: Intensive care unit; MELD: Model for end stage liver disease; UNOS: United Network for Organ Sharing.

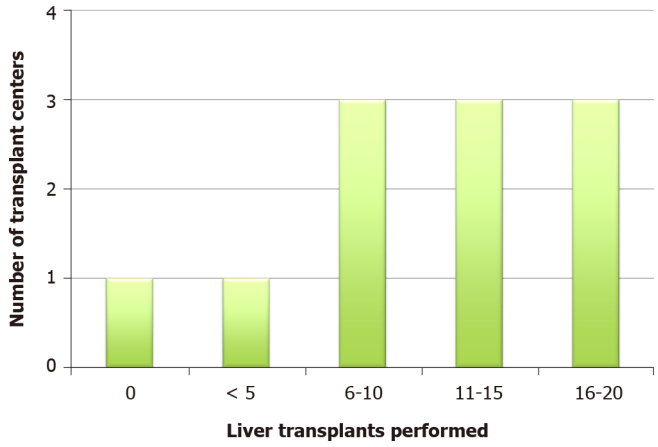

Figure 1 Number of transplants in the preceding 2 mo.

analyzed. The CHERRIES guidelines were used to further describe the methodology and results of our survey.

Results of the questionnaire were analyzed using statistics of central tendency. All data analyses were conducted using SAS version 9.4 (Cary, NC). As this was a survey study without the review of specific patient data, IRB approval was not obtained.

RESULTS

Study population

Of the 15 transplant centers, 11 (73.3%) responded to the questionnaire. All of the centers are academic-based institutions. Nine different cities in 6 different states across the southeastern United States were represented. Ten (91%) of the transplant centers had a dedicated COVID-free space in the intensive care unit (ICU).

Effect of the COVID-19 pandemic on liver transplant centers

Most participating centers performed at least 11 transplants during the preceding 8 wk (Figure 1), ranging from 0 to 20 transplants. Five of 11 centers performed less than 10

transplants. Compared to the previous year, 6 (55%) centers performed less LTs (Figure 2). This included a single center where LT services were stopped altogether. Six (55%) centers had at least 1 recipient infected with COVID-19. During the study period, the mean number of infected transplant recipients per center was 1.8.

Response by liver transplant centers

All centers routinely tested donors and recipients for COVID-19. During the study period, 58% of clinic visits were conducted virtually, and all centers reported at least some degree of telehealth medicine (Figure 3). On average, 73% of each transplant center's staff was assigned to work remotely. Transplant centers attempted to minimize exposure and institutions rotated 72.7% of their providers to minimize exposure. Less than half (45%) of transplant centers had a model for end stage liver disease (MELD) cut-off. For those centers that implemented a cut-off, 25 was the median MELD (Figure 4). All 5 centers that used a MELD cut-off performed less transplants than the year prior. More than half (55%) of the centers continued to fly to procure organs. Centers that continued to fly out for donors performed an average of 15 transplants compared to 9 transplants in centers that stopped flying out for donors. Fifty-five percent of centers had direct communication with United Network for Organ Sharing (UNOS). The centers that did not communicate with UNOS also did not fly out for organs and performed fewer transplants on average (8 *vs* 12).

DISCUSSION

The COVID-19 pandemic presented transplant centers with the unique challenge of providing potential life-saving therapy in the midst of an unprecedented public health crisis. Although several studies have investigated the effects of COVID-19 on rates of transplantation and outcomes in LT recipients[2-5], few have assessed the policy adjustments that centers were forced to implement[6]. To our knowledge, our study is the first to study the early effects of the COVID-19 pandemic, specifically on liver transplant centers, and the steps taken by these centers to provide care to their patients.

The response rate to our survey was at 73%. A recent study that surveyed clinicians on practices and policies at abdominal transplant programs in the United States found a similarly high response rate of 79.3%[6]. This suggests that transplant physicians have a keen interest to improve their understanding and adjust their practice in the midst of the COVID-19 pandemic. At the time of our study, there was limited guidance on appropriate practices and policies for LT programs during the pandemic. In fact, it was not until the third week of April 2020 that the American Association for Study of Liver Disease released a consensus statement from a panel of experts that offered guidance on management during the pandemic[7]. Nearly half of the surveyed centers maintained direct communication with UNOS for guidance[8]. Considering the magnitude of the pandemic and the many challenges that LT programs were therefore forced to manage, we expected more programs to have been in communication with UNOS for guidance during this unprecedented period.

Over the past year, several studies[1] have shown decreases in all types of solid organ transplantation due to the COVID-19 pandemic similar to our findings. The decrease in transplantation is due to many reasons including a paucity of supplies, limited ICU space[6], decreased nursing and medical staff, and the uncertainty of post-transplant care and immunosuppression during the pandemic[9,10]. The majority (90.9%) of centers in our study continued performing LT, albeit often at a limited capacity, thus highlighting the importance of continuing these life-saving procedures. A single center ceased performing all LT. It was also the only center without a dedicated, COVID-free space in the ICU, thus underscoring the tremendous impact that limited resources had on transplant centers during the pandemic. Due to concerns for safety and limited resources, nearly half of centers stopped flying for organ procurement and made use of locally available donors. This may serve as a future impetus for an increased focus on local organ donations.

The safety of liver transplant recipients and hospital staff has been an area of concern since the onset of the COVID-19 pandemic. Nearly 3% of people that have been infected with COVID-19 are healthcare workers[11]. Additionally, several studies have shown that COVID-19 infection rate may be higher in LT recipients, although outcomes are similar when compared to the general population[3,5]. During the study period, a majority (55%) of centers reported at least one transplant recipient with COVID-19 infection. No center reported a COVID-19 related mortality; however, since

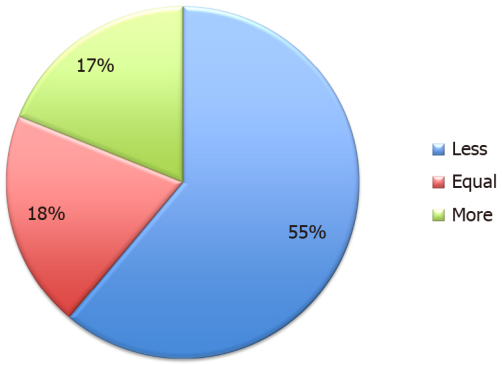

Figure 2 Comparison of liver transplants in 2020 compared to 2019.

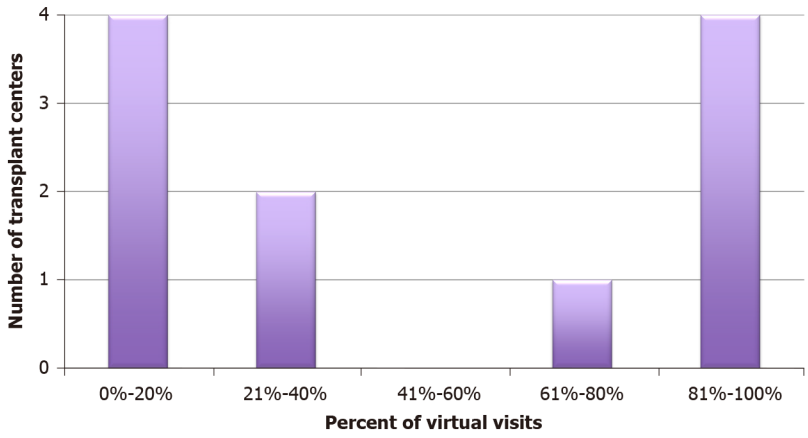

Figure 3 Percent of virtual visits.

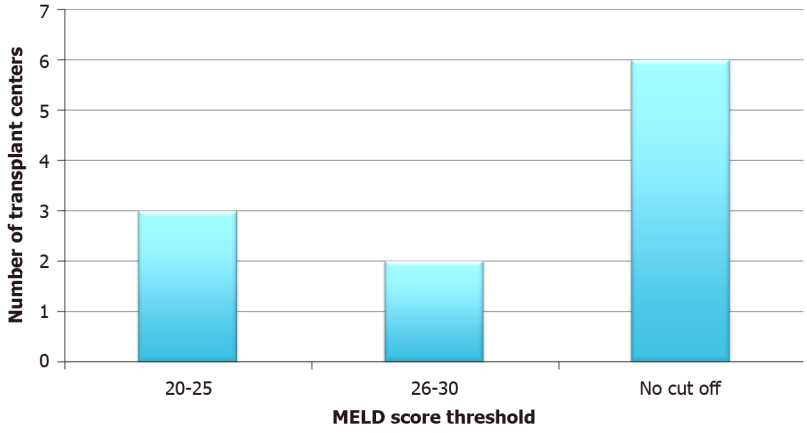

Figure 4 Model for end stage liver disease score cut-off for new evaluation. MELD: Model for end stage liver disease.

the survey was conducted the number of patients infected and the mortality is likely to have changed.

At the onset of the pandemic, transplant centers took steps to ensure the safety of liver transplant staff and recipients. Some of the interventions put in place included testing all LT candidates and donors for COVID-19, utilizing a virtual visit platform, and rotating staff to work remotely. Similar to what was reported in other studies[12, 13], all centers used telemedicine to some capacity. Transplant centers may have been better equipped to adapt to telemedicine due the basic infrastructure that is required for normal operations. Our survey shows that the pandemic changed centers' approach to telemedicine. Though imperfect in many ways, telemedicine has

broadened the reach of transplant programs and has given patients increased access to transplant providers[13].

Our study adds to the growing data[6,14,15] regarding the management and policies of LT during the COVID-19 pandemic. Our study provides a unique perspective to the practice of transplant centers in the Southeast United States, which was a "hotspot" for COVID-19, albeit after the initial wave that affected the New York City region. Also, we had a high response rate to our survey, allowing us to better understand the practices in the majority of centers in the region.

We had several limitations to our study. The primary limitation was the sample size with the inclusion of 11 transplant centers. Though the number of centers was limited, our goal was to highlight the practices of a unique region in the United States. Our survey was only distributed to transplant hepatologists and did not include surgeons and other transplant staff that may have offered more perspective on their centers' practices. Although the peak of the pandemic has passed, this study is a learning opportunity and an encouragement to develop contingency plans for possible future public health emergencies. Finally, due to the nature of the study, there is the possibility of recall bias.

CONCLUSION

COVID-19 changed the practice of medicine across the world, and in our study, we highlight how COVID-19 affected LT practices in the Southeast United States. Our study offers a unique perspective to how individual transplant centers adapted their practice and created their own strategies in response to the COVID-19 public health emergency, despite the lack of clear guidelines. Moving forward, the transplant community should use this experience as an important learning opportunity and as a chance to develop contingency plans for future public health emergencies, natural disasters, and other emergency situations. This may be in the form of specific preemptive guidelines, emergency committees, and resources for communication. These strategies are imperative to continue efficiently performing these life-saving procedures, even during unprecedented situations.

ARTICLE HIGHLIGHTS

Research background

The coronavirus disease-2019 (COVID-19) pandemic greatly affected liver transplant (LT) centers. This is the first study to investigate the effects of COVID-19 specifically on LT centers and the adjustments made by them to provide care to their patients.

Research motivation

There is limited data on policy adjustments made by LT centers during the pandemic. Our findings can help guide transplant centers during future health care emergencies but also to encourage the development of contingency plans for possible future public health emergencies.

Research objectives

Our main aim was to assess the experience of southeastern United States LT centers during the COVID-19 pandemic. Specifically, we wanted to see how the pandemic affected LT centers and the adjustments made by the centers. We were able to realize these objectives.

Research methods

We performed an observation, survey-based study using a 13-question survey. The survey was sent *via* electronic mail to 15 LT centers across the Southeastern United States.

Research results

Eleven of fifteen LT centers responded. 100% of centers made adjustments during the COVID-19 pandemic. Greater than 50% of centers performed fewer LTs. 100% of patients were tested for COVID-19, and most centers implemented a virtual platform.

Research conclusions

LT centers varied in their policy adjustments during the COVID-19 pandemic. This was likely due to the lack of clear guidelines. Going forward, the transplant community should use this experience as an important learning opportunity and galvanize contingency plans for possible future public health emergencies.

Research perspectives

Future studies should assess the most effective way to establish and implement clear guidelines to continue liver transplantation during emergency situations. Future studies should also assess which policy adjustments made during the COVID-19 pandemic were safest and most effective in continuing liver transplantation.

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ORIGINAL ARTICLE

Prospective Study

Accuracy of virtual chromoendoscopy in differentiating gastric antral vascular ectasia from portal hypertensive gastropathy: A proof of concept study

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Abstract

BACKGROUND

Accurate detection of gastric antral vascular ectasia (GAVE) is critical for proper management of cirrhosis-related gastrointestinal bleeding. However, endoscopic diagnosis of GAVE can be challenging when GAVE overlaps with severe portal hypertensive gastropathy (PHG).

AIM

To determine the added diagnostic value of virtual chromoendoscopy to high definition white light for real-time endoscopic diagnosis of GAVE and PHG.

METHODS

We developed an I-scan virtual chromoendoscopy criteria for diagnosis of GAVE and PHG. We tested our criteria in a cross-sectional cohort of cirrhotic adults with GAVE and PHG when high-definition white light endoscopy (HDWLE) diagnosis was in doubt. We then compared the accuracy of I-scan *vs* HDWLE alone to

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histology.

RESULTS
Twenty-three patients were included in this study (65.2% Caucasians and 60.9% males). Chronic hepatitis C was the predominant cause of cirrhosis (43.5%) and seven adults (30.4%) had confirmed GAVE on histology. I-scan had higher sensitivity (100% *vs* 85.7%) and specificity (75% *vs* 62.5%) in diagnosing GAVE compared to HDWLE. This translates into a higher, albeit not statistically significant, accuracy of I-scan in detecting GAVE compared to HDWLE alone (82% *vs* 70%). I-scan was less likely to lead to an accurate diagnosis of GAVE in patients on dialysis ($P < 0.05$) and in patients with elevated creatinine ($P < 0.05$). I-scan had similar accuracy to HDWLE in detecting PHG.

CONCLUSION
This pilot work supports that virtual chromoendoscopy may obviate the need for biopsies when the presence of GAVE is in doubt. Larger studies are needed to assess the impact of virtual chromoendoscopy on success of endoscopic therapy for GAVE.

Key Words: Portal hypertensive gastropathy; Gastric antral vascular ectasia; Virtual chromoendoscopy; Endoscopy

Core Tip: Gastric antral vascular ectasia (GAVE) and portal hypertensive gastropathy (PHG) are two causes of GI bleeding in cirrhosis. Gastric biopsies, which are the gold standard to differentiate the two conditions, may be contraindicated given coagulopathy or thrombocytopenia in cirrhosis. We developed virtual chromoendoscopy (I-scan) criteria for diagnosis of GAVE and PHG. We tested our criteria in a prospective cohort of cirrhotic adults with GAVE and PHG when high-definition white light endoscopy (HDWLE) diagnosis was doubtful. We compared accuracy of I-scan *vs* HDWLE to histology. Compared to HDWLE, I-scan demonstrated superior performance for real-time diagnosis of PHG and GAVE in cirrhosis.

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INTRODUCTION

Gastric antral vascular ectasia (GAVE) and portal hypertensive gastropathy (PHG) account for up to 10% of causes of gastrointestinal bleeding in patients with cirrhosis [1-3]. Management of GAVE is aimed at temporizing bleeding with endoscopic therapy. In contrast, management of PHG is targeted at reducing portal pressure with pharmacologic agents and portosystemic shunting[1-3]. As a result, accurate diagnosis is critical for optimal treatment of GAVE- and PHG-related bleeding[4,5]. Endoscopically, GAVE often manifests as red stripes radiating away from the pylorus commonly referred to as "watermelon stomach" but can also present in a more diffuse, 'honeycomb' pattern[6-8]. Alternatively, PHG usually involves the mucosa in the gastric fundus and body and is characterized by four main features: A mosaic-like pattern, presence of red point lesions, cherry red spots and black brown spots[9]. Despite their typical appearance, distinguishing between GAVE and PHG can be challenging with endoscopy alone as advanced PHG can have similar endoscopic features to GAVE.

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While endoscopic appearance can suggest the diagnosis, gastric biopsies are the current gold standard for differentiating PHG from GAVE. Biopsies may be contraindicated given coagulopathy or thrombocytopenia that are commonly seen with cirrhosis[10,11]. Recently, there has been an increasing interest in the use of digital chromoendoscopy for real-time optical diagnosis of various gastrointestinal pathologies[12]. Utilizing narrow band imaging (Olympus, Tokyo, Japan), Hayashi and Saeki[13], demonstrated that PHG had obscured collecting venules (CVs) and intramucosal hemorrhage as opposed to partial and marked dilation of the capillaries surrounding the gastric pits in patients with GAVE[13]. Achim *et al*[12] demonstrated that the I-scan virtual chromoendoscopy (Pentax, Tokyo, Japan) has an increased sensitivity in the diagnosis of PHG when compared with white light endoscopy[12]. Building on these studies, we aimed to compare the sensitivity, specificity and accuracy of I-scan to high-definition white light endoscopy (HDWLE) in distinguishing between GAVE and PHG. Our main hypothesis is that I-scan virtual chromoendoscopy is more sensitive and specific than HDWLE at diagnosing GAVE when compared to gastric biopsy.

MATERIALS AND METHODS

Study participants

A cross-sectional cohort study was conducted at Saint Louis University-affiliated hospitals in St. Louis Missouri between July 17, 2012 and July 8, 2013. Inclusion and exclusion criteria are highlighted in Figure 1. All adult patients with cirrhosis undergoing an upper endoscopy were considered candidates for this study. Cirrhosis was confirmed on liver biopsy or clinically coupled with laboratory tests (*e.g.* serum albumin less than 3.0 g/dL or blood platelet counts less than 150000 mm^3) and radiologic evidence of cirrhosis. Patients were excluded from the study if GAVE or PHG were absent or had a characteristic endoscopic appearance that could be clearly diagnosed without biopsy. We also excluded pregnant women or if a gastric biopsy did not confirm the diagnosis of GAVE or PHG. The study protocol was approved by the Saint Louis University Institutional Review Board. The study protocol, patient's rights and obligations were reviewed with eligible patients and informed consent was obtained from all participants.

Development of the diagnostic criteria for GAVE and PHG

To create our diagnostic criteria, the author HH prospectively obtained I-scan pictures of the gastric mucosa when endoscopically evaluating classic PHG and GAVE in consenting adults with cirrhosis who underwent esophagogastroduodenoscopy (EGD). Upon review of the images and building on prior studies by Hayashi and Saeki [13] and Achim *et al*[12], the author HH created an I-scan criteria for diagnosis of GAVE and PHG. Gastric pits are usually round, pink, and surrounded by the subepithelial capillary network that drain into CVs. When PHG is present, there is pit edema and capillary engorgement on I-scan which manifests as the snake-skin appearance on HDWLE (Figure 2A). Similarly, CVs appear as dilated star-like dark-red spots with defined borders while intramucosal hemorrhage are typically lighter in color and have a hazier border compared to venules on I-scan (Figure 2B and C). In contrast, the classic appearance of GAVE on I-scan was defined as presence of capillary ectasia characterized by bright red spots with defined borders (Figure 2D)[12, 13]. Additional examples of our PHG and GAVE under HDWLE and I-scan are in Figures 3 and 4. Participating endoscopists were then provided with a PowerPoint presentation explaining the visual appearance of GAVE and PHG with I-scan.

Pre-endoscopic evaluation

Prior to endoscopy, the following data were obtained from the patient once deemed to be eligible for this study: Age, gender, race, history of gastrointestinal bleeding in the past 3 mo, use of certain medications (non-steroidal anti-inflammatory drugs, aspirin, anticoagulants, iron tablets, or beta blockers), alcohol use, and the presence of ascites or lower extremity edema on exam.

Endoscopic examination and specimen collection

All patients underwent an EGD similar to endoscopic evaluation performed in most clinical settings. Upper endoscope (models EG-3470K, EG-2990I, EG-3490K, and EG-2790K) developed by Pentax (Tokyo, Japan) were utilized in this study. Under direct

Figure 1 Study design. PHG: Portal hypertensive gastropathy; GAVE: Gastric antral vascular ectasia; HDWL: High definition white light endoscopy.

visualization, the esophagus was intubated and the endoscope was advanced to the stomach. The gastric mucosa was first inspected using HDWLE for mucosal findings suggestive of GAVE and/or PHG. Patients who had abnormal gastric mucosal findings concerning for GAVE and/or PHG in whom the diagnosis was not certain utilizing HDWLE given lack of classic features underwent further evaluation with I-scan. Areas of abnormal gastric mucosa were carefully examined for 30 to 60 s utilizing HDWLE and the endoscopist determined the following: Visual diagnosis (PHG or GAVE), confidence level about diagnosis (high or low), location (antrum, antrum/body, antrum/body/fundus, antrum/fundus, fundus, or body), PHG severity (mild, moderate, or severe), GAVE appearance (stripped, diffuse, punctate, past previous treatment), stigmata of recent bleeding, and presence of varices. High quality photos were taken. After HDWLE exam was completed, I-scan mode and electronic magnification (× 2) were activated. The tip of the scope was positioned about 2 cm away from the mucosa for careful examination. The endoscopist determined the following: Visual diagnosis (PHG or GAVE), confidence level about diagnosis (high or low), and presence of certain features on I-scan (pit edema, dilated capillaries, dilated venules, or intramucosal hemorrhage). High quality photos were taken. At completion of the visual inspection, biopsies of the abnormal gastric mucosa for histologic confirmation were taken using a standard biopsy forceps (Boston Scientific, Marlborough, MA).

Post-endoscopic evaluation

Biopsy specimens were examined by a gastrointestinal pathologist using hematoxylin and eosin as well as special stains to establish the diagnosis. Pathologist commented on the presence of edema, vascular ectasia, acute and/or chronic inflammation, reactive epithelial cells, smooth fibers, microthrombi, hyalinosis, metaplasia, CD31 and

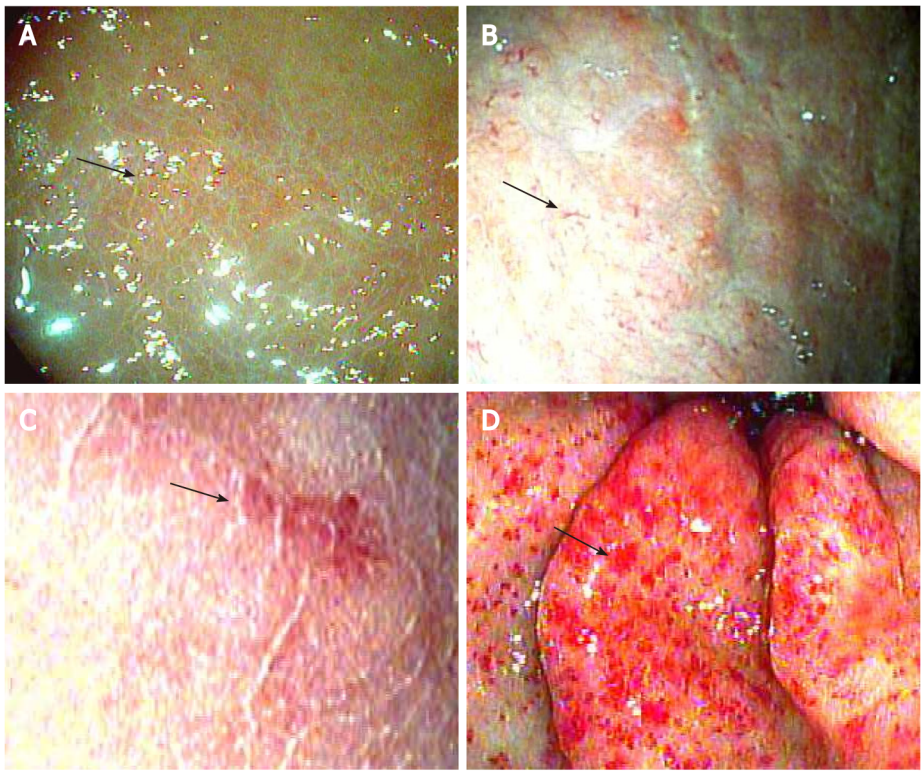

Figure 2 Portal hypertensive gastropathy. A: I-scan with pit edema/capillary engorgement; B: Dilated collecting venules under magnification; C: Intramucosal hemorrhage under magnification; D: Gastric antral vascular ectasia on I-scan defined as presence of capillary ectasia.

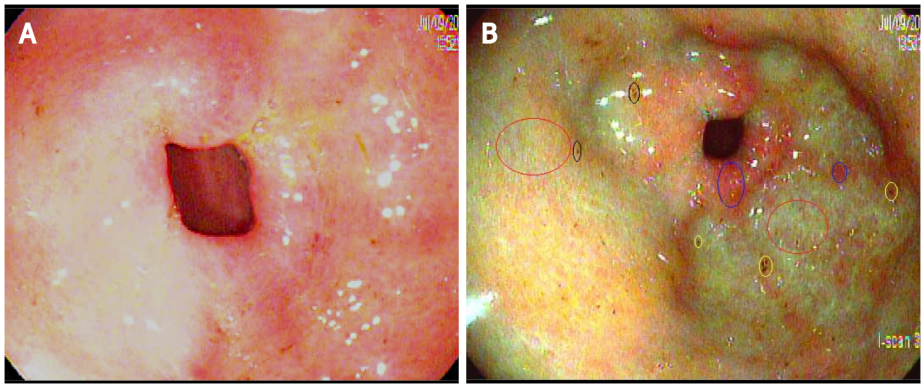

Figure 3 Portal hypertensive gastropathy under high definition white light endoscopy and I-scan Pit edema (red circles), intramucosal hemorrhage (yellow circles), capillary congestion (blue circles), and dilated venules (black circle). A: High definition white light endoscopy; B: I-scan.

CD61 positivity, and pathologic diagnosis. According to Westerhoff *et al*[14], staining for CD61 and CD31 has improved diagnostic accuracy of GAVE and PHG compared to H&E staining[14].

Statistical analysis

Analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC). To characterize the ability of HDWLE and I-scan to diagnose GAVE and PHG, sensitivities and specificities were calculated. Further, the percent accuracy of HDWLE and I-scan in diagnosing GAVE and PHG was compared with Fisher exact tests. Categorical data was summarized with frequencies and percentages while continuous data was summarized with medians and interquartile ranges (IQR). Differences between patients with correct and incorrect I-scan diagnoses of PHG were assessed through the use of Fisher exact tests or Wilcoxon rank-sum tests, as appropriate. Differences between patients with a correct and incorrect I-scan diagnosis of GAVE were analyzed

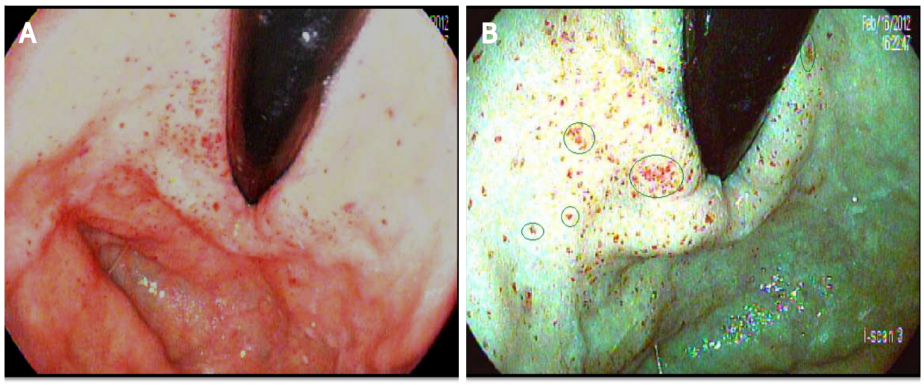

Figure 4 Gastric antral vascular ectasia under high-definition white light and I-scan dilated capillaries (green circles). A: High-definition white light; B: I-scan.

similarly. All statistical tests were evaluated at the α = 0.05 significance level.

Ethics statement

The study protocol was approved by the Saint Louis University Institutional Review Board. The study protocol, patient's rights and obligations were reviewed with eligible patients and informed consent was obtained from all participants.

RESULTS

Patient characteristics

A total of 25 patients met the initial inclusion criteria and were eligible to participate. Two patients were subsequently excluded given biopsies did not show GAVE or PHG. Baseline characteristics of the study cohort including medications and laboratory analysis are summarized in Table 1. The majority of the patients included in this study were Caucasian (65.2%), male (60.9%) and had chronic hepatitis C causing cirrhosis (43.5%). None of the patients were prescribed anticoagulation or antiplatelet agents other than aspirin (31.8%). Median blood work for included patients included hemoglobin 10.6 g/dL, platelets 125000 *per* mm^3, INR 1.1 and creatinine 1.0 mg/dL. The majority of patients underwent an upper endoscopy for management of esophagogastric varices (73.9%). Some patients already had some form of therapy for portal HTN including TIPS (8.7%), history of liver transplantation (13%) or use of beta blockers (45.5%).

Comparing HDWLE and I-scan for diagnosis of GAVE and PHG

Seven adults (30.4%) had confirmed GAVE on histology. HDWLE had a sensitivity of 85.7% and specificity of 62.5% in diagnosing GAVE compared to a sensitivity of 100% and 75% specificity utilizing our I-scan criteria (examples of GAVE and PHG under I-scan are in Supplementary Figures 1 and 2). As a result, utilizing HDWLE alone, the diagnosis of GAVE was accurately made in 69.57% (n = 16) of cases compared to 82.61% (n = 19) when utilizing I-scan technology (P = 0.491; Fisher exact test Table 2). In contrast, HDWLE has a sensitivity of 93.8% and a 75% specificity in diagnosing PHG compared to a sensitivity of 87.5% and specificity of 71.4% utilizing I-scan (accuracy of 82.61% with or without I-scan, P = 1.000 as in Table 3). I-scan was more likely to make an incorrect diagnosis of PHG in patients with alcoholic cirrhosis, alcohol use, or in patients with lower bilirubin levels while a better diagnosis of PHG was made antrum using I-scan when the antrum is involved (P < 0.05) (Supplementary Table 1). I-scan was more likely to make an incorrect diagnosis of GAVE if the patient was on dialysis or an elevated creatinine (P < 0.05) (Supplementary Table 2). Other factors including age, gender, race, ascites, presence of varices, or laboratory findings were no significant.

Table 1 Summary of the patient population

	Overall (*n* = 23)	
Age (median, IQR), *n* (%)	60	
Male	14	60.9
Caucasian	15	65%
Etiology of cirrhosis		
Alcohol (EtOH)	3	13.0
Granulomatous hepatitis	1	4.4
HBV	1	4.4
HCV	10	43.5
HCV, EtOH	1	4.4
Nonalcoholic steatohepatitis	6	26.1
Primary sclerosing cholangitis	1	4.4
Liver biopsy	10	43.5
Liver transplantation	3	13.0
Portal hypertension on imaging	17	73.9
TIPS	2	8.7
Cirrhosis on CT/US	23	100.0
History of connective tissue disease	1	4.4
Dialysis	2	8.7
Endoscopy suite, *n* (%)		
Reason for EGD		
Anemia	1	4.4
GI Bleed	4	17.4
Varices	18	78.2
Anticoagulation	0	0.0
Alcohol use in the past 15 d	5	21.7
ASA in the past 15 d	7	31.8
NSAIDS use in the past 15 d	0	0.0
Plavix	0	0.0
Beta blockers	10	45.5
Labs within 3 mo Pre EGD[1]	**median**	**IQR**
Hemoglobin	10.6	9.5–13.3
Mean corpuscular volume	89.2	87.0–90.5
Platelet count	126.5	68.0–152.0
INR	1.1	1.1–1.2
Serum sodium	139.0	137.0–142.0
Alanine aminotransferase	30.0	25.0–54.0
Aspartate aminotransferase	50.0	32.0–79.0
Total bilirubin	1.6	1.2–2.6
Alkaline phosphatase	108.0	85.0–134.0
Serum albumin	3.2	2.4–3.4
Ferritin	74.3	5.0–2458.0

Creatinine	1.0	0.70–1.47
Labs within 4-8 wk after EGD[1]	**median**	**IQR**
Hemoglobin	11.4	8.9–12.8
Mean corpuscular volume	87.9	84.8–91.6
Platelet count	117.0	63.0–166.0
INR	1.2	1.1–1.3
Serum sodium	140.0	137.0–142.0
Alanine aminotransferase	31.0	21.0–42.0
Aspartate aminotransferase	44.0	29.0–68.0
Total bilirubin	1.2	0.9–1.9
Alkaline phosphatase	132.0	79.0–185.0
Serum albumin	3.0	2.6–3.3
Ferritin	197.4	63.0–199.0
Creatinine	1.0	0.70–1.50

[1]Reference ranges: Hemoglobin 12-15.5 g/dL, mean corpuscular volume 83-11 fL, platelet count 150-400 K/mm^3, INR 0.9-1.2, serum sodium 134-145 mEq/L, alanine aminotransferase 0-61 U/L, aspartate aminotransferase 5-34 U/L, total bilirubin 0.2-1.2 mg/dL, alkaline phosphatase 40-150 U/L, serum albumin 3.4-5 g/dL, ferritin 12-200 ng/mL, and creatinine 0.7-1.3 mg/dL.
CT: Computed tomography; US: Ultrasound; EGD: Esophagogastroduodenoscopy; NSAIDS: Non-steroidal anti-inflammatory drugs; INR: International normalized ratio; IQR: Interquartile ranges; HCV: Hepatitis C; HBV: Hepatitis B.

Table 2 Comparison of white light and I-scan to the gold standard biopsy for diagnosis of gastric antral vascular ectasia

		Biopsy		
		No GAVE	GAVE	
White Light	No GAVE	10	1	Sensitivity: 85.7%
	GAVE	6	6	Specificity: 62.5%
I-Scan	No GAVE	12	0	Sensitivity: 100%
	GAVE	4	7	Specificity: 75.0%

GAVE: Gastric antral vascular ectasia.

Table 3 Comparison of white light and I-scan to the gold standard biopsy for diagnosis of portal hypertensive gastropathy

		Biopsy		
		No PHG	PHG	
White Light	No PHG	4	1	Sensitivity: 93.8%
	PHG	3	15	Specificity: 57.1%
I-Scan	No PHG	5	2	Sensitivity: 87.5%
	PHG	2	14	Specificity: 71.4%

PHG: Portal hypertensive gastropathy.

DISCUSSION

In this pilot study, I-scan with magnification demonstrated a trend towards superior overall performance characteristics for real-time visual diagnosis of PHG and GAVE compared to HDWLE in patients with cirrhosis and ambiguous findings on endoscopic evaluation. This novel method may allow for an accurate, real time

diagnosis in multiple critical clinical situations, such as when biopsy is contraindicated or when more urgent decisions regarding endoscopic management of gastrointestinal bleeding is needed. Therefore, I-scan should be considered a valuable diagnostic tool in such challenging clinical scenarios, although further prospective evaluation is needed.

The superiority of I-scan compared to HDWLE can be contributed to I-scan's ability to provide real-time structural and vascular enhancement of HDWLE images. I-scan image processing involves three algorithms: Surface enhancement (SE), contrast enhancement (CE), and tone enhancement (TE). SE improves the delineation of the examined mucosa by accentuating blood vessels. CE can sharpen the appearance of surface vessels and enhance the visualized details of mucosa surface texture. TE accentuates mucosal patterns and vascular structures to aid in lesion characterization. These enhancements significantly contribute to the endoscopist ability to perform an accurate diagnosis based on the endoscopic appearance which is noted in this study when comparing the ability for the endoscopist to accurately diagnose GAVE based on visual appearance of the gastric mucosa. The utilization of I-scan technology allowed for increased sensitivity and specificity when diagnosing GAVE compared to standard HDWLE. This translated into an accuracy of 82% for I-scan and 70% for HDLWE. While this finding was not statistically significant likely due to small sample size, it does show a trend towards statistical significance. A more recent study using Narrow Band Imaging showed an increased accuracy of virtual chromoendoscopy at diagnosing GAVE. However, our study relied on more extensive advanced imaging diagnosis criteria and used special stains to confirm GAVE[15].

The clinical implications of improved visual diagnosis of GAVE are significant. Utilizing I-scan with magnification may potentially obviate the need for obtaining biopsies when visual diagnosis of GAVE can be made using I-scan. This can be especially helpful in situations where obtaining biopsies is discouraged given coagulopathy or active gastrointestinal bleeding which are relatively common scenarios in patients with cirrhosis. An accurate, real time diagnosis allows the endoscopist to initiate definitive management for gastrointestinal bleeding in a timely manner instead of delaying to confirm diagnosis *via* pathology evaluation. Ultimately, we suspect this will improve patient outcomes and utilization of hospital resources. In addition, an accurate visual diagnosis can obliviate the need to obtain biopsy which will results significant cost savings.

Patients with alcoholic cirrhosis or alcohol use were less likely to have an accurate diagnosis of PHG, suggesting that alcohol may alter the gastric pit and vascular patterns leading to a difficult PHG diagnosis. Indeed, alcohol use is known to alter the upper gastrointestinal mucosa and lead to atrophy and inflammation[16]. In contrast, I-scan had better ability to diagnosis PHG in the antrum and which is the stomach location where GAVE usually appears. These findings highlights the ability of I-scan in making accurate diagnosis of GAVE *vs* PHG in the antrum which is critical for management. We do note that patient with an elevated creatinine, and on dialysis were more likely to have an incorrect diagnosis of GAVE utilizing I-scan technology. At this time, the association between renal dysfunction on incorrect diagnosis using I-scan remain unclear and may have only been noted in this study due to the small sample size or could be due to underlying edema leading to obscured diagnosis. These findings are novel and have not been noted in other studies evaluating the accuracy of I-scan technology in diagnosing gastrointestinal pathology.

In light of the emerging technologies in endoscopic imaging, the preservation and incorporation of valuable endoscopic innovations (PIVI) initiative was developed by the American Society for Gastrointestinal Endoscopy to set thresholds that any new technology should meet before it can replace the current practice of random biopsies. These thresholds have been described for diminutive colonic polyps[17] and Barrett's esophagus[18] but not for PHG or GAVE. This study shows promising results in utilizing I-scan technology to assist with accurate visual diagnosis. Despite the promising results notes in this study, there is limitation to this data. First, the small sample size may have affected the results and these results should be confirmed with a larger study prior to implementing into clinic practice. Given multiple endoscopist performed the procedures after a short PowerPoint presentation on the visual diagnosis of GAVE and PHG utilizing I-scan technology, there was likely some variability in endoscopist's diagnosis. Finally, we could not account for the learning curve leading to more accurate diagnosis for GAVE and PHG with HDWLE later in the study.

CONCLUSION

We conclude that, utilizing I-scan with magnification may obviate the need for biopsies when visual diagnosis of either PHG or GAVE can be made with high confidence. This pilot work supports the further evaluation of I-scan in these challenging clinical situations using a larger sample size and a follow up of outcomes in a randomized fashion.

ARTICLE HIGHLIGHTS

Research background

Gastric antral vascular ectasia (GAVE) and portal hypertensive gastropathy (PHG) are two not uncommon causes of upper gastrointestinal bleeding in patients with cirrhosis. While endoscopic appearance can suggest the diagnosis, gastric biopsies are the current gold standard for differentiating PHG from GAVE.

Research motivation

Distinguishing GAVE from PHG is important as the management is different for the two conditions. Obtaining gastric biopsies to diagnose GAVE and PHG may be contraindicated given coagulopathy or thrombocytopenia which are commonly seen with cirrhosis. Here we hypothesized that I-scan virtual chromoendoscopy is more sensitive and specific than high-definition white light endoscopy (HDWLE) at diagnosing GAVE when compared to gastric biopsy.

Research objectives

The main objective of this work was to determine the added diagnostic value of virtual chromoendoscopy to high definition white light for real-time endoscopic diagnosis of GAVE and PHG.

Research methods

We developed an I-scan virtual chromoendoscopy criteria for diagnosis of GAVE and PHG. We then tested these criteria in a prospective cohort of cirrhotic adults with GAVE and PHG when HDWLE diagnosis was in doubt. We then compared the accuracy of I-scan *vs* HDWLE alone compared to histology.

Research results

I-scan with magnification demonstrated superior overall performance characteristics for real-time visual diagnosis of PHG and GAVE compared to HDWLE in patients with cirrhosis and ambiguous findings on endoscopic evaluation.

Research conclusions

This novel finding allows for an accurate, real time diagnosis in multiple critical clinical situations, such as when biopsy is contraindicated or when more urgent decisions regarding endoscopic management of gastrointestinal bleeding is needed.

Research perspectives

Utilizing I-scan with magnification may obviate the need for biopsies when visual diagnosis of either PHG or GAVE can be made with high confidence. This pilot work supports the further evaluation of I-scan in these challenging clinical situations using a larger sample size and a follow up of outcomes in a randomized fashion.

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ORIGINAL ARTICLE

Prospective Study

Non-alcoholic steatohepatitis in liver transplant recipients diagnosed by serum cytokeratin 18 and transient elastography: A prospective study

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Abstract

BACKGROUND

Nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) seem common after liver transplantation.

AIM

To investigate incidence and predictors of NAFLD and NASH by employing noninvasive testing in liver transplant recipients, namely controlled attenuation parameter (CAP) and the serum biomarker cytokeratin 18 (CK-18). We also evaluated the diagnostic accuracy of CK-18 and CAP compared to liver histology.

METHODS

We prospectively recruited consecutive adult patients who received liver transplant at the McGill University Health Centre between 2015-2018. Serial measurements of CK-18 and CAP were recorded. NAFLD and NASH were diagnosed by CAP ≥ 270 dB/m, and a combination of CAP ≥ 270 dB/m with CK-18 > 130.5 U/L, respectively. Incidences and predictors of NAFLD and NASH were investigated using survival analysis and Cox proportional hazards.

Clinical trial registration statement: The study was registered at ClinicalTrials.gov (NCT03128918).

Informed consent statement: All patients provided their informed written consent prior to participation.

Conflict-of-interest statement: Deschenes M has served as an advisory board member for Merck, Janssen, Gilead; Wong P has acted as consultant for BMS, Gilead, Merck, Novartis; Sebastiani G has acted as speaker for Pfizer, Merck, Novonordisk, Novartis, Gilead and AbbVie, served as an advisory board member for Merck, Gilead, Pfizer, Allergan, Novonordisk, Intercept and Novartis and has received research funding from Merck and Theratec. All other authors have no conflicts of interest to declare.

Data sharing statement: According to stipulations of the patient consent form signed by all study participants, ethical restrictions imposed by our Institutional Ethics review boards (Institutional Ethics Review Board Biomedical B Research Ethics Board of the McGill University Health Centre), and legal restrictions imposed by Canadian law regarding clinical trials, anonymized data are available upon request. Please send data access requests to Sheldon Levy, Biomedical B (BMB) Research Ethics Board (REB) Coordinator Centre for Applied Ethics, 5100, boul. de Maisonneuve Ouest, 5th floor, Office 576, Montréal, Québec, H4A 3T2, Canada.

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RESULTS

Overall, 40 liver transplant recipients (mean age 57 years; 70% males) were included. During a median follow-up of 16.8 mo (interquartile range 15.6-18.0), 63.0% and 48.5% of patients developed NAFLD and NASH, respectively. On multivariable analysis, after adjusting for sex and alanine aminotransferase, body mass index was an independent predictor of development of NAFLD [adjusted hazard ratio (aHR): 1.21, 95% confidence interval (CI): 1.04-1.41; P = 0.01] and NASH (aHR: 1.26, 95%CI: 1.06-1.49; P < 0.01). Compared to liver histology, CAP had a 76% accuracy to diagnose NAFLD, while the accuracy of CAP plus CK-18 to diagnose NASH was 82%.

CONCLUSION

NAFLD and NASH diagnosed non-invasively are frequent in liver transplant recipients within the first 18 mo. Close follow-up and nutritional counselling should be planned in overweight patients.

Key Words: Nonalcoholic steatohepatitis; Nonalcoholic fatty liver disease; Controlled attenuation parameter; Cytokeratin 18; Overweight; Accuracy

Core Tip: This is the first prospective study using cytokeratin 18 in association with transient elastography with controlled association parameter to investigate nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) in liver transplant recipients. NAFLD and NASH diagnosed by non-invasive tests occur frequently in the first 18 mo from liver transplant. Overweight is the main risk factor. Non-invasive liver fibrosis markers have suboptimal accuracy.

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INTRODUCTION

In recent years, there has been a shift in the etiologies of liver diseases leading to liver transplantation (LT): Chronic hepatitis C is declining, while nonalcoholic fatty liver disease (NAFLD) is on the rise. NAFLD affects 25.24% of the general population globally, driven by the epidemic of metabolic conditions such as obesity and type 2 diabetes mellitus[1-3]. NAFLD is an umbrella term encompassing a spectrum of clinical and pathologic features characterized by a fatty overload involving over 5% of the liver weight in the absence of other causes of liver disease. It ranges from simple steatosis or nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH). Without treatment, NAFL can evolve to NASH, liver fibrosis and cirrhosis, eventually resulting in liver failure and hepatocellular carcinoma (HCC)[2,4]. NASH is now the second leading indication for liver transplant in North America and is projected to become the main indication in the next 10 years[5,6].

In contrast to alcoholic liver disease, the mitigation of NASH risk factors is not a requirement for transplant eligibility. Hence, risk factors for NASH may persist or worsen after LT, placing these recipients at risk for recurrence. *De novo* NASH in patients transplanted for other etiologies of liver disease can also occur due to excess of metabolic risk factors following LT, including type 2 diabetes mellitus, rapid weight gain, hypertension, hyperlipidemia. Immunosuppressive medications may also play a role, as both corticosteroids and calcineurin inhibitors promote diabetes, hypertension and hypercholesterolemia[7,8]. About 20% and 10% of LT recipients develop *de novo* NAFLD and NASH, respectively[8]. Recurrent NAFLD and NASH can be as frequent as 62% and 33%, respectively. NAFLD is a common occurrence within 6 mo, whereas

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the onset of NASH occurs in a period of 6 mo to 1 year in several studies[9]. Due to these reasons, LT recipients may require monitoring to detect changes to the liver graft and prevent hepatic failure and mortality. The majority of studies evaluating recurrent NAFLD and NASH in LT recipients have been of retrospective nature, with no serial monitoring. Hence, longitudinal, prospective data on the frequency of NAFLD and NASH are lacking in the first months following LT. Protocol biopsies have long been used to identify liver disease recurrence and guide management. However, liver biopsy is invasive, costly and prone to sampling error[10]. Recent non-invasive tools for the diagnosis of hepatic steatosis and fibrosis include the measurement of liver stiffness by transient elastography (TE) and the associated controlled attenuation parameter (CAP)[2,11-13]. The accuracy of TE for the diagnosis of liver graft fibrosis seems similar to the non-transplant population[14]. Few studies have investigated the accuracy of CAP in the post-transplant setting[15,16]. Serum cytokeratin 18 (CK-18) has been proposed for the non-invasive diagnosis of NASH. CK-18 is the major intermediate filament protein in the liver and one of the most prominent substrates of caspases during hepatocyte apoptosis. Apoptotic cell death of hepatocytes is associated with the release of caspase-cleaved CK-18 fragments into the bloodstream [17]. Apoptotic activity occurs in NASH but not in NAFL, as such the presence of CK-18 fragments in the blood may differentiate the two conditions[17-19]. In a meta-analysis of over 1600 patients, CK-18 predicted the presence of NASH with a pooled area under the curve (AUC) of 0.82[20]. One report suggests that CK-18 could also have a prognostic value in predicting one-year survival post-LT[21]. No study has employed CK-18 to diagnose NASH in LT recipients.

We prospectively investigated incidence and predictors of NAFLD and NASH diagnosed by TE with CAP and CK-18 in LT recipients within the first 18 mo post-transplantation. We also studied the diagnostic accuracy of non-invasive tests compared to paired liver biopsies performed as a part of clinical care.

MATERIALS AND METHODS

Study design and population

This was a prospective, longitudinal study conducted at a single site, the McGill University Health Center (MUHC) Solid Organ Transplant Unit, and it included all eligible and consecutive patients who underwent LT between March 2015 and June 2018. Since 1990, a computerized database on all LT recipients has been maintained into which demographic data, clinical diagnosis, laboratory results, and prescription information had been prospectively entered. In order to be included, patients had to fulfill the following criteria: Age > 18 years; patient and graft survival > 6 mo; a minimum follow-up of 1 year. Exclusion criteria were any of the following: LT due to chronic hepatitis C, genotype 3; patients who received liver grafts involving more than 10% steatosis; failure of TE with CAP examination or unreliable measurement at study entry. The immunosuppressive regimen used as a standard by the LT program is induction with anti-thymocyte globulin, tacrolimus and mycophenolate mofetil as maintenance immunosuppression and rapid prednisone taper. Overweight and obesity were defined as body mass index (BMI) > 25 and > 30 kg/m^2, respectively.

Ethics

The study was approved by the Research Ethics Board of the Research Institute of MUHC (code 15-002-MUHC) and was registered at ClinicalTrials.gov (NCT03128918). The study was conducted according to the Declaration of Helsinki and Good Clinical Practice guidelines. All patients provided their informed written consent prior to participation.

Study assessment

Study visits were scheduled at baseline, month 3, 6, 9, 12 and 18, for a total of 5 visits (Figure 1). The following parameters were collected at each study visit: BMI, laboratory tests for hematology, blood chemistry. The questionnaire Alcohol Use Disorders Identification Test (AUDIT-C) was administered[22]. TE with CAP measurement and plasma to measure CK-18 were also acquired at each study visit. TE examination was performed in patients fasting for at least 3 h using FibroScan 502 Touch (Echosens, Paris, France). The same two experienced operators performed all elastographic measurements. The standard M probe was used in all patients. The XL probe was used in cases of failure of TE with the M probe or if BMI > 30 kg/m^2. The following criteria were applied to define the result of TE as reliable: At least 10

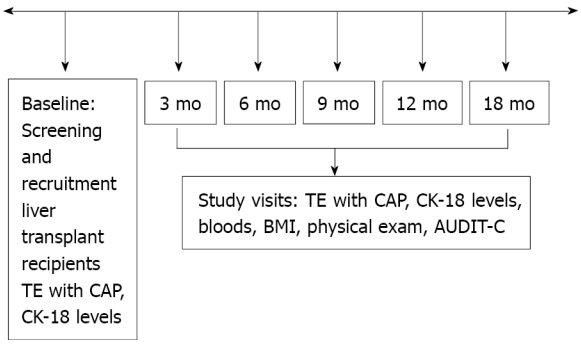

Figure 1 Study design showing baseline and study visit. AUDIT-C: Alcohol Use Disorders Identification Test; BMI: Body mass index; CAP: Controlled attenuation parameter; TE: Transient elastography; CK-18: Cytokeratin 18.

validated measurements and an interquartile range (IQR) < 30% of the median liver stiffness measurement (LSM)[23]. Available liver biopsies were used for the diagnostic accuracy study. Liver biopsy was performed at the discretion of the treating transplant hepatologist, as part of standard of care. All biopsies were obtained with a 16G Tru-Cut type needle and interpreted by two experienced liver pathologists. The stage of fibrosis was reported according to the Kleiner classification[24]. The NAFLD activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3) and hepatocellular ballooning (0-2). A diagnosis of NASH was made if NAS ≥ 5[24]. The CAP cut-off used for diagnosis of NAFLD was 270 dB/m, as recently reported in LT recipients[16]. Plasma stored at -80 °C was used for quantitative measurement of CK-18 levels by the Human cytokeratin ELISA kit (MJS Biolynx inc, Brockville Ontario, Canada). A cut-off of CK-18 > 130.5 U/L was used to indicate significant hepatocyte apoptosis, diagnostic for NASH when combined with CAP > 270 dB/m[25,26]. Liver fibrosis (stage ≥ 1 out of 4) was diagnosed as LSM ≥ 7.4 kPa[16]. The following simple serum fibrosis biomarkers were also computed: Hepatic steatosis index (HSI), defined as 8 × aspartate aminotransferase (AST)/alanine aminotransferase (ALT) + BMI (+ 2, if female; +2, if diabetes mellitus present)[27], fibrosis-4 (FIB-4), calculated as [age (years) × AST]/[platelet count (10^9/L) × ALT][28], and AST to platelet ratio (APRI), calculated as {[AST level/AST (upper limit of normal)]/platelet count (10^9/L) × 100}[29]. Liver fibrosis was defined as FIB-4 > 3.64 and APRI > 1, as previously described in the liver transplant setting[30].

Statistical analysis

The performance of the non-invasive tests to diagnose NAFLD, NASH and liver fibrosis was measured with the following: Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, positive and negative likelihood ratios (LR^+ and LR^-, respectively). Correlation coefficients of TE with CAP with serum biomarkers were calculated using the Pearson correlation analysis. For the longitudinal analysis, baseline (study entry) corresponded to the day of LT. Patients were followed until March 2020 or were censored either when they developed the outcome or at their last study visit (18 mo post-LT). At each visit, complete medical history and physical examination were performed along with routine laboratory work-up. Standard diagnostic and therapeutic management following LT was offered during the follow-up. Continuous variables were expressed as mean (standard deviation), and categorical variables were presented as numbers (%). We estimated incidence rates of NAFLD and NASH by dividing the number of participants developing the outcome by the number of person-years (PY) of follow-up. Poisson count models were used to calculate CI for incidence rates. Multivariable time-dependent Cox regression models were constructed to assess predictors of the development of NAFLD and NASH and included covariates that were determined a priori to be clinically important and with a *P*-value < 0.1 on univariable analysis. The final model was adjusted for sex, BMI and ALT. Robust variance estimation was used in all Cox regression analyses to account for the correlation of data contributed by the same participant at multiple visits. We considered an association with the outcome significant when the 95%CI excluded one. We generated Kaplan-Meier curves to illustrate and compare the cumulative incidence of NAFLD and NASH in overweight *vs* normal weight patients. The log-rank test was used to evaluate differences among incidences. All tests were two-tailed and with a significance level of $\alpha = 0.05$. Statistical analysis was performed using STATA 15 (StataCorp LP, TX, United States).

RESULTS

After applying exclusion criteria, 40 LT recipients were included in this prospective study (Figure 2). The main demographic, clinical and biochemical characteristics of the study population at baseline are summarized in Table 1. Univariable analysis by outcome category of NAFLD and NASH is also reported. Overall, mean age was 57.3 years and 70% of patients were male. The most frequent indications for LT were NASH and HCC. Metabolic comorbidities were frequent, with overweight, type 2 diabetes mellitus and hypertension affecting 40%, 35% and 37.5% of the patients, respectively. Patients who developed NAFLD and NASH during the follow-up period were more frequently transplanted for NASH and on tacrolimus as immunosuppressant.

Diagnostic accuracy of non-invasive tests compared to liver histology and correlation between TE with CAP and serum biomarkers

During the study period, 35 liver biopsies (mean length ± SD: 1.7 ± 0.4 cm) from 24 patients were available. The median time between liver biopsies and non-invasive diagnostic testing was 38.6 ± 30 d. Table 2 shows the performance of non-invasive tests compared to liver histology. The diagnostic accuracy of CAP and HSI for NAFLD was 76% and 45.7%, respectively. The diagnostic accuracy of a combination of CAP ≥ 270 dB/m and CK-18 > 130.5 to diagnose NASH was 82%. The diagnostic accuracy of LSM, FIB-4 and APRI for liver fibrosis was low at 57.8%, 48.7% and 54.1%, respectively. There was a medium positive correlation between CAP and HSI of 0.4. There was a medium positive correlation between LSM and FIB-4 of 0.4, and a weak positive correlation between LSM and APRI of 0.1.

Incidence and predictors of NAFLD and NASH by CAP and CK-18

During a median follow-up of 16.8 mo (IQR: 15.6-18.0), 22 patients (63.0%) developed NAFLD (incidence rate: 71.0 per 100 PY, 95%CI: 45.0-78.0), and 17 patients (48.5%) developed NASH (incidence rate: 48.6 per 100 PY, 95%CI: 31.4-66.0). On multivariate Cox regression analysis, BMI was an independent predictor of both NAFLD (adjusted HR: 1.1, 95%: 1.0-1.2) and NASH (adjusted HR: 1.1, 95%CI: 1.0-1.3) (Table 3). To further elaborate on the effect of high BMI on the incidence of NAFLD and NASH, a hazard plot was performed and showed that overweight was a significant risk factor for both NAFLD and NASH (log-rank, $P < 0.01$, respectively) (Figure 3).

Changes in LSM, FIB-4 and APRI during follow-up

Given the low accuracy for the non-invasive fibrosis tests, we studied changes in LSM, FIB-4 and APRI during the follow-up. While the majority of patients had an LSM ranging from 2.5 to 15 kPa, there were patients who developed marked increases, and these were observed in the first six months of follow-up (Figure 4A). Similarly, while most of the patients had FIB-4 and APRI ranging from 1 to 2.5 and from 0.5 to 1.5, respectively, there were patients who developed marked increases during the first six months of follow-up (Figures 4B and 4C).

DISCUSSION

In this prospective study, we have shown that NAFLD and NASH diagnosed non-invasively are frequent occurrences in the first 18 mo from LT. Similar to results reported in previous retrospective studies, the majority of incident NAFLD and NASH in our population occurred within the first year of LT[31-33]. The main predictor of these events was high BMI, thus underlying the importance of controlling the weight beginning from the first 3 mo post-LT. We also showed that the diagnostic accuracy of non-invasive tests for NAFLD is good and similar to previously reported, while non-invasive fibrosis tests have low accuracy in the first months following LT. Finally, we first report the accuracy of the apoptotic biomarker CK-18 combined with CAP for the diagnosis of NASH.

We compared the performance of non-invasive tests to liver biopsy. We used a CAP cut-off ≥ 270 dB/m, as referenced by Siddiqui *et al*[16], and compared it to the presence of steatosis grade 0 *vs* 1-3 on liver biopsy. Our results showed a lower sensitivity (58% *vs* 74%), however the specificity (86% *vs* 87%), PPV (70% *vs* 78%) and NPV (79% *vs* 84%) were similar. The variations can be explained by the different population sizes, number of available liver biopsies and the timing of the study conducted within the

Table 1 Characteristics of patients at study entry

	Whole cohort	Patients who developed NAFLD	Patients who developed NASH
	n = 40	*n* = 22	*n* = 17
Age (yr)	57.3 ± 8.5	55.5 ± 9.2	56.3 ± 7.9
Male (%)	28 (70)	18 (82)	14 (82)
Ethnicity (%)			
Caucasian	32 (80)	19 (86)	15 (88)
Other (Asian, Black, Arab)	8 (20)	3 (14)	2 (11)
Etiology of liver disease (%)			
NASH	21 (52.5)	13 (52)	12 (70)
HCC	9 (22.5)	2 (9)	2 (12)
HCV (excluding genotype 3)	8 (20)	6 (27)	3 (18)
Alcoholic liver disease	1 (2.5)	1 (4.5)	0
Other	1 (2.5)	0	0
BMI (kg/m^2)	24.8 ± 4.6	26.2 ± 5.1	26.6 ± 4.5
BMI >25 (%)	18 (40)	14 (64)	12 (70)
Comorbidities (%)			
Diabetes	14 (35)	9 (41)	8 (47)
Hypertension	15 (37.5)	7 (32)	8 (47)
Dyslipidemia	6 (15)	6 (27)	5 (29)
MELD-Na Score	< 9	< 9	< 9
Laboratory			
AST (U/L)	27.6 ± 33	31.8 ± 41.2	34.5 ± 45.1
ALT (U/L)	32.8 ± 42.8	37.6 ± 52.6	40.6 ± 57.7
GGT (U/L)	177.5 ± 256.6	177.7 ± 271.4	188.1 ± 297.6
Bilirubin (μmol/L)	17 ± 15.9	18.2 ± 17.3	18 ± 18.2
INR	1.25 ± 1.39	1.05 ± 0.12	1.04 ± 1.3
Albumin (g/L)	39.6 ± 3.69	38.7 ± 4.3	39.4 ± 3.9
Platelets (10^9/L)	172.3 ± 86.9	185 ± 92.5	170.5 ± 93.6

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; GGT: Gamma-glutamyl transpeptidase; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; INR: International normalized ratio; MELD-Na: Model for end stage liver disease-sodium; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

first 18 mo from LT. When HSI was compared to histology, it showed less accuracy than CAP as demonstrated before in other studies on non-LT populations[34,35]. Secondly, we used a combination of CK-18 > 130.5 with CAP ≥ 270 dB/m and compared it to the presence of NASH (NAS ≥ 5 or proven NASH) on liver histology. To our knowledge, this is the first study to use CK-18 to detect NASH in LT patients. Compared to one meta-analysis of over 1600 patients that assessed the accuracy of CK-18 (cut-off range: 121.6-380.2 U/L) in non-transplanted patients with NASH, our results are similar for both sensitivity (75% *vs* 78%) and specificity (83% *vs* 87%)[20]. Compared to another more recent meta-analysis of over 1400 patients that evaluated the diagnostic value of CK-18 for the diagnosis of NASH, our results also reported similar sensitivity (75% *vs* 75%), specificity (83% *vs* 77%), LR^+ (4.5 *vs* 3.3), and LR^- (0.3 *vs* 0.3)[36].

There are two interesting points. Firstly, our cut-off values of all the non-invasive biomarkers reported a higher NPV than PPV which could indicate that these tests are more efficient at ruling-out NAFLD, NASH and liver fibrosis rather than ruling-in these diseases, as previously described[16,37]. However, their ability to minimize the

Table 2 Diagnostic accuracy of non-invasive tests compared to liver histology (*N* = 35 from 24 patients)

	NAFLD		NASH	Liver fibrosis		
	CAP	HSI	CAP + CK-18	LSM	FIB-4	APRI
Sensitivity (%)	58	64.3	75	61.9	7.1	14.3
Specificity (%)	86	33	83	54.2	73.9	78.3
PPV (%)	70	39	37	54.2	14.3	28.6
NPV (%)	79	58	96	61.9	56.7	60
LR^+	4.28	0.96	4.5	1.35	0.27	0.66
LR^-	0.48	1.07	0.3	0.7	1.26	1.1
Accuracy (%)	76	45.7	82	57.8	48.7	54.1

APRI: Aspartate aminotransferase-to-Platelets Ratio Index; CAP: Controlled attenuation parameter; CK-18: Cytokeratin 18; FIB-4: Fibrosis 4 index; HSI: Hepatic steatosis index; LSM: Liver stiffness measurement; LR: Likelihood ratio; MELD-Na: Model for end stage liver disease-sodium; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NPV: Negative predictive value; PPV: Positive predictive value.

Table 3 Risk factors for post-Liver Transplant development of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis using univariate and multivariate Cox regression analysis

	NAFLD				NASH			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	aHR (95%CI)	*P* value	HR (95%CI)	*P* value	aHR (95%CI)	*P* value
Female sex (yes vs no)	0.6 (0.4-1.2)	0.1	0.9 (0.3-1.7)	0.5	0.6 (0.3-1.1)	0.1	0.9 (0.4-2.1)	0.8
Age (per year)	1.0 (0.9-1.0)	0.6			1.0 (0.9-1.0)	0.9		
BMI (per kg/m^2)	1.1 (1.0-1.2)	< 0.01	1.1 (1.0-1.2)	< 0.01	1.1 (1.0-1.2)	0.01	1.1 (1.0-1.3)	< 0.01
Diabetes (yes *vs* no)	1.7 (1.0-2.7)	0.02			1.3 (0.7-2.1)	0.3		
Dyslipidemia (yes *vs* no)	4.6 (1.7-12.8)	< 0.01			4.4 (1.5-13)	0.007		
ALT (per U/L)	1.0 (0.9-1.0)	0.09	1 (0.9-1.0)	0.3	1.0 (1.0-1.0)	0.03	1 (0.9-1.0)	0.1

aHR: Adjusted hazard ratio; ALT: Alanine aminotransferase; BMI: Body mass index; CI: Confidence interval; HR: Hazard ratio; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

need for liver biopsy in this clinical setting still requires further validation. Secondly, while we combined CK-18 with CAP to diagnose NASH, our results are very closely related to those the two meta-analyses which used CK-18 alone to diagnose NASH. This makes us question the role of combining CAP with CK-18 to diagnose NASH. Two studies investigated the combined use of CK-18 with TE to detect fibrosis and found either no significant improvement or only some improvement in AUC by combining CK-18 and TE compared to using a single test[38,39]. Yet, other studies have shown that combining CK-18 with other biomarkers improves the accuracy to diagnose NASH[40,41]. Our analysis must be replicated in a larger sample using different combinations of biomarkers to better understand this.

Our results are comparable to a recent cross-sectional study by Mikolasevic *et al*[15] which reported a prevalence of liver steatosis of 68.6% and severe liver steatosis of 46.8% in LT recipients using CAP and LSM. Our incidence rates are also comparable to previously published meta-analyses and retrospective studies, while minor variations are most likely due to the difference in populations, the cut-off values to define steatosis/NAFLD and NASH, and the absence of the use of CK-18 as a diagnostic tool in those studies[15,31-33]. On multivariate Cox regression analysis, high BMI was the main risk factor for the development of NAFLD and NASH in patients post LT, conceding with results from previous studies[15,31]. Obesity is an independent risk factor for the development of NAFLD and NASH and can occur or continue to be present even during the first months post-LT. Indeed, other studies have shown that the maximum weight gain occurs in the first year post LT mainly because of the use of

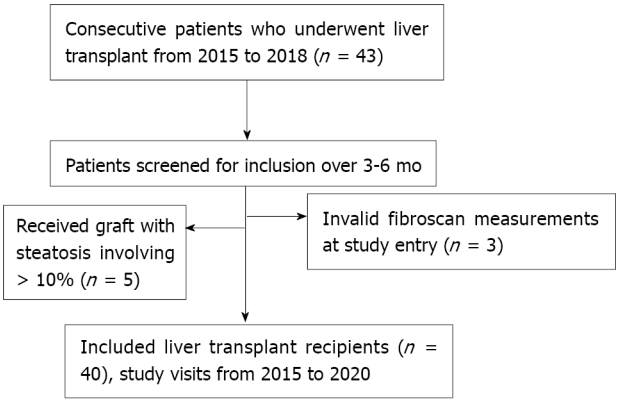

Figure 2 Flow chart displaying the selection of study participants. Of 48 consecutive patients undergoing liver transplant, 3 were excluded because of invalid TE examination and 5 because they received a liver graft with steatosis involving > 10% of hepatocytes. TE: Transient elastography.

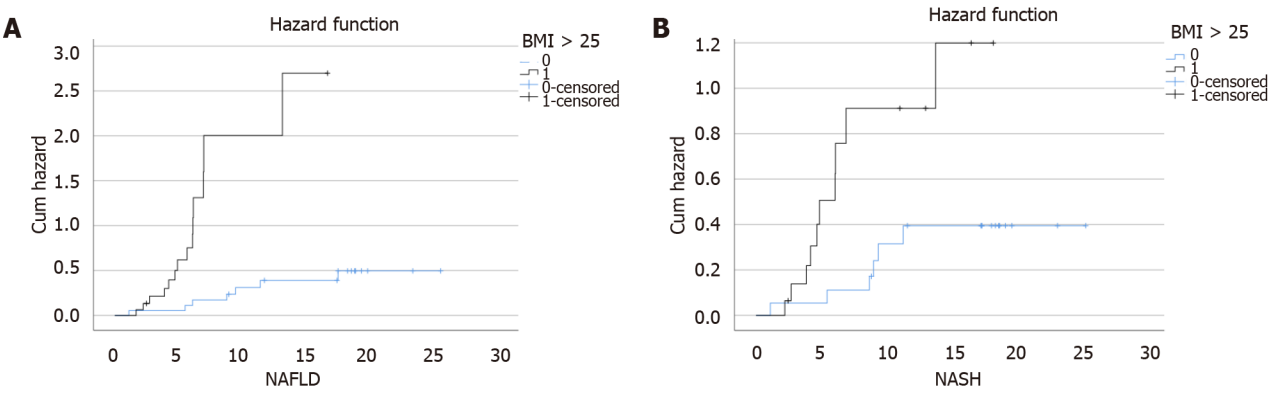

Figure 3 Hazard ratio by body mass index category in nonalcoholic fatty liver disease (log-rank: *P* < 0.0001) and in nonalcoholic steatohepatitis (log-rank: *P* = 0.009). BMI: Body mass index; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

immunosuppressive medications[42,43]. Type 2 diabetes mellitus and dyslipidemia were significant risk factors on univariate analysis, also in line with previous results [15]. The presence of these risk factors poses a risk for the development of fatty deposits in the graft and progression to NAFLD and NASH. Therefore, strategies must be implemented both before and after LT to control and prevent the progression of liver disease. These strategies include weight reduction with a low carbohydrate diet and performing regular exercise, avoiding alcohol and smoking, controlling of comorbid metabolic diseases, and controlling immunosuppression medications post-LT.

We also reported a low performance of non-invasive fibrosis tests during the first 18 mo following LT. Similar findings have been reported previously in post-LT patients with HCV recurrence. El-Meteini *et al*[44] concluded that TE and APRI were not correlated with the degree of fibrosis in liver biopsy done at 3 mo post-LT in 31 patients. Other studies reported a poor diagnostic accuracy of APRI and FIB-4 compared to liver biopsy for the presence of advanced fibrosis post-LT[45,46]. Indeed, some of our patients experienced an important variation in LSM, FIB-4 and APRI particularly during the first 6 mo post-LT. This could be due to several reasons. Inflammation due to congestion or cholestasis is common post-LT and could be one reason for the inaccuracy of fibrosis tests. Fluctuations in liver enzymes and platelets during the first 6 mo may also account for these findings as LT recipients have started receiving and adjusting their immunosuppressive medications. Since a majority of our liver recipients were overweight, this could have interfered with the LSM results[47]. Since our study and the previous studies were performed on small cohorts, a conclusion regarding the accuracy of non-invasive fibrosis tests cannot be made.

There are limitations to our study. The sample size was small which could have interfered with the interpretation of the results. Nevertheless, our incidence rates and predictors are similar to previous retrospective studies[15,31-33]. Additionally, not all patients had available liver biopsy to compare with non-invasive tests. Only 24 out of

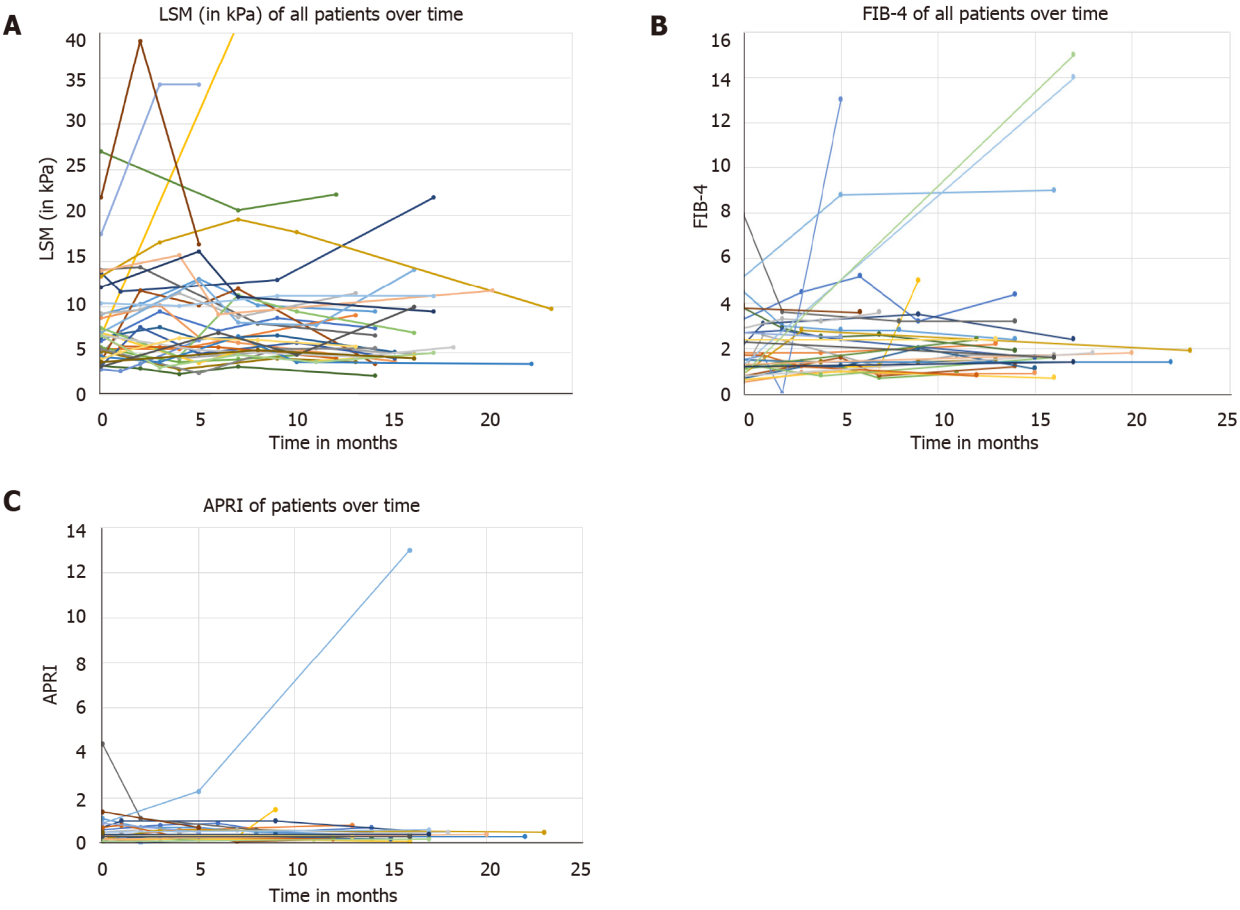

Figure 4 Spaghetti plot of changes. A: Spaghetti plot of changes in liver stiffness measurement during study period; B: Spaghetti plot of changes in fibrosis-4 during study period; C: Spaghetti plot of changes in aspartate aminotransferase-to-Platelets Ratio Index. APRI: Aspartate aminotransferase-to-Platelets Ratio Index; FIB-4: Fibrosis-4; LSM: Liver stiffness measurement.

40 patients required liver biopsy during follow up therefore the comparison was only possible in these patients, for a total of 35 liver biopsies. Regardless of this, the results obtained from our study provide a rationale for the use of non-invasive tests to frequently monitor this patient population, which could not be feasible with liver biopsy, and can be viewed as an opportunity for larger studies to be done on this topic. Another limitation of our study is that CK-18 is not currently a routine test, as such its application to clinical practice should be further explored. The median study length was 16.8 mo, so in the future we plan to continue following these patients for a longer duration by monitoring CAP scores and re-occurrence of steatosis.

CONCLUSION

In conclusion, our study showed that LT recipients have a high risk of developing NAFLD and NASH during the first 18 mo following LT, mainly driven by high BMI. While CAP and CK-18 are promising non-invasive tools for diagnosing NAFLD and NASH, LSM and other fibrosis biomarkers are not reliable tests in detecting liver fibrosis in the first month post-transplant. Larger scale, long-term data on the use of non-invasive tests is needed to determine their accuracy to diagnose and monitor disease progression, as well as their prognostic value. These data may result in the implementation of non-invasive tests and optimization of surveillance.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) is a major indication for liver transplant (LT)

globally. NAFLD and nonalcoholic steatohepatitis (NASH) may occur after LT.

Research motivation

Studies on the incidence of NASH and NAFLD in the first months following LT are limited.

Research objectives

This work aimed to determine the incidence of NASH and NAFLD in the first 18 mo following LT by means of non-invasive diagnostic tests. It also aimed to investigate the diagnostic accuracy of these non-invasive tests compared to liver histology.

Research methods

Consecutive adult patients who received LT at a single center were recruited between 2015-2018. Serial measurements of the biomarker cytokeratin 18 (CK-18) and controlled attenuation parameter (CAP) were recorded. NAFLD and NASH were diagnosed by CAP ≥ 270 dB/m, and a combination of CAP ≥ 270 dB/m with CK-18 > 130.5 U/L, respectively. Incidence and predictors of NAFLD and NASH were investigated using survival analysis.

Research results

During a median follow-up of 16.8 mo, 63% and 48.5% of 40 LT recipients developed NAFLD and NASH, respectively. The diagnostic accuracy for NAFLD and NASH was 76% and 82%, respectively.

Research conclusions

NAFLD and NASH diagnosed by CAP and CK-18 are frequent in LT recipients within the first 18 mo.

Research perspectives

To improve post-transplant outcomes, close follow-up with non-invasive tests and metabolic counselling could be considered.

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CASE REPORT

Rare primary mature teratoma of the liver: A case report

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Abstract

BACKGROUND

Primary liver teratoma is an extremely rare tumor usually affecting children under the age of 3 years. Specific signs of teratoma on ultrasound, computed tomography (CT) or magnetic resonance imaging are lacking, which makes morphology the only diagnostic tool. Misdiagnosis of a mature teratoma may lead to excessive liver resection, whereas misdiagnosis of an immature teratoma may result in spread, causing a life-threatening condition. Consequently, a careful tumor examination is important, and the rarest types of tumors must be accounted for.

CASE SUMMARY

We describe a 52 years old female who presented with a solid mass in the left liver lobe. Contrast-enhanced CT and magnetic resonance imaging (MRI) revealed a round, heterogeneous lesion containing a number of fluid areas and areas of calcification in the middle, and the provisional diagnosis was cholangiocarcinoma. The patient underwent resection of liver segment I. Immunohistochemistry analysis of the resected lesion indicated thyroid follicular epithelium; however, the thyroid gland was intact. 10 years prior to presentation the patient underwent a surgery due to mature teratoma of the right ovary, nevertheless the tumor was benign and could not spread to the liver, in addition teratoma of the liver was also benign. This led to the final diagnosis of primary mature liver teratoma.

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CONCLUSION

Primary hepatic teratoma, including heterotopia of the thyroid gland in the liver, is an extremely rare condition in adults that needs to be considered in the differential diagnosis of solid-cystic neoplasms in the liver and cholangiocarcinoma. This case adds to the limited literature on the patient presentation, clinical workup and management of liver teratomas.

Key Words: Case report; Primary liver teratoma; Ectopic thyroid gland tissue; Mature teratoma; Epidermoi cyst

Core Tip: Primary liver teratoma is an extremely rare tumor. This condition in adults needs to be considered in the differential diagnosis of solid-cystic neoplasms in the liver and cholangiocarcinoma. A careful tumor examination is important, and the rarest types of tumors must be accounted for to allow the diagnosis of heterotopia of the thyroid gland in the liver.

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INTRODUCTION

Teratoma is a rare germ cell tumor (GCT) that comprises at least two of three germ cell layers, the ectoderm, mesoderm and endoderm, and affects both children and adults. Teratomas primarily affect gonadal tissues, as the origin of these tumors is primordial germ cells, which migrate from the allantois to the fetal gonads during the first week of fetal life[1]. Thus, teratomas may also occur along the migration path of primordial germ cells, which can remain in midline extragonadal sites[2]. Consequently, the liver is an extremely rare site for primary teratomas, with an incidence of approximately 1% of all teratomas. Most patients with hepatic teratoma are children under the age of 3 years[3]. Nevertheless, primary or secondary teratomas of the liver can lead to serious health issues and can be a life-threatening condition that claims a comprehensive diagnosis and well-timed therapy. Therefore, our case report and review aim to collect scarce information about hepatic teratomas.

Classification of teratomas

Depending on the differentiation degree of their components, teratomas are classified as mature and immature[1]. Immature teratomas have a tendency for rapid growth, malignant transformation, and metastasis within adults; therefore, the prognosis is very poor[2].

Mature teratomas can be cystic, solid and mixed. According to the reported cases, cystic teratomas of the liver are the most common within mature teratomas. Mature cystic teratomas of the liver represent a mostly unilocular cystic cavity that may have septation and/or calcification and comprise mature elements derived from 3 cell layers, such as thyroid tissue, tooth enamel, hairs, skin, bone, fat, cartilage, neural tissue, or epithelium. The most commonly mature cystic teratomas affect the ovaries; however, approximately 1% of these lesions are found in the liver, usually within females in the right liver lobe[4-6]. The shape and size of mature cystic teratomas on gross appearance are not unique and vary significantly; thus, the largest reported lesion dimensions were 21 cm× 18 cm× 12 cm, and the weight was 1837 g[7]. The symptoms of mature cystic liver teratoma are nonspecific and conditioned by mechanical pressure of the growing tumor, including abdominal distension, constipation, fever, loss of appetite, abdominal pain, a sense of fullness in the right upper quadrant, vomiting, *etc*.[3,8]. Cases of asymptomatic mature teratoma have also been reported[9,10]. Rahmat *et al*[11] described a 46 years old male who presented

with cholangitis caused by a primary benign teratoma of the liver measuring 5.0 cm x 6.5 cm x 8.0 cm and compressing a common bile duct. Despite their high degree of differentiation, cystic teratomas can transform to malignant tumors and harbor other neoplasms; therefore, complete surgical removal is an optimal treatment that can be followed by chemotherapy if necessary[5,12]. Recently, Ramkumar *et al*[13] reported a case of a primary mature teratoma rupture accompanied by acute abdominal pain in a 65 years old female. Surgical removal of the tumor was performed after liquid and antibiotic therapy, and areas of necrosis and hemorrhage were found on histopathology[13].

The differentiation degree of the components of immature teratomas is low, and these tumors may involve any type of tissue, although neurogenic elements are the most common. On histopathology, these teratomas can also be divided into predominantly cystic, solid, and solid with multiple cysts and may contain areas of necrosis and hemorrhage. Immature teratomas tend to show rapid growth with liver capsule invasion and metastasis[2]. Primary immature hepatic teratomas are extremely rare. To the best of our knowledge, only 3 case reports have been published in the English literature up to 2021. The liver is also a rare site of teratoma metastasis; however, secondary immature teratomas are more frequent[14,15]. The symptoms of immature liver teratoma have been described in a few case reports and include pain and sensation of fullness in the right upper quadrant, fatigue, sweating, nausea, vomiting, and weight loss[2,16]. Malek-Hosseini *et al*[16] reported the largest immature liver teratoma, measuring 27 cm in diameter, and the patient recovered completely through surgery with a good follow-up. Immature liver teratomas lead to an elevation in AFP levels, whereas mature teratomas cannot produce AFP; thus, AFP is usually utilized for the differential diagnosis; nevertheless, AFP elevation does not necessarily occur [14,17]. The treatment of immature teratomas includes adjuvant chemotherapy and complete resection of the primary tumor and every metastasis whenever possible[18]. Nonresectable hepatic teratomas require liver transplantation[19].

Diagnosis of hepatic teratomas

The main diagnostic tools for liver teratoma detection are contrast-enhanced CT and MRI, which can show the size, shape, and structure of the tumor and its position related to adjacent elements and organs. CT scans can reveal areas of calcification in teratomas, whereas MRI scans are not sensitive to calcium[3]. Cho *et al*[20] revealed the high sensitivity of attenuation correction CT (AC-CT) acquired during ^{18}F-FDG PET-CT in the diagnosis of immature ovarian teratomas, as their components show significant ^{18}F-FDG uptake. Thus, ^{18}F-FDG PET-CT may be a useful diagnostic tool[20]. Serum AFP, LDH, hCG, CEA, and liver enzymes may be elevated in some patients[2]. However, the final diagnosis of teratoma can be made based only on the histopathology of the tumor samples[9].

Growing teratoma syndrome

Teratomas are usually treated with surgery and chemotherapy. However, metastatic teratomas of nonseminomatous germ cell tumors (NSGCTs) may not respond to chemotherapy and become significantly enlarged even after the original tumor is removed and serum tumor markers (AFP, beta-HCG) and LDH return to normal. This condition is known as *growing teratoma syndrome* (GTS). This syndrome is uncommon, and its etiology and pathogenesis are still unclear; consequently, the diagnosis may be delayed, and the patient's prognosis may become poor[21]. There are two dominant theories on the pathogenesis of GTS: (1) Chemotherapy leads to the survival and subsequent thriving of mature components, whereas immature components are highly sensible; and (2) Chemotherapy results in DNA damage and transformation of the immature teratoma to a mature teratoma[22]. Hiester *et al*[23] suggested a model of GTS development, according to which these tumors comprise meroclones derived from holoclones under chemotherapy. The authors termed these cells "teratoma-forming transit-amplifying cells (TF-TACs)"[23].

GTS should be suspected in every patient with a growing tumor and normal tumor marker levels after chemotherapy of the original NSGCT[21]. The most common sites of original NSGCTs are the ovaries and testis, whereas metastasis usually affects the retroperitoneum; nevertheless, cases of GTS from liver metastasis have also been reported. The common features of the described patients included young age (22 and 24 years old), multiple metastatic deposits among the entire liver, retroperitoneal lymph nodes and kidney from testicular tumors, and elevated AFP levels. Interestingly, the liver teratomas were mature, and there was no evidence of malignancy. Both patients underwent radical orchiectomy, nephrectomy, retroperitoneal lymphadenectomy and chemotherapy, and AFP levels returned to normal.

However, the liver teratomas continued to grow, confirming the GTS diagnosis, and patients were accepted for liver transplantation (LT). After LT, there was no evidence of teratoma recurrence[24,25]. O'Reilly *et al*[22] presented the first case of GTS in a primary liver teratoma in a 22 years old female. AFP levels were elevated (over 18000 cm before chemotherapy) and significantly decreased thereafter, whereas the tumor continued to enlarge up to 31.4 cm x 25.4 cm x 42.1 cm, and GTS was suspected. The patient was discharged after right hepatectomy and resection of the right mediastinal and diaphragmatic metastases, and there was no evidence of teratoma recurrence after 18 mo[22]. Growing teratomas of the liver may cause a disturbance in vital function either by the mechanical compression of contiguous organs and vessels or by hepatic failure; moreover, the incidence of GTS-related malignancy is 2%-8%. As these tumors do not respond to chemo- or radiotherapy, such patients should undergo complete surgical removal of the teratomas, as incomplete resection has a higher rate of tumor recurrence[23].

CASE PRESENTATION

Chief complaints

A 52 years old woman was referred to our hospital by a specialist at the diagnostic center after a solid tumor was detected in the left lobe of the liver with ultrasound (US).

History of present illness

US revealed that the lesion measured 118 mm x 93 mm in size with sharp edges, a heterogeneous and hyperechoic parenchyma and areas of calcification. The patient did not have any complaints associated with this lesion.

History of past illness

The patient underwent right oophorectomy 10 years prior to presentation due to an epidermoid cyst (mature teratoma), and no chemo- or radiotherapy was assigned because the tumor was benign. Apart from that, the medical and family histories were unremarkable.

Personal and family history

Personal and family history is not burdened.

Physical examination

During the general examination, no abnormalities were detected.

Laboratory examinations

The laboratory assessment also did not reveal any pathological findings. The tumor markers CA 19-9 and AFP were not elevated (< 2.5 U/mL and 4.61 U/mL, respectively).

Imaging examinations

Subsequent US with color flow mapping (CFM) revealed moderate vascularization of the lesion and compression of the left portal vein, left hepatic artery and left hepatic vein. Subsequent CT and MRI revealed a heterogeneous lesion 111 mm x 109 mm x 97 mm in size with a round shape containing a number of fluid areas sized from 5 to 12 mm and areas of calcification in the middle of the tumor. The distal intrahepatic bile ducts were dilated, and the inferior vena cava was compressed (Figures 1 and 2). With reference to the CT and MRI scans, the provisional diagnosis was formulated as cholangiocarcinoma of the left hepatic lobe.

MULTIDISCIPLINARY EXPERT CONSULTATION

The histological examination suggested biliary hamartoma, but the lack of bilirubin in the cells lining the cavity did not allow us to exclude lymphangioma or follicular cancer (Figure 3). To reveal the true nature of the tumor and exclude a malignancy, immunohistochemical tests were performed. They demonstrated focal positive expression of thyroglobulin (clone 2H11+6 E1), TTF-1 (clone 8G7G3/1), and galectin-3

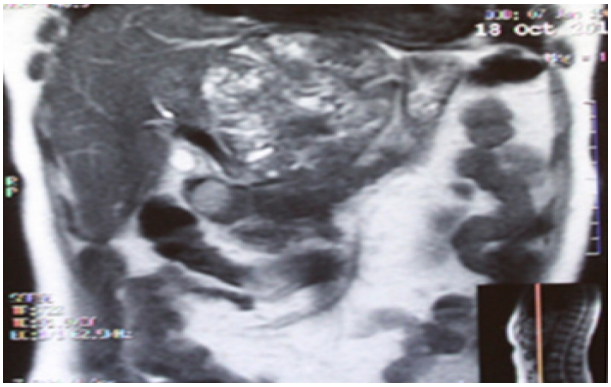

Figure 1 Magnetic resonance imaging of the abdomen: Ill-defined contrast-enhancing, multilobulated cystic lesion involving segments II, III, VI and VIII.

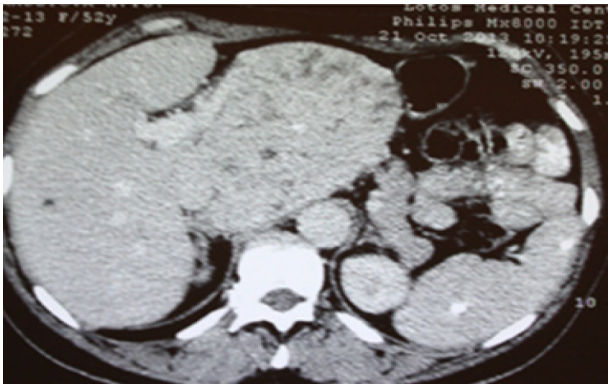

Figure 2 Abdominal computed tomography with contrast enhancement: Tumor invades segment I of the liver (longitudinal section). Ill-defined contrast-enhancing, multilobulated cystic lesion involving segments II, III, VI and VIII.

(clone 9C4), overexpression of cytokeratin 8 and 18 (clones B22.1 + B23.1) and negative expression of CD34 (clone QBEnd/10). The immunophenotype corresponded to the thyroid follicular epithelium. In the postoperative period, we performed ultrasonography, which did not show thyroid gland malignancy and the patient had no endocrine problems.

FINAL DIAGNOSIS

According to the gross appearing, histology and immunohistophenotype the ectopic thyroid gland in the liver (mature teratoma) was finally evident in the patient.

TREATMENT

The patient underwent resection of segment I with the surrounding tumor hepatic parenchyma, D1 Lymphadenectomy and cholecystectomy. The intraoperative inspection revealed an increase in the left liver lobe due to the well-defined encapsulated inhomogeneous tumor in the first segment of the liver (14 cm x 13 cm x 13 cm), crushing atrophied segments 2 and 3 (Figures 4 and 5). The consistency of the tumor was soft, and on its surface, there were twisted veins.

OUTCOME AND FOLLOW-UP

The postoperative period was uneventful. Considering the benign nature of teratoma no complementary treatment was indicated. The patient was discharged from the

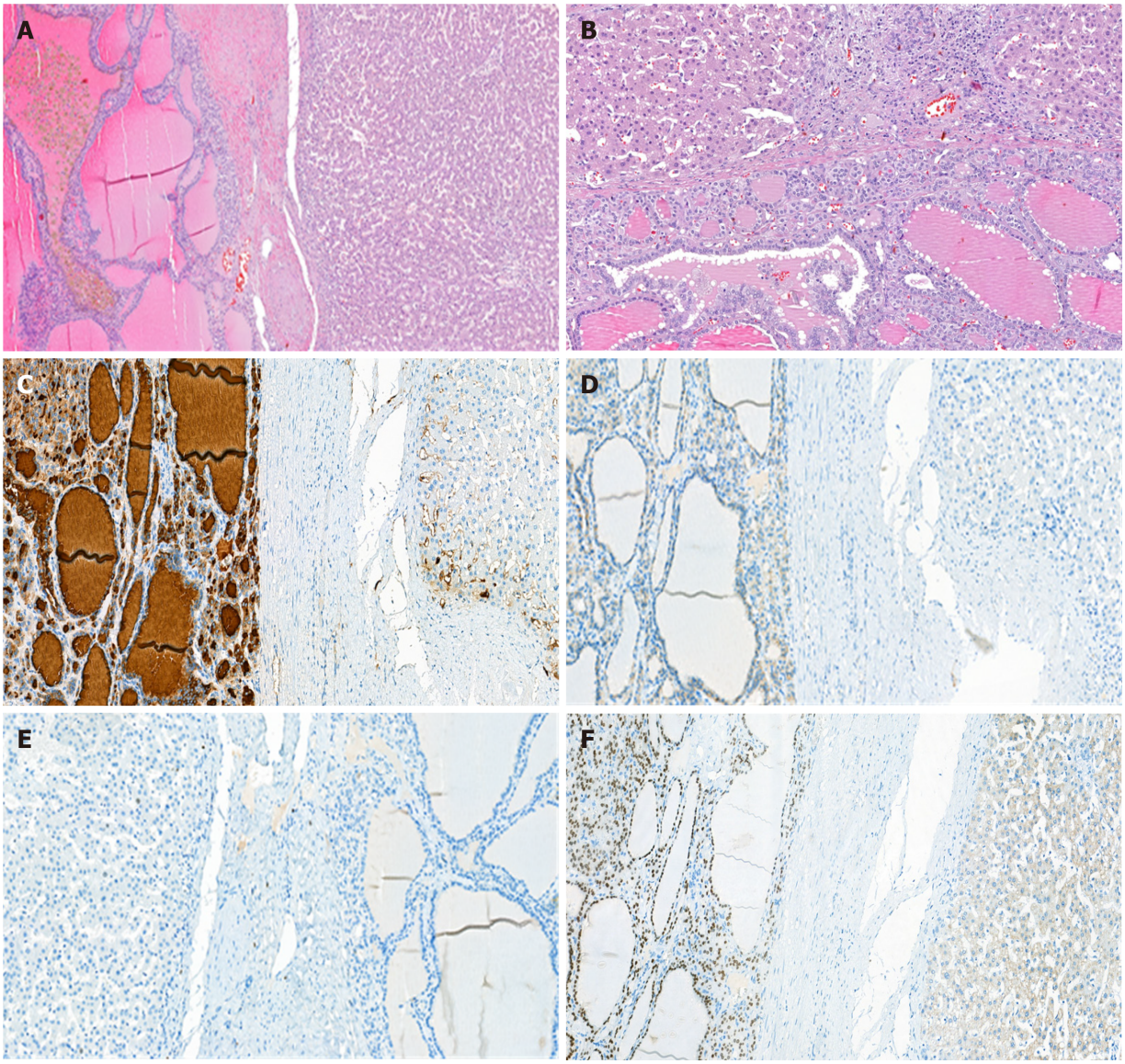

Figure 3 Pathology findings of liver mass. A: Microscopic appearance - the liver node, with shaped borders, is formed from cavities of different sizes filled with eosinophilic fluid, resembling a colloid (100×); B: Cubic single-layered epithelium lining the cavities (200×). Along the apical surface of the cells, there are characteristic vacuoles in the thick colloid; C: Epithelium labeled with anti-thyroglobulin (2H11 + 6 E1) revealing the thyroid origin (200×); D: Membrane CD56 reveals the neuroendocrine nature of tumor cells (200×); E: A single cell within a tumor node labeled with Ki67, the same as the adjacent normal liver (200×); F: Nuclear TTF-1 immunostaining also suggests a thyroid and thyroid-derived tumor origin (200×).

hospital on the 8th day after the operation. Eight years after operation the patient has no complaints, no evidence of teratoma recurrence nor newly formed teratomas were revealed during CT examination in 2021.

DISCUSSION

Hepatic teratoma is rare; to the best of our knowledge, only a small number of case reports exist in the literature (Table 1), and no liver-specific treatment guidelines have been established[5]. The successful treatment of an ectopic thyroid gland in the liver, confirmed by morphological and immunohistochemical tests, described herein was very difficult to correctly diagnose preoperatively due to the highly variable instrumental visualization of the tumor and clinical manifestations of this disease. We managed to find only one similar case of hepatic teratoma in the reviewed literature [26].

The patient's medical history provided no evidence of teratoma in thyroid gland tissue. Before the results of the morphological and immunohistochemical tests became available, the patient was considered to have perihilar cholangiocarcinoma. Bearing in

Table 1 Primary liver teratoma case reports

Ref.	Patient age	Diagnosis	Liver lobe	Treatment	Follow-up
Madan *et al*[8]	34, female	Mature cystic teratoma	Right	Complete resection	Uneventful
Watanabe *et al*[27]	20, female	N/A	Right	Complete resection	N/A
Winter *et al*[28]	61, female	Mature Teratoma	Right	N/A	N/A
Martin *et al*[29]	53, female	Mature cystic teratoma	Right	Complete resection	Uneventful
Nirmala *et al*[6]	36, female	Mature teratoma	Right	Complete resection	Uneventful
O'Reilly *et al*[22]	22, female	Immature teratoma	Right	Complete resection, chemotherapy	Uneventful
Certo *et al*[10]	27, female	Mature teratoma	N/A	Complete resection	N/A
Jaklitsch *et al*[7]	27, female	Mature cystic teratoma	N/A	Complete resection	Uneventful
Cöl *et al*[2]	21, female	Immature teratoma	Right	Complete resection, chemotherapy	Recurrence, death
Xu *et al*[30]	34, male	Immature teratoma	Right	Complete resection, chemotherapy	Recurrence, death
Han *et al*[31]	46, male	Mature cystic teratoma	Quadrant	Complete resection	Uneventful

N/A: Not available.

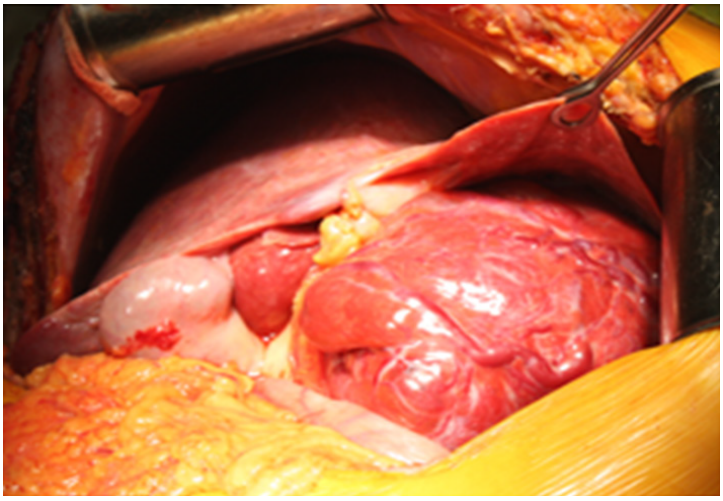

Figure 4 Intraoperative image. Tumor invades segment I of the liver, atrophied left hepatic lobe.

mind the state of our patient, we initially planned hepatectomy with a reconstruction biliary tract live-saving procedure.

The immunohistochemical test results demonstrated thyroid follicular epithelium as a result of the focal positive expression of thyroglobulin (clone 2H11+6 E1), TTF-1 (clone 8G7G3/1), and galectin-3 (clone 9C4), overexpression of cytokeratin 8 and 18 (clones B22.1 + B23.1) and negative expression of CD34 (clone QBEnd/10). This clinical case clearly demonstrates the diagnostic challenge of patients presenting with heterotopia of the thyroid gland in the liver simulating perihilar cholangiocarcinoma. Only a comprehensive examination by clinical, biochemical, and radiological methods makes tumor detection possible and allows the identification of such rare conditions. The diagnostic challenges of this condition can be met with the mass-forming type of cholangiocarcinoma. A proper preoperative evaluation, surgical treatment and preparation facilitate positive treatment outcomes.

The patient underwent ovariectomy due to an epidermoid cyst (mature teratoma) of the right ovary 10 years prior to the detection of the hepatic tumor. Unfortunately, micrographs of the lesion were not available. The ovarian teratoma had no signs of malignancy; therefore, no chemotherapy or radiotherapy was indicated. Nevertheless, hepatic teratomas are not metastases from ovarian teratomas, as mature ovarian teratomas cannot spread. Hepatic teratoma is sometimes misdiagnosed as an immature ovarian teratoma if malignant; however, in the current case, the lesion had

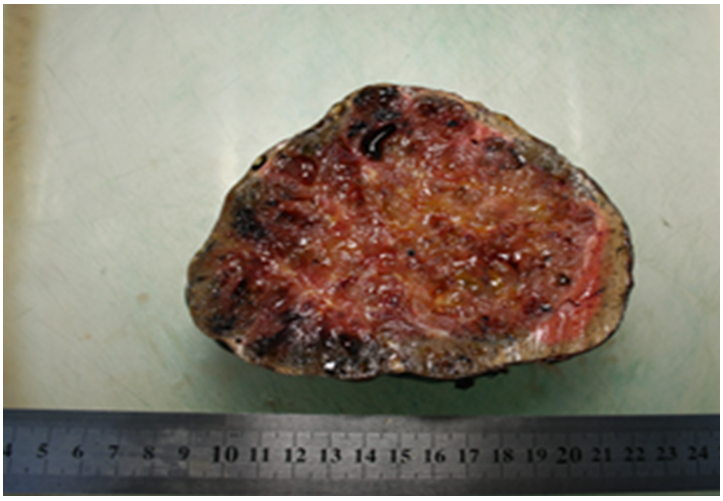

Figure 5 Macroscopic appearance - on the sections, a liver node with areas of reddish-yellow and brown color, with many cavities filled with a brown gelatinous liquid. There are also whitish-gray strands within the tumor.

no signs of malignancy. Consequently, the patient was diagnosed with metachronous teratomas of the right ovary and liver.

In summary, we present an exceedingly rare clinical presentation of heterotopia of the thyroid gland in the liver in an adult patient who underwent surgical resection. The clinical workup included a CT scan, with confirmation of the diagnosis of hepatic teratoma on histopathology. Resection remains the mainstay of treatment.

CONCLUSION

Heterotopia of the thyroid gland in the liver is an extremely rare condition in adults that needs to be considered in the differential diagnosis of solid-cystic neoplasms in the liver and cholangiocarcinoma. Surgical resection remains the mainstay of management, and risk stratification based on histology should determine postoperative surveillance. This case adds to the limited literature on the patient presentation, clinical workup, and management of liver teratomas.

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